CONTEMPORARY CANCER RESEARCH

PROSTATE CANCER

Biology, Genetics, and the New Therapeutics

Second Edition

EDITED BY

LELAND W. K. CHUNG, PhD

WILLIAM B. ISAACS, PhD

JONATHAN W. SIMONS, MD

ASD for AEK fc-original 21

Prostate Cancer

Contemporary Cancer Research

Jac A. Nickoloff, Series Editor

Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition, edited by **Leland W. K. Chung, William B. Isaacs, and Jonathan W. Simons,** 2007

Brain Tumors, edited by **Francis Ali-Osman,** 2005

Cancer Diagnostics: Current and Future Trends, edited by **Robert N. Nakamura, Wayne W. Grody, James T. Wu, and Raymond B. Nagle,** 2004

Cancer Gene Therapy, edited by **David T. Curiel and Joanne T. Douglas,** 2005

DNA Damage and Repair, Volume 3: Advances from Phage to Humans, edited by **Jac A. Nickoloff and Merl F. Hoekstra,** 2001

Prostate Cancer: Biology, Genetics, and the New Therapeutics, edited by **Leland W. K. Chung, William B. Isaacs, and Jonathan W. Simons,** 2001

Chemokines and Cancer, edited by **Barrett J. Rollins,** 1999

Breast Cancer: Moleuclar Genetics, Pathogens, and Therapeutics, edited by **Anne M. Bowcock,** 1999

DNA Damage and Repair, Volume 1: DNA Repair in Prokaryotes and Lower Eukaryotes, edited by **Jac A. Nickoloff** and **Merl F. Hoekstra,** 1998

DNA Damage and Repair, Volume 2: DNA Repair in Higher Eukaryotes, edited by **Jac A. Nickoloff** and **Merl F. Hoekstra,** 1998

Prostate Cancer

Biology, Genetics,

and the New Therapeutics,

Second Edition

Edited by

Leland W. K. Chung, PhD

Emory University School of Medicine, Atlanta, GA

William B. Isaacs, PhD

Johns Hopkins University School of Medicine, Baltimore, MD

and

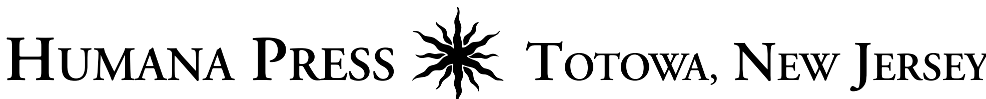
Jonathan W. Simons, MD

Prostate Cancer Foundation, Santa Monica, CA

Foreword by

Donald S. Coffey, PhD

Johns Hopkins University School of Medicine, Baltimore, MD



© 2007 Humana Press Inc.
999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

All rights reserved.

No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording, or otherwise without written permission from the Publisher.

All authored papers, comments, opinions, conclusions, or recommendations are those of the author(s), and do not necessarily reflect the views of the publisher.

This publication is printed on acid-free paper.
ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Printed Library Materials.

Production Editor: Rhukea J. Hussain
Cover artwork supplied by Dr. Leland W. K. Chung

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel: 973-256-1699; Fax: 973-256-8341; E-mail: orders@humanapr.com, or visit our Website: http://humanapress.com

Photocopy Authorization Policy:
Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Humana Press Inc., provided that the base fee of US $30.00 per copy is paid directly to the Copyright Clearance Center at 222 Rosewood Drive, Danvers, MA 01923. For those organizations that have been granted a photocopy license from the CCC, a separate system of payment has been arranged and is acceptable to Humana Press Inc. The fee code for users of the Transactional Reporting Service is: [978-1-58829-696-2/07 $30.00].

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

e-ISBN 13: 978-1-59745-224-3

Library of Congress Cataloging in Publication Data
Prostate cancer : biology, genetics, and the new therapeutics / edited by Leland W.K. Chung, William B. Isaacs, and Jonathan W. Simons; foreword by Donald S. Coffey. -- 2nd ed.
p. ; cm. -- (Contemporary cancer research)
Includes bibliographical references and index.
ISBN 1-58829-696-2 (alk. paper)
1. Prostate--Cancer. 2. Prostate--Cancer--Genetic aspects. 3. Prostate--Cancer--Treatment. I. Chung, Leland W. K. II. Isaacs, William Brewster. III. Simons, Jonathan W. IV. Series.
[DNLM: 1. Prostatic Neoplasms--physiopathology. 2. Prostatic Neoplasms--genetics. 3. Prostatic Neoplasms--therapy. WJ 762 P9661 2007]
RC280.P7P7585 2007
616.99'463--dc22
2006039398

Foreword

The Lance Armstrong Effect:
Turning up the Heat to Cure Other Advanced Cancers

The single most important question in cancer research may be understanding the molecular basis of how Lance Armstrong, with advanced metastatic lesions located at multiple sites throughout his body, was subsequently treated systemically until he could rise from his bed and then return to win so many grueling Tours de France. This fantastic therapeutic success in far-advanced testicular cancer is now common and available to most patients with this type of cancer. In stark contrast, this type of therapeutic success is unavailable to the vast majority of patients with other types of advanced solid cancer such as lung, ovarian, breast, prostate, colon, kidney, and bladder. What can account for this astounding therapeutic success with testicular cancer, and can it be explained both at the cellular and molecular levels? We must resolve this critical question, yet there are only traces of research efforts dedicated to answering it. This Foreword attempts to provide some new insights on these issues.

Understanding the molecular basis for the “Lance Armstrong Effect” may help us unravel the therapeutic cure for the other types of common advanced solid tumors that, at present, remain so recalcitrant to all of our best systemic treatments. In order to reduce the total death rates from cancer, a new therapeutic approach is urgently needed because in the last half century (1956–2006), only marginal inroads have been made in curing advanced metastatic disease of the top five solid tumors. With new drugs, much is often made of partial responses but too often, these are only reflected in very limited changes in survival and usually with a significant trade-off in quality of life. Unfortunately, many of the new molecularly targeted drugs resulting from our biological discoveries come with astounding financial costs to the individual patients, and this will most certainly limit their widespread use. In summary, something must be done to restore to the large number of patients with advanced common solid tumors the quality of life and level of health and vigor that they experienced without cancer. This is what was realized with patients like Lance Armstrong. In addition, the total financial cost of this new cancer therapy must be realistic and not affordable to only a small, select portion of the world’s population.

So, where do we look for leads in explaining the Lance Armstrong effect? It is proposed here that the thermal sensitivity of normal testicular germ cells to the normal body temperature of 98.6°F, when combined with known increases in the thermal sensitivity of most types of cancer cells, has poised testicular cancer at the gateway to destruction through a therapeutically induced apoptosis. Indeed, it is recognized from numerous clinical and basic studies that hyperthermia alone can alter tumor growth and survival in a significant manner both in vivo and in vitro. In addition, hyperthermia is recognized to increase the therapeutic response of tumors to radiation, cytotoxic drugs, and immunotherapy.

Hyperthermia is a very old type of therapy and has been of long-standing interest in medicine and cancer therapy. Although PubMed lists more than 10,000 references to

cancer and hyperthermia, the therapy has not been widely accepted because of limitations in application and understanding. Now, with the new techniques in molecular medicine that can deliver heat to specific tumor cells via nanoparticles, we can move from heating cancer cells externally to heating the cell only internally. In addition, our understanding of how controlled heat affects both tumor cells and host cells at the molecular and cellular level may open the door to specific hyperthermia treatments of other types of advanced solid tumors that, in combination with radiation and chemotherapy, might approach the great therapeutic success realized by Lance Armstrong.

Heat as a Primary Epigenetic Factor in Development and Reproduction

We will now address the importance of heat as an epigenetic factor in biology. In some animals, sex is determined by the environment whereas in others, sex is determined by genetic differences. The sex of amphibian eggs is determined by the specific temperature at which the egg is incubated within the nest. The egg is heated for several weeks by the environment surrounding it. For example, alligator eggs located in the compost-like nature of the nest are subject to different temperatures during the time of their incubation. If the temperature is higher, the eggs hatch as males; if the temperature of incubation is lower, the hatchlets are predominantly females. The effect of heat on sex determination is reversed with turtles, for whom a higher incubation temperature produces females and a lower temperature males. In summary, in amphibians, eggs with the same DNA can hatch either as males or females depending on the nest temperature. Therefore, the genetics of DNA and chromosomes in an amphibian egg do not determine the sex of the hatchling, but rather an epigenetic factor—heat— is responsible for this critical biological determination. Once the heat-determined sex is realized, it is biologically irreversible and silenced, and heat does not alter the sex of the animal throughout its subsequent life. In summary, incubation heat selects and imprints the sex expression in a critical period of time during early development and the results are permanent.

A zygote is a fertilized egg and a single cell, and thus serves as the primary totipotent stem cell for all animals as they develop during life. The fact that, in amphibians, the stem cell is set on two vastly different courses of lifetime development (male or female) based solely on the external application of a specific heat level is an amazing epigenetic feat and deserves to be a model of study for the further resolution of molecular events and systems analysis in stem cell biology. It would be of interest to know when and how chromatin structure is modified by heat. DNA methylation, DNA rearrangements, noncoding RNAs, histone modification, and higher-order DNA loop organization on the nuclear matrix are all concepts of great current interest in molecular development, and many of these factors are interrelated and dynamic. What is the role of temperature after sex is determined and into adulthood?

Once sex is determined, all eggs, either cold-blooded or warm-blooded, must be incubated at a specific temperature to hatching or birth to ensure the organization of the embryo. The second law of thermodynamics governs this process: heat flows from high temperatures to low and the system exhibits an increase in entropy even during this period of self-organization. Even in humans, ovulation is heralded in the female by an increase in body temperature and if the egg is fertilized, the temperature is elevated to birth. In contrast, in the male, spermatogenesis ceases if the testes are subjected to a

body temperature of 98.6°F because the testes are abdominal and not descended. Indeed, if the testes are intra-abdominal, they are infertile and are at increased risk of developing cancer. The mechanism by which heat affects human egg and sperm formation must be resolved.

Evolution, Immunity, and Temperature

Warm-blooded animals evolved basal temperatures far above the ambient temperature of their environments. Maintaining this temperature difference is very costly in terms of energy and must have a large survival benefit to have been selected. We all live within a sea of pathogens that are both deadly and symbiotic. When infected, mammals have adapted to produce fevers as part of an immune response to the pathogens. Temperature is central to the immune process. The antigenicity of an antigenic molecule is increased with temperature, as is the overall immune response through heat-induced immune cell functions. Many have studied the effects of heat on immunity and the involvement of temperature in immunotherapy. The evolution of pathogen-induced fever may have produced biological defense advantages that can be utilized in therapies. Indeed, cold-blooded lizards are more prone to infection when left in the shade but less so when placed in the sun, where their body temperature is increased.

Hyperthermia and Cancer

Cancer cells are, in general, more heat-sensitive. Elevating the temperature of incubation in culture of human and rodent cells and comparing these normal cells with cancer cells indicates that above 42°C, the cancer cells are more sensitive to growth. The response of all cells to heat stress initiates the synthesis, distribution, and function of a family of proteins termed the "heat-shock" proteins. These most interesting proteins are involved in a wide variety of functions including protein folding, chaperoning, and moving throughout the cell and protecting cellular elements from elevated temperatures. Specific heat-shock proteins are appearing as critical factors in cell signaling and have become prime therapeutic targets. The fascinating role of heat-shock proteins and their dynamic properties is now becoming clear, and this should contribute to our understanding of hyperthermia in cancer therapy. The role of heat-shock proteins in apoptosis, mitochondria, and nuclear function are particularly exciting areas. Because heat-shock proteins are involved with the dynamics of protein folding, they should interface with many structures and pathways within the cells.

One of the prime cellular targets of hyperthermia is the nuclear matrix, studies of which have been pioneered by Joseph L. Roti Roti of the Department of Radiation Oncology at Washington University School of Medicine in St. Louis. Variation in changes in nuclear structure are a hallmark of how cancer is diagnosed by the pathologist. The determination of nuclear structure is determined by a residual structure of the nucleus, termed the nuclear matrix, that is composed only 10% of the total nuclear protein and provides a highly dynamic scaffold that is free of lipids, histones, and nucleic acid. The nuclear matrix provides an anchoring site for more than 50,000 loops of DNA. Each loop is approx 60,000 bp in length and represents a replicon comprised of the units of DNA located between replicating forks. These sites of DNA replication are affixed to the matrix at the base of each DNA loop. During DNA replication, the replicon loops are reeled down through the fixed replication sites on the matrix. Near

these sites of DNA replication located on the matrix are co-localized topoisomerases, steroid receptors, and high-mobility group proteins, which are involved in DNA translocations. The nuclear matrix provides many sites for higher-order DNA organization that serve as functional centers for replication, transcription, and alternate splicing.

Proteomic studies have revealed that the composition of the nuclear matrix is both tissue-specific and tumor-specific. Thus, Roti Roti's and J. R. Lepoch's studies of the nuclear matrix as a prime target of hyperthermia are of great potential importance. The observation that loop organization is altered by hyperthermia could be of paramount importance.

Directing Heat to Cancers

Heat can be directed to cancer cells. Magnetic iron particles have been directed to cancer cells by inclusion in nanoparticles or liposomes. Once these iron particles are taken up by the tumors, they are heated by an external magnetic field to frequencies that produce a specific temperature within the tumor cells. This causes intracellular hyperthermia and can be used alone as hyperthermia or in combination with radiation or chemotherapy. Limitations in this approach might be unwanted uptake of the delivery systems by macrophages, fixed reticulo-endothelial cells, or inadvertent lodging of the particles in unintended sites in the body. Specificity might be increased by appropriate placing of magnetic catheters in the urethra or rectum to direct particles to the prostate.

More specific direction of these delivery particles might involve probes that attach to tumor surface markers or to tumor endothelial cell markers. Prostatic specific membrane antigen (PSMA) is an attractive target for prostate cancer cells. PSMA levels are markedly enhanced on the surface of advanced human prostate cancer cells that have failed androgen-deprivation therapy. Antibodies as well as tumor-specific aptamers have been developed to bind to PSMA; including these PSMA-binding agents with nanoparticles or liposomes containing microscopic iron particles would enable us to systemically direct heat to targeted metastatic prostate cancer cells. This hyperthermia within the cells should also enhance the cancer cells' response to other forms of therapy such as radiation, cytotoxicity, and immunotherapy. Hopefully, this type of therapeutic approach may one day extend the Lance Armstrong Effect to other advanced disseminate human solid tumors that are more common.

***Donald S. Coffey,* PhD**
Johns Hopkins University School of Medicine

This article was taken from the *JAMA* paper entitled "*Hyperthermic Biology and Cancer Therapies*": A Hypothesis for the "Lance Armstrong Effect".

Coffey, D. S., Getzenberg, R. H., and DeWeese, T. L. *JAMA* 2006, 296(4), 445–448.

Preface

We are extremely pleased and excited to see the second edition of *Prostate Cancer* reach production, six years after the first edition! Unlike most other prostate cancer books available on the market, this book focuses specifically and in depth on the exciting developments in research, and their translation from bench to bedside, in the areas of: 1) Biology and Imaging; 2) Cancer Genetics; and 3) Novel Therapeutics of human prostate cancer.

The second edition of this book includes a Foreword by Dr. Donald S. Coffey, a preeminent teacher and friend who has inspired, educated and led two generations of prostate cancer researchers. We are grateful for his continued dedication and insight into the deadly disease of prostate cancer. Dr. Coffey creatively describes the Lance Armstrong effect, giving us new and fresh ways to think as well as hope for the development of "curative" therapies for human prostate cancer.

In the new edition, we have expanded the Biology section to include recent exciting developments in the Imaging of prostate cancer. Iyer and Gambhir developed noninvasive imaging methods to track gene expression in living subjects and Nie, Gao and colleagues pioneered the use of quantum dot nanotechnology for the visualization of localized and disseminated tumors in live mice. These exciting imaging modalities will no doubt be translated into the clinic in the near future to ascertain, on a noninvasive and real-time basis, the results of targeted therapies in clinical trials. The new edition also describes exciting recent developments in understanding the biology of prostate cancer, including the roles of inflammation in the pathogenesis of human prostate cancer (DeMarzo), cancer stem cells (Schalken), tumor-stroma interaction (Chung and colleagues), novel xenograft models that have the potential to validate our understanding of prostate cancer biology (Vessella and Corey), and the design of tissue microarrays to confirm basic science studies in clinical specimens (Datta and Kajdacsy-Balla). In addition, big strides were made in understanding signal transduction in the human prostate, including androgen receptor (Klocker and colleagues), hedgehog signaling (Bushman), and cholesterol and lipid rafts (Freeman and colleagues). Understanding fundamental biology has facilitated the development of novel therapeutics. In this new edition, Keller describes the fascinating biology and therapeutic opportunities for treating lethal prostate cancer bone metastasis; Kao, Gardner and colleagues summarize their recent exciting gene therapy approaches for the treatment of advanced prostate cancer; and Lee and colleagues explain their innovative findings exploring the roles of TGFβ as an immune vaccine for the prevention and treatment of human prostate cancer.

In the post-human-genome era, much progress has also been made in understanding the genetics of human prostate cancer. Technological advances in high throughput sequencing, genomics, and bioinformatics have revolutionized the tools available to analyze the cancer cell. Global, genome-wide approaches made possible by the sequencing of the human genome are largely responsible for this revolution. Increasingly appreciated is the role of variations in DNA sequence in human carcinogenesis,

made apparent by projects like HapMap in terms of germline DNA, and The Cancer Genome Atlas in terms of somatic alterations of cancer DNA. It is also increasingly evident that our understanding of the molecular basis of cancer initiation and progression will be critically incomplete without a comprehensive characterization of the cancer epigenome. Tomlins and Chinnaiyan reveal the use of new technologies for gene expression profiling in human prostate cancer upon disease progression, Porkka and Visakorpi define the somatic genetic alterations accompanying clinical progression of human prostate cancer, and Isaacs and Xu present their innovative findings using linkage analysis to identify genes associated with familial and sporadic human prostate cancers. In addition, the significance of epigenetically altered somatic DNA methylation is summarized elegantly by Nelson and colleagues, and the involvement of telomeres, telomerase and chromosomal instability in human prostate cancer is described comprehensively for the first time by Meeker.

New Therapeutics describes new horizons and approaches for potentially curative intervention. Mohler comprehensively reviews epidemiology and gene-environment interaction in human prostate cancer with specific examples focusing on unique features such as racial differences and the contribution of environmental factors. Since the last edition of this book, the standard of care in prostate cancer patients has advanced significantly. DeWeese and Song describe improved dosimetry and precision radiotherapy for the management of prostate cancer patients. Meraney and Heston provide additional insight into the biology and usage of prostate-specific membrane antigen (PSMA) as an effective imaging and therapeutic target for treating prostate cancer patients. As the consequence of our improved understanding of the molecular basis of human prostate cancer progression and drug resistance, Carducci and colleagues introduced a new approach using differentiating agents and epigenomics in therapy. Mohile and Petrylak share their clinical experiences and insights into the use of Food and Drug Administration-approved taxane-based chemotherapy in patients with hormone-refractory prostate cancer. Assikis introduces the concept of dual targeting or co-targeting of both the prostate cancer and its stromal milieu, aimed at both tumor and surrounding interactive stromal compartments. George and Whang provide new insights into possibly druggable intracellular signaling pathways that control lethal prostate cancer metastases. This section ends with a valuable description by Rogatko and Tighiouart of their stepwise approach to trial designs for developing new therapies to treat advanced prostate cancer.

We are looking at a future that seems likely to include reductions in incidence and death rates of human prostate cancer in the western world, but increasing rates of prostate cancer among the Asians and other races in other continents. We are delighted to see the second edition of *Prostate Cancer* reflect so much unfolding and exciting science, as well as newly recruited talents introducing fresh concepts in prostate cancer research. Obviously the entire field of prostate cancer research cannot possibly be covered by a single book. We deeply regret not being able to include all of the exciting new developments in prostate cancer research, and that space limitations prevent us from including all of the superb investigators in the prostate cancer research field. Nevertheless, it is our great pleasure to present to you, through the second edition of this book, a glimpse of the future of prostate cancer research, including much that will

be translated into the clinic to improve the diagnosis, prognosis and treatment of this potentially lethal disease.

The Editors wish to acknowledge the significant contributions of Gary Mawyer for his excellent scientific editing of this book and the tireless and successful efforts of Kristin Gunderson who collected and organized so much fresh material from our busy contributors.

Leland W. K. Chung, Ph.D.
William B. Isaacs, Ph.D.
Jonathan W. Simons, M.D.

Contents

Foreword .... *v*
Preface .... *ix*
List of Contributors .... *xvii*

PART I BIOLOGY AND IMAGING

1 Xenograft Models of Human Prostate Cancer
Eva Corey and Robert L. Vessella ***3***

2 The Pathology of Human Prostatic Atrophy and Inflammation
Angelo M. De Marzo ***33***

3 Tissue Microarrays in Prostate Cancer Research
Milton W. Datta and André A. Kajdacsy-Balla ***49***

4 Prostate (Cancer) Stem Cells
Jack A. Schalken ***63***

5 Cancer–Host Interactions: *A Paradigm Shift Brings New Understanding and New Opportunities*
Leland W. K. Chung, Wen-Chin Huang, Shian-Ying Sung, Daqing Wu, Valerie Odero-Marah, and Haiyen E. Zhau ***73***

6 Androgen Receptor Function in Prostate Cancer Progression
Helmut Klocker, Iris E. Eder, Barbara Comuzzi, Georg Bartsch, and Zoran Culig ***87***

7 Hedgehog Signaling in Development and Cancer
Wade Bushman ***107***

8 Cholesterol, Cell Signaling, and Prostate Cancer
Mohini Lutchman, Keith R. Solomon, and Michael R. Freeman ***119***

9 Gene Therapy for Advanced Prostate Cancer
Juan Antonio Jiménez, Xiong Li, Thomas A. Gardner, and Chinghai Kao ***139***

10 Role of Transforming Growth Factor-β in Immunotherapy of Prostate Cancer
Chung Lee, Ali Shah, Victoria Liu, Irwin Park, Larry Wong, Xuemei Huang, Lijun Huang, Tim Fermin, Tom Jang, Som Kundu, Vivian Zhou, Qiang Zhang, and Norm Smith ***165***

11 Biology and Therapeutic Basis of Prostate Cancer Bone Metastasis
Evan T. Keller ***175***

12 Molecular Imaging: *Noninvasive Strategies to Visualize Gene Expression in Living Subjects*
Meera Iyer and Sanjiv S. Gambhir **193**

13 Quantum Dot Nanotechnology for Prostate Cancer Research
Xiaohu Gao, Yun Xing, Leland W. K. Chung, and Shuming Nie **231**

PART II GENETICS

14 Expression Profiling of Prostate Cancer Progression
Scott A. Tomlins and Arul M. Chinnaiyan **247**

15 Somatic Alterations in Prostate Cancer Progression
Kati P. Porkka and Tapio Visakorpi **269**

16 Linkage Studies of Prostate Cancer Families to Identify Susceptibility Genes
William B. Isaacs and Jianfeng Xu **285**

17 Somatic DNA Methylation Changes and Prostatic Carcinogenesis
William G. Nelson, Srinivasan Yegnasubramanian, Patrick J. Bastian, Masashi Nakayama, and Angelo M. De Marzo **301**

18 Telomeres, Telomerase, Chromosome Stability, and Prostate Cancer
Alan K. Meeker **317**

PART III THERAPEUTICS

19 Racial Differences in Prostate Cancer Mortality
James L. Mohler **355**

20 Radiotherapy for the Treatment of Locally Advanced Prostate Cancer
Danny Y. Song and Theodore L. DeWeese **377**

21 Recombinant Antibody Candidates for Treatment of Prostate Cancer
Anoop M. Meraney and Warren W. D. Heston **397**

22 Differentiation Agents and Epigenomic Therapies: *An Overview and Potential Use for Prostate Cancer*
Madeleine S. Q. Kortenhorst, Michael A. Carducci, and Shabana Shabbeer **411**

23 Taxane-Based Chemotherapy for Prostate Cancer
Supriya G. Mohile and Daniel P. Petrylak **445**

24 Co-Targeting Therapeutics for Hormone Refractory Prostate Cancer
Vasily Assikis **463**

25 Intercellular Targets of Prostate Cancer
Young E. Whang and Daniel J. George **475**

26 Novel and Efficient Translational Clinical Trial Designs in Advanced Prostate Cancer
André Rogatko and Mourad Tighiouart ***487***

Index ***497***

Contributors

VASILY ASSIKIS, MD • *Departments of Medical Oncology, Hematology/Oncology, and Oncology, Piedmont Hospital, Atlanta, GA, and Emory University School of Medicine, Atlanta, GA*

GEORG BARTSCH, MD • *Department of Urology, Innsbruck Medical University, Innsbruck, Austria*

PATRICK J. BASTIAN, MD • *Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Brady Urological Institute, Baltimore, MD*

WADE BUSHMAN, MD, PhD • *Division of Urology, Department of Surgery, University of Wisconsin, Madison, WI*

MICHAEL A. CARDUCCI, MD • *Prostate Cancer Program, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD*

ARUL M. CHINNAIYAN, MD, PhD • *Departments of Pathology and Urology, Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI*

LELAND W. K. CHUNG, PhD • *Molecular Urology and Therapeutics Program, Department of Urology, and Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA*

DONALD S. COFFEY, PhD • *The Catherine Iola and J. Smith Michael Distinguished Professor of Urology and Professor of Oncology and Pharmacology, Johns Hopkins Medical Institutions, Baltimore, MD*

BARBARA COMUZZI, PhD • *Department of Urology, Innsbruck Medical University, Innsbruck, Austria*

EVA COREY, PhD • *Department of Urology, University of Washington Medical Center, Seattle, WA*

ZORAN CULIG, MD • *Department of Urology, Innsbruck Medical University, Innsbruck, Austria*

MILTON W. DATTA, MD • *Hospital Pathology Associates PA, Saint Paul, MN, Departments of Pathology and Urology, Emory University School of Medicine, Atlanta, GA*

ANGELO M. DE MARZO, MD, PhD • *Department of Pathology, Brady Urological Research Institute, and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*

THEODORE L. DEWEESE, MD • *Departments of Radiation Oncology and Molecular Radiation Sciences, Urology, Oncology, and Johns Hopkins School of Public Health, Johns Hopkins University, Baltimore, MD*

IRIS E. EDER, PhD • *Department of Urology, Innsbruck Medical University, Innsbruck, Austria*

TIM FERMIN, BS • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

MICHAEL R. FREEMAN, PhD • *The Urological Diseases Research Center, Department of Urology, and Department of Orthopaedic Surgery, Harvard Medical School, Boston, MA*

SANJIV S. GAMBHIR, MD, PhD • *The Crump Institute for Molecular Imaging and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA and Departments of Radiology and Bioengineering Bio-X Program, Molecular Imaging Program at Stanford, Stanford University, Stanford, CA*

XIAOHU GAO, PhD • *Department of Bioengineering, University of Washington, Seattle, WA*

THOMAS A. GARDNER, MD • *Urology Research Laboratory, Departments of Urology, Microbiology, and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, IN*

DANIEL J. GEORGE, MD • *Divisions of Medical Oncology and Urology, Duke University Medical Center, Durham, NC*

WARREN W. D. HESTON, PhD • *Glickman Urological Institute, The Cleveland Clinic, and Department of Cancer Biology, Lerner Research Institute, Cleveland, OH*

LIJUN HUANG, MD • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

WEN-CHIN HUANG, PhD • *Molecular Urology and Therapeutics Program, Department of Urology, Emory University School of Medicine, Atlanta, GA*

XUEMEI HUANG, MD • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

WILLIAM B. ISAACS, PhD • *Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD*

MEERA IYER, PhD • *The Crump Institute for Molecular Imaging and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA*

TOM JANG, MD • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

JUAN ANTONIO JIMÉNEZ, BS • *Urology Research Laboratory, Departments of Urology, Microbiology, and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, IN*

André A. Kajdacsy-Balla, MD, PhD • *Department of Pathology, Emory University School of Medicine, Atlanta, GA*

Chinghai Kao, PhD • *Urology Research Laboratory, Departments of Urology, Microbiology, and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, IN*

Evan T. Keller, DVM, PhD • *Institute of Gerontology, University of Michigan, Ann Arbor, MI*

Helmut Klocker, PhD • *Department of Urology, Innsbruck Medical University, Innsbruck, Austria*

Madeleine S. Q. Kortenhorst, MD • *Prostate Cancer Program, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD*

Som Kundu, MD • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Chung Lee, PhD • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Xiong Li, MD, PhD • *Urology Research Laboratory, Departments of Urology, Microbiology, and Immunology, Walther Oncology Center, Indiana University Medical Center, Indianapolis, IN*

Victoria Liu, BS • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Mohini Lutchman, PhD • *The Urological Diseases Research Center, Department of Urology, and Department of Surgery, Harvard Medical School, Boston, MA*

Alan K. Meeker, PhD • *Division of Genitourinary Pathology, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD*

Anoop M. Meraney, MD • *Glickman Urological Institute, The Cleveland Clinic, Cleveland, OH*

Supriya G. Mohile, MD, MS • *New York Presbyterian Hospital, New York, NY*

James L. Mohler, MD • *Roswell Park Cancer Institute, Buffalo, NY*

Masashi Nakayama, MD • *Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Brady Urological Institute, Baltimore, MD*

William G. Nelson, MD, PhD • *Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Brady Urological Institute, Baltimore, MD*

Shuming Nie, PhD • *Departments of Biomedical Engineering, Chemistry, Hematology and Oncology, and the Winship Cancer Institute, Emory University School of Medicine and Georgia Institute of Technology, Atlanta, GA*

Valerie Odero-Marah, PhD • *Molecular Urology and Therapeutics Program, Department of Urology, Emory University School of Medicine, Atlanta, GA*

Irwin Park, BS • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Daniel P. Petrylak, MD • *New York Presbyterian Hospital, New York, NY*

Kati P. Porkka, PhD • *Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, Tampere, Finland*

André Rogatko, PhD • *Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA*

Jack A. Schalken, PhD • *Department of Urology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands*

Shabana Shabbeer, PhD • *Prostate Cancer Program, Sidney Kimmel Cancer Center, Johns Hopkins University, Baltimore, MD*

Ali Shah, PhD • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Jonathan W. Simons, MD • *Prostate Cancer Foundation, Santa Monica, CA, Department of Hematology and Oncology, Winship Cancer Institute of Emory University, Atlanta, GA and Department of Biomedical Engineering and Material Sciences Engineering, Georgia Institute of Technology, Atlanta, GA*

Norm Smith, MD • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Keith R. Solomon, PhD • *The Urological Diseases Research Center, Department of Urology; Department of Orthopaedic Surgery, Children's Hospital Boston; and Department of Orthopaedic Surgery, Harvard Medical School, Boston, MA*

Danny Y. Song, MD • *Departments of Radiation Oncology, Urology, and Oncology, The Johns Hopkins Medical Institute, Baltimore, MD*

Shian-Ying Sung, PhD • *Molecular Urology and Therapeutics Program, Department of Urology, Emory University School of Medicine, Atlanta, GA*

Mourad Tighiouart, PhD • *Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA*

Scott A. Tomlins, BA • *Department of Pathology, Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI*

Robert L. Vessella, PhD • *Puget Sound VA Health Care System and Department of Urology, University of Washington Medical Center, Seattle, WA*

Tapio Visakorpi, MD, PhD • *Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, Tampere, Finland*

Young E. Whang, MD, PhD • *Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, NC*

Larry Wong, BS • *Department of Urology, Feinberg School of Medicine, Northwestern University, Chicago, IL*

Daqing Wu, PhD • *Molecular Urology and Therapeutics Program, Department of Urology, Emory University School of Medicine, Atlanta, GA*

Yun Xing, PhD • *Departments of Biomedical Engineering, Chemistry, Hematology and Oncology, and the Winship Cancer Institute, Emory University and Georgia Institute of Technology, Atlanta, GA*

Jianfeng Xu, MD, DrPh • *Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, NC*

Srinivasan Yegnasubramanian, MD, PhD • *Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Brady Urological Institute, Baltimore, MD*

1

Xenograft Models of Human Prostate Cancer

Eva Corey and Robert L. Vessella

Summary

Prostate cancer (CaP) is the second-leading cause of cancer deaths among men in the Western world. Understanding the biology of CaP is essential to the development of novel therapeutic strategies. To do this effectively, researchers need access to cell lines, animal models, and biospecimens. Ideally, these should reflect characteristics of the disease from early diagnosis through the period of androgen-independent metastases. However, the biology of CaP is extraordinarily complex. The primary tumors are heterogeneous and multifocal. They almost uniformly produce a number of biomarkers, such as prostate-specific antigen, prostate acid phosphatase, human kallikrein 2, prostate-specific membrane antigen, and prostate stem cell antigen. The tumors are characteristically androgen dependent, and respond favorably to androgen ablation for a variable period, after which time, they progress to an androgen refractory state, wherein the low androgen levels are no longer growth inhibitory. Progression also involves metastases, primarily to the lymph nodes and bone. Unlike other epithelial tumors that metastasize to bone, CaP results in a notoriously osteoblastic response characterized by the formation of new bone. Thus, CaP presents to the investigator a very dynamic, biologically diverse set of characteristics during its course from early disease to bone metastases. Unfortunately, no single model mimics all of the features of human CaP, but by using a combination of models, nearly all of the biological characteristics can be studied to some degree.

In this chapter, we review the various xenograft models of human CaP that are available for study. We look at the method of generating xenografts, including the host strains and sites of implantation. We report on the models that are best suited for the study of progression from androgen dependence to androgen independence. Unfortunately, spontaneous metastases to bone resulting in an osteoblastic response are an extremely rare event in these models, but several methods that attempt to circumvent this limitation are reviewed. Overall, several examples that demonstrate the wealth of new knowledge being provided by use of these preclinical human CaP xenograft models are provided throughout the chapter.

Key Words: Androgen ablation; androgen independence; bone metastases; metastases; models; prostate cancer; xenografts.

1. INTRODUCTION

Prostate cancer (CaP) is the second-leading cause of cancer deaths among men in the Western world. In 2004, approx 230,000 men were diagnosed and 30,000 men died from advanced disease. Understanding the biology of CaP is essential to the development of novel therapeutic strategies. To do this effectively, researchers need access to cell lines, animal models, and biospecimens. Ideally, these should reflect characteristics of the disease from early diagnosis through the period of androgen-independent metastases.

In general, CaP presents as a multifocal primary tumor with various degrees of aggressiveness, as scored by the Gleason grading system. The primary tumors, being derived from prostate epithelial cells, produce prostate-specific antigen (PSA; also referred to as human kallikrein 3), along with an

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

assortment of other CaP-associated antigens, such as prostate-specific membrane antigen, prostatic acid phosphatase, human kallikrein 2, and prostate stem cell antigen. These tumors are androgen dependent, or at least androgen responsive, with growth regulated in part by the androgen receptor, which may be mutated either in the primary tumor or during the course of disease progression. If treatment of the primary tumor is not curative, androgen ablation becomes the treatment of choice, with chemotherapy often supplementing this course of action. Most frequently, the tumors show a significant response to androgen ablation with a decrease in tumor volume and serum PSA levels. However, during a period ranging from months to years, the tumors progress to an androgen-independent state through a process that is still poorly understood. The appearance of androgen independence is first revealed by an increasing serum level of PSA, followed by resumption of tumor growth. Generally, androgen independence is associated with disseminated disease. Metastases are present in the lymph nodes and skeleton in nearly 100% of men who experience progressive disease. The proclivity for metastasis to bone is a hallmark of CaP. Moreover, unlike nearly all other tumors that metastasize to bone, CaP growth in bone causes a perturbation of normal bone remodeling that results in new bone formation, the characteristic osteoblastic response, whereas other tumor metastases to bone result primarily in resorption of bone (osteolytic response). CaP bone metastases result in significant pain and morbidity before expiration of the patient. Thus, CaP presents to the investigator a very dynamic, biologically diverse set of characteristics during its course from early disease to bone metastases. Unfortunately, no single model mimics all of the features of human CaP, but, by using a combination of models, nearly all of the biological characteristics can be studied to some degree.

In recent years, a number of excellent reviews have detailed the available cell lines and animal models being used for the study of CaP *(1–7)*. Among the most recent and comprehensive of these was the two-part series by Sorbel and Sadar *(6,7)*, which is complemented by a website that provides extensive histories and details of in vitro and in vivo CaP cell lines (http://www.CaPCellLines.com). Because of the availability of this recent series, this chapter will not focus extensively on a compendium of available in vitro cell lines, but will emphasize key characteristics of a range of xenograft lines and present a synopsis of the multiple CaP xenograft lines developed by our laboratory. In addition, we will specifically highlight some methodological considerations, as well as the use of selected xenograft models for studies of metastasis and progression to androgen independence.

2. METHODS OF GENERATING XENOGRAFTS

2.1. Choice of Host

2.1.1. Balb/C Nu/Nu

There is a limited series of immune-compromised mice suitable for xenotransplantation of human cancer tissue or cell lines. Isaacson and Cattanach, in 1962, were the first to report on a mutant mouse (BALB/c Nu/nu) suitable for this purpose *(8)*. The mutation is referred to as *Foxn1*. Because these mice lacked a functional thymus (they are generally referred to as athymic mice), they had a deficient cell-mediated immune response *(9)*. In these mice, the humoral antibody response system is only partially impaired and natural killer cell activity is slightly increased. For the most part, these mice readily accept subcutaneous grafts of human tumors, but there are exceptions, and primary CaP is one of these. To enhance the take rate of CaP in athymic mice, co-injection of a reconstituted basement membrane product (Matrigel) with the tumor cells or pieces of tumor, or supplementation with testosterone or dihydrotestosterone to intact hosts were used. However, Van Weerden and Romijn, in their review of the Rotterdam experience, did not observe any enhancement in tumor take rate with these hormonal manipulations *(1)*. The BALB/c Nu/nu mouse remains the most common host for xenotransplantation of human tumors. The take rate of immortalized cell lines in athymic mice ranges from 50 to 100%. Although the take rate of tumor implants from radical prostatectomy specimens is probably less than 5% overall, in our experience, the take rate of tumors from metastatic sites is approx 25 to 30% initially, and 20% for long-term serial passage.

2.1.2. NMRI/Nu

The NMRI/Nu immune-compromised mouse strain was developed by the Naval Marine Research Institute (Bethesda, MD) and was first described by Festing in 1968 *(10)*. These mice also have the *Foxn1* mutation and are similar in phenotype to the BALB/c Nu/nu mice, that is, the NMRI/Nu mice are athymic and lack hair. Wagner was the first to report an enhanced xenotransplantation take rate in these mice *(11)*. Eventually the Rotterdam group converted from the BALB/c Nu/nu mouse strain to the NMRI/Nu strain and observed an increased take rate of CaP tumors, but no specific side-by-side comparisons were made. They noted that tumors established in the NMRI/Nu mice were readily transplanted into the BALB/c Nu/nu strains, therefore, if there was an advantage, it probably was in the initial growth of the tumor. It is well-accepted that the *Foxn1* mutation does affect the take rate of cell lines and tissues in mice of different backgrounds. The NMRI/Nu mice are relatively susceptible to transplantation-induced sarcomas and lymphomas, making histological monitoring of the xenografts an important aspect of maintenance. Perhaps for this reason, the strain has not gained significant popularity in the United States.

2.1.3. Severely Combined Immune Deficient

The severely combined immune deficient (SCID) mutant mouse was first described by Bosma et al. in 1983 *(12)*. The spontaneous mutation was noted in a colony of specific pathogen-free mice during routine analysis of serum immunoglobulin isotypes. The mice were significantly hypogammaglobulinemic. The trait was found to be heritable and under the control of a recessive gene. Additional work led to the establishment of a colony of specific pathogen-free mice that were homozygous for the defective gene and designated C.B-17*scid*. These mice are deficient for both T- and B-lymphocytes. The authors noted that, on occasion, the mice did produce a single immunoglobulin subtype, and a few developed a T-cell lymphoma. The mice were immediately recognized as valuable models for lymphoid differentiation as well as immune and hematological disorders *(13)*. A few clones of antigen receptor-positive B and T-lymphocytes do appear in a variable proportion (2–25%) of young adult SCID mice and in virtually all old SCID mice; thus, they have been designated as having a “leaky” phenotype *(14,15)*.

2.1.4. RAG and NOD/SCID

Primarily because of the “leaky” phenotype associated with the SCID mice, a small number of new immune-deficient mice have been developed for human immune system reconstitution studies. These mice have also been used by a few investigators for xenograft studies. The RAG knockout mice completely lack functional B- and T-cells and do not display a leaky phenotype *(16)*. Because NK cells are present in SCID mice, efforts were made to develop double-mutant mice for the SCID mutation and NK cell deficiency *(17)*. These mice are referred to as “SCID beige.” Finally, a relatively popular mouse for the study of human hematopoiesis, and on occasion for xenotransplantation, is the non-obese diabetic SCID (NOD/SCID) mouse *(18)*.

2.2. Implantation Site

2.2.1. Subcutaneous Implantation

The subcutaneous implantation of cells or pieces of tissue in the flank or shoulder is by far the most common method of xenotransplantation. For cell lines (such as LNCaP) that are not tumorigenic, most investigators have relied on co-injection with Matrigel to induce tumor formation. Implantation of tumor bits often does not require Matrigel, but some investigators make it a common practice to dip tissue in Matrigel before implantation *(19,20)*. The advantages of subcutaneous implantation are the ease in implantation, monitoring early growth by palpation, and monitoring later growth with calipers.

For many studies, the ability to measure tumor growth directly is an advantage even with tumors that also produce PSA.

2.2.2. Orthotopic Implantation

Orthotopic implantation is a more challenging surgical procedure than subcutaneous implantation, but it is widely thought to be the ideal implantation site for many human cancers, because it mimics the originating environment of the tumor. For example, Fidler et al. *(21)* noted that the subcutaneous microenvironment for human visceral tumors is quite different than their "home" environment, and that this may explain both the paucity of metastases that evolve from subcutaneous implants and the altered drug response. The majority of investigators inject a CaP cell suspension into the orthotopic site. We favor the use of PSA-producing xenografts to enable monitoring of take rate and growth. Without using PSA as a surrogate marker of growth, xenografts can easily exceed the 10% maximum tumor burden recommended by the National Institutes of Health (NIH) guidelines for animal care. An advantage of orthotopic implantation is the generation of spontaneous microscopic metastases, which are only rarely observed with subcutaneous tumors. We have used a modified approach of orthotopic implantation followed by a "prostatectomy" in an effort to achieve a more reproducible metastatic model (*see* Subheading 5.).

2.2.3. Sub-Renal Capsule Implantation

The sub-renal capsule is a highly vascularized site that was first proposed for tissue implantation by Bogden *(22)*. It has not been used frequently for generation of CaP xenografts or for biological studies, although Wang et al. *(23)* have recently demonstrated the attractiveness of this site for both benign and malignant prostate xenotransplantation. This group achieved high take rates of both tissue types and performed a three-way comparison among subcutaneous, orthotopic, and sub-renal capsule implantation sites. They found that sub-renal capsule implantation resulted in the highest take rate (>90%), whereas orthotopic implantation gave the best histopathological differentiation. The high take rates in the sub-renal capsule were achieved with testosterone supplementation. This study was limited to initial take rates and no attempts to establish long-term xenografts were reported. We have also tried to use the sub-renal capsule site for implantation of CaP bone metastases acquired from our rapid autopsy program. However, because of the sharp edges of these tissue fragments, we have found it very difficult to perform the implantation without damage to the capsule or kidney. We have performed approx 12 implantations but have not achieved any tumor takes. However, some of these tissue fragments are so osteoblastic that there is probably more bone than CaP cells in the fragment, and this could explain the lack of tumor growth.

In summary, the investigator has many options for the choice of host and the site of implantation. By far the most common choice for xenotransplantation is the BALB/c Nu/nu mouse, with the SCID mouse chosen frequently for more-specialized studies of metastases or for cell lines that are difficult to work with. Although we have not performed detailed side-by-side comparisons, our data show that some of our more difficult xenografts grow better in SCID than in BALB/c Nu/nu mice, although the take rate of the initial clinical tumor implant does not seem significantly different. Moreover, our studies of metastases seem to be more productive in SCID mice. As presented subsequently, this is especially true when we use intratibial injections for the study of CaP/bone interactions. Similarly, the SCID-hu model using human fetal bone implantation, as developed by the Cher laboratory for the study of CaP bone metastasis, has become a widely accepted model *(24)*. Unfortunately, none of the available hosts are highly suitable for long-term growth of tumor implants from radical prostatectomies. Of the three sites for implantation, subcutaneous, orthotopic, and sub-renal capsule, the subcutaneous site is most often selected. However, both the orthotopic and sub-renal capsule sites can offer distinct advantages in certain situations.

2.3. Sources of Cells and Tissues for Generating CaP Xenografts

It has long been recognized that xenografts can be readily generated from a multitude of tumorigenic CaP cell lines maintained in vitro (see http://www.CaPCellLines.com). Sometimes nontumorigenic cell lines, such as LNCaP, can be induced to produce a xenograft if co-injected with

Matrigel. The biological characteristics of these tumors typically reflect those of the originating cell line. In some respects, this may be considered a limitation, in that the cell lines had undergone selection pressures from years of in vitro culture and are homogeneous in cell type. For this reason, various groups have begun to establish CaP xenografts directly from the subcutaneous implantation of CaP tissue. The Experimental Urology Research Group of the Department of Urology of Erasmus University Rotterdam, headed by Professor Schroder, was the first to report successful generation of a transplantable human CaP xenograft, designated PC-82 *(25)*. Interestingly, only 2 of their next 150 attempts were successful in generating stable xenograft lines. Their persistence ultimately led to the development of a further 10 lines *(1)*. Unfortunately, this highly successful program was unable to continue its efforts beyond the mid 1990s. However, by that time, several other programs, including ours, with similar goals were underway, including ours.

As previously mentioned, long-term CaP xenografts can also be generated, albeit at a frequency of approx 5%, from primary tumors obtained at radical prostatectomy (e.g., CWR22) *(26)*. In a few instances, CaP xenografts have been derived from tissue fragments generated during a transurethral resection of the prostate (e.g., LuCaP 96, Table 1). Clearly, most successes have been achieved from the implantation of metastatic tissue. These tissues, for instance, involved lymph nodes, are obtained most frequently after incidental discovery during a radical prostatectomy. On occasion, they may be the target of direct surgical intervention *(27)*. Because standard autopsies are generally not compatible with acquisition of viable tissue, we implemented a rapid autopsy procedure wherein metastatic tissue is obtained within a few hours of death. This procedure is also being used at the University of Michigan and Johns Hopkins University. In addition to being invaluable sources of tumor for implantation, the metastases are critical for biological investigations of advanced disease *(28)*. In our experience, metastatic samples acquired at autopsies have original take rates of approx 30%, with an overall success rate in establishing long-term xenografts of approx 20%. In general, xenografts derived from tumor implants and maintained by serial passage are more heterogeneous than those established from cell lines. Although this can result in a more varied take rate, growth rate and response to a given treatment (*see* Subheading 4.4.), it is probably more representative of the type of disease seen clinically. Interestingly, the difficulty in establishing in vitro cell lines from primary tumors obtained at radical prostatectomy is recapitulated in similar attempts to establish in vitro cell lines from long-term xenografts. Contrary to expectation, xenografts established from tissue implants are not more easily adapted to tissue culture.

3. CAP XENOGRAFTS MOST FREQUENTLY USED AND NEWLY DERIVED XENOGRAFTS

As previously mentioned, several reviews have provided detailed lists of CaP xenografts derived from cell lines as well as those generated directly from tissue implants. Here, we discuss briefly a few of the xenografts that are most often used in studies of CaP, along with some newly described xenografts, with a brief synopsis of their characteristics (Table 1) *(26,27,29–49)*. These CaP xenografts individually portray many of the characteristics found in the human disease. Some are ideal for studies of progression to androgen independence, whereas others are favored for induction of the osteoblastic response in bone, and still others possess a neuroendocrine phenotype. All of the xenografts in the LuCaP series are maintained by serial passage, because they do not grow in tissue culture. It is important to recognize that CaP does not present itself clinically as a disease with limited, defined characteristics. In fact, it is notoriously heterogeneous in histology, growth, biomarker expression, and response to therapy. It is necessary for the scientific community to derive xenografts and cell lines that portray this vast diversity. Thus, investigators interested in modeling CaP need to determine which of the xenografts and/or cell lines have the characteristics that will best answer the question at hand. We have often provided investigators with an assortment of slides for immunohistology or DNA/RNA from our LuCaP series, so they can determine which xenograft may be best used for their particular studies. One is encouraged to make use of this wide assortment of resources

Table 1
Most Commonly Used Models of Human Prostate Cancer

Xenograft cell line	Origin	In vitro growth	PSA	Androgen sensitivity	AR expression	Reference
LNCaP	Lymph node metastasis	+	+	+	+	*(29)*
C4	LNCaP	+	+	–	+	*(30,31)*
C4-2	C4	+	+	–	+	*(30,31)*
C4-2B	C4-2	+	+	–	+	*(32)*
PC-3	Skull metastasis	+	–	–	–	*(33)*
DU 145	Brain metastasis	+	–	–	–	*(34)*
MDA Pca 2a	Bone metastasis	+	+	+	+	*(35)*
MDA Pca 2b	Bone metastasis	+	+	+	+	*(35)*
MDA Pca 2b HR	MDA Pca2b	+	+	–	+	*(36)*
CWR 22	Primary tumor	+	+	+	+	*(37)*
CWR 22R	CWR 22	+	+	–	+	*(26)*
CWR 22 2Rv1	CWR 22 R	+	+	–	+	*(38)*
VCaP	Vertebrae metastasis	+	+	+	+	*(39)*
DuCaP	Dura metastasis	+	+	+	+	*(40)*
ARCaP	Ascites fluid	+	+	+	+	*(41)*
TEN12	Primary tumor	nd	+	+	+	*(42)*
TEN12F	TEN12	nd	+	–	+	*(42)*
TEN12C	TEN12	nd	+	–	+	*(42)*
HH870	Primary tumor	+	–	–	–	*(43)*
BM18	Bone metastasis	nd	+	+	+	*(44)*
LAPC-4	Lymph node metatasis	+	+	+	+	*(45)*
LACP-4 (squared)	LAPC-4	+	+	–	+	*(46)*
LAPC-9	Bone metastasis	–	+	+	+	*(47)*
LuCaP 23.1	Lymph node metatasis	–	+	+	+	*(48)*
LuCaP 23.1 AI	LuCaP 23.1	–	+	+	+	*(48)*
LuCaP 23.8	Lymph node metatasis	–	+	+	+	*(48)*
LuCaP 23.12	Liver metastasis	–	+	+	+	*(48)*
LuCaP 35	Lymph node metatasis	–	+	+	+	*(27)*
LuCaP 35V	LuCaP 35	–	+	–	+	*(27)*
LuCaP 49	Omental fat metastasis	–	–	–	–	*(49)*
LuCaP 58	Lymph node metatasis	–	+	+	+	
LuCaP 69	Bowel metastasis	–	+	+	+	
LuCaP 70	Liver metastasis	–	+	nd	+	
LuCaP 73	Primary tumor	–	+	+	+	
LuCaP 77	Femur metastasis	–	+	+	+	
LuCaP 78	Lymph node metastasis	–	+	nd	+	
LuCaP 81	Lymph node metastasis	–	+	nd	+	
LuCaP 86.2	Bladder metastasis	–	+	nd	+	
LuCaP 93	Primary tumor	–	–	–	–	
LuCaP 96	Primary tumor	–	+	+	+	
LuCaP 96 AI	LuCaP 96 –	+	–	+		
LuCaP 105	Rib metastasis	–	+	nd	+	
LuCaP 115	Lymph node metastasis	–	+	nd	+	

PSA, prostate-specific antigen; AR, androgen receptor; nd, not determined.

from investigators worldwide and not to rely exclusively on the few cell lines and xenografts that have been available for decades, unless they have been proven in screening tests to have the best characteristics for the contemplated studies.

4. PROGRESSION TO ANDROGEN INDEPENDENCE

The process by which CaP progresses from an androgen-dependent to androgen-independent state is complex and confounding. Although the need to model this progression is urgent, there are, unfortunately, only a few xenograft models that successfully mimic the process as it occurs in man. Clearly, defining ideal characteristics of suitable xenografts is somewhat arbitrary. In our laboratory, we strive for the following: a wild-type androgen receptor, and, after castration, a significant drop in tumor volume and in PSA serum levels (preferably to levels that are undetectable or nearly so), with an increase in PSA levels preceding separate evidence of tumor regrowth.

4.1. LNCaP and PC-346

For many years, LNCaP and PC-346 were the only xenografts available for progression studies *(50–52)*. LNCaP produces PSA but has a mutated androgen receptor. This mutation results in a promiscuous androgen receptor that can bind to other steroids *(53)*. PC-346 also produces PSA and has a wild-type androgen receptor. Although both lines are androgen sensitive, they are not androgen dependent; androgen withdrawal generally resulted in no more than a 10% reduction in tumor volume, although, with PC-346, occasional exceptions were noted in which the tumor volume decrease was more pronounced in some animals, suggesting a heterogeneous tumor cell population in the xenograft. With LNCaP and PC-346 xenografts, significant but transient decreases in PSA production were observed after castration. Tumors of LNCaP and PC-346 classically seemed to stabilize their growth after castration and then reestablish a rapid growth pattern during a period of approx 5 to 6 wk. However, some investigators have not observed a pause in growth after castration. There has been some variation in LNCaP response to castration because the LNCaP cell line does differ slightly between laboratories, probably because of drift during the long period of in vitro maintenance. Androgen-independent variants of LNCaP have been established and are designated C4-2, C4-2B, and CL1 *(31,54–56)*.

4.2. CWR22

CWR22, a CaP xenograft developed in the Pretlow laboratory from a primary CaP *(26,37,57)*, shows the desired response to castration in animal models. With this xenograft, PSA and tumor volume drop significantly after castration. PSA levels begin to rise 2 to 7 months thereafter and tumor growth resumes in 3 to 10 months after androgen withdrawal. Relapsed tumors have been serially passaged in mice and are referred to as CWR22R. Both the CWR22 and CWR22R lines have a mutated androgen receptor, which is activated by testosterone and dihydrotestosterone, as well as by the adrenal androgen dehydroepiandrosterone, estradiol, progesterone, and the antiandrogen hydroxyflutamide *(2,7,58)*. Because a mutated androgen receptor is found in a minority of advanced CaP cases, such promiscuous activity, as also noted in LNCaP, raises concerns regarding whether mechanisms of progression with these xenografts would be reflective of the processes observed in most patients with advanced disease.

4.3. LAPC-4 and LAPC-9

LAPC-4, derived from a lymph node, and LAPC-9, derived from a bone metastasis, are two lines developed by the University of California, Los Angeles group under the direction of Charles Sawyers *(45,47)*. Both xenografts produce PSA and show the desired pattern of significantly decreased PSA and tumor volume after castration, with restoration of PSA and tumor growth over time. Importantly, both of the xenografts express the wild-type androgen receptor. These characteristics make these xenografts good models to study changes associated with development of androgen-independent disease. Both of these xenografts have been used to show involvement of insulin-like growth factor (IGF) signaling in progression to androgen independence *(59)*.

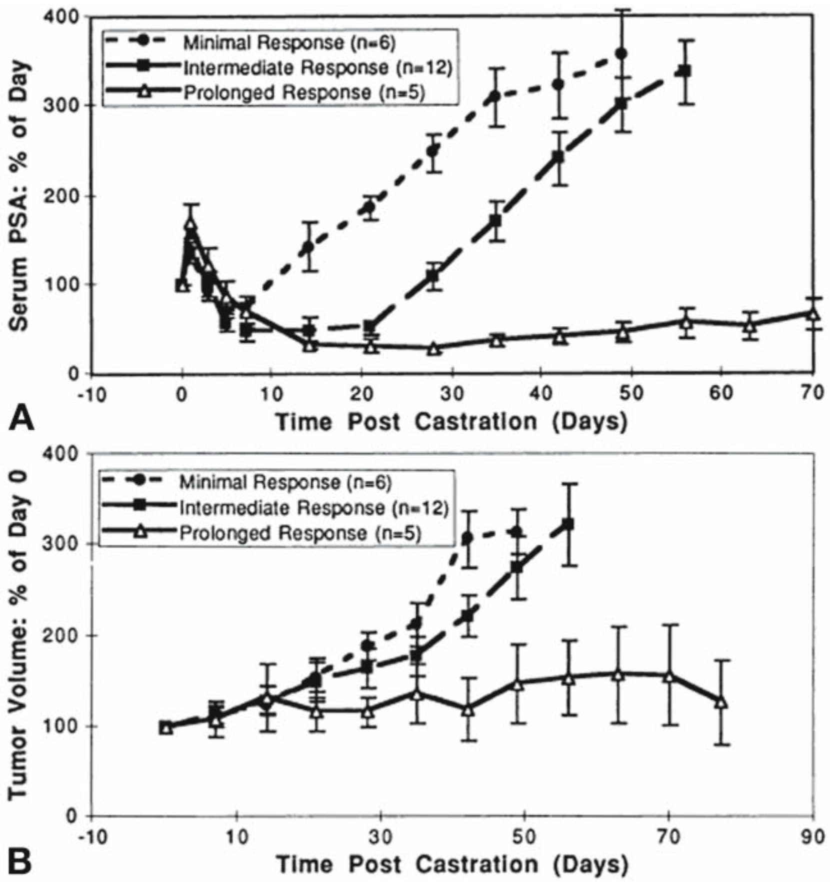


Fig. 1. LuCaP 23.1: response to castration. (**A**) Serum prostate-specific antigen (PSA). LuCaP 23.12 xenografts were subdivided into prolonged (<50 days; △), partial (between 20 and 50 days; ■) and minimal (<20 days; ●) response to castration. (**B**) Tumor volume responses to castration in the same animals from (A). Animals are categorized by PSA response. (Adapted with permission from ref. *48*).

4.4. The LuCaP Series

Our laboratory has been deriving CaP xenografts since approx 1990. Here, we present a few examples of our studies of responses to androgen ablation and progression to androgen independence.

The first set of our xenografts for which we evaluated the response to androgen withdrawal was the LuCaP 23 series *(48)*. Three sublines (23.1, 23.8, and 23.12) of CaP xenografts were established from different metastases of one patient. LuCaP 23.1, which originated from a lymph node metastasis, expresses wild-type androgen receptor and secretes high levels of PSA. This xenograft closely mimics the situation in patients after castration, exhibiting a high heterogeneity of response (Fig. 1). Some of the tumors regressed after androgen withdrawal, whereas the growth of other tumors was inhibited only briefly. From the recurrent LuCaP 23.1, we have established LuCaP 23.1AI, which is androgen independent and is maintained in castrated mice. Another of our xenografts that can show either an androgen-dependent or androgen-sensitive response to castration is LuCaP 35 *(60)*. This xenograft was derived from an androgen-independent left inguinal lymph node metastasis in a patient who had undergone hormonal ablation treatments with diethylstilbestrol, orchiectomy, and flutamide. It has the wild-type androgen receptor and deletions in chromosome 8p. It is also *PTEN* RNA-negative *(61)*. After castration, LuCaP 35 gives the response desired for progression to androgen indepen-

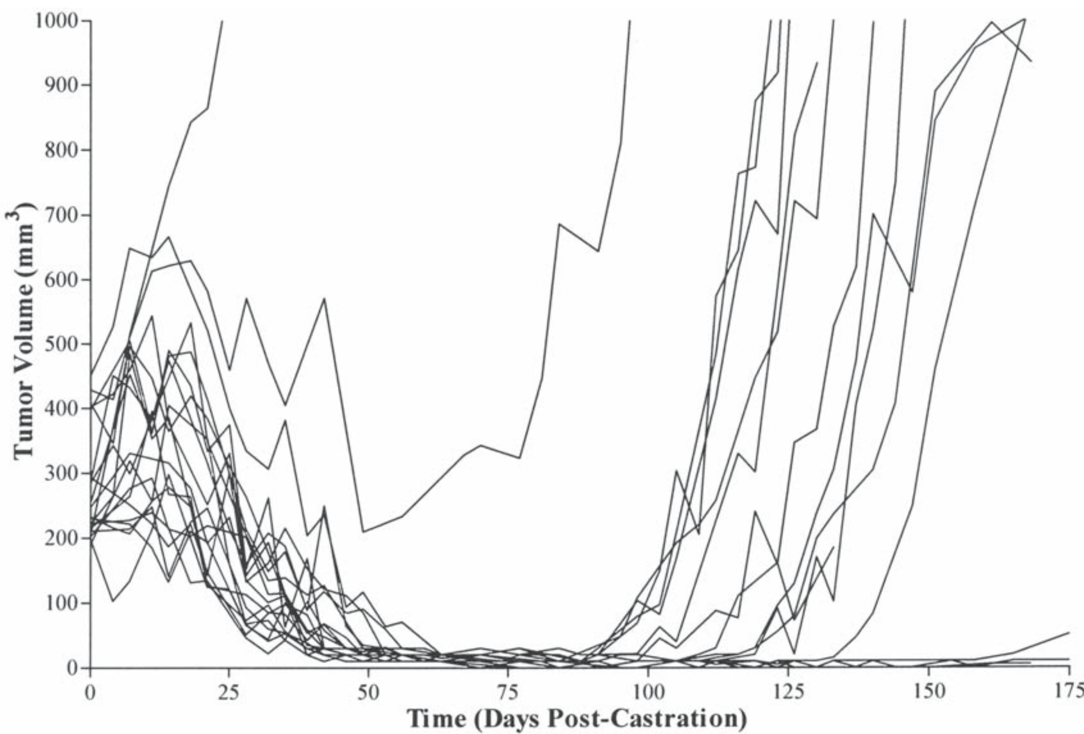


Fig. 2. LuCaP 35: response to castration. (**A**) LuCaP 35 was implanted subcutaneously in intact male mice, which were castrated when tumor volumes reached approx 200 mm^3. The tumor volume of each individual animal is plotted. LuCaP 35 regressed to nonpalpable tumors in nearly all animals by day 30 to 50. Tumors remained dormant for a period of up to 50 days, at which point they began to regrow in the androgen-free environment. (Adapted with permission from ref. *60*).

dence. Initially, PSA serum levels fall to nearly undetectable levels. Subsequently, as illustrated in Fig. 2, tumor volume drops dramatically during a 50-day period. At approximately day 70 to 90 after castration, PSA serum levels begin to rise, followed by measurable increases in tumor volume at approx 100 days. About 70% of LuCaP 35 xenografts exhibited androgen-independent regrowth. The androgen-independent LuCaP 35 xenografts were transplanted into castrated SCID mice, and this led to establishment of the androgen-independent variant designated LuCaP 35V. LuCaP 35V is maintained in castrated SCID male mice. Interestingly, the levels of androgen receptor are increased in LuCaP 35V in comparison with androgen-sensitive LuCaP 35 *(27,62)*.

As indicated, we have derived several xenografts that are androgen dependent or androgen sensitive (Table 1). The response of several of these xenografts to androgen ablation is shown in Fig. 3. These xenografts show a wide spectrum of responses to castration. Again, this is similar to the range of responses seen in men, where some tumors respond for only a few weeks whereas others respond for years.

Several xenografts derived by us and others from androgen-independent clinical specimens revert to an androgen-dependent/sensitive phenotype when implanted into intact mice. Similarly, LuCaP 35V, when reimplanted into intact mice, reverts to the LuCaP 35 androgen-sensitive phenotype. This implies that the androgen-dependent phenotype is the preferred growth state when androgens are present.

Although space does not permit further discussion here, important studies with CaP xenografts are underway to evaluate mechanisms of response to castration and development of androgen indepen-

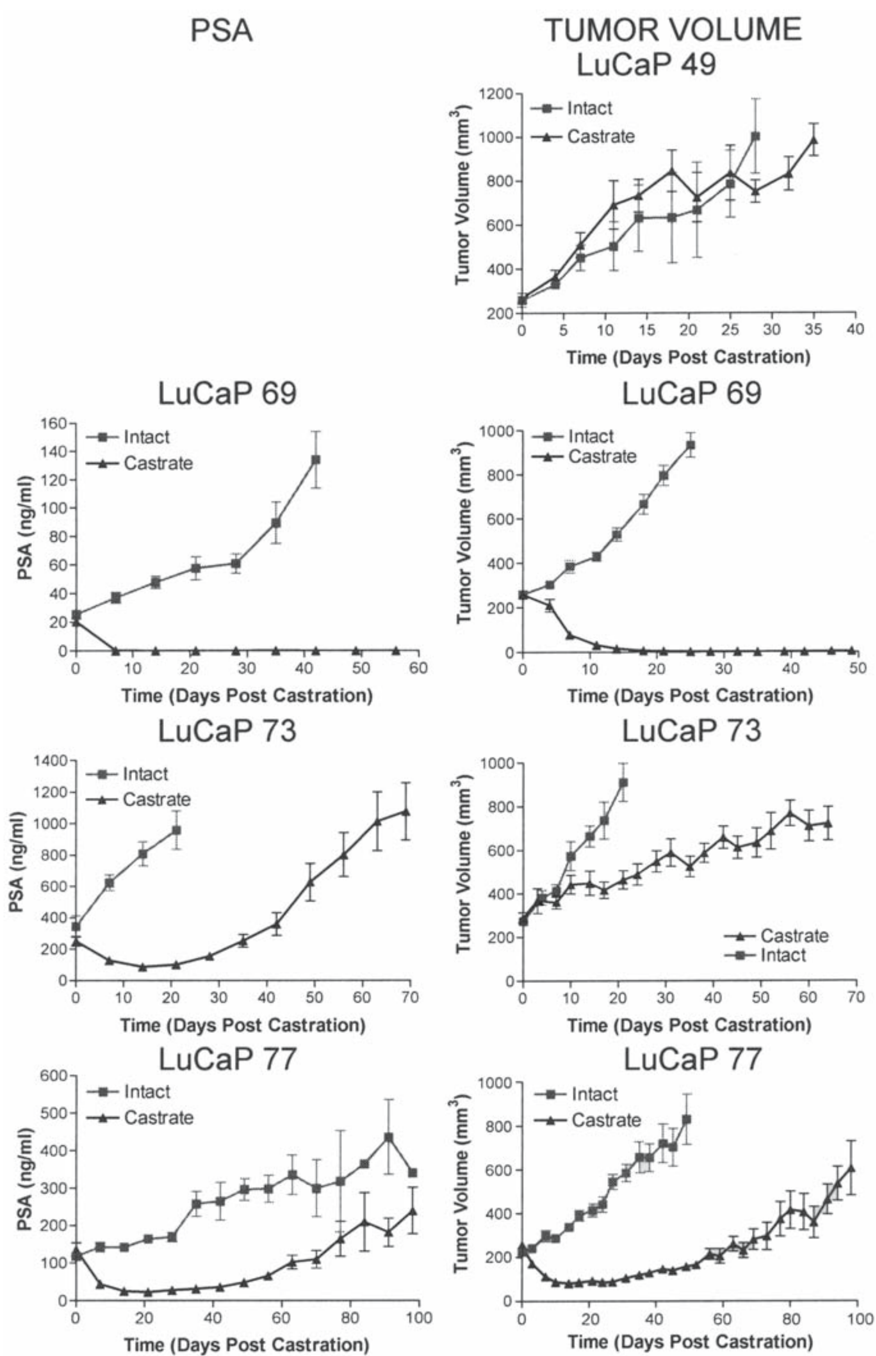


Fig. 3. The response to castration (day 0) of three of our new androgen dependent/sensitive xenografts (LuCaP 69, LuCaP 73, and LuCaP 77) in comparison with LuCaP 49, which is an androgen-independent, neuroendocrine xenograft. Note the differences in tumor volume and prostate-specific antigen (PSA) serum level responses among the three responsive xenografts. LuCaP 69 is the most androgen dependent, with a dramatic drop in tumor volume and PSA serum levels for a period of at least 50 days after castration. LuCaP 73 shows a decrease in tumor growth rate but the tumor volume does not decrease, whereas PSA serum levels drop appreciably for a few weeks before rising again.

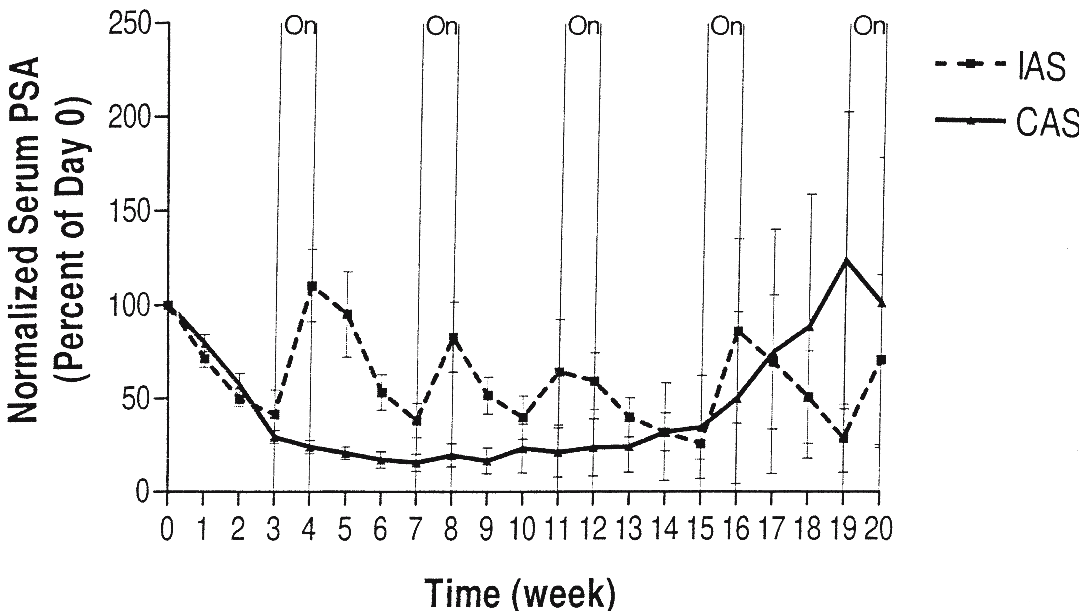


Fig. 4. Normalized mean prostate-specific antigen (PSA) serum levels of intermittent androgen suppression (IAS) vs castrated (CAS) animals (censored data). Normalized mean PSA serum levels are expressed as a percent of serum levels at time of orchiectomy (week 0). "On" androgens signifies when animals are receiving exogenous testosterone via testosterone propionate subcutaneous pellets. (Adapted with permission from ref. *70*).

dence. The availability of multiple models with different characteristics should enable researchers to identify key factors associated with response to castration and development of androgen independence. A number of studies have been published using gene expression array profiling before and at multiple points after castration through the timeframe leading to the androgen-independent state *(63–66)*. Likewise, several of our xenografts are the focus of such studies *(67)*. These studies yield many genes/factors potentially involved in this progression. Further confirmation of altered expression and subsequent experiments to validate the roles of these genes/factors in development of androgen independence are still needed.

Finally, we would like to briefly revisit a study we performed a few years ago that illustrated the concept of androgen intermittent therapy or intermittent androgen suppression (IAS). In the past, androgen ablation was an all-or-none treatment modality imposed either by surgical or by pharmaceutical castration. IAS is a modified approach, promoted initially by Akakura et al. *(50)*, who hypothesized that keeping most of the tumor cells androgen dependent and responsive to androgen ablation would confine overall tumor growth, yield at least equivalent survival, and provide quality-of-life benefits to the patient. This is accomplished by alternating androgen-ablation treatment periods with periods of no treatment, using PSA as a valuable surrogate marker of tumor response *(68,69)*. We modeled this process with our CaP xenograft, LuCaP 23.12 (Fig. 4). Indeed, as hypothesized, Kaplan–Meier log rank analysis of survival showed equivalent survival between IAS and standard castration-induced androgen ablation. Recently, several investigative groups have reported favorable clinical findings using the IAS approach *(71–74)*.

5. METASTASIS

Patients with advanced CaP experience metastases, for which there is currently no curative treatment. The process of tumor metastasis is highly specific and consists of multiple steps. To metasta-

size successfully, the tumor cells must complete all steps of the process: initial transformation and growth, local invasion, survival of immune defense, adhesion to the secondary site, establishment of micrometastases and growth at secondary sites. The mechanisms involved in dissemination of tumor cells and establishment of metastases at secondary sites are not yet understood. Ideally, animal models of metastasis would parallel the human disease, but it has proven to be very difficult to develop models of spontaneous CaP metastasis that mimic human CaP in all aspects. To improve our understanding of CaP metastases and develop new treatment strategies, better animal models of human CaP demonstrating spontaneous metastasis from the orthotopic site are still needed.

The main sites of metastases of CaP are lymph nodes, bone, and lungs. Researchers have used available xenografts (Table 1) to generate lymph node and lung metastases, with varying results, but with very limited success in generating bone metastases. The following section is an overview of various approaches of generating spontaneous non-osseous and osseous metastases and experimental metastases with the most common CaP cells lines and the CaP xenografts developed in our laboratory.

5.1. Non-Osseous Metastasis Models

Standard procedures of growing xenografts have been used in attempts to generate spontaneous CaP metastases, and it has been found that, generally, metastasis from subcutaneous human CaP xenografts occurs very infrequently *(75)*. Implantation of CaP cells into the prostate (orthotopic site) in mice has been used with more success, and has resulted in lymph node and lung metastases.

5.1.1. LNCaP Xenografts

Sato et al. *(76)* reported on generation of LNCaP lymph node metastases in 100% of animals and microscopic lung metastases in 40% of animals, using orthotopic injection of LNCaP cells. Metastases from LNCaP orthotopic tumors were also reported by Rembrink et al. *(77)* and Stephenson et al. *(78)*. Pettaway et al. *(79)* also used orthotopic implantation of LNCaP cells in athymic mice to develop LNCaP sublines with increased metastatic potential; lymph node metastases were harvested and cells were re-injected into the prostate. This process was repeated three to five times. After implantation into the prostate, LNCaP-LN3 cells produced a higher incidence of regional lymph node metastases than LNCaP-Pro5 or LNCaP cells.

Recently, Scatena et al. *(80)* reported on generation of metastases from LNCaP-luc-M6 orthotopic and subcutaneous tumors using SCID-beige mice. In this study, sensitive luminescence imaging was used to detect the metastases. Primary tumors were shielded for this purpose. Lung metastases were detected as early as 7 weeks in 2 of 10 animals, and, at sacrifice, 10 of 10 of animals had lung metastases and 7 of 10 animals had rib metastases. Histology confirmed lung metastases but not rib metastases.

A different approach to generating metastases of LNCaP cells was used by Wang et al. *(81)*. In this study, pieces of LNCaP xenografts grown subcutaneously were used for orthotopic implantation into the ventral lateral lobes of the prostate. The authors used this approach to minimize the possibility that lymph node metastases would be generated from leakage of the cells during injection rather than by a true metastatic process. With this approach, 61% of the animals had lymph node metastases and 44% had lung metastases.

5.1.2. PC-3 Xenografts

PC-3 CaP cells have been used extensively to generate metastases and develop cells with increased metastatic potential. Injection of PC-3 *(79,82)* via the tail vein has led to the establishment of lymph node metastases. Generation of lymph node metastases from PC-3 orthotopic tumors was also reported by Stephenson et al. *(78)* and Rembrink et al. *(77)*. Rubio et al. *(83,84)* used intramuscular injection of PC-3 cells, resulting in metastases in animals. Pettaway et al. *(79)* used PC-3 cells in an attempt to generate sublines of these cells that were more metastatic, parallel to their work with

LNCaP. PC-3M cells were injected orthotopically into athymic mice, lymph node metastases were harvested, and cells were re-injected into the prostate. These experiments yielded PC-3M-Pro4 and PC-3M-LN4. PC-3M-LN4, harvested from a lymph node metastasis, produced more lymph node and bone metastases then cells harvested from prostate PC-3M-Pro4 or parental PC-3M cells.

An et al. *(85)* developed a microsurgical procedure of orthotopic implantation of histologically intact pieces of tumor in SCID mice, to generate reproducible and reliable models of spontaneous metastases of CaP in animals. Intact tissue of the human CaP cell line, PC-3, harvested from a subcutaneous tumor in an athymic mouse, was implanted into the ventral lateral lobes of the prostate gland. A high frequency of lymph node and lung metastasis was noted on histological examination. This was the first report in the literature of the generation of widespread lung metastases after orthotopic implantation of PC-3 cells. In contrast to orthotopic injection of cell suspensions, no multiple metastatic cell selection was necessary to increase the metastatic potential of PC-3. The authors speculated that the stromal tissue architecture maintained in the implanted tumors was important for metastatic potential.

Technical progress has permitted new methodologies to be developed to facilitate detection and quantitative measurements of metastatic spread. This issue is of special importance because the majority of spontaneous metastases of CaP are microscopic. Yang et al. *(86)* used bits of green fluorescent protein-labeled PC-3 tumors that had been implanted orthotopically in nude mice. Metastases were detected in various organs, including liver, lung, kidney, pleural membrane, and adrenal gland. Interestingly, this approach resulted in skeletal micrometastases as well. Luciferase-labeled PC-3 cells grown intramuscularly were also used to monitor trafficking of these cells to lymph nodes *(83)*.

5.1.3. LuCaP 23.8 and LuCaP 35

We have described another model of generating CaP metastases, using the LuCaP 23.8 *(48)* and LuCaP 35 *(27)* CaP xenografts *(60)*. In our efforts to characterize the metastatic potential of xenografts established in our laboratory, we observed small but visible metastases in pelvic lymph nodes of nearly half of the animals harboring various CaP xenografts, along with microscopic lung metastases. We wondered whether the lack of overt metastases in other organs was caused by poor dissemination, poor growth at metastatic sites, or an inadequate period for growth before sacrifice. We addressed this issue by removal of orthotopic tumors after they reached moderate size (250–500 mm^3) to allow time for metastases to grow. The procedure is illustrated in Fig. 5. When we used this procedure, 71% of the LuCaP 23.8 animals and 100% of the LuCaP 35 animals had lymph node metastases. Lymph node metastases were macroscopic, and immunohistochemistry confirmed the prostatic origin of metastases. Seventy-one percent of the LuCaP 23.8 animals and 90% of the LuCaP 35 animals had lung metastases.

In summary, LNCaP, PC-3, and other prostate cells can be used to generate non-osseous metastases, including metastases in lymph nodes and lungs. The challenge of using these models to study mechanisms of metastasis or test new treatment modalities is still in the detection of metastases, which is labor intensive and time consuming. Using new imaging technologies with fluorescent or bioluminescent labeling will help to make evaluation of metastatic spread and effectiveness of new therapeutics more efficient.

5.2. Osseous Metastasis Model

Bone is a very common site of CaP metastasis *(28,87,88)*, and bone metastases are responsible for most of the morbidity associated with the advanced disease. In contrast with bone metastases of breast cancer and myeloma, which are mainly osteolytic, a high percentage of CaP metastases exhibit the radiographic appearance of osteoblastic lesions *(89)*. Histomorphometric studies of CaP bone metastases have shown that some of the sclerotic lesions are actually mixed in nature, with increased activities of both osteoblasts and osteoclasts *(90,91)*.

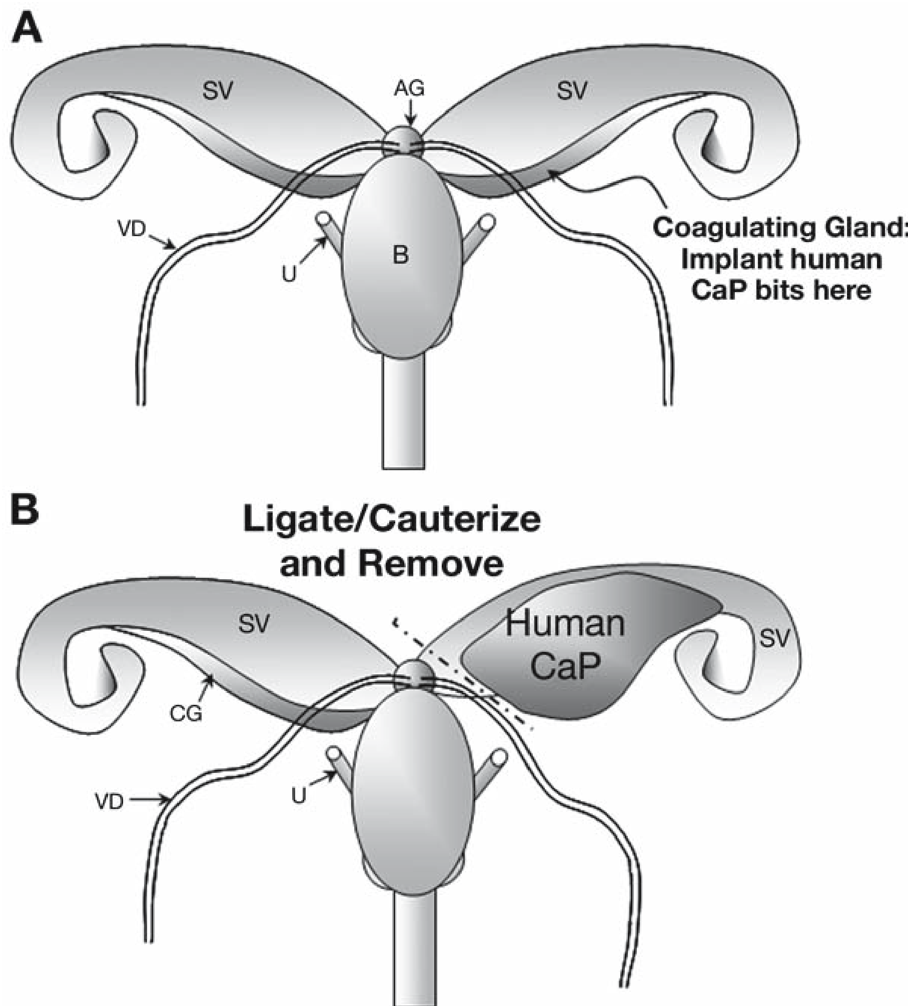


Fig. 5. Scheme of implantation and removal of orthotopic prostate cancer (CaP) tumors. Tumor bits of CaP xenografts were implanted into the coagulating gland (**A**). After tumors reached a size of 250 to 500 mm^3, the animals underwent a second surgery. Tumor and associated seminal vesicle were ligated and removed (**B**). SV, seminal vesicle; VD, vas deferens; U, ureter; B, bladder; AG, ampullary gland; CG, coagulating gland. (Adapted with permission from ref. *60*).

To represent a good model of the human disease, animal models of CaP bone metastasis should exhibit two major characteristics: metastasis to bone, and osteoblastic response in the bone. However, unlike the human disease, human CaP xenograft models rarely metastasize spontaneously to bone from the orthotopic site of primary tumor growth, and, in the few instances in which this has been noted, they did not yield osteoblastic lesions. Although much effort has been spent on the establishment of new CaP xenografts that spontaneously metastasize to bone yielding an osteoblastic reaction, none exists that is reasonably reproducible and at a sufficient frequency to be experimentally useful. Therefore, to study the biology of CaP bone metastases and test new treatment modalities, experimentally induced metastatic models have been used as alternatives. The most commonly used alternatives are intravenous or intracardiac injection of tumor cells, and direct injection of cells into bone.

5.2.1. Intravenous and Cardiac Injection

Shevrin et al. *(82)* used an injection of the CaP cell line PC-3 into the tail veins of athymic mice while the inferior vena cava was occluded. This technique diverted cells into the vertebral venous plexus. Bone lesions developed in 3 of 16 experimental mice. Two tumor sublines were established from explant cultures of bone lesions, and re-injection of these cells into the tail vein resulted in bone metastasis in 19 of 36 animals. The main sites of bone metastasis were lumbar vertebrae, pelvis, and femurs. Wang and Stearns *(92)* selected highly invasive PC-3 sublines based on enhanced capacities to migrate across Matrigel in vitro, and, when these cells were injected intravenously in the tail vein of SCID mice, they metastasized to a wide variety of tissues. Four distinct sublines were isolated that metastasized preferentially to various skeletal sites in approx 80% of animals. Angelucci et al. *(93)* used PC-3 cells that were injected into the left cardiac ventricle of athymic mice, and followed metastatic spread and growth by luminescence. Sixty-four percent of animals developed osteolytic bone metastases. In this study, Angelucci et al. *(93)* isolated PC-3 cells from bone, named PCb2, that exhibited a more invasive phenotype, resulting in higher numbers of bone metastases.

The LNCaP cell line has also been used for studies of interactions between CaP and bone cells *(94)*, although these cells do not metastasize spontaneously to the bone or colonize bone. Thalmann et al. *(30)* developed sublines of LNCaP, of which, one, C4-2, was able to metastasize to bone, although at low frequency (2 of 20). C4-2 sublines, designated B2, B3, B4, and B5, have a higher propensity to metastasize to bone and cause osteoblastic lesions, but, again, the rate of bone metastases was rather low *(31,32,95)*.

In summary, intravenous and cardiac injections can be used to generate experimental bone metastases of CaP. However, these models have critical limitations:

1. PC-3 and its sublines result in osteolytic bone lesions, and do not produce PSA, a marker of prostate epithelial cells, whereas human CaP bone metastases exhibit mainly osteoblastic characteristics.
2. The C4-2b human CaP models *(30–32)* result in bone metastases after injection of the cancer cells, but only after a long delay, and the frequency of these metastases is still too low for preclinical studies.

5.2.2. Direct Bone Injections

Direct injection of tumor cells into the bone marrow has been developed as an alternative to spontaneous metastases for the study of interactions between CaP and bone cells *(24,96–102)*.

5.2.3. SCID-hu Model

The possibility exists that the low frequency of bone metastasis in animal models is caused by incompatibilities between tumor and host environment. Nemeth et al. *(24)* used human fetal bone grafted subcutaneously into SCID mice as a substrate for the growth of human CaP cells PC-3, DU 145, and LNCaP. After 4 weeks of bone growth, tumor cells were injected intravenously or directly into the implanted bone tissues. Only intravenously injected PC-3 cells readily colonized human bone in 5 of 19 animals (Fig. 6) whereas no metastases were observed in implanted human lung or intestinal tissues or mouse bone, demonstrating tissue specificity of the process. Direct injection of tumor cells into the bone implants resulted in tumors of DU 145, PC-3, and LNCaP in 75 to 100% of animals. PC-3 and DU 145 lesions were primarily osteolytic, whereas LNCaP lesions were both osteoblastic and osteolytic (Fig. 7). Davies et al. *(46)* used LAPC-4 CaP cells in the SCID-hu model to establish cells that were more aggressive with stronger metastatic character. LAPC-4 cells were injected near to bone, and LAPC-4 (squared) was selected after growth in the bone environment. These cells form tumors, develop androgen independence, and metastasize to the human bone implants after orthotopic implantation.

5.2.4. Human Adult-Bone Model

The SCID-hu model adapted by Cher's group used human fetal bone as a substrate for human CaP bone metastases. Because CaP affects mainly older men, and bone remodeling is different in fetal and

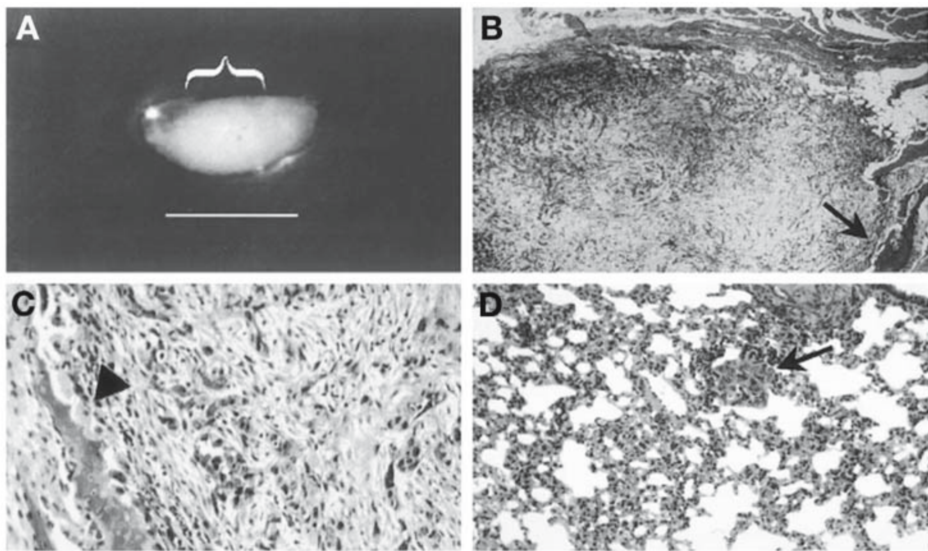


Fig. 6. Circulating cell colonization assay. Representative tumor formed in SCID-hu mouse after intravenous injection of PC-3 cells. (**A**) Gross appearance of tumor in human bone fragment. Tumor appeared as a smooth mass (*bracket*) extending from residual normal bone tissue. Bar = 1 cm. (**B**) Low-power view of tumor mass, showing residual bone tissue at the periphery (*arrow*), original magnification ↔20. (**C**) Close examination revealed cohesive nests of tumor cells in a desmoplastic stromal response. Evidence of osteolytic activity could also be seen (*arrowhead*), original magnification ↔100. (**D**) representative colony of PC3 tumor cells in mouse lung, consisting of a small number of cells (*arrow*), original magnification ↔100. (Adapted with permission from ref. *24*).

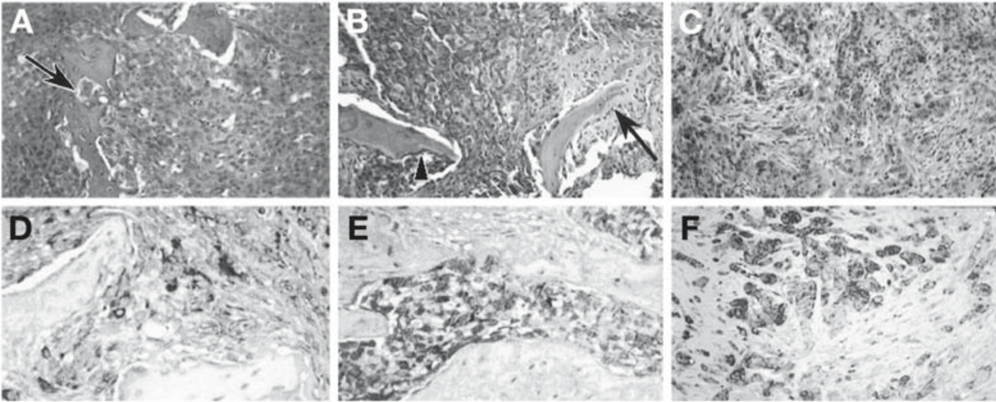


Fig. 7. End-organ growth assay. Representative photomicrographs demonstrating histological features of tumors formed in human bone tissue 6 weeks after the direct injection of human prostate cancer cell lines. (**A**) DU 145 cells formed solid tumors with evidence of osteolytic activity (*arrow*), original magnification ×100. A mild desmoplastic stromal response was visible in some areas of bone tumors and was revealed by red staining of tumor cells with a pancytokeratin antibody (**D**), original magnification ×200. (**B**) Bone tumors formed by LNCaP also showed a desmoplastic stromal response, and evidence of both osteoblastic (*arrow*) and osteolytic activity (*arrowhead*) could be observed by routine staining, original magnification ×100. (**E**) The intermingling of red-stained LNCaP cells and stromal cells could be better seen after cytokeratin immunostaining, original magnification ×200. (**C**) PC3 cells formed solid osteolytic tumors, and bone tissue was often completely destroyed; original magnification ×100. A strong desmoplastic stromal response was visible in these tumors, revealed both by routine H&E staining (C) and by red staining for cytokeratin (**F**). Original magnification ×200. (Adapted with permission from ref. *24*).

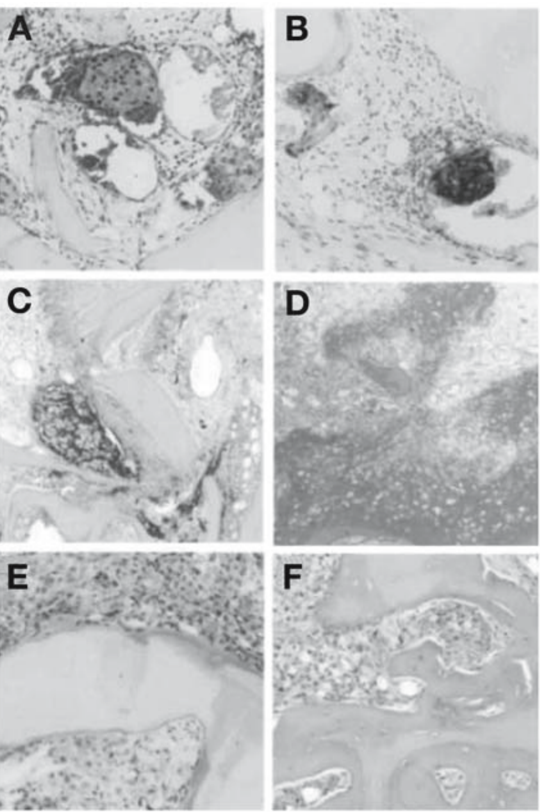


Fig. 8. Histological appearance of LNCaP tumors growing in adult human bone fragments implanted into NOD/SCID mice. Specimens obtained at 2 weeks (**A** and **B**), 4 weeks (**C**), 6 weeks (**D** and **E**), and 8 weeks (**F**) after LNCaP cell injection. (A) Metastatic foci were formed in bone marrow sinuses at 2 weeks after the inoculation of prostate cancer cells. Little initial response of bone marrow stromal cells was detected around the tumors (H&E, original magnification ×100). (B) Prostate-specific antigen (PSA)-positive tumor foci were often found in blood vessels (PSA stain, original magnification ×100). (C) Localized LNCaP tumors were observed in bone fragments at 4 weeks after injection of LNCaP cells. An initial mild response of bone marrow stromal cells was observed around tumor foci (H&E, original magnification ×40). (D) Intertrabecular bone metaplasia. Active bone formation by metaplasia of bone marrow stromal cells adjacent to tumor foci at 6 weeks after injection of LNCaP cells (H&E, original magnification ×100). (E) Appositional bone formation. A minute focus of appositional new bone formation is seen on a trabecula of lamellar bone. The osteoid matrix shows intense eosinophilic staining (Yoshiki stain, original magnification ×100). (F) Reconstructive osteosclerosis in prostate cancer. The intramedullary space has been almost entirely replaced by tumor cells. Although this is a case of osteoblastic metastasis, an irregular cement line suggesting previous bone resorption can be observed (H&E, original magnification ×100). (Adapted with permission from ref. *99*).

adult bone, Yonou et al. *(99)* and Tsingotjidou et al. *(97)* used adult bone as an environment for establishment of CaP bone metastases in immune-compromised mice. Yonou et al. *(98,99)* used non-obese diabetic/severe combined immunodeficient mice, adult human bone, and tail-vein injection of CaP cells. Three to 4 weeks after human bone implantation, PC-3 cells or LNCaP cells were injected. Sixty-five percent and 35% of animals, respectively, developed tumors in the grafted bones. The LNCaP cells colonized the bone marrow cavity, often exhibiting an osteoblastic response at the edges of metastatic foci, with new bone formation adjacent to mature lamellar bone (Fig. 8). Similar to the

findings of Nemeth et al. *(24)*, there was no colonization of human lung tissue implants. Tsingotjidou et al. *(97)* used human adult bone harvested from patients undergoing total joint arthroplasty, which was then implanted intramuscularly in the hind limbs of irradiated SCID mice. Tumor cells PC-3 and LAPC-4 were injected next to the bone 2 months after bone implantation. Both tumor types showed growth in this environment, with no detectable new bone formation, but significant osteolysis.

5.2.5. Intratibial Injection

Another approach for the generation of experimental CaP bone metastases consists of direct injections of CaP tumor cells into mouse bone *(96,100–102)*. This technique was originally developed by Berlin et al. *(103)* in generating spontaneous metastases of osteosarcoma to lungs. We *(96,102)* have used CaP cells PC-3, LNCaP, and C4-2, as well as cells isolated from CaP xenografts LuCaP 23.1 and LuCaP 35, established in our laboratory, to characterize CaP/osseous models derived from the direct injection of tumor cells into tibiae of 4- to 6-week-old SCID mice. We have had considerable success with the intratibial injections. The general protocol, adapted from Berlin et al. *(103)*, is to inject $1–2 \times 10^5$ CaP cells with a 26-gauge needle approx 3 mm into the proximal end of the tibia of 4-week-old SCID male mice. LuCaP 23.1, LNCaP, LuCaP 35, C4-2, and PC-3 cells injected into the tibia all exhibited tumor growth in the bone. PC-3 cells caused an osteolytic response, LNCaP and LuCaP 35 yielded mixed lesions, and LuCaP 23.1 yielded an osteoblastic response (Fig. 9), new bone development was evident throughout the lesions; tumor cells replaced all bone marrow, and extensive new bone formation was observed. We also examined the nature of the interactions of LuCaP 23.1 with bone at early, mid-phase, and late time-points of its growth in the bone environment. Figure 10 shows by radiography representative radiographs of early, mid-phase, and late tumor development. Histology of tibiae with prostate tumor cells was compared with samples of human CaP bone metastases. The LuCaP 23.1 and PC-3 tumored bones, respectively, showed many similarities to human samples of osteblastic and osteolytic bone metastasis of protate cancer (Fig. 11). We also used immunohistochemistry to evaluate the validity of these xenografts as models of human CaP bone metastasis. As in human specimens, we detected osteoprotegerin (OPG), receptor activator of nuclear factor kappa B, (RANKL), parathyroid hormone like protein (PTHrP), and endothelin-1 (ET-1) immunoreactivity in all CaP xenografts growing in the bone.

The C4-2 osseous model and its response to castration were also characterized *(102)*. C4-2 tumors had a 100% take rate in bone and caused radiographically lytic expansile lesions. C4-2 cells decreased bone mineral density and bone volume of the injected tibiae vs normal tibiae. Castration caused a drop in serum PSA with a nadir at day 14, after which it began to rise again. Bone destruction in the tumorous tibiae of castrated animals was decreased by 15.9% vs tumorous tibiae of intact animals.

Simultaneously, Fisher et al. *(101)* published characterization of intratibial injections of PC-3, DU 145 and LNCaP CaP cells into athymic mice. In this report, PC-3 and DU 145 cells had a mixed sclerotic/lytic appearance and caused increases in the number of osteoclasts, whereas LNCaP did not form tumors in the bone environment.

Lee et al. *(100)* used LAPC9 and PC-3 in the tibial injection model. PC-3 caused osteolytic lesions with a large number of osteoclasts. In contrast, LAPC-9 caused osteoblastic lesions at 6 weeks, with osteoclasts detected only rarely. Greater osteoclast activity was detected at 8 weeks when the osteoblastic lesions were well-established (Fig. 12). Immunohistochemical analysis showed that PC-3 produced RANKL, interleukin (IL)-1, and tumor necrosis factor-α, which are associated with osteoclastogenesis. LAPC-9 cells produced no RANKL or IL-1, and minimal amounts of tumor necrosis factor-α, but large quantities of bone morphogenetic protein (BMP) 2, BMP 4, BMP 6, and IL-16, which are associated with bone formation.

Andresen et al. *(104)* injected CWR22 cells into tibiae of athymic rats. Both osteoblastic and osteolytic reactions were detected after 4 to 6 weeks, with the osteosclerotic reaction predominant. Radiological and histological evidence revealed osteosclerotic lesions with trabeculae of newly formed bone lined by active osteoblasts and surrounded by tumor cells. Near the end of the 7-week study, osteolytic bone lesions became more evident on X-rays.

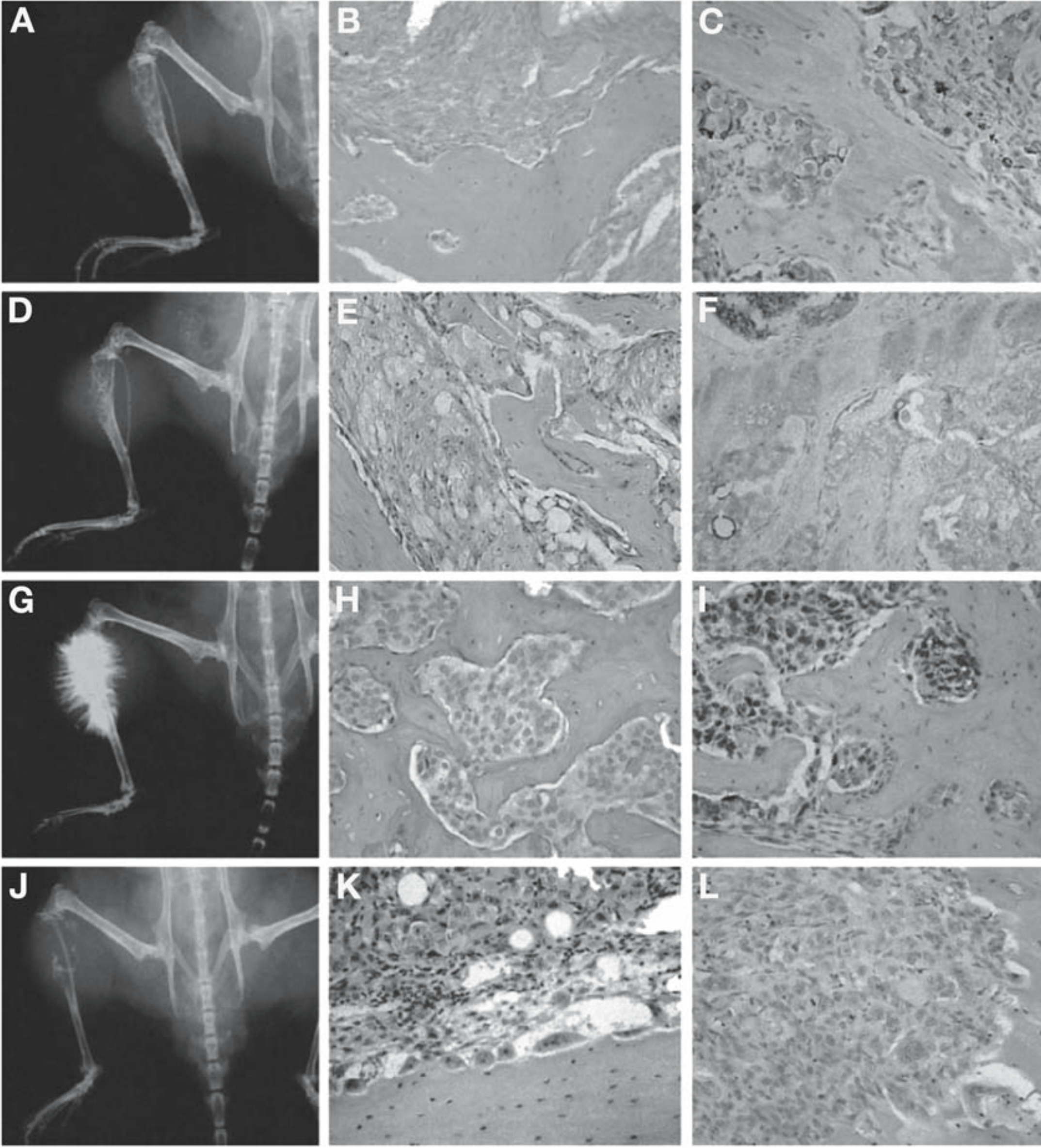


Fig. 9. Characteristics of osseous prostate cancer (CaP) models. (**A**) Radiograph of LNCaP intratibial tumor showing osteolytic expansile lesion with soft-tissue abnormalities. (**B**) H&E of LNCaP intratibial tumor, demonstrating mixed lesion (eroded surface and thickened trabeculae). (**C**) Anti-prostate-specific antigen (PSA) stain of LNCaP in the tibia. (**D**) Radiograph of LuCaP 35 in the tibia showing osteolytic expansile lesion with soft-tissue abnormalities. (**E**) H&E of LuCaP 35 in the tibia demonstrating mixed lesion (eroded surface and thicker trabeculae). (**F**) Anti-PSA stain of LuCaP 35 in the tibia. (**G**) Radiograph of LuCaP 23.1 in the tibia showing osteoblastic growth. (**H**) H&E of LuCaP 23.1 in the tibia. (**I**) Anti-PSA stain of LuCaP 23.1 in the tibia. (**J**) Radiograph of PC-3 in the tibia showing osteolytic lesion with a large reduction in bone mass. (**K**) H&E of PC-3 in the tibia with an increase in osteoclasts lining the bone. (**L**) Anti-PSA IP stain of PC-3 in the tibia showing a lack of reactivity. (Adapted with permission from ref. *96*).

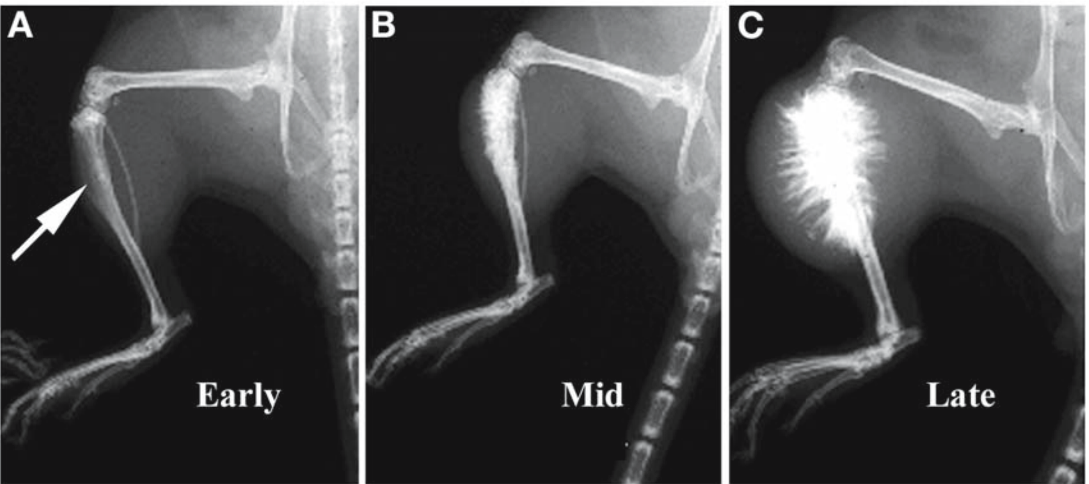


Fig. 10. Progression of LuCaP 23.1 lesions in the tibia. Representative radiographs of (**A**) LuCaP 23.1 in the early phase; (**B**) in the mid phase; and (**C**) in the late stage. (Adapted with permission from ref. *96*).

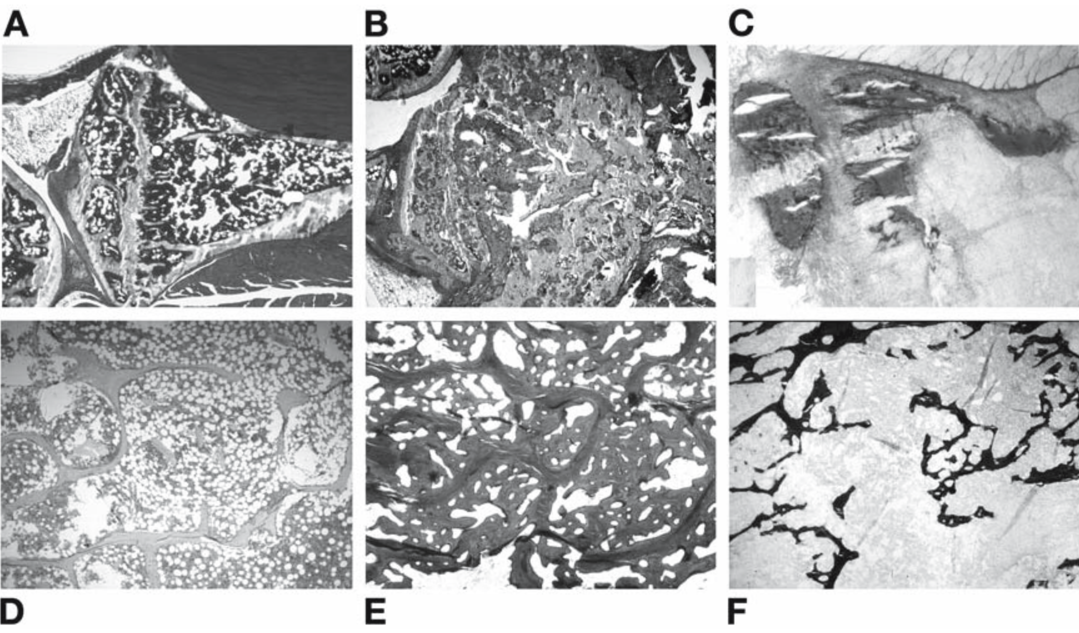


Fig. 11. Goldner's staining of normal tibia, LuCaP 23.1 and PC-3 cells injected into tibia. (**A**) Normal mouse tibia; (**B**) LuCaP 23.1 cells injected into tibia; and (**C**) PC-3 cells injected into tibia; Goldner's staining; original magnification ×6. (**D–F**) Examples of human bone samples of similar character; original magnification ×6. (D) Normal human bone; bony trabeculae and bone marrow are present. (E) Human CaP osteoblastic lesion, with significant increase in mineralized bone (green area), replacement of bone marrow by tumor cells (grey areas). (F) Human CaP osteolytic lesion; most of the bony trabeculae have been lysed and the spaces filled with tumor cells. Green areas are remaining mineralized bone; tumor cells are grey. (Adapted with permission from ref. *96*).

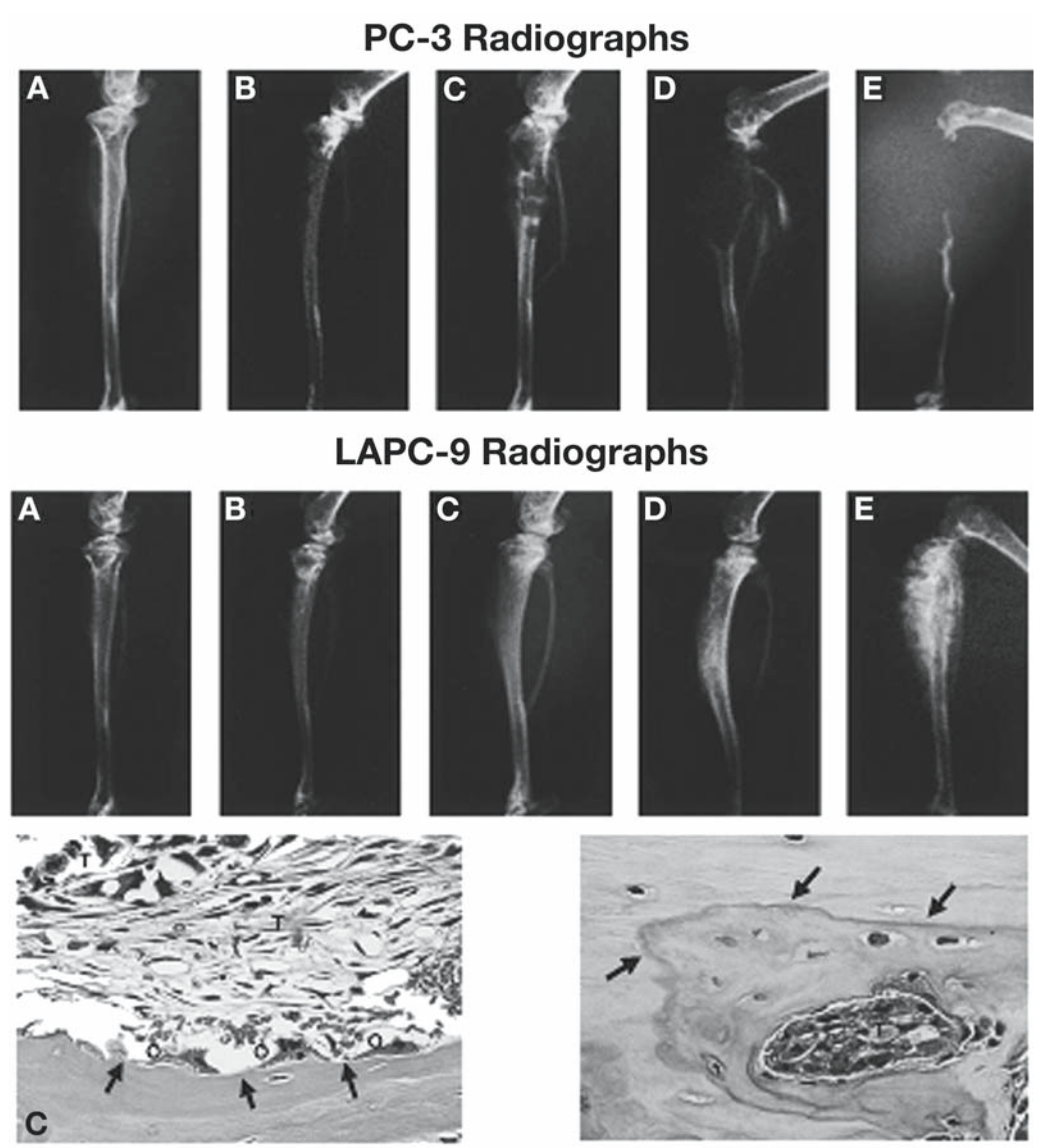


Fig. 12. PC-3 and LAPC-9 in tibiae. (**A**) 1, (**B**) 2, (**C**) 4, (**D**) 6, and (**E**) 8 weeks after injection. Top row: radiographs of PC-3 injected tibias. Osteolytic lesions notable at 2 weeks, with complete destruction of the proximal tibia at 8 weeks. Middle row: radiographs of LAPC-9 injected tibias. Osteoblastic lesions notable at 6 and 8 weeks. Bottom row: Left: H&E of PC-3 bone interphase with osteoclast. Right: H&E of LAPC-9 in bone. (Adapted with permission from ref. *100*).

A similar experimental approach for the study of bone metastasis is injection of tumor cells into the femurs of SCID mice, as used by Navone et al. *(35)* and Pinthus et al. *(105)*. Navone et al. used MDA PCa 2b established from bone metastases, and these cells resulted in an osteoblastic reaction. Pinthus et al. reported that WISH-PC2 produced osteolytic lesions with foci of osteoblastic activity when the cells were injected directly into the femur.

In summary, because of the paucity of models of spontaneous CaP bone metastases, new models have been developed to study interactions of CaP cells with the bone environment, using either human bone or mouse bone and various CaP cell lines. Informative morphological and biochemical findings corresponding to CaP growth in human bone have been observed using all of these models. However, because the bone metastases are experimentally induced by direct bone injection, these models cannot be used effectively for studies of early metastatic events, such as migration, and so on. This is an important limitation on the use of these models for the study of trafficking and migration of CaP, but the results reported herein suggest that this has little impact on the value of the models for investigation of the interactions between CaP and bone and testing of new therapeutic modalities. Another limitation of the existing models is that CaP cells commonly used for these studies result in modest new bone formation (C4-2, C4-2B, CWR22, and MDAPCa2b) or in osteolytic reactions (PC-3). There are only two xenografts, LuCaP 23.1 and LAPC-9, that produce osteoblastic reactions in the bone resembling those in human specimens. Unfortunately, neither of these xenografts grows in vitro, limiting the studies of characterization of phenotypic alterations and effects on signaling pathways.

5.3. Preclinical Use of Experimental Bone Metastasis Models

All of the above-described models of generating experimental bone metastases of CaP have been used to evaluate the effects of new therapeutics and to increase our understanding of the interactions of prostate tumor cells and the bone environment. What follows is a summary of studies from various groups showing the significant usefulness of these models in preclinical testing.

5.3.1. SCID-hu Model

There are multiple reports of use of the SCID-hu model. The first reported use of this model to evaluate new treatment modalities was published by Nemeth et al. *(106)*. The authors showed that matrix metalloproteinase activity was associated with PC-3 cell proliferation in bone, and that daily treatment with batimastat, a broad-spectrum matrix metalloproteinase inhibitor, hampers osteolysis associated with the growth of these cells in the bone environment, as well as tumor growth. Cher et al. *(107)* used this model to show that expression of maspin, when overexpressed in DU 145 cells, caused inhibition of osteolysis, tumor growth, and angiogenesis. The model was also used to evaluate the effects of genistein on PC-3 bone metastases *(108)*; the results showed that genistein inhibits growth of PC-3 cells in the bone environment. Nemeth et al. *(109)* also showed that inhibition of $\alpha_{(v)}$ β-$_3$ integrin reduced PC-3 cell proliferation in the SCID-hu model. Zhang et al. *(110)* blocked RANKL activity with soluble murine RANK-Fc (sRANK-Fc) to prevent progression of CaP cell growth in the bone environment. LuCaP 35 CaP cells, grown in the fetal bone and treated with sRANK-Fc, resulted in fewer osteoblastic lesions, lower bone mineral density, decreased serum PSA levels, and diminished tumor volume. In contrast, sRANK-Fc had no effect on subcutaneously implanted LuCaP 35 cells. The authors concluded that sRANK-Fc is an effective inhibitor of RANKL that diminishes progression of CaP growth in bone through inhibition of bone remodeling.

5.3.2. Adult Bone Model

Models using adult human bone were used by Goya et al. *(111)* and Yonou et al. *(112)*. Goya et al. *(111)* investigated whether a novel antibody directed against human IGF-I and IGF-II (KM1468) inhibits the development and progression of LNCaP bone tumors. KM1468 markedly and dose-dependently suppressed the development of new bone tumors and the progression of established tumor foci, and it also decreased serum PSA levels. These results indicate that the IGF signaling axis is a potential target for prevention and treatment of bone metastases arising from CaP. Yonou et al. *(112)* evaluated whether blockade of osteoclastogenesis by OPG inhibits the development of new bone tumors and the progression of established osteoblastic bone tumors. In this study, OPG reduced the number of osteoclasts and the size of the tumors at the bone sites, but it had no effect on the local

growth of subcutaneous LNCaP tumors. These findings demonstrate that osteoclasts play an important role in bone tumors of CaP, and that OPG decreases the LNCaP CaP burden selectively in bone and prevents the development of new lesions. This suggests that inhibition of osteoclastic bone resorption may be an effective therapy for the treatment of CaP that has colonized bone. Similar findings using intratibial injections have been published by Zhang et al. *(113)* and Kiefer et al. *(114)* (*see* Section 5.3.3).

5.3.3. Direct Injection Into Mouse Bone

Our efforts to treat cancer have focused on compounds that affect growth and apoptosis of tumor cells. Compounds that modulate the host organ microenvironment may provide additional benefits in cancer treatment. Because literature data suggest that increased osteolysis is a key component of CaP bone metastasis, there has been interest in whether inhibitors of osteolysis could slow the growth of these lesions. Zhang et al. *(113)* and Kiefer et al. *(114)* evaluated the effects of OPG, an inhibitor of osteoclastogenesis, on growth of prostate tumor cells in mouse tibiae. Zhang et al. *(113)* used C4-2 cells that cause a moderate osteoblastic reaction, and, in our study, we used our highly osteoblastic xenograft, LuCaP 23.1. Both studies showed that OPG administration inhibited CaP-induced osteoclastogenesis and tumor growth. OPG may, therefore, be beneficial to patients with advanced CaP bone metastases. Using the intratibial model with C4-2 cells overexpressing OPG, we have also shown that OPG may be at least partially responsible for the osteoblastic character of most CaP bone lesions *(115)*.

Bisphosphonates are potent inhibitors of osteolysis, and a new-generation bisphosphonate, zoledronic acid, has been tested as a potential treatment for osteolytic and osteoblastic CaP tumors *(116,117)*. Zoledronic acid inhibits osteoclastogenesis and, therefore, modulates the bone environment, and it may also have direct antitumor effects. Lee et al. *(116)* used osteoblastic LAPC-9 and osteolytic PC-3 cells injected into the tibiae to evaluate the effects of zoledronic acid. Zoledronic acid decreased the number of osteoclasts in PC-3 experimental metastases in bone, but it was not effective in halting blastic lesions of LAPC-9. LAPC-9 lesions were formed even when the number of osteoclasts was significantly reduced. In our study, Corey et al. *(116)*, we used osteoblastic LuCaP 23.1 and osteolytic PC-3 cells in the intratibia model. Growth of osteoblastic and osteolytic lesions was significantly inhibited by administration of zoledronic acid. Both of these studies suggest that the anti-osteolytic activity and the antitumor effects of zoledronic acid could benefit CaP patients with bone metastases. Also, Burton et al. *(118)* used green fluorescent protein imaging to monitor skeletal progression of PC-3 cells injected into the tibia. Subsequently, these authors used this model to evaluate the effects of the bisphosphonate pamidronate on the growth of osteolytic PC-3 bone lesions. As with zoledronic acid, they observed that pamidronate inhibited growth of PC-3 in the bone environment.

Fidler's group has also used the tibial injection model to study the effects of potential new therapeutics on CaP bone metastases *(119–122)*. This group blocked the platelet-derived growth factor receptor (PDGF-R) and the epidermal growth factor receptor (EGF-R) alone or in combination with paclitaxel. PC3-MM2, a metastatic subline of PC-3 cells with higher propensity for bone, was used in these studies. Uehara et al. *(119)* blocked PDGF signaling by the tyrosine kinase inhibitor, STI571, with or without paclitaxel. They observed reductions in tumor incidence and size in animals treated with STI571, and more pronounced inhibition of tumor growth in animals treated with the combination of STI571 plus paclitaxel. Kim et al. *(120)* examined the effects of EGF-R signaling blockade by PKI166, an EGF-R tyrosine kinase inhibitor, alone or in combination with paclitaxel. Administration of PKI166 or the combination reduced the incidence and size of bone tumors and destruction of bone. In subsequent studies, Kim et al. *(121)* used this model to evaluate the combination treatment with CaP bone metastases. Combination therapy using PKI166, STI571, and paclitaxel induced a high level of apoptosis in tumor cells and vascular endothelial cells within the tumor, along with inhibition of tumor growth in bone. In aggregate, these data demonstrate that blockade of EGF-R and PDGF-R

phosphorylation significantly suppresses experimental human CaP bone metastasis, and, when combined with paclitaxel, the therapy is even more effective, indicating significant potential of these treatments against CaP bone metastasis. Finally, because of the heterogeneity of CaP metastasis and the necessity of attacking different pathways to eliminate the advanced disease, Kim et al. *(122)* set out to determine whether systemic administration of zoledronic acid could prevent bone lysis and inhibit progression of PC3-MM2 cells growth in the bone. Zoledronic acid inhibited bone lysis, but did not inhibit the growth of PC-3MM2 cells. Systemic administration of zoledronic acid together with STI571 and paclitaxel yielded significant inhibition of bone lysis and decreases in tumor incidence and tumor growth. These results indicate the potential benefits of a combination of a bisphosphonate with a protein tyrosine kinase inhibitor and a cell-cycle blocking drug for the treatment of CaP bone metastasis.

Gamradt et al. *(123)* used the tibial model to determine whether COX-2 plays a role in the bone formation observed in osteoblastic CaP metastases, using cell lines that produce either osteoblastic lesions (LAPC-9) or osteolytic lesions (PC-3, negative control). Administration of SC-58236, a COX-2-specific inhibitor, significantly reduced the size of osteoblastic lesions after LAPC-9 injection. In contrast, large osteolytic lesions were seen in both control and SC-58236-treated animals after PC-3 cell injections. These findings suggest that progression of osteoblastic metastases induced by injection of human CaP cells may be reduced or delayed by COX-2 inhibitors.

Fizazzi et al. *(124)* directly injected osteoblastic MDA PCa 2b and osteolytic PC-3 CaP cells into the femurs of mice to assess the activity of docetaxel in combination with hormonal therapy on experimental CaP bone metastases. Docetaxel exhibited strong antitumor effects on both osteolytic and osteoblastic lesions. These results provide a strong preclinical rationale for the clinical use of docetaxel in treatment of both locally advanced and disseminated CaP.

Zhang et al. *(110)* used C4-2B cells injected into mouse tibiae to establish a model for detection of prostate tumor cells in the bone environment. The C4-2B cells had been stably transfected with the RANKL promoter driving the luciferase gene. Animals with established tumors were treated with transforming growth factor-β, and bioluminescence was measured. The measurements demonstrated an increase in intraosseous tumor size over time that correlated with serum PSA levels. These observations provide a novel method to use in exploring the biology of CaP.

5.3.4. Cardiac Injection

We found only one published report of intracardiac injections of CaP cells to generate bone metastases as a model for testing new treatment modalities *(125)*. In this report, Sun et al. injected PC-3 cells labeled with luciferase into the left cardiac ventricle of athymic mice, and evaluated the effects of SDF-1/CXCR4 blockade by neutralizing antibodies. The results of this study showed that SDF-1/CXCR4 blockade decreased the number of bone lesions, suggesting that this receptor plays a role in “capturing” CaP cells within the bone marrow. Moreover, a parallel study using the intratibial injection model showed that SDF-1/CXCR4 blockade inhibits growth of these cells in the bone environment.

In summary, experimental models of CaP bone metastasis have given rise to a range of important findings. For example, these models have shown that alteration of the bone environment by administration of compounds inhibiting osteolysis can affect tumor growth. Another general finding of considerable use is that because CaP is nearly always heterogeneous, multimodal therapy often exhibits superior antitumor activities These findings can be used directly in designing clinical trials to attack human CaP more effectively.

6. CHAPTER SUMMARY

In this chapter, we discussed some of the most pertinent aspects of models of human CaP xenografts. We anticipate that there may be readers who are unfamiliar with the different immune-

compromised mice that are available for xenotransplantation and the various methods of implantation of cell lines and clinical tissues, so we have included a brief review of these topics.

Throughout the chapter, we repeatedly referred to the heterogeneity of CaP in humans and the need for multiple models that reflect specific features of this heterogeneity. This was illustrated in both Subheading 4. Progression to Androgen Dependence and Subheading 5. Metastasis. For example, in humans, we observe tumors that do not respond favorably to androgen ablation and rapidly progress to an androgen independent state and tumors that respond with slowed or absent growth for years. It is controversial what role neuroendocrine cells play in this progression, but an assortment of xenografts are now available that model this heterogeneous progression response, including those that have a neuroendocrine phenotype. It is unfortunate that we do not yet have a xenograft model that ideally mimics the spontaneous metastasis pattern observed clinically with an ultimate involvement of the skeleton revealing multiple osteoblastic lesions. However, in our Subheading 5. Metastasis, we summarized a number of approaches used to circumvent the limitations of spontaneous metastases to bone and nonbone sites. In many laboratories, great science is being accomplished using these CaP xenograft models, but more xenografts are still needed to fully understand the biology of this disease and its diversity. Although we have attempted to highlight key findings in the field of CaP xenografts, we apologize for the inevitable and inadvertent omissions of important work, and we hope that this material will nevertheless be of use to those with interest in preclinical models of human CaP.

ACKNOWLEDGMENTS

All of our derived xenografts share the prefix “LuCaP” in appreciation of long-term support from the Richard M. Lucas Foundation. Supplemental support was also provided for many years from the CaP CURE Foundation. The biological studies referred to herein from our group were supported over the years by NIH grants 5-P50-DK47656, 5-PO1-CA085859, and 5-50-CA97186, and several Veteran’s Administration Medical Center Merit Review Grant. We thank the patients who agreed to let us use their tissues for research and the assistance of the many clinicians who facilitated this process. The authors acknowledge Kent Buhler, Janna Quinn, and Austin Odman, who oversaw the management of our animal facility. Finally, the authors thank Michael Corey, Ph.D. for his editorial assistance.

REFERENCES

1. van Weerden, W. M. and Romijn, J. C. (2000). Use of nude mouse xenograft models in prostate cancer research. *Prostate* **43**, 263–271.
2. van Bokhoven, A., Varella-Garcia, M., Korch, C., et al. (2003). Molecular characterization of human prostate carcinoma cell lines. *Prostate* **57**, 205–225.
3. Rosol, T. J., Tannehill-Gregg, S. H., LeRoy, B. E., Mandl, S., and Contag, C. H. (2003). Animal models of bone metastasis. *Cancer* **97**, 748–757.
4. Navone, N. M., Logothetis, C. J., von Eschenbach, A. C., and Troncoso, P. (1998). Model systems of prostate cancer: uses and limitations. *Cancer Metastasis Rev.* **17**, 361–371.
5. Russell, P. J. and Voeks, D. J. (2003). Animal models of prostate cancer. *Methods Mol. Med.* **81**, 89–112.
6. Sobel, R. E. and Sadar, M. D. (2005). Cell lines used in prostate cancer research: a compendium of old and new lines—part 1. *J. Urol.* **173**, 342–359.
7. Sobel, R. E. and Sadar, M. D. (2005). Cell lines used in prostate cancer research: a compendium of old and new lines—part 2. *J. Urol.* **173**, 360–372.
8. Isaacson, J. H. and Cattanach, B. M. (1962). Mouse News Letter. *Report* 27–31.
9. Pandelouris, E. (1968). Absence of thymus in a mouse mutant. *Nature* **217**, 370–371.
10. Festing, M. F. W. (1968). International index of laboratory animals. Leicester: University of Leicester.
11. Wagner, B., Otto, U., Becker, H., Schroder, S., and Klosterhalfen, H. (1989). Transplantation of human prostate or BHP tissue into NMRI nu/nu mice: reliability of an experimental model. *Urol. Res.* **17**, 340.
12. Bosma, G. C., Custer, R. P., and Bosma, M. J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature* **301**, 527–530.
13. Leblond, V., Autran, B., and Cesbron, J. Y. (1997). The SCID mouse mutant: definition and potential use as a model for immune and hematological disorders. *Hematol. Cell Ther.* **39**, 213–221.

14. Bosma, G. C., Fried, M., Custer, R. P., Carroll, A., Gibson, D. M., and Bosma, M. J. (1988). Evidence of functional lymphocytes in some (leaky) scid mice. *J. Exp. Med.* **167**, 1016–1033.
15. Bosma, M. J. and Carroll, A. M. (1991). The SCID mouse mutant: definition, characterization, and potential uses. *Annu. Rev. Immunol.* **9**, 323–350.
16. Shinkai, Y., Rathbun, G., Lam, K. P., et al. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**, 855–867.
17. Mosier, D. E., Stell, K. L., Gulizia, R. J., Torbett, B. E., and Gilmore, G. L. (1993). Homozygous scid/scid;beige/beige mice have low levels of spontaneous or neonatal T cell-induced B cell generation. *J. Exp. Med.* **177**, 191–194.
18. Shultz, L. D., Schweitzer, P. A., Christianson, S. W., et al. (1995). Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J. Immunol.* **154**, 180–191.
19. Presnell, S. C., Werdin, E. S., Maygarden, S., Mohler, J. L., and Smith, G. J. (2001). Establishment of short-term primary human prostate xenografts for the study of prostate biology and cancer. *Am. J. Pathol.* **159**, 855–860.
20. Gray, D. R., Huss, W. J., Yau, J. M., et al. (2004). Short-term human prostate primary xenografts: an in vivo model of human prostate cancer vasculature and angiogenesis. *Cancer Res.* **64**, 1712–1721.
21. Fidler, I. J. (1990). Critical factors in the biology of human cancer metastasis: twenty-eighth G. H. A. Clowes memorial award lecture. *Cancer Res.* **50**, 6130–6138.
22. Bogden, A. E., Kelton, D. E., Cobb, W. R., and Esber, H. J. (1978). A Rapid Screening Method for Testing Chemotherapeutic Agents Human Tumor Xenografts. Gustav Fisher, New York, NY.
23. Wang, Y., Revelo, M. P., Sudilovsky, D., et al. (2005). Development and characterization of efficient xenograft models for benign and malignant human prostate tissue. *Prostate* **64**, 149–159.
24. Nemeth, J. A., Harb, J. F., Barroso, U. J., He, Z., Grignon, D. J., and Cher, M. L. (1999). Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone. *Cancer Res.* **59**, 1987–1993.
25. Hoehn, W., Schroeder, F., Riemann, J., Joebsis, A., and Hermanek, P. (1980). Human prostatic adenocarcinoma: some characteristics of a serially transplantable line in nude mice (PC 82). *Prostate* **1**, 95–104.
26. Nagabhushan, M., Miller, C. M., Pretlow, T. P., et al. (1996). CWR22: The first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. *Cancer Res.* **56**, 3042–3046.
27. Corey, E., Quinn, J. E., Buhler, K. R., et al. (2003). LuCaP 35: a new model of prostate cancer progression to androgen independence. *Prostate* **55**, 239–246.
28. Roudier, M. P., True, L. D., Higano, C. S., et al. (2003). Phenotypic heterogeneity of end-stage prostate carcinoma metastatic to bone. *Hum. Pathol.* **34**, 646–653.
29. Horoszewicz, J. S., Leong, S. S., Kawinski, E., et al. (1983). LNCaP model of human prostatic carcinoma. *Cancer Res.* **43**, 1809–1818.
30. Thalmann, G. N., Anezinis, P. E., Chang, S., et al. (1994). Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res.* **54**, 2577–2581.
31. Thalmann, G. N., Sikes, R. A., Wu, T. T., et al. (2000). LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. *Prostate* **44**, 91–103.
32. Wu, T. T., Sikes, R. A., Cui, Q., et al. (1998). Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen- producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *Int. J. Cancer* **77**, 887–894.
33. Kaighn, M., Shakar Narayan, K., Ohnuki, Y., Lechner, J., and Jones, L. (1979). Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Urology* **17**, 16–23.
34. Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., and Paulson, D. F. (1978). Isolation of a human prostate carcinoma cell line (DU 145). *Int. J. Cancer* **21**, 274–281.
35. Navone, N. M., Olive, M., Ozen, M., et al. (1997). Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin. Cancer Res.* **3**, 2493–2500.
36. Hara, T., Nakamura, K., Araki, H., Kusaka, M., and Yamaoka, M. (2003). Enhanced androgen receptor signaling correlates with the androgen-refractory growth in a newly established MDA PCa 2b-hr human prostate cancer cell subline. *Cancer Res.* **63**, 5622–5628.
37. Wainstein, M. A., He, F., Robinson, D., et al. (1994). CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. *Cancer Res.* **54**, 6049–6052.
38. Tepper, C. G., Boucher, D. L., Ryan, P. E., et al. (2002). Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer Res.* **62**, 6606–6614.
39. Korenchuk, S., Lehr, J. E., MClean, L., et al. (2001). VCaP, a cell-based model system of human prostate cancer. *In Vivo* **15**, 163–168.
40. Lee, Y. G., Korenchuk, S., Lehr, J., Whitney, S., Vessela, R., and Pienta, K. J. (2001). Establishment and characterization of a new human prostatic cancer cell line: DuCaP. *In Vivo* **15**, 157–162.
41. Zhau, H. Y., Chang, S. M., Chen, B. Q., et al. (1996). Androgen-repressed phenotype in human prostate cancer. *Proc. Natl. Acad. Sci. USA* **93**, 15,152–15,157.
42. Harper, M. E., Goddard, L., Smith, C., and Nicholson, R. I. (2004). Characterization of a transplantable hormone-responsive human prostatic cancer xenograft TEN12 and its androgen-resistant sublines. *Prostate* **58**, 13–22.

43. Selvan, S. R., Cornforth, A. N., Rao, N. P., et al. (2005). Establishment and characterization of a human primary prostate carcinoma cell line, HH870. *Prostate* **63**, 91–103.
44. McCulloch, D. R., Opeskin, K., Thompson, E. W., and Williams, E. D. (2005). BM18: a novel androgen-dependent human prostate cancer xenograft model derived from a bone metastasis. *Prostate* **65**, 35–43.
45. Klein, K. A., Reiter, R. E., Redula, J., et al. (1997). Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nat. Med.* **3**, 402–408.
46. Davies, M. R., Lee, Y. P., Lee, C., Zhang, X., Afar, D. E., and Lieberman, J. R. (2003). Use of a SCID mouse model to select for a more aggressive strain of prostate cancer. *Anticancer Res.* **23**, 2245–2252.
47. Craft, N., Chhor, C., Tran, C., et al. (1999). Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res.* **59**, 5030–5036.
48. Ellis, W. J., Vessella, R. L., Buhler, K. R., et al. (1996). Characterization of a novel androgen-sensitive, prostate-specific antigen-producing prostatic carcinoma xenograft: LuCaP 23. *Clin. Cancer Res.* **2**, 1039–1048.
49. True, L. D., Buhler, K. R., Quinn, J., et al. (2002). A neuroendocrine/small cell prostate carcinoma xenograft: LuCaP 49. *Am. J. Pathol.* **161**, 705–715.
50. Akakura, K., Bruchovsky, N., Goldenberg, S. L., Rennie, P. S., Buckley, A. R., and Sullivan, L. D. (1993). Effects of intermittent androgen suppression on androgen-dependent tumors. Apoptosis and serum prostate-specific antigen. *Cancer* **71**, 2782–2790.
51. Zhao, X., van Steenbrugge, G. J., and Schroder, F. H. (1992). Differential sensitivity of hormone-responsive and unresponsive human prostate cancer cells (LNCaP) to tumor necrosis factor. *Urol. Res.* **20**, 193–197.
52. van Steenbrugge, G. J., van Weerden, W. M., de Ridder, C. M. A., van der Kwast, T. H., and Schroder, F. H. (1994). Development and Application of Prostatic Xenograft Models for the Study of Human Prostate Cancer. Elsevier, Amsterdam, Holland, pp. 11–12.
53. Veldscholte, J., Ris Stalpers, C., Kuiper, G. G., et al. (1990). A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **173**, 534–540.
54. Wu, H. C., Hsieh, J. T., Gleave, M. E., Brown, N. M., Pathak, S., and Chung, L. W. (1994). Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. *Int. J. Cancer* **57**, 406–412.
55. Tso, C. L., McBride, W. H., Sun, J., et al. (2000). Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J.* **6**, 220–233.
56. Patel, B. J., Pantuck, A. J., Zisman, A., et al. (2000). CL1-GFP: an androgen independent metastatic tumor model for prostate cancer. *J. Urol.* **164**, 1420–1425.
57. Pretlow, T. G., Wolman, S. R., Micale, M. A., et al. (1993). Xenografts of primary human prostatic carcinoma. *J. Natl. Cancer Inst.* **85**, 394–398.
58. Tan, J., Sharief, Y., Hamil, K. G., et al. (1997). Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. *Mol. Endocrinol.* **11**, 450–459.
59. Nickerson, T., Chang, F., Lorimer, D., Smeekens, S. P., Sawyers, C. L., and Pollak, M. (2001). In vivo progression of LAPC-9 and LNCaP prostate cancer models to androgen independence is associated with increased expression of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR). *Cancer Res.* **61**, 6276–6280.
60. Corey, E., Quinn, J. E., and Vessella, R. L. (2003). A novel method of generating prostate cancer metastases from orthotopic implants. *Prostate* **56**, 110–114.
61. Whang, Y. E., Wu, X., Suzuki, H., et al. (1998). Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc. Natl. Acad. Sci. USA* **95**, 5246–5250.
62. Chen, C. D., Welsbie, D. S., Tran, C., et al. (2004). Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* **10**, 33–39.
63. Amler, L. C., Agus, D. B., LeDuc, C., et al. (2000). Dysregulated expression of androgen-responsive and nonresponsive genes in the androgen-independent prostate cancer xenograft model CWR22-R1. *Cancer Res.* **60**, 6134–6141.
64. Mousses, S., Bubendorf, L., Wagner, U., et al. (2002). Clinical validation of candidate genes associated with prostate cancer progression in the CWR22 model system using tissue microarrays. *Cancer Res.* **62**, 1256–1260.
65. Sirotnak, F. M., She, Y., Khokhar, N. Z., Hayes, P., Gerald, W., and Scher, H. I. (2004). Microarray analysis of prostate cancer progression to reduced androgen dependence: studies in unique models contrasts early and late molecular events. *Mol. Carcinog.* **41**, 150–163.
66. Gu, Z., Rubin, M. A., Yang, Y., et al. (2005). Reg IV: a promising marker of hormone refractory metastatic prostate cancer. *Clin. Cancer Res.* **11**, 2237–2243.
67. Porkka, K. P., Tammela, T. L., Vessella, R. L., and Visakorpi, T. (2004). RAD21 and KIAA0196 at 8q24 are amplified and overexpressed in prostate cancer. *Genes Chromosomes Cancer* **39**, 1–10.
68. Gleave, M., Goldenberg, S. L., Bruchovsky, N., and Rennie, P. (1998). Intermittent androgen suppression for prostate cancer: rationale and clinical experience. *Prostate Cancer Prostatic. Dis.* **1**, 289–296.
69. Bruchovsky, N., Klotz, L. H., Sadar, M., et al. (2000). Intermittent androgen suppression for prostate cancer: Canadian Prospective Trial and related observations. *Mol. Urol.* **4**, 191–199.

70. Buhler, K. R., Santucci, R. A., Royai, R. A., et al. (2000). Intermittent androgen suppression in the LuCaP 23.12 prostate cancer xenograft model. *Prostate* **43**, 63–70.
71. Sato, N., Akakura, K., Isaka, S., et al. (2004). Intermittent androgen suppression for locally advanced and metastatic prostate cancer: preliminary report of a prospective multicenter study. *Urology* **64**, 341–345.
72. Lane, T. M., Ansell, W., Farrugia, D., et al. (2004). Long-term outcomes in patients with prostate cancer managed with intermittent androgen suppression. *Urol. Int.* **73**, 117–122.
73. Higano, C., Shields, A., Wood, N., Brown, J., and Tangen, C. (2004). Bone mineral density in patients with prostate cancer without bone metastases treated with intermittent androgen suppression. *Urology* **64**, 1182–1186.
74. Peyromaure, M., Delongchamps, N. B., Debre, B., and Zerbib, M. (2005). Intermittent androgen deprivation for biologic recurrence after radical prostatectomy: long-term experience. *Urology* **65**, 724–729.
75. Ware, J. L., Paulson, D. F., Mickey, G. H., and Webb, K. S. (1982). Spontaneous metastasis of cells of the human-prostate carcinoma cell-line PC-3 in athymic nude-mice. *J. Urol.* **128**, 1064–1067.
76. Sato, N., Gleave, M. E., Bruchovsky, N., Rennie, P. S., Beraldi, E., and Sullivan, L. D. (1997). A metastatic and androgen-sensitive human prostate cancer model using intraprostatic inoculation of LNCaP cells in SCID mice. *Cancer Res.* **57**, 1584–1589.
77. Rembrink, K., Romijn, J. C., van der Kwast, T. H., Rubben, H., and Schroder, F. H. (1997). Orthotopic implantation of human prostate cancer cell lines: a clinically relevant animal model for metastatic prostate cancer. *Prostate* **31**, 168–174.
78. Stephenson, R. A., Dinney, C. P., Gohji, K., Ordonez, N. G., Killion, J. J., and Fidler, I. J. (1992). Metastatic model for human prostate cancer using orthotopic implantation in nude mice. *J. Natl. Cancer Inst.* **84**, 951–957.
79. Pettaway, C. A., Pathak, S., Greene, G., et al. (1996). Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin. Cancer Res.* **2**, 1627–1636.
80. Scatena, C. D., Hepner, M. A., Oei, Y. A., et al. (2004). Imaging of bioluminescent LNCaP-luc-M6 tumors: a new animal model for the study of metastatic human prostate cancer. *Prostate* **59**, 292–303.
81. Wang, X., An, Z., Geller, J., and Hoffman, R. M. (1999). High-malignancy orthotopic nude mouse model of human prostate cancer LNCaP. *Prostate* **39**, 182–186.
82. Shevrin, D. H., Kukreja, S. C., Ghosh, L., and Lad, T. E. (1988). Development of skeletal metastasis by human prostate cancer in athymic nude mice. *Clin. Exp. Metastasis* **5**, 401–409.
83. Rubio, N., Villacampa, M. M., and Blanco, J. (1998). Traffic to lymph nodes of PC-3 prostate tumor cells in nude mice visualized using the luciferase gene as a tumor cell marker. *Lab. Invest.* **78**, 1315–1325.
84. Rubio, N., Villacampa, M. M., El Hilali, N., and Blanco, J. (2000). Metastatic burden in nude mice organs measured using prostate tumor PC-3 cells expressing the luciferase gene as a quantifiable tumor cell marker. *Prostate* **44**, 133–143.
85. An, Z., Wang, X., Geller, J., Moossa, A. R., and Hoffman, R. M. (1998). Surgical orthotopic implantation allows high lung and lymph node metastatic expression of human prostate carcinoma cell line PC-3 in nude mice. *Prostate* **34**, 169–174.
86. Yang, M., Jiang, P., Sun, F. X., et al. (1999). A fluorescent orthotopic bone metastasis model of human prostate cancer. *Cancer Res.* **59**, 781–786.
87. Rubin, M. A., Putzi, M., Mucci, N., et al. (2000). Rapid ("warm") autopsy study for procurement of metastatic prostate cancer. *Clin. Cancer Res.* **6**, 1038–1045.
88. Bubendorf, L., Schopfer, A., Wagner, U., et al. (2000). Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. *Hum. Pathol.* **31**, 578–583.
89. Shimazaki, J., Higa, T., Akimoto, S., Masai, M., and Isaka, S. (1992). Clinical course of bone metastasis from prostatic cancer following endocrine therapy: examination with bone x-ray. *Adv. Exp. Med. Biol.* **324**, 269–275.
90. Urwin, G. H., Percival, R. C., Harris, S., Beneton, M. N., Williams, J. L., and Kanis, J. A. (1985). Generalised increase in bone resorption in carcinoma of the prostate. *Br. J. Urol.* **57**, 721–723.
91. Clarke, N. W., McClure, J., and George, N. J. (1991). Morphometric evidence for bone resorption and replacement in prostate cancer. *Br. J. Urol.* **68**, 74–80.
92. Wang, M. and Stearns, M. E. (1991). Isolation and characterization of PC-3 human prostatic tumor sublines which preferentially metastasize to select organs in S.C.I.D. mice. *Differentiation* **48**, 115–125.
93. Angelucci, A., Gravina, G. L., Rucci, N., et al. (2004). Evaluation of metastatic potential in prostate carcinoma: an in vivo model. *Int. J. Oncol.* **25**, 1713–1720.
94. Gleave, M., Hsieh, J. T., Gae, C., von Eschenback, A. C., and Chung, L. W. K. (1991). Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res.* **51**, 3753–3761.
95. Koeneman, K. S., Yeung, F., and Chung, L. W. (1999). Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* **39**, 246–261.
96. Corey, E., Quinn, J. E., Bladou, F., et al. (2002). Establishment and characterization of osseous prostate cancer models: intra-tibial injection of human prostate cancer cells. *Prostate* **52**, 20–33.
97. Tsingotjidou, A. S., Zotalis, G., Jackson, K. R., et al. (2002). Development of an animal model for prostate cancer cell metastasis to adult human bone. *Anticancer Res.* **21**, 971–978.
98. Yonou, H., Ochiai, A., Goya, M., et al. (2004). Intraosseous growth of human prostate cancer in implanted adult human bone: relationship of prostate cancer cells to osteoclasts in osteoblastic metastatic lesions. *Prostate* **58**, 406–413.
99. Yonou, H., Yokose, T., Kamijo, T., et al. (2001). Establishment of a novel species- and tissue-specific metastasis model

of human prostate cancer in humanized non-obese diabetic/severe combined immunodeficient mice engrafted with human adult lung and bone. *Cancer Res.* **61**, 2177–2182.

100. Lee, Y., Schwarz, E., Davies, M., et al. (2003). Differences in the cytokine profiles associated with prostate cancer cell induced osteoblastic and osteolytic lesions in bone. *J. Orthop. Res.* **21**, 62–72.
101. Fisher, J. L., Schmitt, J. F., Howard, M. L., Mackie, P. S., Choong, P. F., and Risbridger, G. P. (2002). An in vivo model of prostate carcinoma growth and invasion in bone. *Cell Tissue Res.* **307**, 337–345.
102. Pfitzenmaier, J., Quinn, J. E., Odman, A. M., et al. (2003). Characterization of C4-2 prostate cancer bone metastases and their response to castration. *J. Bone Miner. Res.* **18**, 1882–1888.
103. Berlin, O., Samid, D., Donthineni-Rao, R., Akeson, W., Amiel, D., and Woods, V. L., Jr. (1993). Development of a novel spontaneous metastasis model of human osteosarcoma transplanted orthotopically into bone of athymic mice. *Cancer Res.* **53**, 4890–4895.
104. Andresen, C., Bagi, C. M., and Adams, S. W. (2003). Intra-tibial injection of human prostate cancer cell line CWR22 elicits osteoblastic response in immunodeficient rats. *J. Musculoskelet. Neuronal Interact.* **3**, 148–155.
105. Pinthus, J. H., Waks, T., Schindler, D. G., et al. (2000). WISH-PC2: a unique xenograft model of human prostatic small cell carcinoma. *Cancer Res.* **60**, 6563–6567.
106. Nemeth, J. A., Yousif, R., Herzog, M., et al. (2002). Matrix metalloproteinase activity, bone matrix turnover, and tumor cell proliferation in prostate cancer bone metastasis. *J. Natl. Cancer Inst.* **94**, 17–25.
107. Cher, M. L., Biliran, H. R., Jr., Bhagat, S., et al. (2003). Maspin expression inhibits osteolysis, tumor growth, and angiogenesis in a model of prostate cancer bone metastasis. *Proc. Natl. Acad. Sci. USA* **100**, 7847–7852.
108. Li, Y., Che, M., Bhagat, S., et al. (2004). Regulation of gene expression and inhibition of experimental prostate cancer bone metastasis by dietary genistein. *Neoplasia* **6**, 354–363.
109. Nemeth, J. A., Cher, M. L., Zhou, Z., Mullins, C., Bhagat, S., and Trikha, M. (2003). Inhibition of alpha(v)beta3 integrin reduces angiogenesis, bone turnover, and tumor cell proliferation in experimental prostate cancer bone metastases. *Clin. Exp. Metastasis* **20**, 413–420.
110. Zhang, J., Dai, J., Yao, Z., Lu, Y., Dougall, W., and Keller, E. T. (2003). Soluble receptor activator of nuclear factor kappaB Fc diminishes prostate cancer progression in bone. *Cancer Res.* **63**, 7883–7890.
111. Goya, M., Miyamoto, S., Nagai, K., et al. (2004). Growth inhibition of human prostate cancer cells in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice by a ligand-specific antibody to human insulin-like growth factors. *Cancer Res.* **64**, 6252–6258.
112. Yonou, H., Kanomata, N., Goya, M., et al. (2003). Osteoprotegerin/osteoclastogenesis inhibitory factor decreases human prostate cancer burden in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice. *Cancer Res.* **63**, 2096–2102.
113. Zhang, J., Dai, J., Qi, Y., et al. (2001). Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. *J. Clin. Invest.* **107**, 1235–1244.
114. Kiefer, J. A., Vessella, R. L., Quinn, J. E., et al. (2004). The effect of osteoprotegerin administration on the intra-tibial growth of the osteoblastic LuCaP 23.1 prostate cancer xenograft. *Clin. Exp. Metastasis* **21**, 381–387.
115. Corey, E., Brown, L. G., Kiefer, J. A., et al. (2005). Osteoprotegerin in prostate cancer bone metastasis. *Cancer Res.* **65**, 1710–1718.
116. Lee, Y. P., Schwarz, E. M., Davies, M., et al. (2002). Use of zoledronate to treat osteoblastic versus osteolytic lesions in a severe-combined-immunodeficient mouse model. *Cancer Res.* **62**, 5564–5570.
117. Corey, E., Brown, L. G., Quinn, J. E., et al. (2003). Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. *Clin. Cancer Res.* **9**, 295–306.
118. Burton, D. W., Geller, J., Yang, M., et al. (2005). Monitoring of skeletal progression of prostate cancer by GFP imaging, X-ray, and serum OPG and PTHrP. *Prostate* **62**, 275–281.
119. Uehara, H., Kim, S. J., Karashima, T., et al. (2003). Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastases. *J. Natl. Cancer Inst.* **95**, 458–470.
120. Kim, S. J., Uehara, H., Karashima, T., Shepherd, D. L., Killion, J. J., and Fidler, I. J. (2003). Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. *Clin. Cancer Res.* **9**, 1200–1210.
121. Kim, S. J., Uehara, H., Yazici, S., et al. (2004). Simultaneous blockade of platelet-derived growth factor-receptor and epidermal growth factor-receptor signaling and systemic administration of paclitaxel as therapy for human prostate cancer metastasis in bone of nude mice. *Cancer Res.* **64**, 4201–4208.
122. Kim, S. J., Uehara, H., Yazici, S., et al. (2005). Modulation of bone microenvironment with zoledronate enhances the therapeutic effects of STI571 and paclitaxel against experimental bone metastasis of human prostate cancer. *Cancer Res.* **65**, 3707–3715.
123. Gamradt, S. C., Feeley, B. T., Liu, N. Q., et al. (2005). The effect of cyclooxygenase-2 (COX-2) inhibition on human prostate cancer induced osteoblastic and osteolytic lesions in bone. *Anticancer Res.* **25**, 107–115.
124. Fizazi, K., Sikes, C. R., Kim, J., et al. (2004). High efficacy of docetaxel with and without androgen deprivation and estramustine in preclinical models of advanced prostate cancer. *Anticancer Res.* **24**, 2897–2903.
125. Sun, Y. X., Schneider, A., Jung, Y., et al. (2005). Skeletal localization and neutralization of the SDF-1(CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites in vivo. *J. Bone Miner. Res.* **20**, 318–329.

2
The Pathology of Human Prostatic Atrophy and Inflammation

Angelo M. De Marzo

Summary

Focal atrophy is an extremely common histological alteration in the human prostate. Although most investigators during the last several decades have assumed that it is not relevant to prostate cancer, some pathologists suggested that prostate cancers might arise from atrophy as early as the 1930s *(1)*. Chronic inflammation is a major contributing cause of cancer in many organ systems. Only in the last few years have investigators begun to examine whether chronic inflammation, which is virtually always associated with prostate tissue that is atrophic, may be also involved in the pathogenesis of prostate cancer. Recently, a model of prostatic carcinogenesis involving a pathway of tissue injury, focal atrophy, regeneration, and specific molecular alterations occurring in the setting of chronic inflammation has been presented *(2–4)*. In addition, the epidemiology of the potential role of inflammation and infection in prostate cancer has been reviewed recently *(5–8)*. In this chapter, data regarding the histological features as well as the cellular and molecular biology of prostate atrophy and inflammation are examined.

Key Words: Focal atrophy; PIA; proliferative inflammatory atrophy; prostate atrophy; prostate cancer; prostate inflammation.

1. HISTOPATHOLOGY OF PROSTATE ATROPHY

1.1. Classification of Focal Atrophy Lesions

There are several morphological variants of focal prostate atrophy. These were first classified in detail by Franks *(9)*, who described five patterns. There were two patterns of "simple atrophy": "simple atrophy" and "simple atrophy with cyst formation." A third pattern was referred to as "sclerotic atrophy." Finally, there were two patterns of "post-atrophic hyperplasia": "lobular hyperplasia," and "sclerotic atrophy with hyperplasia." These descriptions served as the basis for studies of prostate atrophy for many years. Unfortunately, these terms, and several others related to them, have been used during the last few decades in inconsistent ways. For example, lesions referred to by some pathologists as post-atrophic hyperplasia *(10–12)* would mostly likely be referred to by other pathologists as lobular atrophy *(13–15)*. Additionally, some lesions referred to by Epstein et al. as partial atrophy *(16)* would seem to be referred to by others as post-atrophic hyperplasia *(13–15)*. To facilitate the comparison of future studies of prostate atrophy, and its potential relation to prostate cancer and other prostate disease, such as "chronic prostatitis" and benign prostatic hyperplasia (BPH), it was important to develop standardized terminology. A Working Group Classification of Focal Atrophy of the Prostate was developed and a manuscript describing this system and the results of a study that show good interobserver reliability of pathologists to classify these lesions has been shed *(17)*. The major features of this histological classification are presented next.

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

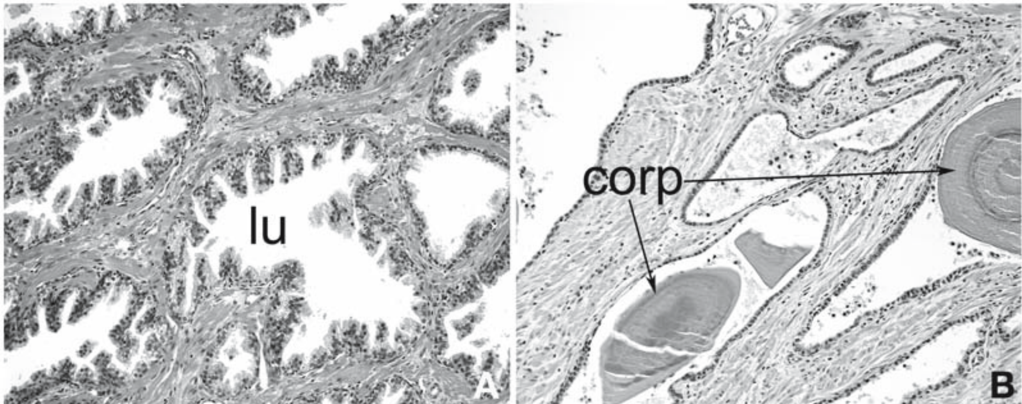


Fig. 1. Normal prostate vs simple atrophy. **(A)** Normal prostate epithelium. Lu, lumen. H&E original magnification ×100. **(B)** Simple atrophy lesion. Corp, corpora amylacea in lumens. Note lack of papillary infoldings, decreased cytoplasmic volume with lack of clear cytoplasm, and scattered mononuclear cells in the stromal and luminal compartments. H&E; original magnification ×100.

1.1.1. Working Group Classification of Prostate Atrophy Lesions

For the investigator to diagnose focal atrophy of the prostate, some general knowledge of the normal glandular histology is required. Although normal histology is briefly described, a more comprehensive description has been presented by McNeal *(18)*.

1.1.1.1. Normal Epithelium

Although there are some differences between the appearance of the epithelium in the different prostate zones, there are many features shared by all zones. Unless indicated, this description will focus on the peripheral zone. In all zones, it is not possible to distinguish acini from ducts unless one happens to have sampled an elongated open channel that can be observed branching proximally to distally. The epithelium in prostate ducts and acini are thought to be histologically identical *(18)*.

Normal epithelium, as observed in routine hematoxylin and eosin (H&E) sections of formalin-fixed, paraffin-embedded tissues, contains acini lined by luminal cells that are tall and columnar (Fig. 1A). The cytoplasm is generally clear/pale with a slightly reticular appearance at high power. The clear appearance is an artifact of tissue processing after formaldehyde fixation, in which "prostate secretory granules" are inadvertently removed. When fixed in a strong glutaraldehyde solution, these granules, which contain prostate-specific antigen, prostate-specific acid phosphatase, and polyamines, are largely retained *(19,20)*. In the central zone, the luminal, cells tend to contain darker eosinophilic cytoplasm.

The luminal cell nuclei are round and generally polarized towards the basement membrane side of the cell, although, at times, they can appear pseudostratified *(18)* which occurs more in the central zone. The chromatin is finely granular and evenly distributed and nucleoli are generally not visible or are small (<1 μm). The luminal cells overlie a continuous basal cell layer, in which the cells contain scant cytoplasm and nuclei that are small, oval, and frequently oriented perpendicular to the basement membrane. At times, in immunostained sections, cytoplasmic projections are observed that extend up in between the luminal cells. The basal layer may be only partially visible without immunostaining, although it is often more "prominent" in the transition zone in nodules of BPH, and, at times, in the central zone. The prominence results from the size of the basal cells, which are larger and more rounded. Neuroendocrine cells are not visible without immunostaining, but are present as scattered single cells that can be found in the basal, luminal, or both compartments *(21–23)*.

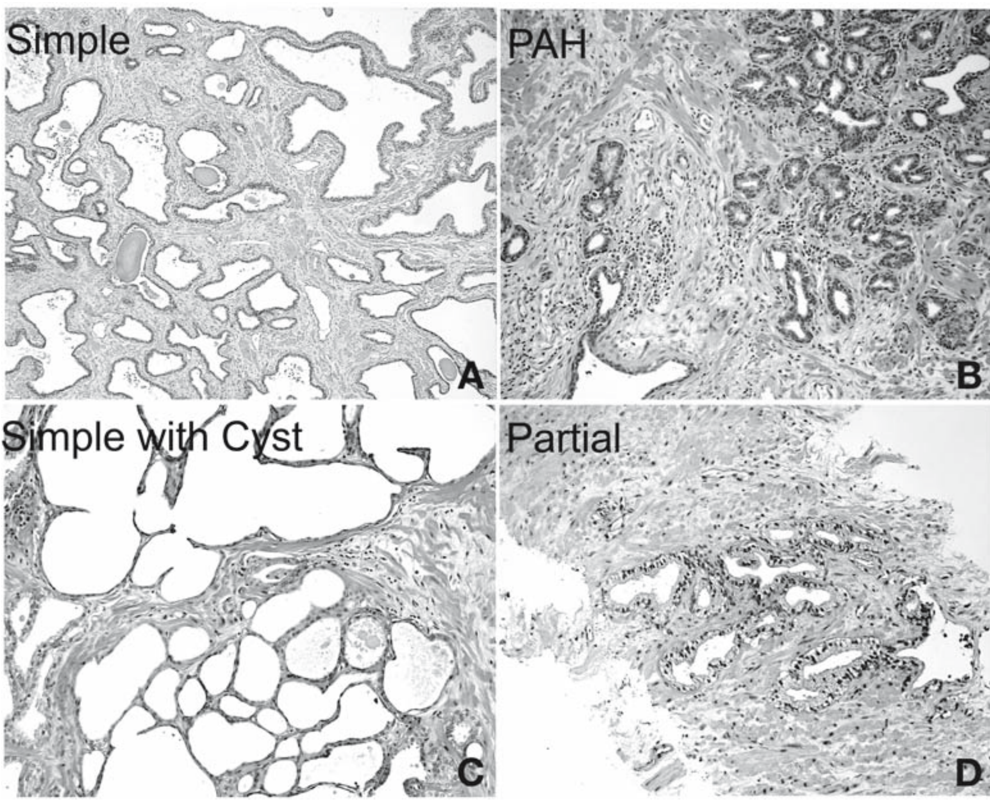


Fig. 2. Four patterns of focal prostate atrophy, as indicated. PAH, post-atrophic hyperplasia. H&E; original magnifications: **(A)** ×40; **(C)** and **(D)** ×100.

Prostatic acini and ducts are spaced at nearly regular intervals and are embedded in a stroma consisting largely of smooth muscle (Fig. 1A), which varies in character depending on zonal location *(18)*. The stroma also contains nerves, small blood vessels, collagen, and variable numbers of inflammatory cells. The acini contain multiple infoldings that are true "papillae" because they contain vascular channels. The acini in the central zone tend to have more complex arborization *(18)* and tend to contain benign "roman bridges" *(24)*. The acini in BPH observed in the transition zone are more variable but may contain even longer papillae.

1.1.1.2. Focal Atrophy

By definition, the luminal cells in atrophic regions contain reduced amounts of cytoplasm compared with normal epithelium. The reduction in cytoplasm is quite variable, ranging from being extremely scant and barely visible in flattened cells, to more abundant in cuboidal cells. The epithelium in all types of atrophy is composed of two layers consisting of basal cells and luminal cells—although two layers may be difficult to discriminate at times by standard H&E staining. The two layers are apparent after immunohistochemical staining for basal cell specific cytokeratins *(10,13–15,25)* (Fig. 6) or p63 *(26)*. Although the basal layer may be visible in focal atrophy, it is often very patchy.

1.1.1.2.1. Architectural Patterns/Subtypes of Focal Atrophy. Focal atrophy lesions can be classified into one of four subtypes, as described in Table 1 (Fig. 2). Although stereotypical cases of each of these occur, in practice, more than one type is often found within a given region *(9,10,11,27)*.

Table 1
Four Subtypes of Focal Atrophy Lesions. Adapted with permission from De Marzo et al. J. Surg. Pathol. 30:1281–1291, 2006

Simple atrophy: Although there is some variability, the acini are of relatively normal caliber and are generally spaced apart in a configuration similar to that of normal epithelium, and the number of acini per unit area does not seem to be increased relative to normal acini *(11)*. Glands that are dilated but not rounded are also considered simple atrophy. The acini are somewhat stellate in shape and may be angulated and, at times, seem to be compressed. Unlike normal glands, the atrophic glands lack vascularized papillary infoldings. Most of these lesions contain at least some chronic inflammatory cells in the stroma, epithelium, or lumen. Acute inflammatory cells may also be present, but these are much more variable. At times there seems to be drop-out of acini and ducts in the atrophic regions, in that only few glands remain in relatively large areas of stroma. Thus, in addition to the term "atrophy" referring to the appearance of the individual cells (relative lack of cytoplasm) and acini (changed architecture), there may also be loss of previously present acini and/or ducts in some focal prostate atrophy (Figure). *The frequency and extent of this loss is presently unknown.*

Post-atrophic hyperplasia (PAH): This subtype in the current classification is most like the lesions referred to by Franks as lobular hyperplasia, which was described as a subtype of PAH *(9)*. The morphology of PAH lesions in the current classification is also quite consistent with those described by several authors *(10–12,27,102)* (Fig. 2B).

PAH consists of acini that are small and mostly round that are arranged in a lobular distribution. Often these acini seem to be surrounding a somewhat dilated "feeder" duct. Many of these lesions frequently resemble normal-appearing resting breast lobules, and are referred to by some authors as lobular atrophy *(14)*. Most of these lesions contain low cuboidal cells with very scant cytoplasm, both lateral and apical to the nucleus. The close packing of multiple small acini suggests that there is an increase in their number compared with normal tissue *(11)*. Some of the cells in PAH may show mild-to-moderate nucleolar enlargement, which can lead to diagnostic confusion with adenocarcinoma. Similar to simple atrophy, most of these lesions contain at least some chronic inflammatory cells in the stroma, epithelium, or lumen. Acute inflammatory cells may also be present.

Simple Atrophy with Cyst Formation: This is considered a subtype of focal atrophy as delineated originally by Franks *(9)*. However, the current classification is more specific. Two general patterns are now encompassed: those containing very large diameter rounded acini (>1 mm), and those containing smaller, rounded acini (Fig. 2). In simple atrophy with cyst formation, the acini are not simply dilated, but *must be rounded and appear cyst-like*. Many of the acini in this pattern are arranged in a back-to-back configuration, with little intervening stroma. At times, the amount of cytoplasm may be so attenuated as to be nearly invisible, even at high power. When there is visible cytoplasm in the luminal cells, it tends to be somewhat clear (Fig. 2). Simple atrophy with cyst formation lesions tend to have little or only very few inflammatory cells. At times, the glands may be quite small and appear in a lobular configuration, similar to PAH.

Partial Atrophy: In partial atrophy *(16)*, most of the luminal cells contain less cytoplasm than normal, but not so little as the cells in the other subtypes of atrophy, and the cytoplasm in most cells must be clear, such that, unlike most simple atrophy and PAH lesions, partial atrophy does not seem basophilic at low magnification. Often, there is more cytoplasm lateral to the nucleus. The architectural arrangement of acini can be similar to that of either simple atrophy or PAH. Some partial atrophy lesions contain glands with the appearance of more "fully developed" atrophy *(16)*. In addition, partial atrophy may show moderate nucleolar enlargement, which can lead to diagnostic confusion with adenocarcinoma *(16)*.

Descriptive Qualifiers: In addition to the unique architectural patterns of the acini and epithelial cells in prostate atrophy, a number of other pathological processes, such as the type and extent of inflammation, fibrosis (at times referred to as sclerosis), and relation to other nearby lesions may be apparent in prostate atrophy. Some of these features, such as fibrosis, were used in the past to help define some atrophy subtypes. For example, Franks referred to lesions with abundant stromal fibrosis as "sclerotic atrophy." In the present classification, however, all of these are viewed as secondary features that may be expressed using descriptive qualifiers, and observers were not asked to comment on these features. The presence and extent of these changes are very variable. For studies that wish to compare type, extent, and location of inflammation, a National Institutes of Health panel scoring system has been devised *(58)*.

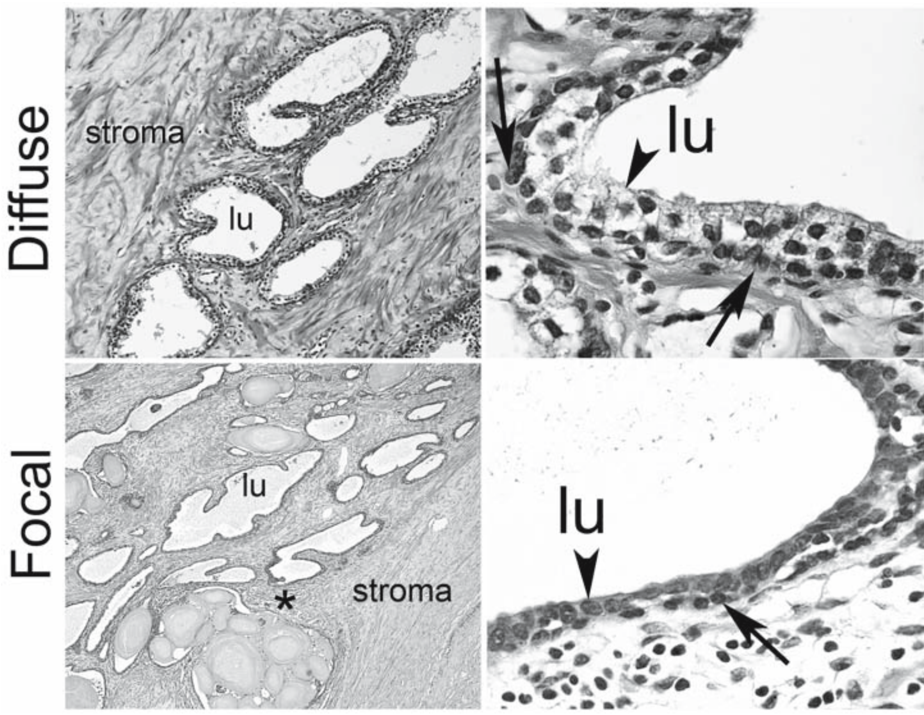


Fig. 3. Diffuse/hormonal atrophy vs focal atrophy. H&E. **(A)** Diffuse/hormonal atrophy from a patient receiving leuprolide. Note the lack of true papillae, and the reduced cytoplasmic volume in luminal cells. Original magnification ×100. **(B)** Higher-power view of **(A)** showing cytoplasmic clearing (*arrowhead*) in luminal cells and prominence of basal cell nuclei (*arrows*). Original magnification ×600. **(C)** Focal atrophy from the same patient. *Asterisk* indicates mononuclear inflammatory cells. Note also the corpora amylacea. Original magnification ×40. **(D)** Higher-power view of **(C)** showing nonprominent basal layer and luminal cells with scant dark cytoplasm. Original magnification ×600. Lu, lumen.

There may also be merging of one pattern into another, and these are considered mixed lesions. Although there is little data on the relative amounts of the different types of focal prostatic atrophy, simple atrophy, as described here, is the most frequent and extensive type (A.M. De Marzo and C. Magi-Galluzzi, unpublished observations).

1.1.2. Proliferative Inflammatory Atrophy

McNeal indicated that many foci of atrophy contain mild chronic inflammation, and referred to these lesions as "post-inflammatory atrophy" to indicate that they likely represent remnants of more severe inflammatory reactions that occurred sometime in the past *(28)*. To highlight the fact that these atrophic foci generally harbor associated inflammation *(18,29,30)*, which ranges from very mild to severe, and increased epithelial proliferation *(11,12,31–36)*, it was proposed to refer to most simple atrophy and post-atrophic hyperplasia lesions as "proliferative inflammatory atrophy" (PIA) *(33)*. This also serves to emphasize the unique epithelial changes in inflammatory lesions in the prostate (*see* below). Thus, many lesions considered PIA by our group would be considered by some authors as "chronic prostatitis" *(29,37)* or "lymphocytic prostatitis" *(38)*.

Some focal prostate atrophy lesions do not contain an apparent increase in inflammation. Because many of these foci also generally contain an increased proliferative fraction compared with normal-appearing epithelium, these may be considered proliferative atrophy (PA). Whether these lesions

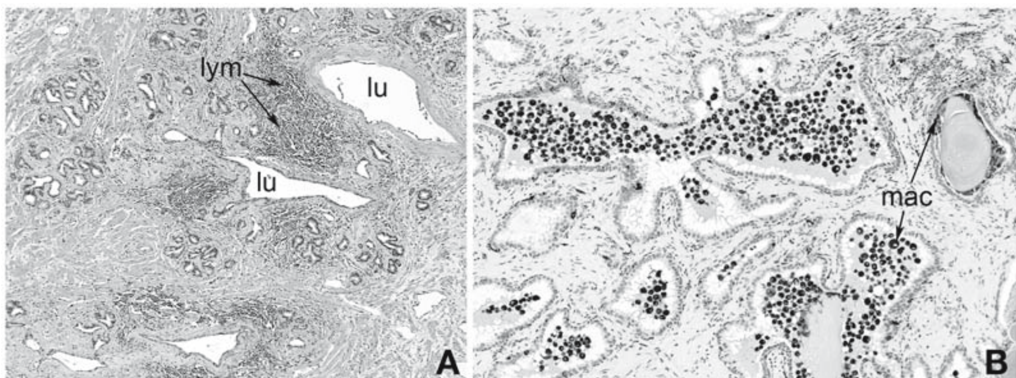


Fig. 4. Prostate atrophy with inflammation (PIA). **(A)** Post-atrophic hyperplasia with moderate chronic inflammation of the stromal compartment. Lym, predominantly lymphocytes; lu, lumen. H&E; original magnification ×40. **(B)** Abundant macrophages in the lumen in a simple atrophy lesion. Immunohistochemical stain for CD68 (KP1). Original magnification ×100. Mac, macrophages.

contain a more subtle increase in inflammatory cells that can be revealed by immunostaining is currently unknown, and whether, in three dimensions, these noninflamed lesions would be found to contain visible inflammation on further sectioning, is also unknown. Simple atrophy with cyst formation and partial atrophy tend to be associated with very little inflammation. Whether there is increased proliferation in simple atrophy with cyst formation and partial atrophy remains undetermined. Thus, these latter two forms are not currently considered PIA or PA. The Working Group Classification of prostate atrophy suggests that the use of the terms PIA or PA is optional.

1.3. Diffuse/Hormonal Atrophy Vs Focal Atrophy

Thus far, only focal prostatic atrophy has been discussed. Yet, two major types of epithelial atrophy occur in the prostate—diffuse and focal. Although these are not usually regarded as separate entities in the literature, and have not been clearly distinguished in most previous studies, they seem to be quite distinct in how they are induced and in their morphology. Diffuse atrophy is hormonally induced, and, as such, can be considered an "involutional" process. Although focal atrophy clearly increases in extent with age *(9,39)*, it does occur in young men (age 20–29 yr) who died with intact androgenicity *(40)*. This implies that focal atrophy is not related to systemic androgen deprivation. Nevertheless, both forms generally coexist in prostates that have been removed after patients were treated with androgen-deprivation therapy (Fig. 3).

Diffuse/hormonal atrophy develops as a result of androgen deprivation, which is most commonly induced after "total androgen blockade" involving luteinizing hormone–releasing hormone superagonist and an anti-androgen, or, by castration and an anti-androgen *(41–45)*. It has been reported that finasteride, a 5-α reductase type II inhibitor, and dutasteride, an inhibitor of both the type I and type II enzymes, produce histological changes in the benign regions of the prostate that are similar to, albeit less extensive than, the changes produced by combined androgen blockade *(46–49)*.

1.3.1. Distribution of Diffuse/Hormonal Atrophy and Focal Atrophy

The distribution of hormonally induced atrophy involves the organ in a more uniform manner than focal atrophy *(43)*. With total androgen blockade, there are virtually no areas of the prostate that would be considered completely unaffected. Although focal atrophy may involve large areas of the prostate, by definition, its distribution is patchy. Focal atrophy is most commonly found in the peripheral zone *(9,18)*. It also occurs frequently in the transition zone, in and around nodules of BPH. The

central zone may contain focal atrophy, although, compared with the peripheral and transition zones, the prevalence and extent is much less.

1.3.2. Cellular and Architectural Features of Prostate Atrophy

1.3.2.1. Similarities Between Focal and Diffuse/Hormonal Atrophy

In terms of cellular structure, as compared with normal-appearing epithelium, or hyperplastic epithelium in BPH, both types of atrophy show a reduction in the volume of the cytoplasm. In terms of glandular/acinar architecture, both types show a loss of papillae, which is complete in focal atrophy, and marked in diffuse/hormonal atrophy. The papillae that remain in diffuse/hormonal atrophy are generally blunted (Fig. 3).

1.3.2.2. Differences Between Focal and Diffuse/Hormonal Atrophy

Two key histological features separate diffuse/hormonal atrophy from focal atrophy, as well as from BPH and normal prostate epithelium: the luminal epithelial cells in diffuse/hormonal atrophy contain clear vacuolated cytoplasm, and the basal cell layer is usually more prominent in diffuse/hormonal atrophy *(41–44)* (Fig. 3). Along with the more prominent basal cell layer, it has been noted that after androgen deprivation there is an increase in the extent of basal cell hyperplasia *(44)*. Rather than having a prominent basal layer, focal atrophy usually contains a relatively normal-appearing basal layer, or an attenuated and sparse basal layer *(10,26)*. Occasionally, focal atrophy occurs amid regions of basal cell hyperplasia, or shows some piling up of the basal cell layer.

1.3.2.2.1. Cytoplasmic Features. At low magnification, many focal atrophy lesions seem hyperchromatic as compared with normal, BPH, and diffuse/hormonal atrophy. This is the result of three characteristic features of most focal atrophy lesions (except simple atrophy with cyst formation, and partial atrophy). First, the nuclear-to-cytoplasmic ratio in focal atrophy is usually high, imparting a dark blue/purple appearance at low power because most of what is observed is nuclei. Second, the cytoplasm that remains in many focal atrophy lesions is darkly stained and not clear. If darkly staining epithelium is present in diffuse/hormonal atrophy, it tends to be focal even within a given acinus. Third, in focal atrophy, the nuclei are often crowded, nearly abutting against one another. Interestingly, although compared with normal there is less cytoplasm in the luminal cells of diffuse/hormonal atrophy, the nuclear-to-cytoplasmic ratio is generally not increased. This results from the well-known finding that androgen deprivation also produces a relative decrease in nuclear size in some of the luminal cells, which often appear pyknotic (Fig. 3).

1.3.3. Historical Considerations of Diffuse Atrophy of the Prostate

The recognition of the distinction between the different types of atrophy, diffuse and focal, seems to have been first documented by McNeal *(50)*; he indicated that these two entities "did not appear to be part of the same process. In almost all cases, focal atrophy was sharply limited to the ramifications of one or a few adjacent main ducts, and the involved tissue usually showed evidence of chronic prostatitis as previously defined" *(50)*.

The description of diffuse atrophy that was associated with aging by McNeal was related to levels of "glandular activity" in which he described "a progressive decrease in duct size and prominence of the luminal ridges. Concomitantly the epithelium often is altered to a pattern designated as involutional epithelium. Here there is a reduction in cellular crowding to a uniformly simple columnar epithelium whose cells have very pale to clear cytoplasm with sharp outlines" *(37)*.

Although there may be some similarities between the "diffuse" atrophy of aging described by McNeal to the hormonally induced atrophy described above, it is clear that there are differences. For instance, although he did describe luminal epithelial cell clearing, he did not describe the features considered pathopneumonic of androgen deprivation, such as a marked reduction in luminal cell volume, a prominent basal layer, loss of most papillary infoldings, and cytoplasmic vacuolization *(18,37,50,51)*. Future studies directly comparing "involutional" histological changes associated with

aging to hormonal manipulation will shed light on these issues, as long as focal and diffuse atrophy are considered separately.

2. PATTERNS OF INFLAMMATION IN THE PROSTATE

2.1 . General Considerations

In normal-appearing prostate tissue without frank inflammation, there are lymphocytes that are scattered as individual cells in the epithelium, peri-acinar region, and stroma *(52,53)*. Endogenous macrophages can also be identified and these are usually in the stroma *(54)*. Both resident T cells and macrophages generally cannot be observed unless highlighted by immunohistochemical staining. The T cells tend to be $CD8^+$ in the intraepithelial compartment and $CD4^+$ in the stromal compartment *(52,53)*.

The majority of the prostates of older men obtained either by radical prostatectomy for prostate cancer, radical cystoprostatectomy for bladder cancer, transurethral resection for benign hyperplasia, or needle biopsy, show evidence of foci of mononuclear inflammatory cell infiltrates, which are generally referred to as "chronic" inflammation *(55–59)*. A large number of these specimens also show foci of polymorphonuclear neutrophil infiltrates, which are traditionally referred to as "acute" inflammation. Although these descriptions of acute and chronic patterns of inflammation are often representative of temporal events, even "acute" (as in time) viral infections are often followed by predominantly mononuclear infiltrates, and, recurrent or "chronic" bacterial infections are generally characterized by neutrophilic infiltrates. In several organ systems, the more modern phrasing is that neutrophilic infiltrates represent "active" inflammation or "activity" rather than a temporal event. At times, both patterns of inflammation occur together. For the purposes of this discussion, acute and chronic will be considered as traditionally defined. In addition, although the word "prostatitis" literally means inflammation of the prostate, most genitourinary pathology experts do not use this term in pathology reports because this connotes the clinical syndrome of "chronic prostatitis," which is not a diagnosis that can be made by histopathology examination of tissue sections.

In terms of the localization within the tissue, both acute and chronic inflammatory cells may be found in any or all of the following four "compartments": stromal, periglandular, intraepithelial, and intraluminal. A grading system that classifies prostatic inflammation according to its extent and grade/severity in each tissue compartment has been presented *(58)*.

2.2. Chronic Inflammation

Most prostate chronic inflammation consists of variable amounts of collections of lymphocytes and macrophages that are primarily centered in the peri-acinar region and stroma around acini and ducts (Fig. 4). The extent of these infiltrates ranges from only a few small foci, covering much less than 1 mm in area, to extensive numbers of foci covering large portions of the peripheral zone and/or the transition zone. The central zone only rarely shows inflammation. The mononuclear cells may also involve the epithelium to varying degrees and may also be intraluminal. At times, a variable number of acini show large collections of foamy macrophages involving the lumens, the intraepithelial compartment, or both (Fig. 4B). Often these are found near corpora amylacea. Although occasional formation of lymphoid follicles is observed, most of these chronic inflammatory infiltrates do not contain large follicles (i.e., greater than a few hundred cells), nor do they contain germinal centers. Eosinophils may be present in low numbers, but are rarely a prominent feature of prostate inflammation. Plasma cells may be present in chronic inflammation of the prostate, but tend to be present mostly in lesions with marked chronic inflammation, or in granulomatous lesions.

2.3. "Acute" Inflammation

Acute inflammation can vary from essentially none to extensive regions of abscess formation with massive tissue destruction that is associated with acute bacterial prostatitis. More often, there are small foci of acute inflammation involving small groups of acini that may contain frank epithelial

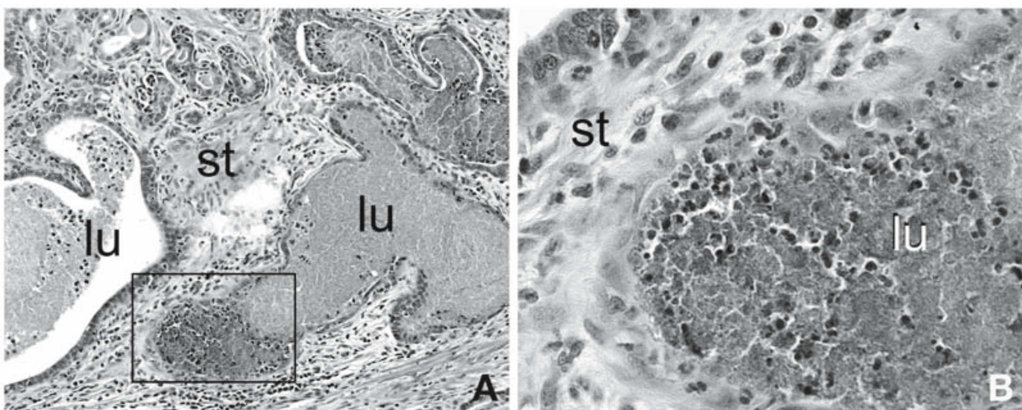


Fig. 5. Acute inflammation in the prostate. H&E. **(A)** Simple atrophy with polymorphonuclear neutrophils infiltration lumen (lu), stroma (st), and causing localized epithelial necrosis. Original magnification ×100. **(B)** Higher-power image from boxed regions in **(A)** showing neutrophils admixed with luminal debris with erosion of epithelial surface. Original magnification ×400.

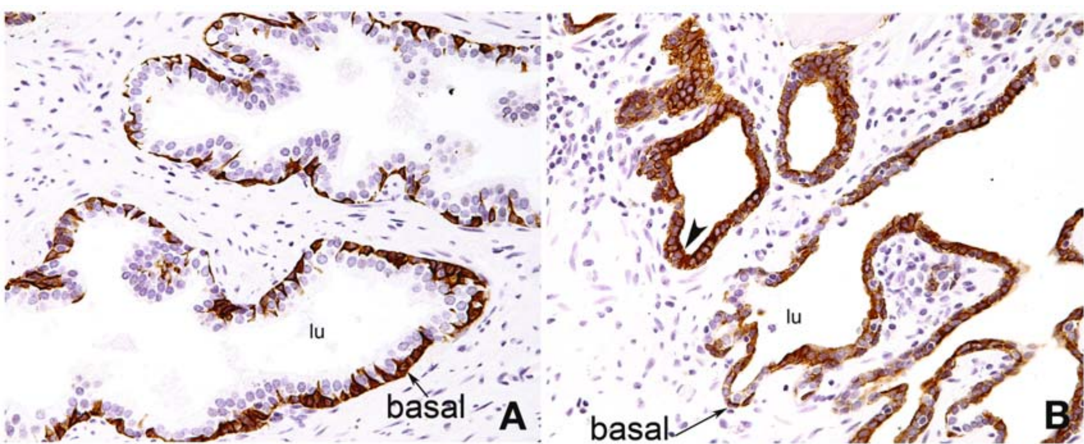


Fig. 6. Immunohistochemical staining for 34βE12 using heat-induced antigen retrieval. **(A)** Normal prostate showing exclusive staining for basal cells. Original magnification ×200. **(B)** Simple atrophy showing that the majority of luminal cells as well as basal cells stain positively. Original magnification: A, ×200. Lu, lumen.

destruction (Fig. 5). This is commonly observed in the transition zone and peripheral zone, but almost never in the central zone.

2.4. Granulomatous Inflammation

Granulomatous inflammation may accompany fungal or mycobacterial infections of the prostate *(24,60)*. Nonspecific granulomatous prostatitis is a rare self-limiting condition of unknown etiology that is not associated with known infectious agents. It often contains severe inflammatory infiltrates consisting of loosely formed granulomata with associated foamy macrophages, neutrophils, plasma cells, and eosinophils *(24)*. Allergic granulomatous prostatitis is an exceedingly rare condition associated with systemic generalized allergic conditions in which the granulomas are associated with abundant eosinophils *(24)*.

3. MOLECULAR BIOLOGY OF PROSTATE ATROPHY

Several groups have found molecular changes in prostate atrophy. These include an increase in the chromosome 8 centromere number *(34,61)*, an increase in nonclonal p53 mutations *(12)*, an increase in nonclonal androgen receptor mutations *(62)*, hypermethylation of the CpG island of the *GSTP1* promoter region in a small fraction of cases *(63)*, and the preferential accumulation of BK virus DNA sequences, large T antigen protein, and p53 protein *(64)*.

4. CELLULAR BIOLOGY OF AND PHENOTYPE OF NORMAL PROSTATE AND FOCAL PROSTATE ATROPHY

4.1. Keratin Expression

Prostate epithelial cell types are defined by location, morphology, and the expression of specific gene products (reviewed in refs. *65–68*). Focal atrophy lesions are very heterogeneous in morphology and in terms of which molecular markers they express. In terms of keratins, normal luminal cells contain strong staining for keratins 8 and 18 and absent staining for keratins 5 and 14. Basal cells, by contrast, stain strongly for keratins 5 and most, albeit not all, also stain strongly for keratin 14 *(25,69)*. Although many consider basal cells to be negative for keratins 8 and 18, it is clear that they express low levels of these molecules *(69)*. Keratin 19 staining has been reported to be uniformly expressed in prostate ducts *(70)*, but is generally heterogeneous in both basal and luminal cells in acini *(70,71)*.

Atrophic glands contain both a basal and luminal layer, although, at times, this can only be demonstrated by staining for basal cell-specific markers, such as p63 *(26)*. In terms of keratins, basal cells in focal atrophy seem to be similar to normal epithelium. However, in focal atrophy, a great deal of the luminal cells express keratin 5 *(25)* (Figure 6). This is in sharp contrast to normal luminal cells that do not stain for keratin 5. In addition, the keratin 5 that is present in the majority of the luminal cells in focal atrophy seems to be in an unstable form. This was determined by noting that luminal cell staining in atrophy is present after heat-induced epitope retrieval without protease, whereas it is not observed after antigen retrieval using protease treatment *(72)*. This contrasts with the keratin 5 present in basal cells, which is protease resistant and has its recognition by antibodies in immunohistochemistry enhanced by protease treatment. The most common antibody used for these types of studies is the mouse monoclonal antibody, 34βE12, which is thought to recognize keratin 1, 5, 10, and 14, although the exact keratin molecules recognized by this antibody have not been fully elucidated *(73)*.

The vast majority of luminal cells in focal atrophy also stain for keratin 8 (A.M. De Marzo, unpublished observations) as well as the monoclonal antibody Cam 5.2 *(33)*, which recognizes keratins 8, 18, and 19. Thus, these luminal cells in prostate focal atrophy are unique in their keratin expression in that they show “dual” strong staining for keratin 5 and keratin 8, imparting an “intermediate” phenotype between basal and luminal cells for these atrophic luminal cells *(25)*. Double-label immunofluorescence has shown that at least some keratin 5-positive luminal cells stain for Ki-67 in prostate atrophy *(25)*.

4.2. Other Differentiation and Cell Regulatory Proteins in Focal Prostate Atrophy

Further molecular support to indicate that may of the luminal cells in prostate atrophy are intermediate in phenotype between normal basal and luminal cells, and are perhaps in a transient state, is provided by the finding that they generally show low, albeit variable levels of staining for androgen receptors and prostate-specific antigen *(33)*, which are much more highly expressed in the luminal cells of normal glands. Similarly to keratin 5, many of the atrophic luminal cells stain strongly for Bcl-2 *(33,35,55)*. Interestingly, there is an inverse relation between staining intensity for Bcl-2 and androgen receptors, even within the same acini. Both C-met *(25,74)* and hepatocyte activator inhibi-

tor-1 *(74)* also show strong staining in normal basal cells, weak/negative staining in normal secretory cells, and moderate-to-strong staining in many of the atrophic luminal cells in focal atrophy.

4.3. Proliferation and Differentiation Related Proteins

4.3.1. Ki-67 and other Proliferation Markers

Several proliferation markers have been examined in human prostate, including immunohistochemical analysis with antibodies against Ki-67, proliferating cell nuclear antigen, and topoisomerase II-a *(33)*. Most studies have used Ki-67. In the normal human prostate, the majority of proliferation occurs in the basal cell layer *(75,76)*, which is considered by most to be the reserve layer of the prostate epithelium. Bonkhoff et al. *(75)* indicated that the relative distribution of Ki-67 positive cells was approx 70% in the basal layer and 30% in the luminal layer. This does imply, however, that some luminal cells can regenerate themselves. In focal atrophy, it is clear that the increase in the proliferative fraction, as measured by KI-67 staining in the epithelium *(11,12,32–36)*, which ranges from approx 3- to 80-fold higher than the adjacent normal epithelium, occurs mostly in the luminal compartment *(25)*. Proliferation in diffuse/hormonal atrophy has been studied in a few experiments and it seems that it decreases dramatically after a few days, but comes back after approx 1 week to levels observed without androgen blockade *(77)*. This is similar to what has been reported for castrated rodents (see ref. 77). Interestingly, it seems that the proliferation in diffuse/hormonal atrophy also occurs more commonly in the luminal compartment *(78)*.

4.3.2. NKX3.1

NKX3.1 encodes a homeodomain protein that is selectively expressed at high levels in normal prostate luminal epithelial cells, is a potential target for deletion on chromosome 8p, and is downregulated in some prostate cancers *(79–83)*. Targeted disruption of this gene in mouse models results in prostatic hyperplasia and prostatic intraepithelial neoplasia (PIN), and *Nkx3.1* disruption in combination with *Pten* deletion or *Cdkn1b* (encoding p27) deletion results in invasive carcinoma. Recently, we have observed that many focal human atrophy lesions contain a marked downregulation of NKX3.1 protein in the luminal compartment, as compared with normal luminal cells *(84)*.

4.3.3. $P27^{Kip1}$

P27 is highly expressed and localized to the nuclei of normal luminal cells *(85)* in the prostate, such that upwards of 80 to 85% of these cells are strongly positive *(86)*. The protein is downregulated in many prostate cancers and decreased expression may imply a worse prognosis *(85–92)*. The basal cells, by contrast, are very variable and are much more frequently negative for p27 *(86)*. At times, in p27-stained sections, one can see a third layer of cells that is located between the basal and luminal cells and, when this occurs, the basal-most cells and the luminal-most cells are positive but the cells occupying the intermediate location show an absence of staining *(86)*. This is found most commonly in the periurethral region and in prostates treated with androgen ablation therapy, but can also be observed in benign normal-appearing glands and in focal atrophy glands. A similar, although not identical, pattern of staining has been shown for involucrin, a protein associated with squamous differentiation *(93)*.

4.4. Stress Response Proteins

Glutathione-*S*-transferase (GST) P1 is a stress response protein that is constitutively expressed in the human prostate basal cells, but infrequently expressed in normal-appearing luminal cells *(48,94–96)*. By contrast, this protein is upregulated in focal prostate atrophy, in which many, albeit not all, of the luminal cells stain positively *(33)*. GST-α is another stress-induced member of the GST family. It is only very infrequently expressed in the normal prostate, but is upregulated in most focal atrophy lesions *(97)*. Cyclooxygenase 2 is another stress response gene that is upregulated in many of

the luminal cells in focal atrophy lesions *(98)*, and it has been shown that the frequency of staining in the luminal cells is higher in areas of inflammation *(35)*.

5. CONCLUSIONS AND THE "INJURY AND REGENERATION" HYPOTHESIS OF PROSTATE CARCINOGENESIS

Focal prostate atrophy has several recognizable morphological patterns. It is rapidly becoming of great interest to many investigators. Although its etiology remains unknown, its relation to inflammation, PIN, BPH, and carcinoma is being explored by a number of new molecular approaches, as well as by traditional pathological association studies. Prostate inflammation also has several morphological patterns and is exceedingly common. Except in relatively rare cases in which infection can be demonstrated, prostate inflammation is also of largely unknown etiology. Animal studies have implicated dietary factors, such as lack of soy, and neonatal estrogen exposure, as potential factors that trigger an autoimmune response *(99–101)*.

A model has been proposed *(3,4)* that suggests that repeated bouts of injury to the prostate epithelium, presumably as a result of inflammation in response to unknown pathogens or autoimmune disease and/or dietary factors, result in proliferation of epithelial cells that possess a phenotype intermediate between basal cells and mature luminal cells. These cells are hypothesized to be attempting tissue repair. This is supported by the finding that several proteins known to be involved in tissue repair, such as C-met *(25)* and hepatocyte activator inhibitor-1 *(74)*, show elevated expression in focal atrophy. The model predicts that in a small subset of cells somatic genome alterations occur, such as such as cytosine hypermethylation within the CpG island of the *GSTP1* gene and telomere shortening, that drive genetic instability and initiate high-grade PIN and prostate cancer formation. A classification system of focal atrophy lesions *(17)* was recently validated that should aid in further testing this model by various groups in human patho-epidemiological and ecological studies. In addition, an improved understanding of the molecular pathogenesis of prostate atrophy and inflammation and new animal models of prostate inflammation are needed to further test this model vigorously in experimental settings.

ACKNOWLEDGMENTS

Supported by Funded by Public Health Services NIH/NCI # RO1CA84997, NIH/NCI Specialized Program in Research Excellence (SPORE) in Prostate Cancer #P50CA58236 (Johns Hopkins), The Donald and Susan Sturm Foundation Dr. De Marzo is the recipient of the Bing Scholar Award as part of The Patrick C. Walsh Prostate Cancer Research Fund.

REFERENCES

1. Rich, A. R. (1934). On the frequency of occurrence of occult carcinoma of the prostate. *J. Urol.* **33**, 215–223.
2. De Marzo, A. M., Putzi, M. J., and Nelson, W. G. (2001). New concepts in the pathology of prostatic epithelial carcinogenesis. *Urology* **57**, 103–114.
3. Nelson, W. G., De Marzo, A. M., and Isaacs, W. B. (2003). Prostate cancer. *N. Engl. J. Med.* **349**, 366–381.
4. De Marzo, A. M., DeWeese, T. L., Platz, E. A., et al. (2004). Pathological and molecular mechanisms of prostate carcinogenesis: implications for diagnosis, detection, prevention, and treatment. *J. Cell Biochem.* **91**, 459–477.
5. Platz, E. A. and De Marzo, A. M. (2004). Epidemiology of inflammation and prostate cancer. *J. Urol.* **171**, S36–S40.
6. Lucia, M. S. and Torkko, K. C. (2004). Inflammation as a target for prostate cancer chemoprevention: pathological and laboratory rationale. *J. Urol.* **171**, S30–S34; discussion S35.
7. Palapattu, G. S., Sutcliffe, S., Bastian, P. J., et al. (2005). Prostate carcinogenesis and inflammation: emerging insights. *Carcinogenesis* **26**, 1170–1181.
8. Sutcliffe, S. and Platz, E. A. (2006). Inflammation in the etiology of prostate cancer: an epidemiologic perspective. *Urologic Oncology: Seminars and Original Investigations*, in press.
9. Franks, L. M. (1954). Atrophy and hyperplasia in the prostate proper. *J. Pathol. Bacteriol.* **68**, 617–621.
10. Cheville, J. C. and Bostwick, D. G. (1995). Postatrophic hyperplasia of the prostate. A histologic mimic of prostatic adenocarcinoma. *Am. J. Surg. Pathol.* **19**, 1068–1076.
11. Ruska, K. M., Sauvageot, J., and Epstein, J. I. (1998). Histology and cellular kinetics of prostatic atrophy. *Am. J. Surg. Pathol.* **22**, 1073–1077.

12. Tsujimoto, Y., Takayama, H., Nonomura, N., Okuyama, A., and Aozasa, K. (2002). Postatrophic hyperplasia of the prostate in Japan: histologic and immunohistochemical features and p53 gene mutation analysis. *Prostate* **52**, 279–287.
13. Srigley, J. R. (1988). Small-acinar patterns in the prostate gland with emphasis on atypical adenomatous hyperplasia and small-acinar carcinoma. *Semin. Diagn. Pathol.* **5**, 254–272.
14. Srigley, J. R. (2004). Benign mimickers of prostatic adenocarcinoma. *Mod. Pathol.* **17**, 328–348.
15. Amin, M. B., Tamboli, P., Varma, M., and Srigley, J. R. (1999). Postatrophic hyperplasia of the prostate gland: a detailed analysis of its morphology in needle biopsy specimens. *Am. J. Surg. Pathol.* **23**, 925–931.
16. Oppenheimer, J. R., Wills, M. L., and Epstein, J. I. (1998). Partial atrophy in prostate needle cores: another diagnostic pitfall for the surgical pathologist. *Am. J. Surg. Pathol.* **22**, 440–445.
17. De Marzo, A. M., Platz, E. A., Epstein, J. I., et al. (2006). A working group classification of focal prostate atrophy lesions, Am. J. Surg. Pathol. **30**,1281–1291.
18. McNeal, J. E. (1997). Prostate. In Histology for Pathologists, 2nd ed. (Sternberg, S. S., ed.), Lippincott-Raven, Philadelphia, PA, pp. 997–1017.
19. Cohen, R. J., Fujiwara, K., Holland, J. W., and McNeal, J. E. (2001). Polyamines in prostatic epithelial cells and adenocarcinoma; the effects of androgen blockade. *Prostate* **49**, 278–284.
20. Cohen, R. J., McNeal, J. E., Edgar, S. G., Robertson, T., and Dawkins, H. J. (1998). Characterization of cytoplasmic secretory granules (PSG), in prostatic epithelium and their transformation-induced loss in dysplasia and adenocarcinoma. *Hum. Pathol.* **29**, 1488–1494.
21. Bonkhoff, H., Stein, U., and Remberger, K. (1995). Endocrine-paracrine cell types in the prostate and prostatic adenocarcinoma are postmitotic cells [see comments]. *Hum. Pathol.* **26**, 167–170.
22. Cohen, R. J., Glezerson, G., Taylor, L. F., Grundle, H. A., and Naude, J. H. (1993). The neuroendocrine cell population of the human prostate gland. *J. Urol.* **150**, 365–368.
23. Abrahamsson, P. A. (1999). Neuroendocrine cells in tumour growth of the prostate. *Endocr. Relat. Cancer* **6**, 503–519.
24. Epstein, J. I. and Yang, X. J. (2002). Prostate Biopsy Interpretation, 3rd ed. Biopsy interpretation series, Lippincott Williams & Wilkins, Philadelphia, PA.
25. van Leenders, G. J., Gage, W. R., Hicks, J. L., et al. (2003). Intermediate cells in human prostate epithelium are enriched in proliferative inflammatory atrophy. *Am. J. Pathol.* **162**, 1529–1537.
26. Parsons, J. K., Gage, W. R., Nelson, W. G., and De Marzo, A. M. (2001). p63 protein expression is rare in prostate adenocarcinoma: implications for cancer diagnosis and carcinogenesis. *Urology* **58**, 619–624.
27. Billis, A. (1998). Prostatic atrophy: an autopsy study of a histologic mimic of adenocarcinoma. *Mod. Pathol.* **11**, 47–54.
28. McNeal, J. E. (1988). Normal histology of the prostate. *Am. J. Surg. Pathol.* **12**, 619–633.
29. Bennett, B. D., Richardson, P. H., and Gardner, W. A. (1993). Histopathology and cytology of prostatitis. In Prostate Diseases (Lepor, H. and Lawson, R. K., eds.), W.B. Saunders Co., Philadelphia, PA, pp. 399–414.
30. McNeal, J. E. (1988). Normal histology of the prostate. *Am. J. Surg. Pathol.* **12**, 619–633.
31. Liavag, I. (1968). Mitotic activity of prostatic epithelium. A study by means of Colcemid. *Acta Pathol. Microbiol. Scand.* **73**, 19–28.
32. Feneley, M. R., Young, M. P., Chinyama, C., Kirby, R. S., and Parkinson, M. C. (1996). Ki-67 expression in early prostate cancer and associated pathological lesions. *J. Clin. Pathol.* **49**, 741–748.
33. De Marzo, A. M., Marchi, V. L., Epstein, J. I., and Nelson, W. G. (1999). Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. *Am. J. Pathol.* **155**, 1985–1992.
34. Shah, R., Mucci, N. R., Amin, A., Macoska, J. A., and Rubin, M. A. (2001). Postatrophic hyperplasia of the prostate gland: neoplastic precursor or innocent bystander? *Am. J. Pathol.* **158**, 1767–1773.
35. Wang, W., Bergh, A., and Damber, J. E. (2004). Chronic inflammation in benign prostate hyperplasia is associated with focal upregulation of cyclooxygenase-2, Bcl-2, and cell proliferation in the glandular epithelium. *Prostate* **61**, 60–72.
36. Faith, D., Han, S., Lee, D. K., et al. (2005). p16 Is upregulated in proliferative inflammatory atrophy of the prostate. *Prostate* **65**, 73–82.
37. McNeal, J. E. (1965). Morphogenesis of prostatic carcinoma. *Cancer* **18**, 1659–1666.
38. Blumenfeld, W., Tucci, S., and Narayan, P. (1992). Incidental lymphocytic prostatitis. Selective involvement with nonmalignant glands. *Am. J. Surg. Pathol.* **16**, 975–981.
39. Liavag, I. (1968). Atrophy and regeneration in the pathogenesis of prostatic carcinoma. *Acta Path. Microbiol. Scandinav.* **73**, 338–350.
40. Gardner, W. A., Jr. and Culberson, D. E. (1987). Atrophy and proliferation in the young adult prostate. *J. Urol.* **137**, 53–56.
41. Murphy, W. M., Soloway, M. S., and Barrows, G. H. (1991). Pathologic changes associated with androgen deprivation therapy for prostate cancer. *Cancer* **68**, 821–828.
42. Tetu, B., Srigley, J. R., Boivin, J. C., et al. (1991). Effect of combination endocrine therapy (LHRH agonist and flutamide) on normal prostate and prostatic adenocarcinoma. A histopathologic and immunohistochemical study. *Am. J. Surg. Pathol.* **15**, 111–120.
43. Civantos, F., Marcial, M. A., Banks, E. R., et al. (1995). Pathology of androgen deprivation therapy in prostate carcinoma. A comparative study of 173 patients. *Cancer* **75**, 1634–1641.

44. Reuter, V. E. (1997). Pathological changes in benign and malignant prostatic tissue following androgen deprivation therapy. *Urology* **49**, 16–22.
45. Montironi, R. and Schulman, C. C. (1998). Pathological changes in prostate lesions after androgen manipulation. *J. Clin. Pathol.* **51**, 5–12.
46. Bostwick, D. G., Qian, J., Civantos, F., Roehrborn, C. G., and Montironi, R. (2004). Does finasteride alter the pathology of the prostate and cancer grading? *Clin. Prostate Cancer* **2**, 228–235.
47. Pomante, R., Santinelli, A., Muzzonigro, G., and Montironi, R. (1999). Nodular hyperplasia of the prostate. Quantitative evaluation of secretory cell changes after treatment with finasteride. *Anal. Quant. Cytol. Histol.* **21**, 63–69.
48. Montironi, R., Mazzucchelli, R., Pomante, R., et al. (1999). Immunohistochemical expression of pi class glutathione S-transferase in the basal cell layer of benign prostate tissue following chronic treatment with finasteride. *J. Clin. Pathol.* **52**, 350–354.
49. Iczkowski, K. A., Qiu, J., Qian, J., et al. (2005). The dual 5-alpha-reductase inhibitor dutasteride induces atrophic changes and decreases relative cancer volume in human prostate. *Urology* **65**, 76–82.
50. McNeal, J. E. (1968). Regional morphology and pathology of the prostate. *Am. J. Clin. Pathol.* **49**, 347–357.
51. McNeal, J. E. (1984). Ageing and the prostate. In Urology in the Elderly (Brocklehurst, J. C., ed.), Churchill Livingstone, Edinburgh, UK, pp. 193–202.
52. Bostwick, D. G., de la Roza, G., Dundore, P., Corica, F. A., and Iczkowski, K. A. (2003). Intraepithelial and stromal lymphocytes in the normal human prostate. *Prostate* **55**, 187–193.
53. Liu, A. Y. and True, L. D. (2002). Characterization of prostate cell types by CD cell surface molecules. *Am. J. Pathol.* **160**, 37–43.
54. Shimura, S., Yang, G., Ebara, S., Wheeler, T. M., Frolov, A., and Thompson, T. C. (2000). Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. *Cancer Res.* **60**, 5857–5861.
55. Gerstenbluth, R. E., Seftel, A. D., MacLennan, G. T., et al. (2002). Distribution of chronic prostatitis in radical prostatectomy specimens with up-regulation of bcl-2 in areas of inflammation. *J. Urol.* **167**, 2267–2270.
56. Nickel, J. C., Downey, J., Young, I., and Boag, S. (1999). Asymptomatic inflammation and/or infection in benign prostatic hyperplasia. *BJU Int.* **84**, 976–981.
57. Schatteman, P. H., Hoekx, L., Wyndaele, J. J., Jeuris, W., and Van Marck, E. (2000). Inflammation in prostate biopsies of men without prostatic malignancy or clinical prostatitis: correlation with total serum PSA and PSA density. *Eur. Urol.* **37**, 404–412.
58. Nickel, J. C., True, L. D., Krieger, J. N., Berger, R. E., Boag, A. H., and Young, I. D. (2001). Consensus development of a histopathological classification system for chronic prostatic inflammation. *BJU Int.* **87**, 797–805.
59. Boag, A. H., and Young, I. D. (1999). Pathology of acute and chronic prostate inflammation. In Textbook of prostatitis (Nickel, J. C., ed.). Isis Medical Media Ltd., Oxford, UK, pp. 57–68.
60. Epstein, J. I. and Hutchins, G. M. (1984). Granulomatous prostatitis: distinction among allergic, nonspecific, and post-transurethral resection lesions. *Hum. Pathol.* **15**, 818–825.
61. Macoska, J. A., Trybus, T. M., and Wojno, K. J. (2000). 8p22 loss concurrent with 8c gain is associated with poor outcome in prostate cancer. *Urology* **55**, 776–782.
62. Tsujimoto, Y., Takakuwa, T., Takayama, H., et al. (2004). In situ shortening of CAG repeat length within the androgen receptor gene in prostatic cancer and its possible precursors. *Prostate* **58**, 283–290.
63. Nakayama, M., Bennett, C. J., Hicks, J. L., et al. (2003). Hypermethylation of the human GSTP1 CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using Laser-Capture Microdissection. *Am. J. Pathol.* **163**, 923–933.
64. Das, D., Shah, R. B., and Imperiale, M. J. (2004). Detection and expression of human BK virus sequences in neoplastic prostate tissues. *Oncogene* **23**, 7031–7046.
65. Bonkhoff, H. and Remberger, K. (1996). Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model. *Prostate* **28**, 98–106.
66. De Marzo, A. M., Nelson, W. G., Meeker, A. K., and Coffey, D. S. (1998). Stem cell features of benign and malignant prostate epithelial cells. *J. Urol.* **160**, 2381–2392.
67. van Leenders, G. J. and Schalken, J. A. (2001). Stem cell differentiation within the human prostate epithelium: implications for prostate carcinogenesis. *BJU Int* **88(Suppl. 2)**, 35–42; discussion 49–50.
68. Litvinov, I. V., De Marzo, A. M., and Isaacs, J. T. (2003). Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling? *J. Clin. Endocrinol. Metab.* **88**, 2972–2982.
69. van Leenders, G., Dijkman, H., Hulsbergen-van de Kaa, C., Ruiter, D., and Schalken, J. (2000). Demonstration of intermediate cells during human prostate epithelial differentiation in situ and in vitro using triple-staining confocal scanning microscopy. *Lab. Invest.* **80**, 1251–1258.
70. Yang, Y., Hao, J., Liu, X., Dalkin, B., and Nagle, R. B. (1997). Differential expression of cytokeratin mRNA and protein in normal prostate, prostatic intraepithelial neoplasia, and invasive carcinoma. *Am. J. Pathol.* **150**, 693–704.
71. Peehl, D. M., Sellers, R. G., and McNeal, J. E. (1996). Keratin 19 in the adult human prostate: tissue and cell culture studies. *Cell Tissue Res.* **285**, 171–176.

72. Bennett, C. J., Hicks, J. L., Gage, W. R., and De Marzo, A. M. (2003). "False positive" immunostaining for 34BE12 in prostate cancer: Systematic study of the effect of antigen retrieval conditions using high density tissue microarrays. *Mod. Pathol.* **16**, 141A–142A.
73. Gown, A. M. and Vogel, A. M. (1984). Monoclonal antibodies to human intermediate filament proteins. II. Distribution of filament proteins in normal human tissues. *Am. J. Pathol.* **114**, 309–321.
74. Knudsen, B. S., Lucas, J. M., Fazli, L., et al. (2005). Regulation of hepatocyte activator inhibitor-1 expression by androgen and oncogenic transformation in the prostate. *Am. J. Pathol.* **167**, 255–266.
75. Bonkhoff, H., Stein, U., and Remberger, K. (1994). The proliferative function of basal cells in the normal and hyperplastic human prostate. *Prostate* **24**, 114–118.
76. McNeal, J. E., Haillot, O., and Yemoto, C. (1995). Cell proliferation in dysplasia of the prostate: analysis by PCNA immunostaining. *Prostate* **27**, 258–268.
77. Ohlson, N., Wikstrom, P., Stattin, P., and Bergh, A. (2005). Cell proliferation and apoptosis in prostate tumors and adjacent non-malignant prostate tissue in patients at different time-points after castration treatment. *Prostate* **62**, 307–315.
78. van der Kwast, T. H., Tetu, B., Suburu, E. R., Gomez, J., Lemay, M., and Labrie, F. (1998). Cycling activity of benign prostatic epithelial cells during long-term androgen blockade: evidence for self-renewal of luminal cells. *J. Pathol.* **186**, 406–409.
79. Bowen, C., Bubendorf, L., Voeller, H. J., et al. (2000). Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression [In Process Citation]. *Cancer Res.* **60**, 6111–6115.
80. Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., et al. (1999). Roles for Nkx3.1 in prostate development and cancer. *Genes Dev.* **13**, 966–977.
81. Xu, L. L., Srikantan, V., Sesterhenn, I. A., et al. (2000). Expression profile of an androgen regulated prostate specific homeobox gene NKX3.1 in primary prostate cancer. *J. Urol.* **163**, 972–979.
82. Ornstein, D. K., Cinquanta, M., Weiler, S., et al. (2001). Expression studies and mutational analysis of the androgen regulated homeobox gene NKX3.1 in benign and malignant prostate epithelium. *J. Urol.* **165**, 1329–1334.
83. Korkmaz, C. G., Korkmaz, K. S., Manola, J., et al. (2004). Analysis of androgen regulated homeobox gene NKX3.1 during prostate carcinogenesis. *J. Urol.* **172**, 1134–1139.
84. Douglas, C., Gage, W. R., Bieberich, C. J., and De Marzo, A. M. (2006). Decreased nkx 3.1 protein expression in atrophy, high grade PIN and adenocarcinoma of the prostate association with Gleason Score and Chromosome 8p deletion. *Cancer Res.* **66**, 10,683–10,690.
85. Guo, Y. P., Sklar, G. N., Borkowski, A., and Kyprianou, N. (1997). Loss of the cyclin-dependent kinase inhibitor P27(Kip1) protein in human prostate cancer correlates with tumor grade. *Clin. Cancer Res.* **3**, 2269–2274.
86. De Marzo, A. M., Meeker, A. K., Epstein, J. I., and Coffey, D. S. (1998). Prostate stem cell compartments: expression of the cell cycle inhibitor p27Kip1 in normal, hyperplastic, and neoplastic cells. *Am. J. Pathol.* **153**, 911–919.
87. Cheville, J. C., Lloyd, R. V., Sebo, T. J., et al. (1998). Expression of p27kip1 in prostatic adenocarcinoma. *Mod. Pathol.* **11**, 324–328.
88. Cordon-Cardo, C., Koff, A., Drobnjak, M., et al. (1998). Distinct altered patterns of p27KIP1 gene expression in benign prostatic hyperplasia and prostatic carcinoma. *J. Natl. Cancer Inst.* **90**, 1284–1291.
89. Cote, R. J., Shi, Y., Groshen, S., et al. (1998). Association of p27Kip1 levels with recurrence and survival in patients with stage C prostate carcinoma. *J. Natl. Cancer Inst.* **90**, 916–920.
90. Fernandez, P. L., Arce, Y., Farre, X., et al. (1999). Expression of p27/Kip1 is down-regulated in human prostate carcinoma progression. *J. Pathol.* **187**, 563–566.
91. Tsihlias, J., Kapusta, L. R., DeBoer, G., et al. (1998). Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma. *Cancer Res.* **58**, 542–548.
92. Yang, R. M., Naitoh, J., Murphy, M., et al. (1998). Low p27 expression predicts poor disease-free survival in patients with prostate cancer. *J. Urol.* **159**, 941–945.
93. Garraway, L. A., Lin, D., Signoretti, S., et al. (2003). Intermediate basal cells of the prostate: in vitro and in vivo characterization. *Prostate* **55**, 206–218.
94. Cookson, M. S., Reuter, V. E., Linkov, I., and Fair, W. R. (1997). Glutathione S-transferase PI (GST-pi) class expression by immunohistochemistry in benign and malignant prostate tissue. *J. Urol.* **157**, 673–676.
95. Lee, W. H., Morton, R. A., Epstein, J. I., et al (1994). Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc. Natl. Acad. Sci. USA* **91**, 11,733–11,737.
96. Moskaluk, C. A., Duray, P. H., Cowan, K. H., Linehan, M., and Merino, M. J. (1997). Immunohistochemical expression of pi-class glutathione S-transferase is down-regulated in adenocarcinoma of the prostate. *Cancer* **79**, 1595–1599.
97. Parsons, J. K., Nelson, C. P., Gage, W. R., Nelson, W. G., Kensler, T. W., and De Marzo, A. M. (2001). GSTA1 expression in normal, preneoplastic, and neoplastic human prostate tissue. *Prostate* **49**, 30–37.
98. Zha, S., Gage, W. R., Sauvageot, J., et al. (2001). Cyclooxygenase-2 is up-regulated in proliferative inflammatory atrophy of the prostate, but not in prostate carcinoma. *Cancer Res.* **61**, 8617–8623.

99. Naslund, M. J., Strandberg, J. D., and Coffey, D. S. (1988). The role of androgens and estrogens in the pathogenesis of experimental nonbacterial prostatitis. *J. Urol.* **140**, 1049–1053.
100. Sharma, O. P., Adlercreutz, H., Strandberg, J. D., Zirkin, B. R., Coffey, D. S., and Ewing, L. L. (1992). Soy of dietary source plays a preventive role against the pathogenesis of prostatitis in rats. *J. Steroid Biochem. Mol. Biol.* **43**, 557–564.
101. Prins, G. S. (1992). Neonatal estrogen exposure induces lobe-specific alterations in adult rat prostate androgen receptor expression. *Endocrinology* **130**, 2401–2412.
102. Billis, A. and Magna, L. A. (2003). Inflammatory atrophy of the prostate. Prevalence and significance. *Arch. Pathol. Lab. Med.* **127**, 840–844.

3

Tissue Microarrays in Prostate Cancer Research

Milton W. Datta and André A. Kajdacsy-Balla

Summary

The goal of translating basic findings in prostate cancer research into clinically applicable biomarker tests and therapeutics has led to an increased need and use of prostate cancer tissue specimens. Many of the needs in these studies can be met through the use of archived prostate tissue specimens placed in tissue microarrays. In this chapter, we describe the development and use of tissue microarrays. Issues related to tissue microarray construction and design, with a specific emphasis on prostate cancer, are reviewed and discussed. We also examine the different types of tissue microarray designs, including general tissue microarrays, progression tissue microarrays, and outcomes-based tissue microarrays. Current and developing methods in tissue microarray analysis, including archived imaging and automated image analysis are described. Finally, we examine the statistical analyses needed for the optimal use of the associated tissue microarray data. Scientists will gather an understanding of the uses and limitations of tissue microarrays for prostate cancer studies from this chapter.

Key Words: Image analysis; pathology; prostate tissues; statistics; tissue microarray.

1. INTRODUCTION

The emphasis on translational cancer research has led to a dramatic increase in research on patient tissue samples. The initial wave of tissue-based studies used tumor samples for large-scale genomic- and proteomic-based biomarker identification programs. The completion of many of these experiments has provided many investigators with sets of genes that need refined tissue studies for biomarker validation. In addition, genes and pathways discovered through basic research on cancer cell lines and animal models use tissue specimens for validation of experimental data. Yet, the amount of cancer tissue available for research remains limited. The use of less-invasive biopsy techniques has led to smaller tissue samples from patients, and the development of alternative therapies, such as radiation or chemotherapy, has led to decreasing numbers of patients having definitive surgery for their tumors. Thus, there is a need for techniques that optimize the use of small specimens. Tissue microarray technologies have been developed to address these specific concerns *(1)*. Through the use of tissue microarrays, small portions of patient tissue samples can be used in the study of genes and proteins involved in cancer biology, thus, conserving the amounts of available patient tissues. The small size of the tissues means that multiple samples can be placed on a single glass microscope slide and examined with small quantities of antibodies or probes. Subsequent experimental data is then analyzed with respect to patient demographic, clinical, pathological, and outcomes data with sufficient statistical power to infer clinical significance of the genes and proteins being studied. In this chapter, we will provide an overview of tissue microarray technology, and discuss advantages and issues in its use, with specific emphasis on prostate cancer research.

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

2. ARCHIVED PATIENT SPECIMENS: A REPOSITORY FOR BIOMEDICAL RESEARCH

Tissue microarray technology is predicated on the large repositories of archived patient specimens present in most hospitals and academic medical centers. These specimens represent residual patient tissues that have been fixed in formaldehyde, processed, and embedded into paraffin blocks. After the preparation of glass slides for clinical diagnosis, the remaining tissues are stored in hospitals for a minimum of 5 years, and then discarded because of the high cost of storage. Yet, in many academic medical centers, these archived tissue blocks are stored for many years (often back to the 1940s or earlier) and represent an invaluable repository of pathological disease specimens. If approval is received from the patients through the human subjects research committee or institutional review board, then biomedical researchers can collect hundreds to thousands of tumor specimens and study them for a given gene or protein. Additional clinical and outcomes history can be obtained either through the patient medical record or by contacting the patient. Many of the specimens were collected over years and represent patients with varying outcomes, therefore, allowing experimental data to be correlated with patient outcomes and treatment response. Thus, these archived specimen repositories represent a great resource for biomarker validation studies. The specimens are often small and limited, therefore, the judicious use of the materials for research is paramount. A typical prostate surgery specimen in a paraffin block may measure only 1.5 × 1.5 × 0.2 cm, and, thus, studies that require large amounts of tissue cannot be typically performed. A diagnostic needle prostatic biopsy is much smaller, with enough tissue to prepare only 5 to 10 microscopy slides Techniques have been optimized for the isolation of small amounts of DNA and its subsequent PCR amplification for genetic mapping or sequencing *(2,3)*, or the amplification of fragments of RNA for RT-PCR-based sequencing or expression analysis *(4–6)*. The most common use has been the preparation of individual slides from the tissues for immunohistochemistry or *in situ* hybridization studies. In each case, although the studies are feasible, they use large amounts of the specimen archive, and, thus, limit the number of future studies that can be performed. Methods that can extend the life of the specimen archive while allowing for the continued use of specimens in biomedical research are of utmost value.

3. TISSUE MICROARRAY DEVELOPMENT

Tissue microarrays were first developed by Kononen *(1)* and were demonstrated in the use of breast cancer tissues. The concept was to use a small representative portion of the patient specimen for a research study, thus, conserving the remaining specimen for future studies. In the process, a cylindrical core is removed from the specimen block and placed in a second paraffin (array) block. Preliminary studies have suggested that four to five cores provide representative data on par with traditional whole-slide sections, although differences with respect to tissue and tumor type still need to be evaluated. This is discussed in detail for prostate cancer studies. Similarly, cores are taken from other specimen blocks and placed in the array block following a grid format (Fig. 1). In this tissue microarray block, one also includes appropriate positive and negative control tissues. Using these techniques, and by taking small tissue cores of 0.6 to 2.0 mm in diameter, up to 1000 tissue cores can be placed in a single paraffin array block. Once completed, the array block is then cut to prepare slides for studies. Each slide contains circular pieces of tissue from each patient core arranged in the grid format (Fig. 2). The slide can then be stained, used for immunohistochemistry, *in situ* hybridization, or other studies. The presence of all of the tissue samples on a single slide provides uniformity to the staining of samples. Analysis is based on the evaluation of each individual tissue piece using a microscope and a corresponding slide map and spreadsheet (Fig. 3), with correlation of the results to the initial patient sample and (if available) patient data. In this way, a single slide can be used to study multiple patient samples with a single antibody or probe.

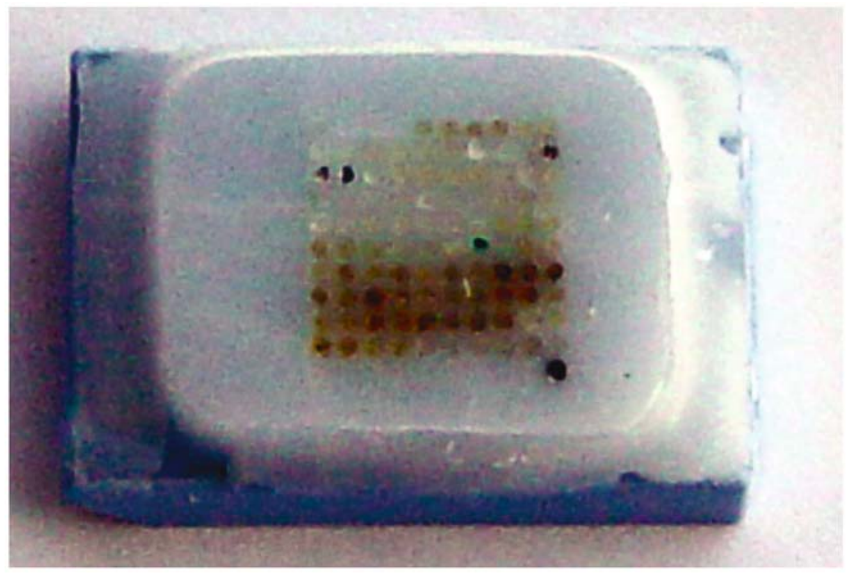


Fig. 1. Tissue microarray block.

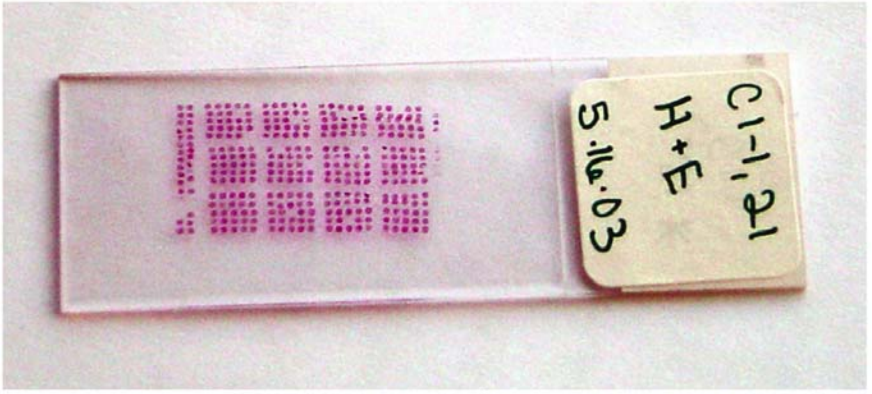


Fig. 2. Tissue microarray slide.

4. MAKING TISSUE MICROARRAYS

The technology for making tissue microarrays has been automated *(7,8)*, and many pathology departments have access to both manual and automated equipment for the production of tissue microarrays from specimen block archives. Commercially available patented equipment can be obtained from Beecher Instruments (Sun Prairie, WI; http://www.beecher.com), Chemicon (Temecula, CA), and others. Articles providing detailed guides for tissue microarray production are also available *(9,10)*. Key components are the presence of archived specimens, human subjects or institutional review board approval, and histology and pathology support. Most of the technical aspects associated with tissue microarray production and use include the manipulation of specimen paraffin blocks, slide preparation and staining, immunohistochemistry, and *in situ* hybridization. These skills are usually within the skill set of a well-trained histology technician. Collaboration with a trained his-

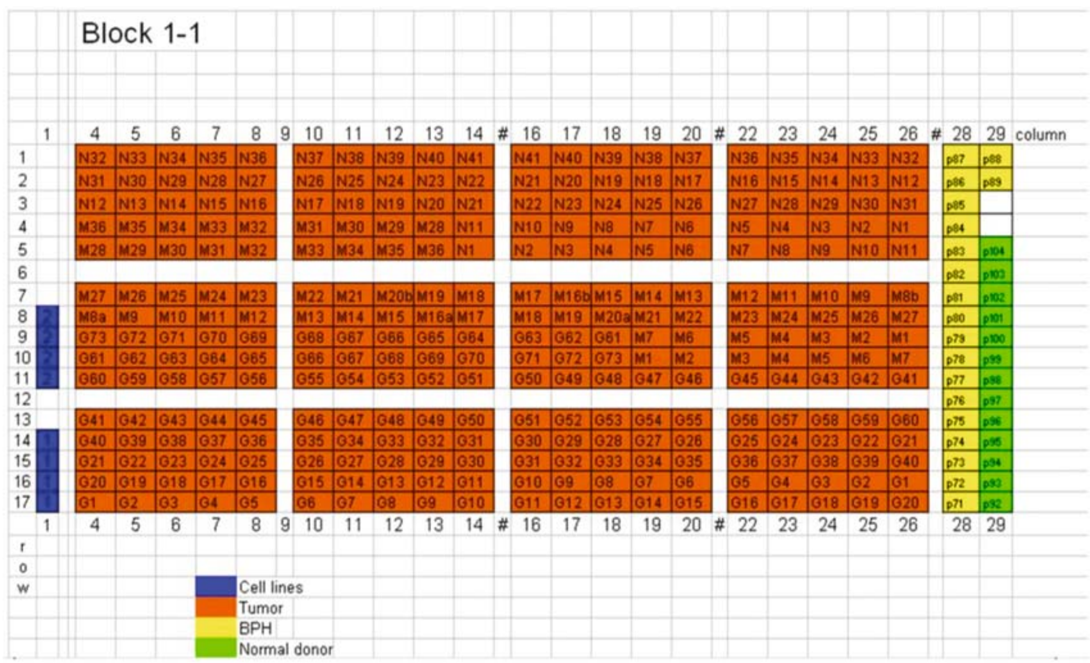


Fig. 3. Tissue microarray map. BPH, benign prostatic hyperplasia.

tologist and pathologist is crucial in determining whether the portion of the archived specimen block being used for the tissue microarray is present and representative of the area or interest (tumor, normal tissue, etc.). They can also help in the subsequent interpretation of the immunohistochemical results. In our experience, specimen preparation (identification of archived specimen blocks, and marking of the blocks for the histology technician so they know where on the block they should remove a core of tissue) is the most time-consuming component of the tissue microarray construction process and can take days to weeks of dedicated personnel time. Once this is completed, the physical production of a tissue microarray can be performed in as little as 2 or 3 days. The cutting and storage of tissue microarray slides has been an area of disagreement, with some advocating the storage of intact array blocks and fresh cutting of "as needed" slides, whereas others have recommended the cutting of slides and subsequent storage *(11)*. Cutting slides on an "as needed" basis results in the loss of material from the tissue microarray blocks, because each time the block must be realigned on the microtome and several sections cut before a suitable tissue microarray slide can be prepared. The storage of the cut slides has ranged from vacuum dessicator storage at 4°C or desiccator storage in the dark to paraffin coating of slides and dessicator storage in the dark. Although some studies have shown a dramatic difference in antigen preservation on tissue microarray slides preserved during 3 months *(11)*, our recent studies comparing these methods has shown no significant differences compared with freshly cut slides for the androgen receptor, p53, p27, and high molecular weight cytokeratin (A.A. Kajdacsy-Balla and M.W. Datta, unpublished observations). Because we prepare and ship large numbers of tissue microarray slides, we currently store all tissue microarray slides in a vacuum dessicator at 4°C.

For biomedical researchers who do not wish to construct their own tissue microrarrays, several companies have commercially available tissue microarray slides (please see list of many of these companies at the end of this chapter, Subheading 12.). The National Cancer Institute has sponsored multi-institutional cooperative groups to make tissue microarrays from prostate, breast, and other specimen archives (*see* Subheading 12.). Specifically, information regarding prostate tissue

microarrays prepared by the Cooperative Prostate Cancer Tissue Resource is available at its website (www.prostatetissues.org). Tissue microarrays have mainly been used for immunohistochemical studies of potential biomarkers. Additional studies using *in situ* hybridization have also been successful *(12,13)*. Immunohistochemical techniques for use on tissue microarray slides are essentially the same as those used for standard specimen slides, and require the availability of an antibody that will work in formalin-fixed paraffin-embedded tissues, previous optimization of antigen retrieval and antibody hybridization conditions, and a method for the interpretation of the subsequent staining data. Similar uses and precautions apply to the use of tissue microarrays for *in situ* hybridization.

There are key differences that need to be taken into account when using tissue microarrays as opposed to conventional specimen slides. Because the specimens come from different archived paraffin blocks, there may be differences in the fixation, processing, or storage of the materials. This may lead to differences in staining across the cores of a tissue microarray. Often these differences are small, but, whenever possible, specimen core staining should be checked by staining a separate tissue microarray slide with a well-characterized antibody to identify fixation heterogeneity. It is often recommended that tissue microarray data be validated on a subset of the tissues represented on the tissue microarray by repeating the staining on whole-specimen slides. Care must also be taken in correlating the staining of individual tissue pieces with the correct patient data, and often involves the use of spreadsheets and databases *(14–18)*. In addition, the small size of the tissue piece often means that representational bias may be present in the data. The small piece of tissue may not be representative of the entire tumor, or the degree of heterogeneity of the tumor may not be represented by a single small piece of tissue. Alternatively, during the experimental study, the small piece of tissue from a given patient may be lost. These caveats are often overcome by the use of multiple (three to five) tissue pieces from a single patient specimen to guarantee that the representative nature of the tumor is present (*see* Subheading 5.).

5. TISSUE MICROARRAYS IN PROSTATE CANCER RESEARCH

Many of the initial studies that use tissue microarrays have been performed on prostate cancer tissue *(12,19–39)*, and, therefore, much has been learned regarding optimizing tissue microarray production and use for prostate cancer research. This is reflected in the large number of prostate cancer tissue microarrays in production or already developed. In each case, the tissue microarrays address different scientific questions related to prostate cancer, and, therefore, have been designed to answer such questions. During the preparation and use of tissue microarrays for prostate cancer, specific issues related to this tumor type have been identified and addressed. For example, the architectural features of prostate cancer and the tendency to infiltrate between normal prostate gland tissues results in most cores being composed of both normal and cancer cells. This also makes it difficult for the technician to take cores from an area of tumor and guarantee that tumor is present on each small tissue core. They may have inadvertently taken an area of normal prostate within the tumor nodule, or the portion of prostate cancer may disappear after multiple slides have been prepared from the tissue core. This has led some investigators to question the value of tissue microarrays, in particular for biomarkers that require quantitative data, such as p53 staining *(40)*. However, others have clearly demonstrated that well-designed tissue microarrays can be used for quantitative biomarker studies *(41–44)*. These issues have been addressed by two approaches:

1. Larger tissue cores of up to 2.0 mm are taken from the tumor nodule.
2. Multiple small cores of 0.6 mm are taken from a single tumor nodule.

In both cases, the specimens on the tissue microarray slide become more representative of the original specimen. During staining, some core pieces may be lost from the slide, therefore most tissue microarrays use multiple tissue cores from the same patient tumor on the array. Initial studies have suggested that four to five cores from a single patient specimen may provide sufficient representation of a patient prostate cancer *(43)*. To further improve the chances that tissues from a single patient are

not completely lost during slide preparation and staining, the multiple tissue cores are spread over different tissue microarray blocks, thus, guaranteeing that a completed experiment will have sufficient material for evaluation. Quality control is performed, with selected slides (often every 10th or 20th) stained and evaluated for the presence of tissue and tumor *(45,46)*. Using these techniques, up to 200 high-quality tissue microarray slides can be produced from limited specimen stores. Examples of different types of arrays are presented in the following sections.

6. THE GENERAL TISSUE OR TUMOR MICROARRAYS

For most prostate cancer research, the initial question is often “does my gene/protein of interest have any significance in cancer?” This is, in essence, two questions:

1. Where is this gene/protein found?
2. Is this gene/protein involved in cancer?

To answer the first question, one will often wish to examine the biomarker in a series of different tissues to understand the specificity of expression. This is the ideal use of a tissue microarray, which allows one to survey a large number of tissues on a single slide. Similarly, the use of a general cancer tissue microarray that is composed of many different types of cancers will provide the biomedical researcher with a sense of the types of tumors in which the biomarker may be present, thus, providing tumor specificity. This has been effectively used in numerous publications *(1,47,48)*. No associated patient data is needed, because the simple question of biomarker presence or absence in tissues is being addressed. Both of these types of arrays are readily available through commercial vendors (*see* Subheading 12.). Data analysis is usually limited to the qualitative or semiquantitative expression and correlation with various tissue or tumor types. Statistical analysis is usually limited to simple χ^2 or Fisher’s exact tests because of the small sample size. However, from these studies, a biomedical researcher can get an idea of the degree to which their biomarker is tumor or tissue specific.

7. PROSTATE CANCER PROGRESSION TISSUE MICROARRAYS

There has been a strong scientific interest in the stepwise progression of prostate cancer from precancerous high-grade prostatic intraepithelial neoplasia to invasive prostate cancer to metastatic prostate cancer. Biomarkers that can be shown to mark various stages of tumor progression are of interest both for prostate cancer research and to predict patient progression and possibly determine the timing of treatment intervention. Tissue microarrays have been designed for the validation of biomarkers of prostate cancer disease progression, centering around two general formats:

1. The normal to high-grade prostatic intraepithelial neoplasia to invasive cancer progression.
2. The normal to cancer to metastatic cancer progression.

The first type contains samples from nonneoplastic prostate, high-grade prostatic intraepithelial neoplasia, and invasive prostate cancer (confined to the prostate), and examines the transition to invasive tumor. This allows for the identification of biomarkers that predict the earliest shift to invasive tumor, and, thus, could provide biomarkers for use in chemopreventive studies. Examples of these types of arrays have been extensively used in prostate cancer studies *(21,33–35,49)*. The second type of progression array tracks tumors from invasive clinically localized cancers to metastatic prostate cancer. Biomarkers tested in this format focus on genes that predict tumor spread, and, thus, would help target aggressive therapy in patients likely to die of their disease. Because of the difficulty in obtaining specimens from tumor metastases, these tissue microarrays are more difficult to construct, but have been used in prostate cancer studies *(12,32,50,51)*. In both cases, the tissue microarrays may come with associated patient outcomes data and can be analyzed using qualitative or semiquantitative methods and simple statistical associations. The identification of a statistical association with disease progression is taken as transitive evidence of biomarker correlation with disease progression and outcome. This is because the advanced disease state is a known marker of

poor outcome. Yet, when one identifies a biomarker that is associated with specific stages of disease progression, this does not indicate that the biomarker is an independent predictor of patient outcome, and, therefore, additional studies are still needed to validate that biomarker.

8. PROSTATE CANCER OUTCOME TISSUE MICROARRAYS

Once a biomarker is associated with prostate cancer, the ability of the biomarker to provide additional prognostic data is often desired. In this situation, the biomarkers are tested on tissue microarrays that contain a large number of prostate cancers with associated pathological and outcomes data. Using an outcomes-based tissue microarray, one compares the expression of a marker against the patient's final clinical outcome (prostate-specific antigen recurrence, development of metastatic disease, or death from tumor). Alternatively, a biomarker can detect patients with high probability of early or rapid recurrence, even in a disease in which most patients have a recurrence, such as hepatocellular or pancreatic carcinoma. In the literature, most outcomes-based prostate cancer tissue microarrays are mixed outcomes and progression arrays and typically contain a series of prostate cancers and associated groups of nonmalignant prostate tissue. These mixed arrays are the most commonly used tissue microarrays *(19,23–26,28,31,38,40,50,52–56)*. There are different types of nonmalignant prostate tissues, which may range from nodular hyperplasia (benign prostatic hypertrophy) to histologically normal prostate tissue from prostates isolated from organ donors. Some tissue microarrays also provide positive control cores that are composed of formalin-fixed pellets of human prostate cancer cell lines *(46)*. The tumors cores are typically taken from radical prostatectomy specimens and represent the dominant tumor nodule from the prostate. These tissue microarrays typically contain 190 to 550 patients and the associated controls. The most important component is the associated patient data, which can vary from simple Gleason score to detailed patient clinical, demographic, pathological, and outcomes information. These data are usually supplied in a Microsoft Excel file format, although a recently released xml file format for tissue microarray data exchange is gaining popularity for data standardization and transfer *(57,58)*. General prostate cancer tissue microarrays are currently available through collaboration with the National Cancer Institute's (NCI) Prostate Specialized Program in Research Excellence (SPORE) program or the NCI Cooperative Prostate Cancer Tissue Resource. Similar to general tissue and tumor arrays, data analysis is limited to qualitative and semiquantitative means, and simple statistical correlations can be made to the associated patient data. Although these results will provide a general idea of the role of a given biomarker in prostate cancer, the use of a general prostate cancer tissue microarray is best for the generation of clinically significant hypotheses and to provide initial frequency data for a subsequent large-scale case–control or cohort prospective validation study.

9. SPECIALIZED PROSTATE CANCER TISSUE MICROARRAYS

Once the general importance of a biomarker has been established for prostate cancer, there is often interest in further analyzing the biomarker in subsets of prostate cancer patients. For example, if an association is found between a biomarker and the Gleason grade of prostate cancer, further studies may be performed using tissue microarrays that contain specific Gleason grade tumors, thus, further defining the relationship between the biomarker and Gleason grade. Alternatively, there may be a significant scientific effort to identify biomarkers that relate to a clinically significant issue in prostate cancer (metastatic tumor potential or hormone response, for example), and, thus, only biomarkers that can be validated for that hypothesis are studied. In these cases, a carefully designed case–control tissue microarray can be used to support or refute the previous findings. Examples of such specific tissue microarrays that have been made or are in production are listed in Table 1. In many of the specialty arrays, a case–control design has been used with attempts made to maximize the statistical power of the array while working within the constraints of specimen availability and space on the array block.

Table 1
Specialized Prostate Cancer Tissue Microarrays

Array type	References
Metastatic prostate cancer array	*(51,73)*
Gleason grade array	*(13,46)*
Androgen status array	*(36,39)*
Ethnicity array	*(20,46)*
Perineural invasion	*(30)*

10. ADDITIONAL TYPES OF TISSUE MICROARRAYS

Tissue microarray technology has now been used for longer than 5 years, and during this time we have begun to see translation of the technology for other applications. Some of these are of direct benefit to prostate cancer research, and include the production of tissue microarrays from frozen tissues, cell line samples, animal tumors or tumor xenografts, prostate needle biopsies, and specimens collected as a part of clinical trials.

Many antibodies will not stain formalin-fixed paraffin embedded tissues, but will work on frozen tissue sections. Although technically challenging, tissue microarrays have been designed using frozen tissues *(59,60)*. This also relies on the presence of a large series of banked frozen prostate specimens, something that is not routinely stored in most hospitals. Many academic centers have been collecting frozen prostate cancer specimens, and their availability facilitates the production of these arrays *(46)*. The key is the ability to maintain the frozen specimens while constructing the tissue microarray block. If successful, such arrays open up a large number of experimental antibodies for high-throughput studies. One of the limitations in the use of frozen tissue microarrays is the difficulty in determining whether the tissue available in a specific core contains benign or malignant components because of tissue freezing-based morphological artifacts.

The use of cell lines in tissue microarrays has been performed since 2001, with the cells placed on the tissue microarray as positive controls for the experimental tissues (Fig. 4) *(35,61–63)*. The cells are grown in culture and, when sufficient cells are present, they are collected and spun into 15-mL tubes to create a cell pellet. The pellet is hardened in gelatin and perfused with formalin. The fixed cell pellet is embedded in a paraffin block, which is used to generate cores for placement in the tissue microarray block. Sectioning of the tissue microarray block places a circle of cells on the slide, which is stained along with the adjacent tissue cores. The dedicated construction of cell line tissue microarrays has also been performed and can be used for the rapid evaluation of a biomarker across multiple cell lines *(61)*. Likewise, cells transfected or treated in multiple different ways can be rapidly assessed using a cell line tissue microarray.

Although tissue microarrays were first used for human specimens, the small nature of animal model specimens is ideally suited for tissue microarrays *(64,65)*. These samples can be used for both primary and metastatic tumors in genetically modified animals, but also for tumor xenografts and their associated metastatic tumor deposits isolated from different organ sites. In each of these tissue microarrays, multiple studies can be carried out on limited specimens.

The advent of improved radiologic imaging has allowed for extremely accurate image guided needle biopsies of suspicious lesions. In prostate cancer, small prostate needle biopsies may represent the only material available from a patient as they continue with definitive radiation or chemotherapy. Ongoing prostate cancer clinical trials to test chemopreventive nutrients or drugs or to evaluate tumor progression are based around the collection of prostate needle biopsies. We have developed a method whereby these specimens can be used in the production of tissue microarrays *(45)*. Although technically challenging, these microarrays can maximize the very limited material present in a prostate needle biopsy, such that multiple studies can be performed from the limited material.

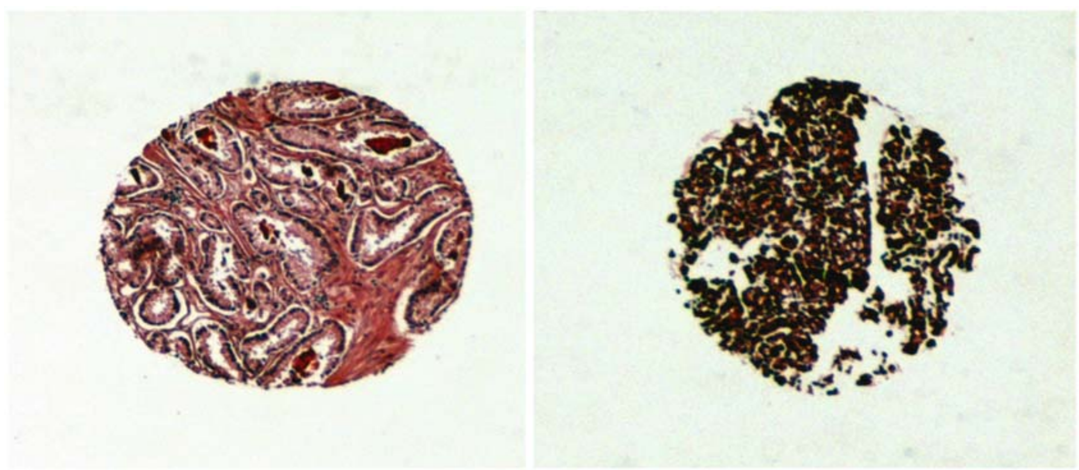


Fig. 4. Cell block from a tissue microarray.

11. ADVANCED ANALYSIS OF TISSUE MICROARRAY DATA

The majority of the analyses performed with tissue microarrays use single tissue microarray slides and a single biomarker. As the number of available tissue microarrays increases, scientists are running groups of biomarkers on sequential tissue microarray slides composed of the same specimens. This ability to associate specimen data to data from multiple biomarkers presents the opportunity to perform complex biomarker and molecular pathway analysis. From this, one can identify significant patterns of complex biomarker expression that predict patient outcome more accurately than any single biomarker. Likewise, a series of genes within a given pathway can be analyzed on tissue microarrays to identify the role of the pathway in prostate cancer progression or outcome. Such data might indicate that disruption of the pathway correlates with tumor progression, regardless of where the disruption occurs.

Although most immunohistochemical staining performed on tissue microarrays has been analyzed using qualitative or semiquantitative means, it is clear that some of the differences present for biomarkers may require quantitative methods that are more rigorous. This is particularly true for biomarker candidates identified through gene expression profiling methods, which emphasize statistically significant differences in transcript levels. These differences may translate to significant differences in protein expression that cannot be elucidated using the simple methods currently available for immunohistochemical analysis. During the last 10 years, there have been multiple efforts directed at the development of quantitative image analysis of immunohistochemical staining results *(66,67)*. This includes commercial products, such as the Automated Cellular Imaging System and Automated Quantitative Analysis systems *(32,68–70)*. These both provide some degree of automation and limit the required pathologist-specific dedicated time. The difficulty lies in the complex nature of the data. Within the tissue, the staining pattern depends on cell type identification, cellular and subcellular localization, staining intensity, and background staining intensity. For a prostate tissue, this may mean separating the tumor from the benign glands, counting the number of cells with appropriate subcellular localization of the protein, and calculating the differential staining intensity by subtracting the staining present in the surrounding stromal background. Only recently have we seen analysis systems that are sufficiently robust to handle such analyses, with some of the initial success occurring in prostate cancer studies *(14,71,72)*. This area will continue to develop during the next 5 years. However, even with a perfect image analysis system, there will be unavoidable variation in the preanalytical factors, such as duration and type of tissue fixation, tissue processing, age of the specimen, and uneven distribution of staining reagents from one core to another.

Table 2
Ready-to-Use Tissue Microarrays

Company or Organization	Web site	Description
Invitrogen	http://www.resgen.com	General tissues tissue microarrays
Imgenex	http://www.imgenex.com	General tissues and cancers tissue microarrays
Asterand	http://www.asterand.com	General tissues and cancers tissue microarrays
Lifespan Biosciences	http://www.lsbio.com	General tissues and cancers tissue microarrays
Deutsches Ressourcenzentrum fur Genomforschung GmbH	http://www.rzpd.de General tissues and cancers tissue microarrays	General tissues and cancers tissue microarrays
Chemicon	http://www.chemicon.com	
Telechem International Inc.	http://arrayit.com	General tissues, general cancers, and prostate cancer tissue microarrays, with pathological grade and stage
Folio	http://www.foliobio.com	General tissues, general cancers, and prostate cancer
progression tissue microarrays		
Nexgen Biosciences	http://www.nxgenbiosciences.com	Prostate cancer progression tissue microarrays
NCI Tissue Array Research Program	http://ccr.cancer.gov/tech_initiatives/tarp	General cancer and prostate cancer tissue microarrays, with some pathological data
NCI Cooperative Prostate Cancer Tissue Resource	http://www.prostatetissues.org	Prostate cancer outcomes-based, ethnicity-based, hormone refractory cases, metastasis cases, and Gleason grade tissue microarrays
NCI Cooperative Breast Cancer Tissue Resource	http://cbctr.nci.nih.gov	Breast cancer outcome and progression tissue microarrays

For the majority of current image analysis, quantitation can rely on the pathological interpretation of microarray data by pathologists. This often relies on the scanning of the tissue microarray slides and subsequent storage of the digital images on image servers for subsequent web-based viewing and scoring. Groups have developed a series of web-based software programs that allow the tissue microarray images to be broken into separate tissue cores, stored, and shared across institutions for review by panels of trained pathologists *(14,15)*. Commercial systems have also been developed or are in development. The subsequent scoring data can then be collated and analyzed to determine the reproducibility of the pathological staining interpretation. This ability to develop reproducible scoring systems for immunohistochemical staining is essential for the broad applicability of a tissue-based biomarker. Until robust and readily available methods for automated quantitative immunohistochemical interpretation are developed, shared scoring systems will be essential for the broad generalization of biomarker data from tissue specimens.

Automated and web-based analysis methods generate large amounts of data that need specialized databases for storage. Such systems have been developed and are in use, in particular, for prostate cancer *(15–18)*. These systems are helped by the development of general guidelines for tissue microarray data exchange that standardize data in xml format *(57,58)*. Such methods are already in use for the transfer of data associated with prostate cancer tissue microarrays developed by the NCI Cooperative Prostate Cancer Tissue Resource. As experimental data is collected from tissue microarrays, the ability to perform higher-level combinatorial analysis with other predictors of outcome (tumor grade and stage, and patient ethnicity or age) will become a reality. These new methods are expected to yield sets of biomarkers that will become the final panels for predicting patient outcome and treatment-based disease response. These studies will require the ongoing development of mathematical and statistical methods that are more robust, much of which is already occurring because of the explosion of gene expression microarray data during the last few years.

In summary, tissue microarrays have become a useful method for the generation of significant amounts of experimental data from limited specimen resources. This technique and its associated methodology provide the ability to rapidly determine the sensitivity and specificity of a potential prostate cancer biomarker. Once this preliminary data is generated, more-detailed prospective case–control studies can be defined based on the initial data generated from tissue microarrays. Additional tissue microarrays may also be used as validation sets for tissue biomarker discovery at this time. As methods such as automated image analysis and data sharing are developed, combinatorial analysis will be possible. These analyses will link to patient demographic, clinical, pathological, and outcomes data, allowing for the development of tailored biomarker panels that predict patient response to therapy or outcome.

12. READY-TO-USE TISSUE MICROARRAYS

For investigators who do not have access to local specimen archives, both commercial and publicly supported sources of tissue microarrays exist and provide tissue microarrays for a set cost. These resources often are the easiest source of general tissue microarrays, and can provide additional specific tissue microarrays for different communities. These resources are listed in Table 2.

REFERENCES

1. Kononen, J., Bubendorf, L., Kallioniemi, A., et al. (1998). Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med.* **4(7)**, 844–847.
2. Blomeke, B., Bennett, W. P., Harris, C. C., and Shields, P. G. (1997). Serum, plasma and paraffin-embedded tissues as sources of DNA for studying cancer susceptibility genes. *Carcinogenesis* **18(6)**, 1271–1275.
3. Neubauer, A., Neubauer, B., He, M., et al. (1992). Analysis of gene amplification in archival tissue by differential polymerase chain reaction. *Oncogene* **7(5)**, 1019–1025.
4. Mies, C. (1994). A simple, rapid method for isolating RNA from paraffin-embedded tissues for reverse transcription-polymerase chain reaction (RT-PCR). *J. Histochem. Cytochem.* **42(6)**, 811–813.

5. Godfrey, T. E., Kim, S. H., Chavira, M., et al. (2000). Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. *J. Mol. Diagn.* **2(2)**, 84–91.
6. Bledsoe, B., Groshart, K., Zhang, Q., Quasney, M., and Dosanjh, A. (2004). The microextraction of RNA from archival cardiac allografts embedded in paraffin. *Clin. Transplant.* **18(5)**, 591–595.
7. Kallioniemi, O. P., Wagner, U., Kononen, J., and Sauter, G. (2001). Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum. Mol. Genet.* **10(7)**, 657–662.
8. Packeisen, J., Buerger, H., Krech, R., and Broecker, W. (2002). Tissue microarrays: a new approach for quality control in immunohistochemistry. *J. Clin. Pathol.* **55(8)**, 613–615.
9. Fedor, H. L. and De Marzo, A. M. (2005). Practical methods for tissue microarray construction. *Methods Mol. Med.* **103**, 89–101.
10. Jensen, T. A. and Hammand, M. E. H. (2001). The tissue microarray—a technical guide for histologists. *J. Histotech.* **24**, 283–287.
11. DiVito, K. A., Charette, L. A., Rimm, D. L., and Camp, R. L. (2004). Long-term preservation of antigenicity on tissue microarrays. *Lab. Invest.* **84(8)**, 1071–1078.
12. Bubendorf, L., Kononen, J., Koivisto, P., et al. (1999). Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. *Cancer Res.* **59(4)**, 803–806.
13. Skacel, M., Ormsby, A. H., Pettay, J. D., et al. (2001). Aneusomy of chromosomes 7, 8, and 17 and amplification of HER-2/neu and epidermal growth factor receptor in Gleason score 7 prostate carcinoma: a differential fluorescent in situ hybridization study of Gleason pattern 3 and 4 using tissue microarray. *Hum. Pathol.* **32(12)**, 1392–1397.
14. Bova, G. S., Parmigiani, G., Epstein, J. I., Wheeler, T., Mucci, N. R., and Rubin, M. A. (2001). Web-based tissue microarray image data analysis: initial validation testing through prostate cancer Gleason grading. *Hum. Pathol.* **32(4)**, 417–427.
15. Coombes, K. R., Zhang, L., Bueso-Ramos, C., et al. (2002). TAD: a web interface and database for tissue microarrays. *Appl. Bioinformatics* **1(3)**, 155–158.
16. Manley, S., Mucci, N. R., De Marzo, A. M., and Rubin, M. A. (2001). Relational database structure to manage high-density tissue microarray data and images for pathology studies focusing on clinical outcome: the prostate specialized program of research excellence model. *Am. J. Pathol.* **159(3)**, 837–843.
17. Shaknovich, R., Celestine, A., Yang, L., and Cattoretti, G. (2003). Novel relational database for tissue microarray analysis. *Arch. Pathol. Lab. Med.* **127(4)**, 492–494.
18. Sharma-Oates, A., Quirke, P., and Westhead, D. R. (2005). TmaDB: a repository for tissue microarray data. *BMC Bioinformatics* **6(1)**, 218.
19. Bowen, C., Bubendord, L., Voeller, H. J., et al. (2000). Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. *Cancer Res.* **60(21)**, 6111–6115.
20. Perrone, E. E., Theoharis, C., Mucci, N. R., et al. (2000). Tissue microarray assessment of prostate cancer tumor proliferation in African- American and white men. *J. Natl. Cancer Inst.* **92(11)**, 937–939.
21. Chaib, H., Rubin, M. A., Mucci, N. R., et al. (2001). Activated in prostate cancer: a PDZ domain-containing protein highly expressed in human primary prostate tumors. *Cancer Res.* **61(6)**, 2390–2394.
22. Rubin, M. A., Mucci, N. R., Figurski, J., Fecko, A., Pienta, K. J., and Day, M. L. (2001). E-cadherin expression in prostate cancer: a broad survey using high-density tissue microarray technology. *Hum. Pathol.* **32(7)**, 690–697.
23. Saramaki, O., Willi, N., Bratt, O., et al. (2001). Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. *Am. J. Pathol.* **159(6)**, 2089–2094.
24. Srivastava, M., Bubendorf, L., Srikantan, V., et al. (2001). ANX7, a candidate tumor suppressor gene for prostate cancer. *Proc. Natl. Acad. Sci. USA* **98(8)**, 4575–4580.
25. Ayala, G., Wang, D., Wulf, G., et al. (2003). The prolyl isomerase Pin1 is a novel prognostic marker in human prostate cancer. *Cancer Res.* **63(19)**, 6244–6251.
26. Freedland, S. J., Seligson, D. B., Liu, A. Y., et al. (2003). Loss of CD10 (neutral endopeptidase) is a frequent and early event in human prostate cancer. *Prostate* **55(1)**, 71–80.
27. Gelmann, E. P., Bowen, C., and Bubendorf, L. (2003). Expression of NKX3.1 in normal and malignant tissues. *Prostate* **55(2)**, 111–117.
28. Halvorsen, O. J., Haukaas, S. A., and Akslen, L. A. (2003). Combined loss of PTEN and p27 expression is associated with tumor cell proliferation by Ki-67 and increased risk of recurrent disease in localized prostate cancer. *Clin. Cancer. Res.* **9(4)**, 1474–1479.
29. Kiyama, S., Morrison, K., Zellweger, T., et al. (2003). Castration-induced increases in insulin-like growth factor-binding protein 2 promotes proliferation of androgen-independent human prostate LNCaP tumors. *Cancer Res.* **63(13)**, 3575–3584.
30. Li, R.,Wheeler, T., Dai, H., Ayala, G. (2003). Neural cell adhesion molecule is upregulated in nerves with prostate cancer invasion. *Hum. Pathol.* **34(5)**, 457–461.

31. Richardsen, E., Ukkonen, T., Bjornsen, T., Mortensen, E., Egevad, L., Busch, C. (2003). Overexpression of IGBFB2 is a marker for malignant transformation in prostate epithelium. *Virchows Arch.* **442(4)**, 329–335.
32. Faith, D. A., Isaacs, W. B., Morgan, J. D., et al. (2004). Trefoil factor 3 overexpression in prostatic carcinoma: prognostic importance using tissue microarrays. *Prostate* **61(3)**, 215–227.
33. Hofer, M. D., Kuefer, R., Varambally, S., et al. (2004). The role of metastasis-associated protein 1 in prostate cancer progression. *Cancer Res.* **64(3)**, 825–829.
34. Garraway, I. P., Seligson, D., Said, J., Harvath, S., and Reiter, R. E. (2004). Trefoil factor 3 is overexpressed in human prostate cancer. *Prostate* **61(3)**, 209–214.
35. Rehman, I., Azzouzi, A. R., Cross, S. S., et al. (2004). Dysregulated expression of S100A11 (calgizzarin) in prostate cancer and precursor lesions. *Hum. Pathol.* **35(11)**, 1385–1391.
36. Rocchi, P., So, A., Kojima, S., et al. (2004). Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. *Cancer Res.* **64(18)**, 6595–6602.
37. Rubin, M. A., Varambally, S., Beroukhim, R., et al. (2004). Overexpression, amplification, and androgen regulation of TPD52 in prostate cancer. *Cancer Res.* **64(11)**, 3814–3822.
38. Li, H., Zhang, Y., Glass, A., et al. (2005). Activation of signal transducer and activator of transcription-5 in prostate cancer predicts early recurrence. *Clin. Cancer Res.* **11(16)**, 5863–5868.
39. Titus, M. A., Gregory, C. W., Ford, O. H., Schell, 3rd, M. J., Maygarden, S. J., Mohler, J. L., et al. (2005). Steroid 5alpha-reductase isozymes I and II in recurrent prostate cancer. *Clin. Cancer Res.* **11(12)**, 4365–4371.
40. Merseburger, A. S., Kuczyk, M. A., Serth, J., et al. (2003). Limitations of tissue microarrays in the evaluation of focal alterations of bcl-2 and p53 in whole mount derived prostate tissues. *Oncol. Rep.* **10(1)**, 223–228.
41. Camp, R. L., Charette, L. A., and Rimm, D. L. (2000). Validation of tissue microarray technology in breast carcinoma. *Lab. Invest.* **80(12)**, 1943–1949.
42. Torhorst, J., Bucher, C., Kononen, J., et al. (2001). Tissue microarrays for rapid linking of molecular changes to clinical endpoints. *Am. J. Pathol.* **159(6)**, 2249–2256.
43. Rubin, M. A., Dunn, R., Strawderman, M., and Pienta, K. J., et al. (2002). Tissue microarray sampling strategy for prostate cancer biomarker analysis. *Am. J. Surg. Pathol.* **26(3)**, 312–319.
44. Nocito, A., Bubendorf, L., Tinner, E. Maria, et al. (2001). Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. *J. Pathol.* **194(3)**, 349–357.
45. Datta, M. W., Kahler, A., Macias, V., Brodzeller, T., and Kajdacsy-Balla, A. (2005). A simple inexpensive method for the production of tissue microarrays from needle biopsy specimens: examples with prostate cancer. *Appl. Immunohistochem. Mol. Morphol.* **13(1)**, 96–103.
46. Melamed, J., Datta, M. W., Becich, M. J., et al. (2004). The cooperative prostate cancer tissue resource: a specimen and data resource for cancer researchers. *Clin. Cancer Res.* **10(14)**, 4614–4621.
47. Frierson, H. F., Moskaluk, Jr., C. A., Powell, S. M., et al. (2003). Large-scale molecular and tissue microarray analysis of mesothelin expression in common human carcinomas. *Hum. Pathol.* **34(6)**, 605–609.
48. Reis-Filho, J. S., Simpson, P. T., Martins, A., Preto, A., Gartner, F., and Schmitt, F. C. (2003). Distribution of p63, cytokeratins 5/6 and cytokeratin 14 in 51 normal and 400 neoplastic human tissue samples using TARP-4 multi-tumor tissue microarray. *Virchows Arch.* **443(2)**, 122–132.
49. Tuxhorn, J. A., Ayala, G. E., Smith, M. J., Smith, V. C., Dang, T. D., and Rowley, D. R. (2002). Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin. Cancer Res.* **8(9)**, 2912–2923.
50. Zhou, M., Shah, R., Shen, R., and Rubin, M. A. (2003). Basal cell cocktail (34betaE12 + p63) improves the detection of prostate basal cells. *Am. J. Surg. Pathol.* **27(3)**, 365–371.
51. Assikis, V. J., Do, K. A., Wen, S., et al. (2004). Clinical and biomarker correlates of androgen-independent, locally aggressive prostate cancer with limited metastatic potential. *Clin. Cancer Res.* **10(20)**, 6770–6778.
52. Inoue, T., Segawa, T., Shiraishi, T., et al. (2005). Androgen receptor, Ki67, and p53 expression in radical prostatectomy specimens predict treatment failure in Japanese population. *Urology* **66(2)**, 332–337.
53. Zellweger, T., Ninck, C., Mirlacher, M., et al. (2003). Tissue microarray analysis reveals prognostic significance of syndecan-1 expression in prostate cancer. *Prostate* **55(1)**, 20–29.
54. Rubin, M. A., Zhou, M., Dhanasekaran, S. M., et al. (2002). alpha-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. *JAMA* **287(13)**, 1662–1670.
55. Mousses, S., Bubendorf, L., Wagner, U., et al. (2002). Clinical validation of candidate genes associated with prostate cancer progression in the CWR22 model system using tissue microarrays. *Cancer Res.* **62(5)**, 1256–1260.
56. Li, R., Younes, M., Frolov, A., et al. (2003). Expression of neutral amino acid transporter ASCT2 in human prostate. *Anticancer Res.* **23(4)**, 3413–3418.
57. Berman, J. J., Datta, M., Kajdacsy-Balla, A., et al. (2004). The tissue microarray data exchange specification: implementation by the Cooperative Prostate Cancer Tissue Resource. *BMC Bioinformatics* **5**, 19.
58. Berman, J. J., Edgerton, M. E., and Friedman, B. A. (2003). The tissue microarray data exchange specification: a community-based, open source tool for sharing tissue microarray data. *BMC Med. Inform. Decis. Mak.* **3**, 5.

59. Fejzo, M. S. and D. J. Slamon. (2001). Frozen tumor tissue microarray technology for analysis of tumor RNA, DNA, and proteins. *Am. J. Pathol.* **159(5)**, 1645–1650.
60. Karsten, S. L., Van Deerlin, V. M., Sabatti, C., Gill, L. H., and Geschwind, D. H., et al. (2002). An evaluation of tyramide signal amplification and archived fixed and frozen tissue in microarray gene expression analysis. *Nucleic Acids Res.* **30(2)**, E4.
61. Abbott, R. T., Tripp, S., Perkins, S. L., Elenitoba-Johnson, K. S., and Lim, M. S. (2003). Analysis of the PI-3-Kinase-PTEN-AKT pathway in human lymphoma and leukemia using a cell line microarray. *Mod. Pathol.* **16(6)**, 607–612.
62. Meeker, A. K., Gage, W. R., Hicks, J. L., et al. (2002). Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. *Am. J. Pathol.* **160(4)**, 1259–1268.
63. Zha, S., Gage, W. R., Sauvageot, J., et al. (2001). Cyclooxygenase-2 is up-regulated in proliferative inflammatory atrophy of the prostate, but not in prostate carcinoma. *Cancer Res.* **61(24)**, 8617–8623.
64. Tasheva, E. S., Ke, A., and Conrad, G. W. (2004). Analysis of the expression of chondroadherin in mouse ocular and non-ocular tissues. *Mol. Vis.* **10**, 544–554.
65. Xu, Z. R., Du, H. K., Wang, S. X., Liu, D. W., and Wang, J. (2005). [Effects of taurine on the expression of nitric oxide synthase in lung of rats exposed to silica]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* **23(2)**, 116–118.
66. Chen, W., Reiss, M., and Foran, D. J. (2004). A prototype for unsupervised analysis of tissue microarrays for cancer research and diagnostics. *IEEE Trans. Inf. Technol. Biomed.* **8(2)**, 89–96.
67. Chen, W., Foran, D. J., and Reiss, M. (2002). Unsupervised imaging, registration and archiving of tissue microarrays. *Proc. AMIA Symp.* 136–139.
68. Camp, R. L., Dolled-Filhart, M., King, B. L., and Rimm, D. L. (2003). Quantitative analysis of breast cancer tissue microarrays shows that both high and normal levels of HER2 expression are associated with poor outcome. *Cancer Res.* **63(7)**, 1445–1448.
69. Berger, A. J., Camp, R. L., Divito, K. A., Kluger, H. M., Halaban, R. and Rimm, D. L. (2004). Automated quantitative analysis of HDM2 expression in malignant melanoma shows association with early-stage disease and improved outcome. *Cancer Res.* **64(23)**, 8767–8772.
70. Harigopal, M., Berger, A. J., Camp, R. L., Rimm, D. L., and Kluger, H. M. (2005). Automated quantitative analysis of E-cadherin expression in lymph node metastases is predictive of survival in invasive ductal breast cancer. *Clin. Cancer Res.* **11(11)**, 4083–4089.
71. Haedicke, W., Popper, H. H., Buck, C. R., and Zatloukal, K. (2003). Automated evaluation and normalization of immunohistochemistry on tissue microarrays with a DNA microarray scanner. *Biotechniques* **35(1)**, 164–168.
72. Rubin, M. A., Zerkowski, M. P., Camp, R. L., et al. (2004). Quantitative determination of expression of the prostate cancer protein alpha-methylacyl-CoA racemase using automated quantitative analysis (AQUA): a novel paradigm for automated and continuous biomarker measurements. *Am. J. Pathol.* **164(3)**, 831–840.
73. Shah, R. B., Mehra, R., Chinnaiyan, A. M, et al. (2004). Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **64(24)**, 9209–9216.

4
Prostate (Cancer) Stem Cells

Jack A. Schalken

Summary

The prostate stem cell concept described in this chapter was studied in our laboratory, where we provided experimental immunophenotypic evidence of the presence of keratin, androgen receptor, neuroendocrine markers, and c-Met protooncogene in unique populations of normal developing and malignant human prostate epithelial cells. The characteristic responses of these stem cells to growth factors and neoadjuvant hormonal therapy supported the cancer stem cell concept, and, if successfully applied to human prostate cancer specimens, could reveal new therapeutic targets.

Key Words: Androgen receptor; cancer stem cells; c-Met protooncogene; hepatocyte growth factor/ scatter factor; HGF/SF; immunophenotype; keratin 14, 5, and 18; neuroendocrine factors; primary; recurrent and metastatic prostate cancer; serotonin and chromogranin A.

1. INTRODUCTION

Solid tumors are heterogeneous, typically containing varied populations of cells that differ in the specific proteins or phenotypic markers they express. The cancer stem cell (CSC) hypothesis suggests that neoplastic clones are maintained exclusively by a rare fraction of cells with stem cell properties *(1,2)*. As early as in 1976, Fialkow *(3–5)* identified the CSC for chronic myelocytic leukemia, and further evidence was obtained independently by others *(5)*. For leukemia, the CSC hypothesis is now generally accepted *(6)*. For solid tumors in general, significant progress has been made in recent years. A candidate CSC population was identified in breast cancer *(7–9)*. For brain tumor, initiating cells were isolated and characterized *(10,11)*. Singh and colleagues *(11)* described how they isolated a minority population of human brain cancer cells based on the expression of a cell surface marker called CD133. They report that, when injected into the brains of mice, this subpopulation of $CD133^+$ cells could, by itself, drive tumor growth and dissemination. As few as 100 of the $CD133^+$ cells formed tumors that could be serially transmitted from mouse to mouse, whereas tens of thousands of cancer cells lacking CD133 failed to do so. When tumors that arose from the injected $CD133^+$ cells were examined, the cellular heterogeneity and architecture closely resembled that of the human tumors from which the cells had originally been taken.

In the normal brain, neuronal stem cells as well as early progenitor cells, but not their fully mature progeny, express the CD133 marker. In the brain tumors examined, Singh et al. *(11)* found distinct subpopulations of cells that expressed either CD133 or various markers of mature brain cells. Thus, the cellular architecture of the brain tumors may be a caricature of that of the normal brain, with brain CSCs, probably derived from normal $CD133^+$ stem or progenitor cells, giving rise to aberrantly differentiated progeny.

Stem cells have two unique properties that make it likely that they are involved in cancer development. First, they are often the only long-lived cells that have the ability to replicate in a tissue. Mul-

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

tiple mutations, occurring over many years, are necessary before a cell becomes cancerous. Thus, the implication is that cancer-inducing mutations accumulate in the long-lived, normal stem cells. Second, through a process called self-renewal, stem cells generate new stem cells with similar proliferation and differentiation capacities as their parental cell. By contrast, with each round of replication, progenitor cells become progressively more differentiated and are eventually destined to stop proliferating. Predictably, self-renewal is an essential property of some cancer cells, and at least some genes that regulate normal stem cell self-renewal also do so in cancer cells *(12,13)*. There seem to be common signaling pathways implicated in stem cell expansion and CSC growth, such as wingless *(14,15)*, and hedgehog *(16)*; for a review see Rizvi *(17)*. Interestingly, Beachy and colleagues have recently provided evidence for hedgehog involvement in normal and malignant prostate development *(18,19)*. This suggests that cancers arise either from normal stem cells or from progenitor cells in which self-renewal pathways have become activated.

These observations provide a mechanism by which breast and prostate cancer patients with cancer cells in their marrow can remain progression-free for a prolonged time *(20,21)*. One explanation for tumor dormancy is that the microscopic clusters of cancer cells did not contain CSCs and therefore, like the $CD133^+$ brain cancer cells, were unable to grow further. Taken together with the observation that circulating cancer cells in the blood are an indicator of prognosis in breast cancer patients *(22,23)*, this suggests that the use of markers to reveal CSCs could help in making decisions regarding treatments. The identification of CSCs is a significant step in the fight against these dreaded diseases. Because self-renewal is essential if tumors are to grow, agents that target such cells may be effective treatments. A possible complication is that the mechanisms known to regulate CSC self-renewal also regulate the same process in normal stem cells. Unlike normal stem cells *(24,25)*, however, the expansion of CSCs is not tightly regulated, implying that there are significant differences between normal and cancerous self-renewal pathways. This gives hope that the isolation of CSCs, coupled with our knowledge of the mutations causing cancer, will result in ways to eliminate cancer cells while sparing normal tissues. *The identification and functional characterization of CSCs can contribute significantly to improved methods for prognosis, better treatment decisions, and new treatments for cancer.*

2. PROSTATE EPITHELIAL STEM CELLS

The existence of prostate epithelial stem cells and their putative role in prostate cancer development was proposed by Isaacs and Coffey *(26–28)*. The stem cell model described in this paper is very similar to the one described above. Prostate cancers arise from prostate secretory acini. These acini are characterized by two cell layers that can be discriminated morphologically as undifferentiated basal cells and luminal cells primarily composed of terminally differentiated exocrine (prostate-specific antigen producing) cells. The neuroendocrine cells are found “supra” basally, with protrusions through the epithelium. The first evidence for a hierarchical relation between the basal cells and the luminal cells was provided by our group *(29,30)*, using keratin antibodies as differentiation markers. We and others found further indications that the neuroendocrine and exocrine cells have a common progenitor, termed the transiently amplifying (TRANSIT) cell *(31–34)*.

Clearly, most of these studies are descriptive and enable discrimination between the various cell types based on specific immunophenotypes. The location of the cells, as well as hormone manipulation studies *(30)*, suggest a hierarchical relation between the basal cells and the luminal cells. The early and late progenitors are characterized by “intermediate” immunophenotypes (Fig. 1; Table 1). The first evidence for a hierarchical relation using primary epithelial cell cultures was described by our group *(35)*. More recently, Collins and colleagues succeeded in isolating a purer candidate prostate epithelial stem cell, using CD133 selection *(36)*. Thus, a very specific immunophenotype of the stem cell, the early and late progenitor cell populations, and the terminally differentiated exocrine and neuroendocrine cells emerges (*see* Table 1).

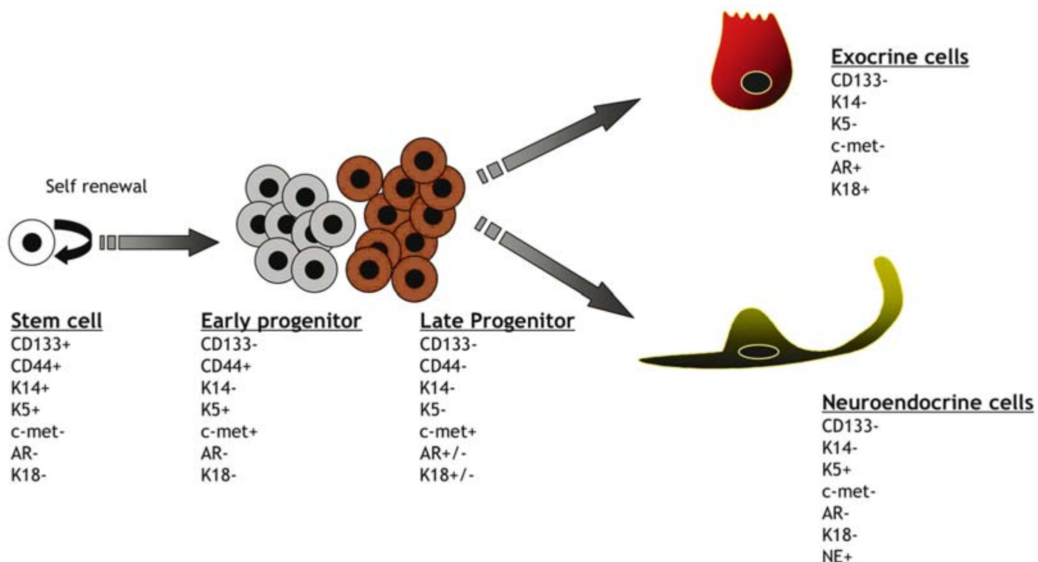


Fig. 1. Prostate epithelial cell hierarchy. The stem cells divide and give rise to a new stem cell by self-renewal and move committed progenitor cells)early and late) for the functional exocrine and neuroendocrine cell lineages. The exocrine lineage is critically dependent on DHT, and in fact this population represents > 90% of all epithelial cells in the adult prostate gland.

Table 1
Immunophenotype of the Cell Types in Nonmalignant Prostate Acini

Cell type/marker	CD133	K14	CD44	K5	c-met	K18	AR	NE
Stem cell								
Early Progenitor								
Late Progenitor								
Exocrine cells								
Neuroendocrine cells								

K14, keratin 14; K5, keratin 5; K18, keratin 18; AR, androgen receptor; NE, neuroendocrine markers (e.g., serotonin and chromogranin A).

The hierarchical relation between the cell types is schematically illustrated in Fig. 1. The exocrine lineage, resulting in the tall columnar prostate-specific antigen-producing cells, is critically dependent on the hormone, dihydrotestosterone. After castration, more than 90% of epithelial cells die

through apoptosis *(37,38)*. The remaining cells have renewal capacity, because the kinetics of regrowth after implanting testosterone-containing slow-release devices is independent of the time interval between castration and implantation of the silastic testosterone-containing devices *(26,30)*. The population of cells remaining after castration are thought to represent the stem and early progenitor cells. In the developing prostate (embryogenesis and peripubertal), there is a relative enrichment in the progenitor cell populations *(31)*. The early and late progenitors are thought to play a pivotal role in the development of benign and malignant prostate neoplasms *(39–41)*. Implicit evidence that the remaining cell population after castration has renewal ability was provided by early experiments by Coffey and Isaacs *(26)* showing that the kinetics of prostate regrowth are independent of the time interval between castration and testosterone re-administration. Long-term culture experiments using CD133-selected cells proliferate and can be maintained for longer than 140 days. CSC reside in a "niche," and our understanding of the regulation of the expansion into the various epithelial lineages of differentiation is growing steadily through the pioneering work of Fuchs and colleagues *(42–47)*. Unfortunately, relatively little is known on the location/niche of the prostate epithelial stem cell. Collins and colleagues show that there seems to be a niche in which the prostate epithelial stem cells are more firmly attached to the basement membrane *(48)*. The branching morphology of the secretory ductal system is rather complex, and 3D reconstruction is difficult for the human prostate secretory system. The combined data indicate that the branching points and the tips of the acini are the candidate niche for the stem cell *(31,39,49)*. The stroma plays an essential role in the induction of branching morphogenesis and essential mediators seem to be hepatocyte growth factor and fibroblast growth factor-10 *(50–53)*. In general, despite the unique characteristics of the various specialized epithelial cells, common signaling mechanisms seem to play a role, such as wingless-, notch-hedgehog, and bone morphogenetic protein signaling (for a recent review, *see* ref. *17*).

3. PROSTATE CSC

Most studies, thus far, shed light on the candidate prostate CSC through phenotyping with the markers discussed in the previous section. Although a number of similarities between the brain stem cell model and the prostate stem cell model are apparent, there are also clear differences. The most striking feature is that the stem cell markers K14 and CD133 are not reported to be expressed in primary or metastatic prostate cancer *(36,54–58)*, suggesting that the prostate CSC is most similar to the early or late progenitors. Alternatively, the frequency may be so low that sampling artifacts can prevent identification of prostate CSC *in situ*.

The model describing the ontogeny of prostate CSCs from early or late progenitors, whereby genetic changes have accumulated in the stem cell or early progenitor, is schematically outlined in Fig. 2. On basis of the CSC phenotype, at least three different types of prostate cancers can be discriminated on basis of their progenitor phenotype, as illustrated in Fig. 2 *(57–59)*. This minority population of cells in these specimens are the candidate CSCs. Comparative analysis of primary cancers before and after endocrine therapy, resulting in impaired exocrine differentiation, shows a strong relative increase in cells with the progenitor phenotype (*see* also preliminary investigations) *(57,60,61)*.

These data suggest that a CSC population for prostate cancer can be identified as most similar to the early and late progenitors of the exocrine and neuroendocrine differentiation lineages. The unique ability for self-renewal by these cells makes them the most challenging target for therapeutic intervention. To join the growing number of targeted therapies for prostate cancer, functional validation of the relevant target in the stem cell model is critical.

3.1. Identification and Isolation of Prostate Epithelial Stem Cells

Our group was one of the first to develop markers that could discriminate cell types in the prostate epithelium *(30)*. We initially immunophenotyped prostate epithelial cells with a panel of antibodies

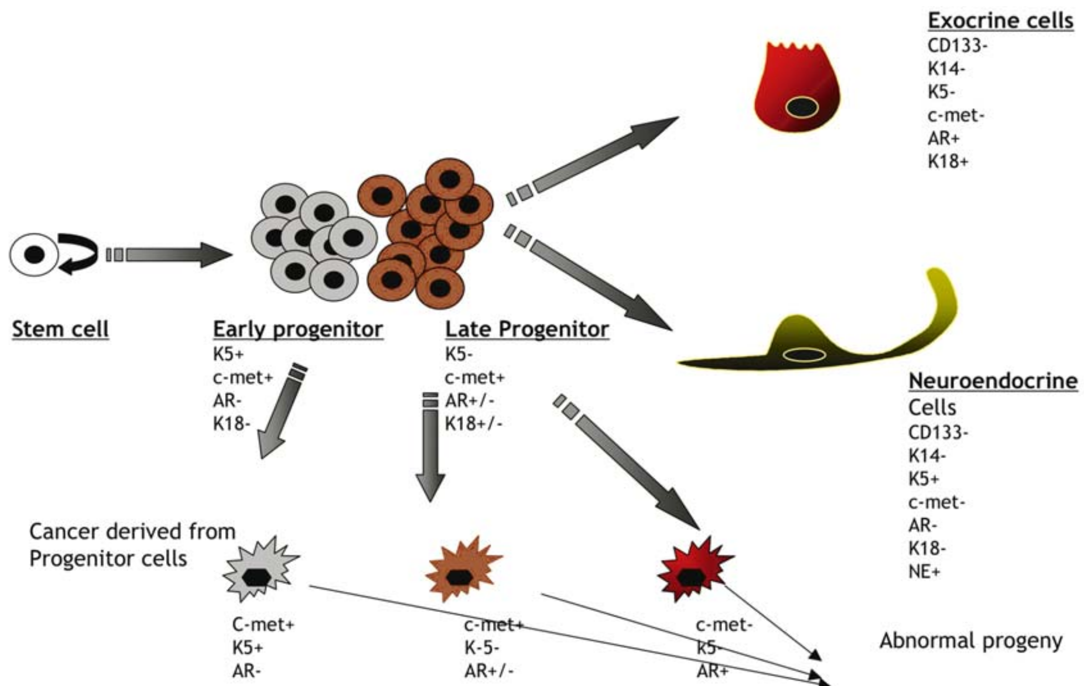


Fig. 2. Ontogeny of prostate cancer stem cells. In primary human prostate tumors, three classes can be discriminated; those with a minority of cells (candidate stem cells) similar to the early or late progenitors, and the pre-terminally differentiated phenotype.

against keratins. Keratins represent a family of intermediate filament proteins known to be useful as differentiation markers. We extended the panel with the proto-oncogene c-met *(62,63)*. Combined with the investigations of our collaborators and in the literature, a reasonably comprehensive marker set became available, as described in Table 1.

To challenge the stem cell hypothesis, we developed a procedure to culture prostate epithelial cells from human prostate tissue. We are using a procedure modified from Lang and colleagues *(36,64)*, and we are now able to routinely establish primary explant cultures. When the cells grow to confluence, they form spheroids that, after detailed analysis, seem to mimic glandular morphogenesis *(65)*.

We therefore refer to these spheroids as "glandular buds." In particular, the expression of basal cell cytokeratins 14 and 5 at the periphery of the bud and the luminal type cytokeratin 18 in the center of the bud illustrate that, in this system, glandular morphogenesis is mimicked to a reasonable extent. More recently, we refined the system by plating the primary epithelial cells *as single cells* onto a matrix of growth factor-deprived Matrigel. We have shown that spheroids are formed from single cells that, similar to the initial experiments, mimic glandular morphogenesis. Moreover, when we added the ligand for c-met, hepatocyte growth factor, we were able to induce branching morphogenesis in these cultures (Fig. 3). These cultures can be maintained under certain conditions for longer than 120 days, suggesting renewal ability.

Thus, we have established a primary 3D culture system starting with human prostate tissue to induce budding and branching morphogenesis. Methods to analyze these structures were also developed. The cultures are either stained in situ *and subsequently analyzed using confocal scanning laser microscopy, or, alternatively, individual buds are "hand-picked" and embedded in tissue tech, and frozen sections are stained with various differentiation markers (data not shown).*

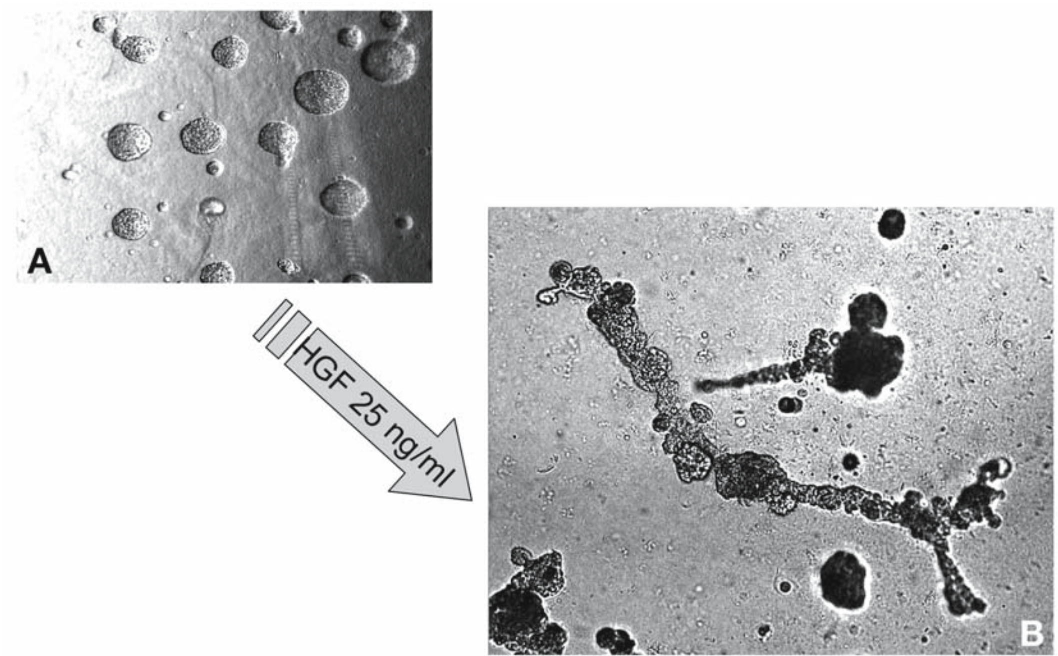


Fig. 3. (A) Spheroids/glandular buds develop 10 to 14 days after primary prostate epithelial cells (PrECs) are seeded onto growth factor-deprived Matrigel. Typically, 100 to 200 buds develop from 1×10^5 PrEcs. **(B)** When hepatocyte growth factor is added at a concentration of 25 ng/mL, branching structures develop.

3.2. Characterization of Prostate CSCs in Primary, Recurrent, and Metastatic Prostate Cancer

Using the marker panel described in Table 1, we analyzed a large series of human prostate cancer specimens before hormonal therapy. Furthermore, we analyzed a select group of patients who underwent neoadjuvant hormonal therapy before radical prostatectomy, and also immunophenotyped those specimens. We found, similar to many other groups, that the pure basal marker keratin 14 is not expressed in our series. There is only one report by Epstein and colleagues of keratin 14-expressing cells found in a very small fraction of patients with prostate cancer.

We were also able to confirm the results of Collins and colleagues *(36)* that CD133, the candidate adult stem cell marker, is not expressed in human prostate cancers. Markers for the early and late progenitor cells that are expressed in prostate cancer are CD44v3-v10, K19 *(66)*, and keratin 5 (35% of the cancers) and c-met. Obviously, the vast majority of cells have the characteristics of (pre-) terminally differentiated secretory cells, and neuroendocrine cells are also found in human prostate cancer specimens. Considering the proposed hierarchical relation between early and late progenitors and the terminally differentiated exocrine and neuroendocrine cells, and the fact that we are able to identify a subpopulation of prostate cancer cells with markers of the early and late progenitor cells (Fig. 4A,B) *(29)*, we, therefore, conclude that these are the candidate CSCs (Fig. 4B). In this model, one would predict that when the endocrine/exocrine differentiation pathway is abrogated through castration, the relevant percentage of CSCs increases. This was indeed found by Van Leenders and colleagues (Fig. 5) *(67)*.

On the basis of these experiments, we conclude that prostate cancers can arise from early progenitors (CD44, K5, c-met), late progenitors (c-met positive), or very late progenitors. We have to assume that the latter phenotype exists because a significant percentage (30%) does not express mark-

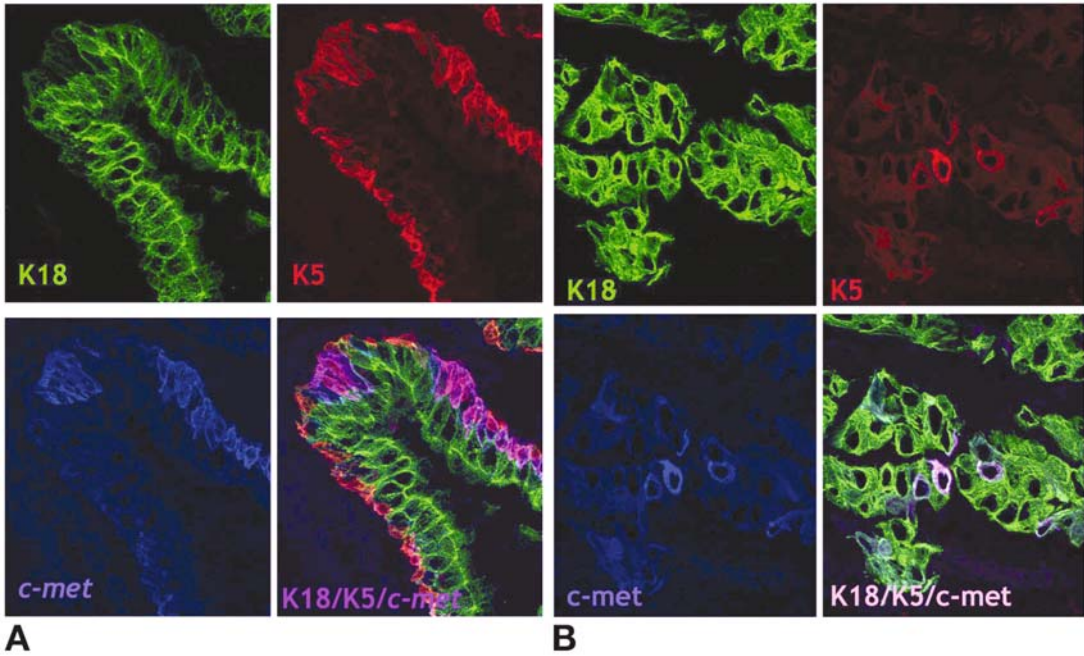


Fig. 4. (A) Immunophenotype of nonmalignant prostate epithelial cells assessed by triple staining with antibodies against K5, K18, and c-met. At least two intermediate cell types occur between the K5- and the K18-positive cells. **(B)** In prostate cancer, the cells with an immunophenotype associated with the least differentiated/progenitor cells expressing K5/K18 and c-met (candidate cancer stem cell phenotype)

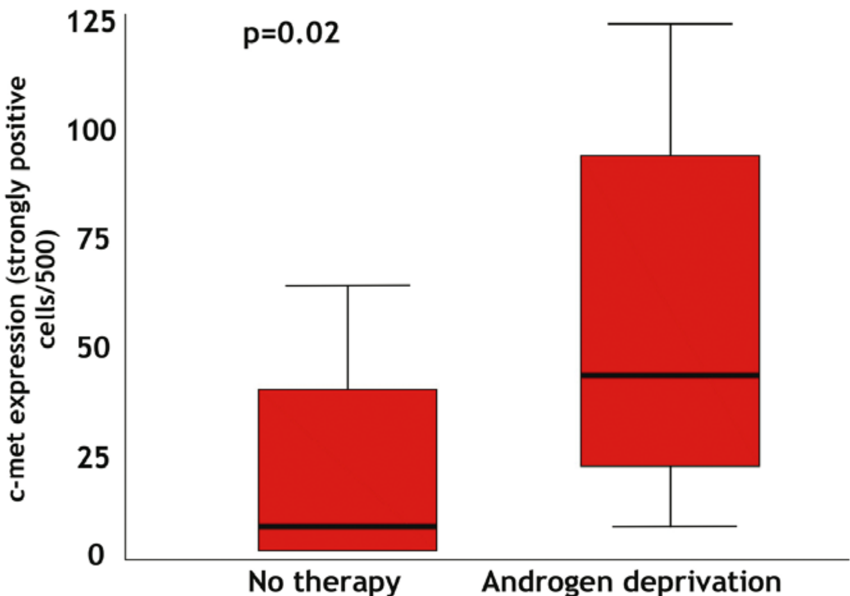


Fig. 5. Number of cancer cells with an immunophenotype similar to early/late progenitors before and after neoadjuvant hormonal therapy (maximal androgen blockade). There is a significant increase in c-met-positive cells, supporting the cancer stem cell concept.

ers from the early or late progenitor cells. Our observations are in agreement with a model in which the accumulation of genetic damage associated with cancer development occurs in the lineage from stem cell to early/very late progenitor. Recently, Collins et al. (68) were the first to report the prospective identification of prostate cancer stem cells after selection based on high expression of $\alpha 2\beta 1$ and CD133.

CONCLUSION

The ability to isolate prostate epithelial cells with the potential to grow into morphological structures resembling glandular budding indicates that stem cells exist in adult prostate tumors. Extensive immunophenotyping of primary prostate cancers has further shown that a phenotype for a prostate CSC exists, thus, allowing us to test the CSC hypothesis for prostate cancer. This effort, if successful, will lead to models that can be used to validate new therapeutic targets.

REFERENCE

1. Reya T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414(6859)**, 105–111.
2. Pardal, R., Clarke, M. F., and Morrison, S. J. (2003). Applying the principles of stem-cell biology to cancer. *Nat. Rev. Cancer* **3(12)**, 895–902.
3. Fialkow, P. J., Jacobson, R. J., and Papayannopoulou, T. (1977). Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am. J. Med.* **63(1)**, 125–130.
4. Fialkow, P. J. (1976). Human tumors studied with genetic markers. *Birth Defects Orig. Artic. Ser.* **12(1)**, 123–132.
5. Lapidot, T., Sirard, C., Vormoor, J., et al. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367(6464)**, 645–648.
6. Bonnet, D. and Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Med.* **3**, 730–737.
7. Al Hajj, M. and Clarke, M. F. (2004). Self-renewal and solid tumor stem cells. *Oncogene* **23(43)**, 7274–7282.
8. Al Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **100(7)**, 3983–3988.
9. Dontu, G., Al Hajj, M., Abdallah, W. M., Clarke, M. F., and Wicha, M. S. (2003). Stem cells in normal breast development and breast cancer. *Cell Prolif.* **36(Suppl. 1)**, 59–72.
10. Singh, S. K., Clarke, I. D., Hide, T., and Dirks, P. B. (2004). Cancer stem cells in nervous system tumors. *Oncogene* **23(43)**, 7267–7273.
11. Singh, S. K., Hawkins, C., Clarke, I. D., et al. (2004). Identification of human brain tumour initiating cells. *Nature* **432(7015)**, 396–401.
12. Lessard, J., Faubert, A., and Sauvageau, G. (2004). Genetic programs regulating HSC specification, maintenance and expansion. *Oncogene* **23(43)**, 7199–7209.
13. Lessard, J. and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* **423(6937)**, 255–260.
14. Pinto, D. and Clevers, H. (2005). Wnt, stem cells and cancer in the intestine. *Biol. Cell* **97(3)**, 185–196.
15. Sancho, E., Batlle, E., and Clevers, H. (2003). Live and let die in the intestinal epithelium. *Curr. Opin. Cell Biol.* **15(6)**, 763–770.
16. Taipale, J. and Beachy, P. A. (2001). The Hedgehog and Wnt signalling pathways in cancer. *Nature* **411(6835)**, 349–354.
17. Rizvi, A. Z. and Wong, M. H. (2005). Epithelial stem cells and their niche: there's no place like home. *Stem Cells* **23(2)**, 150–165.
18. Berman, D. M., Desai, N., Wang, X., et al. (2004). Roles for Hedgehog signaling in androgen production and prostate ductal morphogenesis. *Dev. Biol.* **267(2)**, 387–398.
19. Karhadkar, S. S, Bova, G. S., Abdallah, N., et al. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* **431(7009)**, 707–712.
20. DiStefano, A., Tashima, C. K., Yap, H. Y., and Hortobagyi, G. N. (1979). Bone marrow metastases without cortical bone involvement in breast cancer patients. *Cancer* **44(1)**, 196–198.
21. Horan, A. H. (2004). Prostate cancer cells: detection and isolation from peripheral blood and bone marrow. *Urology* **64(1)**, 187.
22. Cristofanilli, M. (2005). The "microscopic" revolution in breast carcinoma. *Cancer* **103,** 877–880.
23. Cristofanilli, M., Budd, G. T., Ellis, M. J., et al. (2004). Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N. Engl. J. Med.* **351(8)**, 781–791.

24. Morrison, S. J., Qian, D., Jerabek, L., et al. (2002). A genetic determinant that specifically regulates the frequency of hematopoietic stem cells. *J. Immunol.* **168(2)**, 635–642.
25. Chen, J. K., Taipale, J., Cooper, M. K., and Beachy, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. **16**, 2743–2748.
26. Coffey, D. S. and Isaacs, J. T. (1981). Prostate tumor biology and cell kinetics—theory. *Urology* **17(Suppl. 3)**, 40–53.
27. English, H. F., Santen, R. J., and Isaacs, J. T. (1987). Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. *Prostate* **11**, 229–242.
28. Isaacs, J. T. and Coffey, D. S. (1989). Etiology and disease process of benign prostatic hyperplasia. *Prostate* **2(Suppl.)**, 33–50.
29. Verhagen, A. P., Ramaekers, F. C., Aalders, T. W., Schaafsma, H. E., Debruyne, F. M., and Schalken, J. A. (1992). Colocalization of basal and luminal cell-type cytokeratins in human prostate cancer. *Cancer Res.* **52(22)**, 6182–6187.
30. Verhagen, A. P., Aalders, T. W., Ramaekers, F. C., Debruyne, F. M., and Schalken, J. A. (1988). Differential expression of keratins in the basal and luminal compartments of rat prostatic epithelium during degeneration and regeneration. *Prostate* **13(1)**, 25–38.
31. Xue, Y., Smedts, F., Verhofstad, A., Debruyne, F., de la, R. J., and Schalken, J. (1998). Cell kinetics of prostate exocrine and neuroendocrine epithelium and their differential interrelationship: new perspectives. *Prostate* **8(Suppl.)**, 62–73.
32. Bonkhoff, H. (1998). Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. *Prostate* **8(Suppl.)**, 18–22.
33. Huss, W. J., Gray, D. R., Werdin, E. S., Funkhouser, W. K., Jr., and Smith, G. J. (2004). Evidence of pluripotent human prostate stem cells in a human prostate primary xenograft model. *Prostate* **60(2)**, 77–90.
34. Schalken, J. A. and van Leenders, G. (2003). Cellular and molecular biology of the prostate: stem cell biology. *Urology* **62(5 Suppl. 1)**, 11–20.
35. van Leenders, G. J. and Schalken, J. A. (2001). Stem cell differentiation within the human prostate epithelium: implications for prostate carcinogenesis. *BJU Int.* **88(Suppl. 2)**, 35–42; discussion 49–50.
36. Richardson, G. D., Robson, C. N., Lang, S. H., Neal, D. E., Maitland, N. J., and Collins, A. T. (2004). CD133, a novel marker for human prostatic epithelial stem cells. *J. Cell. Sci.* **117(Pt 16)**, 3539–3545.
37. Schalken, J. A., and van Leenders, G. (2003). Cellular and molecular biology of the prostate: stem cell biology. *Urology* **62(5 Suppl 1)**, 11–20.
38. van Leenders, G. J. and Schalken, J. A. (2001). Stem cell differentiation within the human prostate epithelium: implications for prostate carcinogenesis. *BJU Int.* **88(Suppl 2)**, 35–42; discussion 49–50.
39. Xue, Y., Smedts, F., Umbas, R., et al. (1997). Changes in keratin expression during the development of benign prostatic hyperplasia. *Eur. Urol.* **32(3)**, 332–338.
40. De Marzo, A. M., Nelson, W. G., Meeker, A. K., and Coffey, D. S. (1998). Stem cell features of benign and malignant prostate epithelial cells. *J. Urol.* **160(6 Pt 2)**, 2381–2392.
41. Long, R. M., Morrissey, C., Fitzpatrick, J. M., and Watson, R. W. (2005). Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies. *Clin. Sci. (Lond)* **108(1)**, 1–11.
42. Alonso, L. and Fuchs, E. (2003). Stem cells of the skin epithelium. *Proc. Natl. Acad. Sci. USA* **100(Suppl 1)**, 11,830–11,835.
43. Alonso, L. and Fuchs, E. (2003). Stem cells in the skin: waste not, Wnt not. *Genes Dev.* **17(10)**, 1189–1200.
44. Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L., and Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* **118(5)**, 635–648.
45. Fuchs, E., Tumbar, T., and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell* **116(6)**, 769–778.
46. Hong, K. U., Reynolds, S. D., Watkins, S., Fuchs, E., and Stripp, B. R. (2004). Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am. J. Pathol.* **164(2)**, 577–588.
47. Tumbar, T., Guasch, G., Greco, V., et al. (2004). Defining the epithelial stem cell niche in skin. *Science* **303(5656)**, 359–363.
48. Collins, A. T., Habib, F. K., Maitland, N. J., and Neal, D. E. (2001). Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. *J. Cell. Sci.* **114(Pt 21)**, 3865–3872.
49. Kinbara, H., Cunha, G. R., Boutin, E., Hayashi, N., and Kawamura, J. (1996). Evidence of stem cells in the adult prostatic epithelium based upon responsiveness to mesenchymal inductors. *Prostate* **29(2)**, 107–116.
50. Cunha, G. R. (1985). Mesenchymal-epithelial interactions during androgen-induced development of the prostate. *Prog. Clin. Biol. Res.* **171**, 15–24.
51. Cunha, G. R., Shannon, J. M., Taguchi, O., Fujii, H., and Chung, L. W. (1982). Mesenchymal-epithelial interactions in hormone-induced development. *J. Anim. Sci.* **55(Suppl 2)**, 14–31.
52. Freestone, S. H., Marker, P., Grace, O. C., et al. (2003). Sonic hedgehog regulates prostatic growth and epithelial differentiation. *Dev. Biol.* **264(2)**, 352–362.
53. Marker, P. C., Donjacour, A. A., Dahiya, R., and Cunha, G. R. (2003). Hormonal, cellular, and molecular control of prostatic development. *Dev. Biol.* **253(2)**, 165–174.
54. Schalken, J. A. and van Leenders, G. (2003). Cellular and molecular biology of the prostate: stem cell biology. *Urology* **62(5 Suppl 1)**, 11–20.

55. van Leenders, G., van Balken, B., Aalders, T., Hulsbergen-van de Kaa, C, Ruiter, D., and Schalken, J. (2002). Intermediate cells in normal and malignant prostate epithelium express c-MET: implications for prostate cancer invasion. *Prostate* **51(2)**, 98–107.
56. van Leenders, G., Dijkman, H., Hulsbergen-van de Kaa, C., Ruiter, D., and Schalken, J. (2000). Demonstration of intermediate cells during human prostate epithelial differentiation in situ and in vitro using triple-staining confocal scanning microscopy. *Lab. Invest.* **80(8)**, 1251–1258.
57. van Leenders, G. J., Gage, W.R., Hicks, J. L., et al. (2003). Intermediate cells in human prostate epithelium are enriched in proliferative inflammatory atrophy. *Am. J. Pathol.* **162(5)**, 1529–1537.
58. van Leenders, G. J., Aalders, T. W., Hulsbergen-van de Kaa, C. A., Ruiter, D. J., and Schalken J. A. (2001). Expression of basal cell keratins in human prostate cancer metastases and cell lines. *J. Pathol.* **195(5)**, 563–570.
59. van Leenders, G, van Balken, B., Aalders, T., Hulsbergen-van de Kaa, C., Ruiter, D., and Schalken, J. (2002). Intermediate cells in normal and malignant prostate epithelium express c-MET: implications for prostate cancer invasion. *Prostate* **51(2)**, 98–107.
60. De Marzo, A. M., Meeker, A. K., Epstein, J. I., and Coffey, D. S. (1998). Prostate stem cell compartments: expression of the cell cycle inhibitor p27Kip1 in normal, hyperplastic, and neoplastic cells. *Am. J. Pathol.* **153(3)**, 911–919.
61. van Leenders, G., van Balken, B., Aalders, T., Hulsbergen-van de Kaa, C., Ruiter, D., and Schalken, J. (2002). Intermediate cells in normal and malignant prostate epithelium express c-MET: implications for prostate cancer invasion. *Prostate* **51(2)**, 98–107.
62. Schalken, J. A. and van Leenders, G. (2003). Cellular and molecular biology of the prostate: stem cell biology. *Urology* **62(5 Suppl 1)**, 11–20.
63. van Leenders, G., van Balken, B., Aalders, T., Hulsbergen-van de Kaa, C., Ruiter, D., and Schalken, J. (2002). Intermediate cells in normal and malignant prostate epithelium express c-MET: implications for prostate cancer invasion. *Prostate* **51(2)**, 98–107.
64. Lang, S. H., Stark, M., Collins, A, Paul, A. B., Stower, M. J., and Maitland N. J. (2001). Experimental prostate epithelial morphogenesis in response to stroma and three-dimensional matrigel culture. *Cell Growth Differ.* **12(12)**, 631–640.
65. van Leenders, G., Dijkman, H., Hulsbergen-van de Kaa, C., Ruiter, D., and Schalken, J. (2000). Demonstration of intermediate cells during human prostate epithelial differentiation in situ and in vitro using triple-staining confocal scanning microscopy. *Lab. Invest.* **80(8)**, 1251–1258.
66. Alam, T. N., O'Hare, M. J., Laczko, I., et al. (2004). Differential expression of CD44 during human prostate epithelial cell differentiation. *J. Histochem. Cytochem.* **52(8)**, 1083–1090.
67. van Leenders, G., van Balken, B., Aalders, T., Hulsbergen-van de Kaa, C., Ruiter, D., and Schalken, J. (2002). Intermediate cells in normal and malignant prostate epithelium express c-MET: implications for prostate cancer invasion. *Prostate* **51(2)**, 98–107.
68. Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., and Maitland, N. J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* **65(23)**, 10,946–10,951

5
Cancer–Host Interactions

A Paradigm Shift Brings New Understanding and New Opportunities

Leland W. K. Chung, Wen-Chin Huang, Shian-Ying Sung, Daqing Wu, Valerie Odero-Marah, and Haiyen E. Zhau

Summary

Cancer is not a single cell disease and its existence and behavior are constantly modulated by the host. Cancer gene expression and genetics are also highly dynamic and are regulated epigenetically by the host. In this chapter, we describe the molecular pathways leading to an unusual property of cancer cells: the ability to mimic the host microenvironment, and, in particular, the characteristics of osteomimicry and vasculogenic mimicry. We also discuss the importance of host inflammatory and stem cells, which contribute to the growth and survival of cancer cells. By understanding the salient features of cancer–host interaction, novel therapeutics may be developed to target both cancer and host in the treatment of lethal prostate cancer metastases.

Key Words: Bone metastasis; cell signaling; epigenetic modulation of gene expression; epithelial-to-mesenchymal transition; extracellular matrices; growth factors; inflammation; molecular targeting and therapy; osteomimicry; stem cells; tumor stromal interaction.

1. INTRODUCTION

It is now well-accepted that cancer progression depends not only on the genetic constituents and modifications of the cancer cells, but also on the genetics and epigenetic factors contributed by the host *(1–5)*. Although genetic changes in the cancer cells are required for cancer to occur, these changes are insufficient to induce the entire spectrum of a progressive cancer *(6,7)*. It has been amply demonstrated that intimate interaction between cancer cells and their host microenvironment greatly influences the growth and subsequent dissemination of cancer cells *(8–11)*. The work of Paget, more than a hundred years ago, epitomizes the tumor ("seed") and host ("soil") relationship that determines the patterns of cancer dissemination in patients *(12)*. Recent work, however, showed that this interaction is far more dynamic than previously thought, including the ability of the host *to alter the genetics of the cancer cells* and the ability of cancer cells *to reciprocally modify the genetics of the host (5,13–16)*. Host cells are not static, but can be recruited to or modified at the site of cancer cell growth, and greatly influence the behaviors of cancer cells *(5,13,17)*. The homeostasis between cancer cells and their immediate microenvironment, including inflammatory cells and bone marrow-derived stem cells recruited to sites of cancer growth, the levels of hypoxia and circulating hormones surrounding the cancer cells, and the stress conditions, reactive oxygen species (ROS) induced by cell crowding and changing pH, and the osmolarity of the tumor-adjacent microenvironment are all known to contribute to changing cancer behaviors *(18–21)*. Through constant contact and interaction with the rich milieu of the microenvironment, including soluble growth factors, extracellular matrices and ROS, cancer cells gain additional genetic modifications and behavioral changes that drive them to migrate and

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

invade. Ultimately, the host factors determine the patterns of cancer cell dissemination *(10,12,22–24)*. The present review focuses on the contribution of host stromal fibroblasts and osteoblasts to local cancer growth, progression, and its final metastasis to the skeleton and visceral organs. The converging signaling pathways that respond to factors that confer growth and lead to the survival and metastasis of cancer cells, and the therapeutic opportunities arising from this paradigm shift, are emphasized. Specifically, the following topics and molecular events are reviewed and discussed:

1. The plasticity of cancer and stromal fibroblasts that together contribute to cancer growth and progression.
2. The molecular basis of mimicry by cancer cells, with gene expression and cellular functions guided by the host/cancer microenvironment and vascular endothelial and osteoblastic cells that support the growth and survival of cancer cells.
3. How cancer cells orchestrate gene expression profile changes of the host cells that together participate in the genesis and progression of cancer.
4. The opportunity for therapeutic co-targeting of both the cancer and the tumor microenvironment, disrupting the evolutionary continuum of the cancer and stromal cells that leads to the uncontrolled growth of cancer cells and their resistance to therapy.

2. THE PLASTICITY OF CANCER AND STROMAL FIBROBLASTS

The developmental fate of a normal cell is regulated temporally and spatially by precise inductive cues from the cell microenvironment *(25)*. In response to the signaling molecules, the gene expression profiles and the behaviors of a differentiating cell are subjected to changes allowing the normal differentiation program of developing organs to result. The genomic stability of the normal cell is desired and expected, and essential for survival *(26)*. However, in the case of cancer, many of the physiological processes are "hijacked" by cancer cells, and mechanisms controlling growth and survival in the normal physiological context are lost *(27–29)*. For example, the plasticity of cancer cells and stromal fibroblasts could fuel tumor growth through the production of excessive amounts of angiogenic substances leading to the ingrowth of new blood vessels, mimicking the normal physiological processes of wound repair *(30,31)*. Cancer cells have been described as a wound that fails to heal. This refers to the persistent stromal response to the invading cancer epithelium and the secreted local factors, such as plasminogen activator, that prevented the blood-clotting and wound-healing processes *(32,33)*. Cancer cells can be immune-evasive "outlaws" that have lost the major histocompatibility (MHC) class 1 antigen and are no longer recognized by the host cytotoxic T cells *(34)*. Cancer cells overcome and resist the physiologically programmed apoptotic response to the developmental signals, tissue injuries and stress responses induced by hypoxia, hormone withdrawal, chemotherapy, and radiation therapy through a variety of mechanisms related to increased proliferative and decreased apoptotic mechanisms that lead to tumor growth and survival *(35,36)*.

The prostate gland is derived from the embryonic urogenital sinus, with the growth and differentiation of the glandular epithelium specified and maintained by its adjacent mesenchyme under tight control of the male steroid hormone, testosterone *(37–39)*. When prostate epithelial cells are undergoing neoplastic transformation, initially the growth of glandular epithelium is still stimulated and maintained by testicular androgen, with its action mediated by the androgen receptor (AR) in the glandular epithelium and stromal fibroblasts *(39–41)*. After androgen-deprivation therapy for the treatment of prostate cancer, prostate epithelial growth becomes androgen refractory and is no longer controlled by androgenic hormones, but is instead controlled by yet-to-be-defined factors secreted by cancer cells as well as cells in the cancer microenvironment *(42–45)*. This altered control mechanism, with inherited and epigenetically driven intrinsic genomic instability, DNA repair defects, and acquisition of multiple survival mechanisms by the cancer cells, allows cancer cells to escape from normal developmental constraints and undergo a dedifferentiation process with possible nonrandom genetic changes *(15,16,46,47)*. Remarkably, despite genetic alterations in cancer cells, they often remain nontumorigenic in mice and, even when tumorigenic, seldom acquire metastatic potential *(48,49)*. We think that certain undefined host factors could contribute largely to cancer development

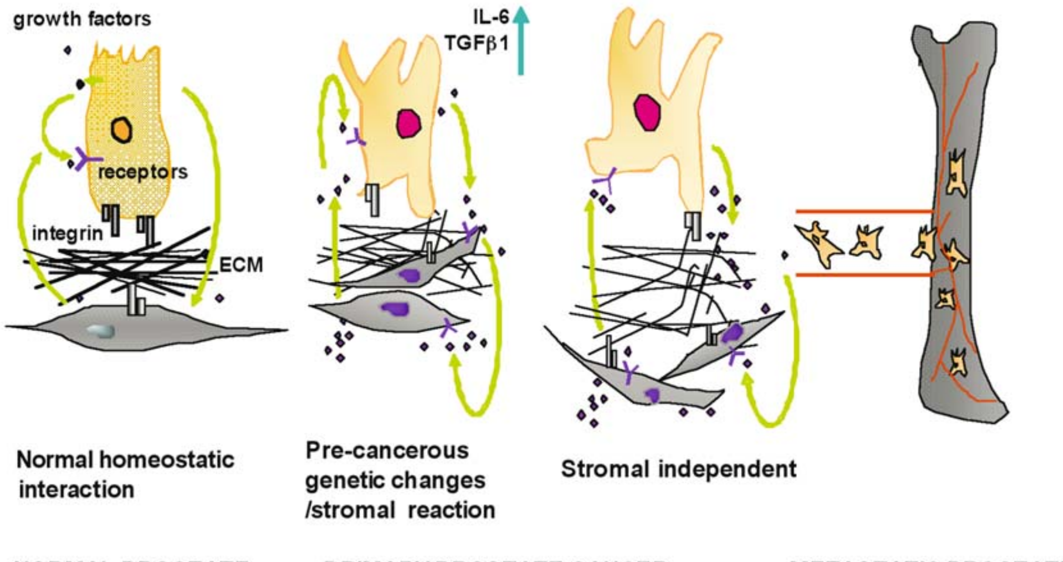


Fig. 1. Dynamic reciprocal genetic changes in prostate cancer and bone stromal cells through cellular interaction in a "vicious cycle" manner could contribute to the invasiveness and metastasis of human prostate cancer cells to bone. The normal prostate epithelial cell homeostatic interaction with its adjacent stroma through growth factors and extracellular matrix (ECM), via growth factor receptors or integrins, is deranged because of genetic modifications that occur in the epithelium. The genetic altered prostate epithelial cell provokes a stromal reaction, which sets off a chain reaction, such as elevated transforming growth factor (TGF)-β1 and interleukin (IL)-6, in modulating reciprocally the cancer epithelium, which becomes prostate stroma-independent and eventually invades and migrates to bone and exerts strong reciprocal interaction with the bone cells.

and progression. For example, host inflammatory cytokines could play a positive and directive role in tumor growth and progression, and indirectly affect the host microenvironment to facilitate tumor growth and expansion. Tumor-derived and host stroma-derived factors, such as transforming growth factor (TGF)-β1, are known to induce tumor angiogenesis and elicit stromal reactions and deposition of extracellular matrices *(50)*. The altered stromal microenvironment could help drive local tumorigenesis and subsequently enhance tumor cell distant dissemination through a positive feedback mechanism, as depicted in Fig. 1. The progression of cancer cells, therefore, can occur in three steps:

1. Genetic instability of precancerous epithelial cells contributes to altered cell behaviors, including increased cell proliferation, decreased apoptosis, and increased cell motility.
2. The behaviorally altered precancerous or cancerous epithelial cells could trigger a stromal response, or desmoplastic reaction in the stroma, with morphological and gene expression changes that particularly increase the deposition of extracellular matrices and also the secretion of growth and angiogenic factors that collectively induce genetic changes in the stromal fibroblasts.
3. In consequence, the changing stromal microenvironment could induce additional genetic modifications of the cancer epithelial cells which become stromal independent and develop the propensity for bone metastasis.

The resulting cancer cells could become highly unstable with increased motility and invasive and metastatic potential. This interaction between stroma and epithelium is a "vicious cycle" maintained by permanent genetic changes within both the tumor and stromal cell compartments, ultimately contributing to the progression of an invasive cancer *(5,51)*.

3. HOW CANCER CELLS RESPOND TO A CHANGING TUMOR MICROENVIRONMENT

3.1. Osteomimicry

Cancer cells are capable of mimicking the characteristics of cells in the tumor microenvironment. Dramatic examples include osteomimicry, the ability of cancer cells to express genes normally highly restricted to bone cells before, during, or after metastasis through the synthesis, secretion, and accumulation of bone-like proteins, such as osteocalcin (OC), osteopontin, bone sialoprotein (BSP), and osteonectin, even forming mineralizing bone under certain culture conditions *(29,52,53)*. Cancer cells are also capable of expressing receptor activator of NF-κB (RANK) ligand and parathyroid hormone related peptide, which are known to directly or indirectly increase bone turnover through increased RANK ligand (associated with cancer cells)–RANK (associated with osteoclasts) interaction and activation of osteoclastogenesis *(54,55)*. These unusual characteristics are caused by the ability of cancer cells to respond to factors secreted by cancer cells or by host cells in the immediate microenvironment. Using human prostate cancer and bone cells as models to define the molecular basis of osteomimicry, we observed a unique key switch controlling OC and BSP gene expression that is operative in prostate cancer but not in bone cells *(56,57)*. This switch resides at the 8-base nucleotides sequences, called the cyclic AMP (cAMP)-responsive element (CRE), within both OC and BSP promoters, and is responsible for the regulation of both endogenous OC and BSP as well as their promoter activities *(57,58)*. We further showed that CRE activation is under the control of a soluble factor secreted by prostate cancer and host cells, with its action mediated by cAMP-dependent protein kinase A (PKA) activation. The activation of CRE binding protein (CREB), was demonstrated by the observation of CREB phosphorylation after activation of cAMP-PKA, phosphorylated CREB translocation into the cell nucleus, and the subsequent binding of CREB to CRE as shown by gel shift and supershift assays *(57)*. Based on these data, Fig. 2 depicts a number of possible molecular pathways mediating osteomimicry in human prostate cancer cells *(58)*:

1. The binding of a soluble factor to a putative cell surface receptor, linking with the activation of intracellular cAMP-PKA signaling pathway. The putative receptor can be a G protein-coupled receptor that mediates downstream signaling via PKA, or, alternatively, can be linked to MHC class 1 antigen or a yet-to-be-identified receptor that binds to the soluble factor and transmits intracellular signaling through the cAMP-PKA system.
2. The participation by a soluble factor, β2 microglobulin (β2M), which was shown to activate the cAMP-PKA system *(58)*. Because β2M is known to complex with a classic MHC class 1 antigen, this could implicate the role of this complex in the downstream intracellular signaling of osteomimicry in human prostate cancer cells.
3. The direct action of β2M in activating the cAMP-PKA system, or the direct participation by β2M in CREB downstream activation of target genes.

The biological consequences of osteomimicry could be numerous. For example, the activation of OC and BSP expression could result in the recruitment of bone cells, such as osteoclasts and osteoblasts that participate in enhanced osteoclastogenesis, that is, increased bone turn-over, or bone pitting to create new sites in support of cancer cell attachment, growth, and colonization in bone *(59,60)*. OC and BSP activation could contribute to new bone formation and mineralization *(61)*. Prostate cancer cells derived from LNCaP cells with increased bone metastatic potential, such as C4-2 and C4-2B, have activated OC and BSP gene expression and also are capable of forming bone nodules when subjected to mineralizing cell culture conditions in vitro *(62)*. Activation of CREB could result in marked gene expression changes in cancer cells and cells in the cancer microenvironment that could facilitate cancer cell growth, survival, and colonization in bone. It is interesting to note that, of approx 4000 potential CREB target genes, only a fraction are expressed in a cell context-dependent manner *(63,64)*. This again supports the concept of the importance of the host contribution to cancer cell growth, resistance to apoptosis, and resistance to therapy. A large number of other downstream genes unrelated to osteomimicry were also found to be regulated by cAMP-PKA activation. For

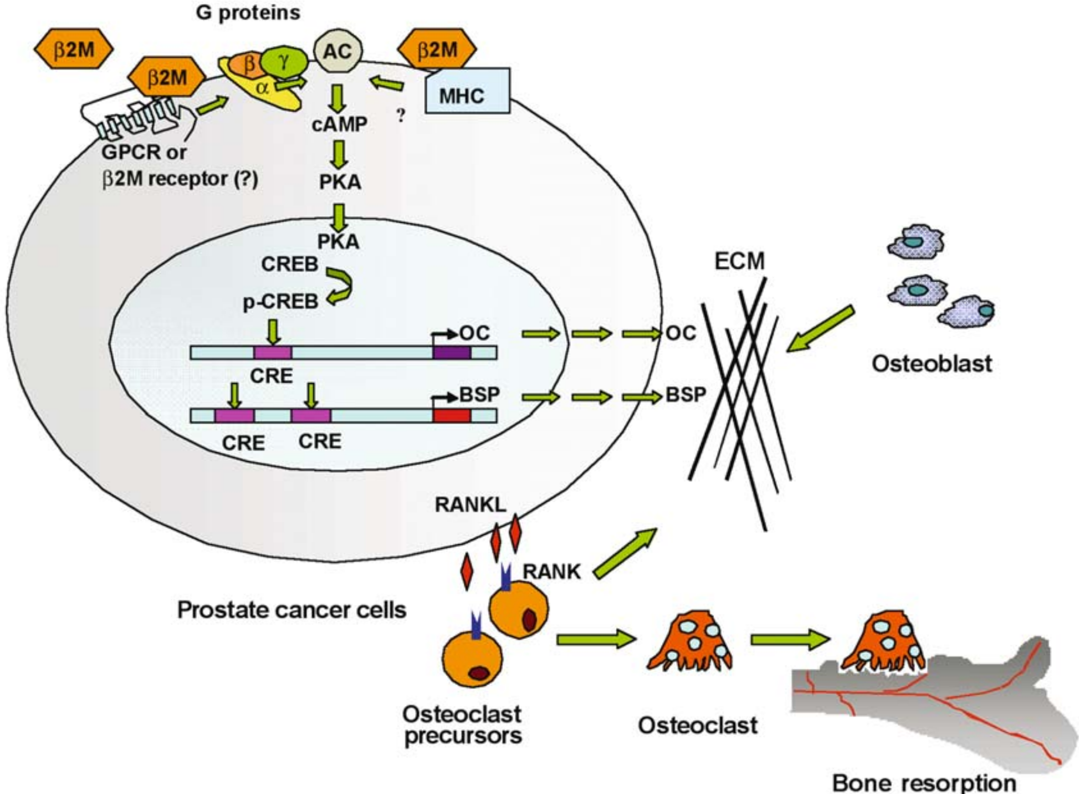


Fig. 2. Molecular mechanisms of osteomimicry in prostate cancer cells. Prostate cancer cells have the ability to mimic gene expression and behaviors of bone cells by synthesizing and depositing bone-like proteins, such as osteocalcin (OC) and bone sialoprotein (BSP) *(57)*. Among factors that could regulate the expression of these proteins, we found that β2 microglobulin (β2M) induces the expression of OC and BSP through an activation of the cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway with its downstream activation of a cAMP-responsive element (CRE)-binding protein (CREB). β*2M* is considered as a housekeeping gene with uniform expression of its mRNA in many cells. Interestingly, β2M protein expression varied widely between cells. β2M could exert its action via a number of membrane receptors, such as a G protein-coupled receptor (GPCR), a major histocompatibility antigen complex (MHC), or a yet-to-be identified β2M receptor. Alternatively, β2M could also act intracellularly by modulating directly CREB downstream target gene expression. The resulting increased expression and deposition of OC and BSP in bone matrix could recruit osteoblasts and osteoclasts and initiate increased bone turnover (i.e., increased bone resorption and formation) through osteoclastogenesis, which facilitates prostate cancer bone colonization. RANK, receptor activator of NF-κB and RANKL, RANK ligand *(58)*.

example, after cAMP-PKA/CREB activation in cancer cells, the phosphorylated CREB is responsible for the recruitment of CBP/p300 to activate downstream genes such as vascular endothelial growth factor (VEGF), cyclins, and survival factors aiding the growth and survival of cancer cells *(65,66)*. It has been shown that removing androgen from cultured prostate cancer cells elicits a neuroendocrine phenotype caused by CREB activation *(42,66,67)*. As expected, neuroendocrine differentiation, which has been associated with increased invasion, migration, and metastasis of prostate cancer cells, was also activated by cAMP mimetics, such as dibutyl cAMP or forskolin *(42,67)*. Activation of G protein, via cAMP-activated β2 adrenergic receptor, has been shown to compensate for the requirement of androgen to activate AR downstream genes *(67)*. This phenomenon could have clinical importance, because it has been proposed that the activation of AR by suboptimal concentrations

of androgen could be responsible for the survival of prostate cancer cells in patients subjected to androgen-withdrawal treatment regimens *(45)*.

3.2. Vasculogenic Mimicry

Cancer cells often grow under stress conditions, because of the lack of oxygen, increased cell crowding, and the lack of sufficient nutrients. In response to these conditions, adaptive changes by cancer cells have been observed to create their own blood vessels, such as ischemia-induced vasculogenic mimicry in melanoma, breast, and prostate cancer *(61,68,69)*. Hendrix and colleagues *(31)* demonstrated the plasticity of melanoma cells, which formed tubular structures with patterned matrix deposition including laminin, heparan sulfate proteoglycans, and collagens IV and VI, expressing genes normally expressed by vascular endothelial cells and interconnecting with the preexisting blood vessels *(31)*. This adaptive capability of cancer cells toward changes in microenvironmental cues could sustain their growth and survival at metastatic sites and allow them to gain further invasive and migratory potentials. A direct link between vasculogenic mimicry, the activation of focal adhesion kinase (FAK) through phosphorylation of tyrosine-397 and -576, and decreased plasminogen activation through decreased urokinase activity have been reported *(70)*. Because FAK activation has been associated with increased cell invasion and migration, and plasminogen activation is crucial for blood clotting, the ability of cancer cells to alter FAK and plasminogen activation is consistent with observations of an increased ability to metastasize along with aberrant wound-healing properties *(30,71,72)*.

3.3. Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a fundamental cellular process whereby an epithelial cell undergoes a structural and functional transition to assume the phenotype and behavior of a mesenchymal cell, characterized by increased migratory and invasive properties in embryonic development and also on neoplastic progression *(73)*. It is now well-accepted that EMT occurs in a number of human cancers, including prostate cancer, and that EMT is associated with increased cancer invasion and metastasis. EMT is a highly dynamic process that signals the plasticity of cancer cells (Fig. 3). Cancer metastasis is often preceded by EMT, but, on the completion of the metastatic process, cancer cells can revert their phenotype and behavior by undergoing mesenchymal-to-epithelial transition (MET), presumably to increase cancer cell adhesion to each other and to cells in host microenvironment. This suggests the importance of the epigenetic host microenvironment that could trigger EMT and its reversal, MET *(74)*.

There are numerous well-characterized molecular pathways that describe the underlying key regulatory processes of EMT in human cancer cells. Activation of TGF-β signaling enhances receptor tyrosine kinases and Ras activities that together can drive the translocation of Smad and Snail transcription factors from the cytoplasmic to the nuclear compartment, and the activation or suppression of downstream target genes associated with EMT have been widely proposed as the key regulatory mechanisms underlying EMT in human cancer cells *(75,76)*. Other molecular mechanisms include the activation of Wnt and β-catenin signaling, which suppresses E-cadherin and initiates the early step of EMT *(77,78)*; activation of the Hedgehog pathway, which contributes to increased EMT and stem cell differentiation *(73,79)*, both important features shared by invasive cancer cells; and activation of NF-κB transcription factor, which translocates into the cell nucleus, improves the survival of cancer cells, and allows them to resist apoptotic death after therapeutic intervention *(80,81)*. Figure 3 depicts selective growth control signaling pathways of EMT and MET as a continuum of prostate cancer progression.

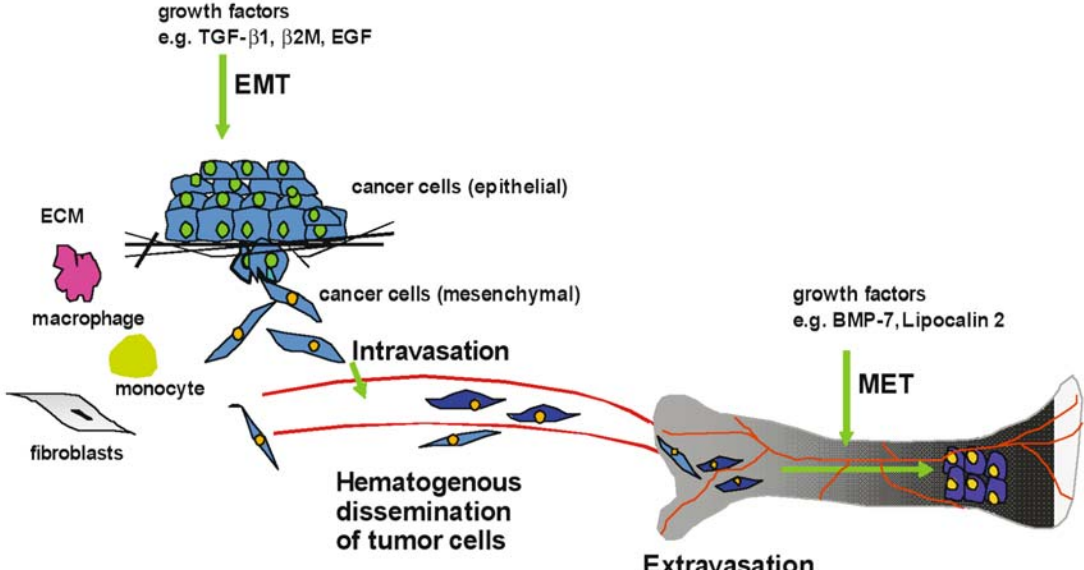


Fig. 3. The proposed epithelial-to-mesenchymal transition (EMT) and its reversal of mesenchymal-to-epithelial transition (MET) during the progression of human prostate cancer. Increased migration and invasion of prostate cancer cells before bone metastasis can be initiated through EMT under the influence of increased growth factor signaling (e.g., transforming growth factor [TGF]-β1, epidermal growth factor [EGF], or β2 microglobulin [β2M]). The sources of growth factors and cytokines can originate from resident fibroblasts or inflammatory cells within cancer-associated stroma, and these factors can complex with extracellular matrices (ECM). Cancer cells can metastasize to bone through hematogenous spread and after arriving at the bone, cancer cells have been observed to undergo MET under the influence of bone morphogenetic protein (BMP)-7 and lipocalin 2, by reexpressing epithelial cell-associated markers, such as E-cadherin, and decreased expression of vimentin and N-cadherin and increased cancer cell adhesion and colonization at metastatic sites.

4. HOW HOST CELLS CONTRIBUTE TO THE GENESIS OF CANCER

4.1. Host Infiltrating Inflammatory Cells

Cancer development often coincides with active chronic and recurrent inflammatory responses caused by innate immune responses to the presence of altered cancer epithelial cells and bacterial or viral infections at the site of cancer origin. The infiltrating inflammatory cells have been shown to release ROS and reactive nitrogen species, such as hydrogen peroxide, superoxide, and nitric oxide, to protect the host cells and eradicate the "foreign" cells and invading organisms *(82,83)*. Responses to the presence of these highly reactive oxygen and nitrogen radicals released by the inflammatory cells and cancer cells could induce DNA damage to cancer cells and host stroma, activating DNA repair and cell proliferation programs to compensate for the cell loss resulting from failure to repair and subsequent cell death *(84,85)*. An extensive literature suggests the possible functional and signaling roles of oxygen and nitrogen radicals in eliciting cell responses to stress and escape mechanisms for survival *(86)*. These observations collectively support the important role of the inflammatory cascade in carcinogenesis. De Marzo et al. *(87,88)* proposed a role for inflammation in prostate cancer development when they found foci of proliferative inflammatory atrophy (PIA) as precursor lesions before the detection of prostate intraepithelial neoplasia (PIN), a known early pathological lesion associated with human prostate cancer development. They reported compelling epidemiological evidence to suggest a link between prostate inflammation and prostate cancer in men *(87)*. For example, a positive correlation was found between prostatitis and sexually transmitted infections

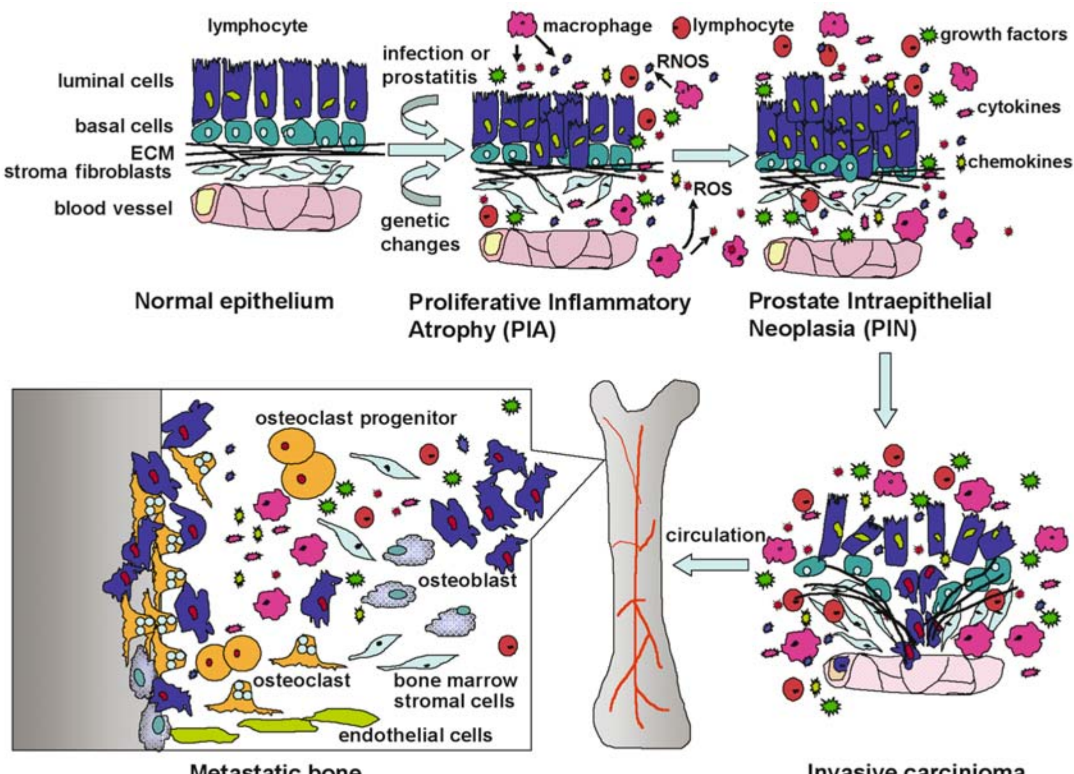


Fig. 4. The contribution of inflammatory cells to the progression of prostate cancer. Recruitment of inflammatory cells to the prostate gland can occur under a variety of physiological and pathophysiological conditions, such as wound, infection, prostatitis, and cancer. Genetically altered prostate epithelial cells, under the influence of resident fibroblasts, inflammatory (such as macrophage and lymphocytes) cells, and endothelial cells progress further through additional genetic changes triggered by reactive oxygen species (ROS) or reactive nitrogen species (RNOS). The genetically unstable prostate cancer cell clusters can form proliferative inflammatory atrophy and then proceed to prostate intraepithelial neoplasia before becoming prostate cancer cells with increased malignant potential. After metastasizing to bone, prostate cancer cells interact with bone cells, such as osteoblasts and marrow stromal cells, to increase their growth and survival in bone. Through cellular interaction with osteoclasts, prostate cancer cells also promote osteoclastogenesis and bone turnover by increased osteoclast maturation via receptor activator of NF-κB (RANK) ligand (localized on prostate cancer cell surface) and RANK (localized on the cell surface of osteoclasts) interaction. Because of increased bone turnover, there is increased release of soluble growth factors, cytokines and extracellular matrices (ECMs), which promote further prostate cancer growth and survival in bone.

and increased prostate cancer risk *(88–90)*. Intake of anti-inflammatory drugs and antioxidants has been shown to decrease prostate cancer risk *(91–94)*. Genetic studies revealed further supportive evidence that *RNASEL*, encoding an interferon-inducible ribonuclease *(95,96)*, and *MSR1*, encoding subunits of the macrophage scavenger receptor *(96–98)*, are candidates as inherited susceptibility genes for familial prostate cancer. Conversely, the loss of *GSTP1*, encoding a glutathione-*S*-transferase capable of inactivating ROS and, thus, reducing genome damage, has been found to occur frequently in human prostate cancer *(87,98–100)*. Figure 4 emphasizes the potential roles of inflammatory processes and the ways to antagonize them in prostate cancer development.

4.2. Host Stem Cells

There are two pools of stem cells that are thought to contribute to local cancer growth and its distant metastasis, and one of these stem cell populations serves as progenitors. With their uncontrolled growth potential and self-renewal property, they become a constant source of cancer cells and populate the tumor mass *(101–103)*. For example, the basal cells in the prostate gland have been referred to as stem cells that share gene expression profiles with cancer cells and could be considered as the progenitor cells of prostate cancer *(102,104)*. Another pool of stem cells could originate from the host *(22,105)*. Bone marrow-derived progenitor stem cells have migratory and invasive potential *(106,107)*. On cancer cell growth and metastasis, this pool of cells has been shown to migrate into primary or metastatic cancers, creating a rich source of growth factor and a cytokine niche supporting the growth and expansion of cancer cells *(25)*. Although there is no concrete example of this kind of mechanism in prostate cancer, a recent work by Kaplan et al. *(108)* showed that the ability of tumor cells to metastasize to a predetermined location can be explained by the previous "marking" of the metastatic site by bone marrow–hematopoietic progenitor cells that express VEGF receptor 1 (VEGF1, or Flt1) and VLA-4 (integrin $\alpha 4\beta 1$; refs. *109* and *110*). The expression of $\alpha 4\beta 1$, a known receptor of fibronectin produced by resident fibroblasts, in response to tumor-specific factors, creates a permissive niche for the incoming migrating tumor cells *(109,110)*. If this mechanism has general applicability, it can be proposed that a previously established bone marrow stem cell niche, in response to tumor-derived factors by resident marrow stromal cells, could also be responsible for attracting prostate cancer bone metastasis *(108–111)*. It has already been proposed that the homing mechanism of prostate cancer cells may involve:

1. Chemokines (SDF-1 or CXCR12) derived from marrow stromal cells and chemokine receptors (CXCR4) on the cell surface of prostate cancer cells *(112)*.
2. Cell adhesion molecules on marrow endothelial cells and integrins ($\alpha v\beta 3$ and $\alpha 4\beta 1$) on the cell surface of prostate cancer cells *(113,114)*.
3. Hedgehog produced by cancer cells, which triggers a host stromal response mediated by paracrine interaction with cell surface receptors, patched1 *(115–117)*.
4. Complementary growth factors/growth factor receptors and/or extracellular matrices/integrins produced by prostate cancer cells and bone cells, such as marrow stromal cells, osteoblasts, osteoclasts, or bone marrow progenitor stem cells.

Understanding the molecular mechanisms at the interface of prostate cancer cells and host cells could help in the future development of novel therapies for the treatment of prostate cancer bone metastasis *(5,45,60)*.

5. STRATEGIES TO CO-TARGET BOTH CANCER AND HOST FOR THE TREATMENT OF LETHAL PROSTATE CANCER METASTASIS

Increasing evidence suggests that an intimate interaction between cancer and its host contributes to local prostate cancer growth and distant metastasis. Cancer and host interaction has also been shown to support the survival of prostate cancer cells when subjected to hormone therapy, chemotherapy, and radiation therapy *(5,118,119)*. Through epigenetic cancer and host interaction, additional genetic changes can be introduced into both of the interactive cell types and further evolve the tumor phenotypes and genotypes *(5,10,13,15)*. This could be the molecular basis for cancer's status as a constantly moving target for which therapy must be tailored on an individual basis. For these reasons, the most effective means of controlling prostate cancer local growth and distant metastasis may be co-targeting strategies that eliminate cancer cell growth and also deprive cancer cells of their support systems from the host. This will hopefully eliminate or minimize the ability of cancer cells to survive and undergo continuous genetic and behavioral evolution by escaping previously effective therapies. The best-known example is the androgen-independent progression of prostate cancer cells, by which they escape hormonal dependency and become unresponsive to androgen

withdrawal *(45,120)*. The concept of co-targeting cancer and host has already been backed up by a number of examples. These are:

1. The use of an antiangiogenic drug (e.g., thalidomide) to target the endothelium in combination with chemotherapy targeting prostate cancer cells *(121,122)*.
2. The combined use of a radionuclide, Sr89 or Sm, to target the osteoblasts with chemotherapy targeting prostate cancer cells *(123)*.
3. The use of an endothelin (ET)-1 receptor antagonist, atrasentan, to block the paracrine interaction between prostate cancer cells, which produce ET-1, with its action mediated by ET-1A receptor on the cell surface of osteoblasts *(124)*.
4. Interrupting paracrine/autocrine growth factors and growth factor receptors or extracellular matrix and integrin interactions by the use of antibodies, such as insulin-like growth factor-1R or platelet-derived growth factor receptor antibodies, integrin isotype-specific antibodies, and antibodies to the stem cell hedgehog signaling pathway “hijacked” by cancer cells *(5,125,126)*.
5. Targeting the interphase between prostate cancer and bone cell interaction by slowing down bone turnover with bisphosphonates, and osteoblast/prostate cancer interaction with a decoy RANK receptor, osteoprotegerin *(54,127)*.
6. Co-targeting prostate cancer and bone stroma using an adenoviral-based gene therapy with either therapeutic cytotoxic *herpes simplex virus* (hsv)-thymidine kinase or viral replication controlled by tissue-specific and tumor-restrictive promoter, hOC or hBSP *(119,128,129)*.

The rationales of these strategies are to interrupt cancer–host interaction and communication and make the host microenvironment hostile to cancer growth and survival. A broad range of experimental approaches holds promise and could someday change how cancer metastasis is evaluated and could lead to its treatment on an individual basis.

REFERENCES

1. Hunter, K. (2006). Host genetics influence tumour metastasis. *Nat. Rev. Cancer* **6(2)**, 141–146.
2. Bissell, M. J. and Labarge, M. A. (2005). Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? *Cancer Cell* **7(1)**, 17–23.
3. Haslam, S. Z. and Woodward, T. L. (2003). Host microenvironment in breast cancer development: epithelial-cell-stromal-cell interactions and steroid hormone action in normal and cancerous mammary gland. *Breast Cancer Res.* **5(4)**, 208–215.
4. Lynch, C. C. and Matrisian, L. M. (2002). Matrix metalloproteinases in tumor-host cell communication. Differentiation **70(9–10)**, 561–573.
5. Chung, L. W., Baseman, A., Assikis, V., and Zhau, H. E. (2005). Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J. Urol.* **173(1)**, 10–20.
6. Baker, S. G., Lichtenstein, P., Kaprio, J., and Holm, N. (2005). Genetic susceptibility to prostate, breast, and colorectal cancer among Nordic twins. *Biometrics* **61(1)**, 55–63.
7. Heavey, P. M., McKenna, D., and Rowland, I. R. (2004). Colorectal cancer and the relationship between genes and the environment. *Nutr. Cancer* **48(2)**, 124–141.
8. Gallagher, P. G., Bao, Y., Prorock, A., et al. (2005). Gene expression profiling reveals cross-talk between melanoma and fibroblasts: implications for host-tumor interactions in metastasis. *Cancer Res.* **65(10)**, 4134–4146.
9. Gleave, M., Hsieh, J. T., Gao, C. A., von Eschenbach, A. C., and Chung, L. W. (1991). Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res.* **51(14)**, 3753–3761.
10. Thalmann, G. N., Anezinis, P. E., Chang, S. M., et al. (1994). Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res.* **54(10)**, 2577–2581.
11. Yoneda, T. and Hiraga, T. (2005). Crosstalk between cancer cells and bone microenvironment in bone metastasis. *Biochem. Biophys. Res. Commun.* **328(3)**, 679–687.
12. Paget, S. (1889). The distribution of secondary growths in cancer of the breast. *Cancer Metastasis Rev.* **8(2)**, 98–101.
13. Hill, R., Song, Y., Cardiff, R. D., and Van Dyke, T. (2005). Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. *Cell* **123(6)**, 1001–1011.
14. Pathak, S., Nemeth, M. A., Multani, A. S., Thalmann, G. N., von Eschenbach, A. C., and Chung, L. W. (1997). Can cancer cells transform normal host cells into malignant cells? *Br. J. Cancer* **76(9)**, 1134–1138.
15. Rhee, H. W., Zhau, H. E., Pathak, S., et al. (2001). Permanent phenotypic and genotypic changes of prostate cancer cells cultured in a three-dimensional rotating-wall vessel. *In Vitro Cell Dev. Biol. Anim.* **37(3)**, 127–140.
16. Thalmann, G. N., Sikes, R. A., Wu, T. T., et al. (2000). LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. *Prostate* **44(2)**, 91–103.

17. Tuxhorn, J. A., Ayala, G. E., and Rowley, D. R. (2001). Reactive stroma in prostate cancer progression. *J. Urol.* **166(6)**, 2472–2483.
18. Bedogni, B., Welford, S. M., Cassarino, D. S., Nickoloff, B. J., Giaccia, A. J., and Powell, M. B. (2005). The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation. *Cancer Cell* **8(6)**, 443–454.
19. Lewis, C. E. and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* **66(2)**, 605–612.
20. Radisky, D. C., Levy, D. D., Littlepage, L. E., et al. (2005). Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* **436(7047)**, 123–127.
21. Zou, W. (2005). Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat. Rev. Cancer* **5(4)**, 263–274.
22. Fidler, I. J. (2002). The organ microenvironment and cancer metastasis. *Differentiation* **70(9–10)**, 498–505.
23. Horak, C. E. and Steeg, P. S. (2005). Metastasis gets site specific. *Cancer Cell* **8(2)**, 93–95.
24. Minn, A. J., Kang, Y., Serganova, I., et al. (2005). Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J. Clin. Invest.* **115(1)**, 44–55.
25. Constantinescu, S. N. (2000). Stem cell generation and choice of fate: role of cytokines and cellular microenvironment. *J. Cell. Mol. Med.* **4(4)**, 233–248.
26. Nojima, H. (1997). Cell cycle checkpoints, chromosome stability and the progression of cancer. *Hum. Cell* **10(4)**, 221–230.
27. Blackburn, E. H. (2005). Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. *FEBS Lett.* **579(4)**, 859–862.
28. Blasco, M. A. (2005). Telomeres and human disease: ageing, cancer and beyond. *Nat. Rev. Genet.* **6(8)**, 611–622.
29. Koeneman, K. S., Yeung, F., and Chung, L. W. (1999). Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* **39(4)**, 246–261.
30. Dvorak, H. F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* **315(26)**, 1650–1659.
31. Hendrix, M. J., Seftor, E. A., Hess, A. R., and Seftor, R. E. (2003). Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat. Rev. Cancer* **3(6)**, 411–421.
32. Bajou, K., Noel, A., Gerard, R. D., et al. (1998). Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat. Med.* **4(8)**, 923–928.
33. Boccaccio, C. and Comoglio, P. M. (2005). A functional role for hemostasis in early cancer development. *Cancer Res.* **65(19)**, 8579–8582.
34. Lassam, N. and Jay, G. (1989). Suppression of MHC class I RNA in highly oncogenic cells occurs at the level of transcription initiation. *J. Immunol.* **143(11)**, 3792–3797.
35. Gatti, L. and Zunino, F. (2005). Overview of tumor cell chemoresistance mechanisms. *Methods Mol. Med.* **111**, 127–148.
36. Moreno, J., Krishnan, A. V., and Feldman, D. (2005). Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer. *J. Steroid. Biochem Mol. Biol.* **97(1–2)**, 31–36.
37. Chung, L. W. (1995). The role of stromal-epithelial interaction in normal and malignant growth. *Cancer Surv.* **23**, 33–42.
38. Cunha, G. R. (1994). Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer* **74(3 Suppl)**, 1030–1044.
39. Cunha, G. R., Donjacour, A. A., Cooke, P. S., et al. (1987). The endocrinology and developmental biology of the prostate. *Endocr. Rev.* **8(3)**, 338–362.
40. Chung, L. W., Anderson, N. G., Neubauer, B. L., Cunha, G. R., Thompson, T. C., and Rocco, A. K. (1981). Tissue interactions in prostate development: roles of sex steroids. *Prog. Clin. Biol. Res.* **75A**, 177–203.
41. Thompson, T. C., Cunha, G. R., Shannon, J. M., and Chung, L. W. (1986). Androgen-induced biochemical responses in epithelium lacking androgen receptors: characterization of androgen receptors in the mesenchymal derivative of urogenital sinus. *J. Steroid Biochem.* **25(5A)**, 627–634.
42. Amorino, G. P. and Parsons, S. J. (2004). Neuroendocrine cells in prostate cancer. *Crit. Rev. Eukaryot. Gene Expr.* **14**(4), 287–300.
43. Culig, Z. (2004). Androgen receptor cross-talk with cell signalling pathways. *Growth Factors* **22(3)**, 179–184.
44. Sanchez, P., Clement, V., and Ruiz i Altaba, A. (2005). Therapeutic targeting of the Hedgehog-GLI pathway in prostate cancer. *Cancer Res.* **65(8)**, 2990–2992.
45. Scher, H. I. and Sawyers, C. L. (2005). Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J. Clin. Oncol.* **23(32)**, 8253–8261.
46. Charames, G. S. and Bapat, B. (2003). Genomic instability and cancer. *Curr. Mol. Med.* **3(7)**, 589–596.
47. Mendez, O., Fernandez, Y., Peinado, M. A., Moreno, V., and Sierra, A. (2005). Anti-apoptotic proteins induce non-random genetic alterations that result in selecting breast cancer metastatic cells. *Clin. Exp. Metastasis* **22(4)**, 297–307.
48. Hunter, K. W. (2004). Host genetics and tumour metastasis. *Br. J. Cancer* **90(4)**, 752–755.
49. Koop S., MacDonald, I. C., Luzzi, K., et al. (1995). Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate. *Cancer Res.* **55(12)**, 2520–2523.

50. Bhowmick, N. A., Chytil, A., Plieth D., et al. (2004). TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* **303(5659)**, 848–851.
51. Mundy, G. R. (1997). Mechanisms of bone metastasis. *Cancer* **80(8 Suppl)**, 1546–1556.
52. Lecrone, V., Li, W., Devoll, R. E., Logothetis, C., and Farach-Carson, M. C. (2000). Calcium signals in prostate cancer cells: specific activation by bone-matrix proteins. *Cell Calcium* **27(1)**, 35–42.
53. Thomas, R., True, L. D., Bassuk, J. A., Lange, P. H., and Vessella, R. L. (2000). Differential expression of osteonectin/SPARC during human prostate cancer progression. *Clin. Cancer Res.* **6(3)**, 1140–1149.
54. Keller, E. T. (2002). The role of osteoclastic activity in prostate cancer skeletal metastases. *Drugs Today* (Barc) **38(2)**, 91–102.
55. Zhang, J., Dai, J., Qi, Y., et al. (2001). Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. *J. Clin. Invest.* **107(10)**, 1235–1244.
56. Yeung, F., Law, W. K., Yeh, C. H., et al. (2002). Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. *J. Biol. Chem.* **277(4)**, 2468–2476.
57. Huang, W. C., Xie, Z., Konaka, H., Sodek, J., Zhau, H. E., and Chung, L. W. (2005). Human osteocalcin and bone sialoprotein mediating osteomimicry of prostate cancer cells: role of cAMP-dependent protein kinase A signaling pathway. *Cancer Res.* **65(6)**, 2303–2313.
58. Huang, W. C., Wu, D., Xie, Z., et al. (2006). B2-Microglobulin is a signaling and Growth-Promoting Factor for Human Prostate Cancer Bone Metastasis. **16(18)**, 9108–9116.
59. Keller, E. T. and Brown, J. (2004). Prostate cancer bone metastases promote both osteolytic and osteoblastic activity. *J. Cell Biochem.* **91(4)**, 718–729.
60. Logothetis, C. J. and Lin, S. H. (2005). Osteoblasts in prostate cancer metastasis to bone. *Nat. Rev. Cancer* **5(1)**, 21–28.
61. Xiao, Y., Haase, H., Young W. G., and Bartold, P. M. (2004). Development and transplantation of a mineralized matrix formed by osteoblasts in vitro for bone regeneration. *Cell Transplant.* **13(1)**, 15–25.
62. Lin, D. L., Tarnowski, C. P., Zhang, J., et al. (2001). Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes in vitro. *Prostate* **47(3)**, 212–221.
63. Louie, M. C., Yang, H. Q., Ma, A. H., et al. (2003). Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. *Proc. Natl. Acad. Sci. USA* **100(5)**, 2226–2230.
64. Zhang, X., Odom, D. T., Koo S. H., et al. (2005). Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proc. Natl. Acad. Sci. USA* **102(12)**, 4459–4464.
65. Harris, A. L. (2002). Hypoxia—a key regulatory factor in tumour growth. *Nat. Rev. Cancer* **2(1)**, 38–47.
66. Mayo, L. D., Kessler, K. M., Pincheira, R., Warren, R. S., and Donner, D. B. (2001). Vascular endothelial cell growth factor activates CRE-binding protein by signaling through the KDR receptor tyrosine kinase. *J. Biol. Chem.* **276(27)**, 25,184–25,189.
67. Kasbohm, E. A., Guo, R., Yowell, C. W., et al. (2005). Androgen receptor activation by G(s) signaling in prostate cancer cells. *J. Biol. Chem.* **280(12)**, 11,583–11,589.
68. Carmeliet, P., Dor, Y., Herbert, J. M., et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394(6692)**, 485–490.
69. Lee, M., Hwang, J. T., Lee, H. J., et al. (2003). AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *J. Biol. Chem.* **278(41)**, 39,653–39,661.
70. Hess, A. R., Postovit, L. M., Margaryan, N. V., et al. (2005). Focal adhesion kinase promotes the aggressive melanoma phenotype. *Cancer Res.* **65(21)**, 9851–9860.
71. Hauck, C. R., Hsia, D. A., and Schlaepfer, D. D. (2002). The focal adhesion kinase—a regulator of cell migration and invasion. *IUBMB Life* **53(2)**, 115–119.
72. Kornberg, L. J. (1998). Focal adhesion kinase and its potential involvement in tumor invasion and metastasis. *Head Neck* **20(8)**, 745–752.
73. Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat. (Basel)* **154(1)**, 8–20.
74. Thiery, J. P. (2003). Epithelial-mesenchymal transitions in development and pathologies. *Curr. Opin. Cell Biol.* **15(6)**, 740–746.
75. Davies, M., Robinson, M., Smith, E., Huntley, S., Prime, S, and Paterson, I. (2005). Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways. *J. Cell Biochem.* **95(5)**, 918–931.
76. Janda, E., Lehmann, K., Killisch, I., et al. (2002). Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J. Cell Biol.* **156(2)**, 299–313.
77. Brabletz, T., Hlubek, F., Spaderna, S., et al. (2005). Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. *Cells Tissues Organs* **179(1–2)**, 56–65.
78. Liebner, S., Cattelino, A., Gallini, R., et al. (2004). Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. *J. Cell Biol.* **166(3)**, 359–367.

79. Huber, M. A., Kraut, N., and Beug, H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr. Opin. Cell Biol.* **17**(5), 548–558.
80. Bharti, A. C. and Aggarwal, B. B. (2002). Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem. Pharmacol.* **64(5–6)**, 883–888.
81. Kucharczak, J., Simmons, M. J., Fan, Y., and Gelinas, C. (2003). To be, or not to be: NF-kappaB is the answer—role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene* **22(56)**, 8961–8982.
82. Bauer, G. (2000). Reactive oxygen and nitrogen species: efficient, selective, and interactive signals during intercellular induction of apoptosis. *Anticancer Res.* **20(6B)**, 4115–4139.
83. Ernst, P. (1999). Review article: the role of inflammation in the pathogenesis of gastric cancer. *Aliment Pharmacol. Ther.* **13(Suppl. 1)**, 13–18.
84. Boonstra, J. and Post J. A. (2004). Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene* **337**, 1–13.
85. Slupphaug, G., Kavli, B., and Krokan, H. E. (2003). The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat. Res.* **531(1–2)**, 231–251.
86. Fehrenbach, E., Northoff, H. (2001). Free radicals, exercise, apoptosis, and heat shock proteins. *Exerc. Immunol. Rev.* **7**, 66–89.
87. De Marzo, A. M., Meeker, A. K., Zha, S., et al. (2003). Human prostate cancer precursors and pathobiology. *Urology* **62(5 Suppl 1)**, 55–62.
88. Platz, E. A. and De Marzo, A. M. (2004). Epidemiology of inflammation and prostate cancer. *J. Urol.* **171(2 Pt 2)**, S36–S40.
89. Mehik, A., Hellstrom, P., Sarpola, A., Lukkarinen, O., and Jarvelin, M. R. (2001). Fears, sexual disturbances and personality features in men with prostatitis: a population-based cross-sectional study in Finland. *BJU Int.* **88(1)**, 35–38.
90. Peyromaure, M., Ravery, V., Messas, A., Toublanc, M., Boccon-Gibod, L., and Boccon-Gibod, L. (2002). Pain and morbidity of an extensive prostate 10-biopsy protocol: a prospective study in 289 patients. *J. Urol.* **167(1)**, 218–221.
91. Fleshner, N. E. and Klotz, L. H. (1998). Diet, androgens, oxidative stress and prostate cancer susceptibility. *Cancer Metastasis Rev.* **17(4)**, 325–330.
92. Hussain, T., Gupta, S., Adhami, V. M., and Mukhtar, H. (2005). Green tea constituent epigallocatechin-3-gallate selectively inhibits COX-2 without affecting COX-1 expression in human prostate carcinoma cells. *Int. J. Cancer* **113(4)**, 660–669.
93. Lin, D. W. and Nelson, P. S. (2003). The role of cyclooxygenase-2 inhibition for the prevention and treatment of prostate carcinoma. *Clin. Prostate Cancer* **2(2)**, 119–126.
94. Nelson, W. G., De Marzo, A. M., Deweese, T. L., et al. (2001). Preneoplastic prostate lesions: an opportunity for prostate cancer prevention. *Ann. NY Acad. Sci.* **952**, 135–144.
95. Maier, C., Haeusler, J., Herkommer, K., et al. (2005). Mutation screening and association study of RNASEL as a prostate cancer susceptibility gene. *Br. J. Cancer* **92(6)**, 1159–1164.
96. Nelson, W. G., De Marzo, A. M., DeWeese, T. L., and Isaacs, W. B. (2004). The role of inflammation in the pathogenesis of prostate cancer. *J. Urol.* **172(5 Pt 2)**, S6–S11; discussion S11–S12.
97. Seppala, E. H., Ikonen, T., Autio, V., et al. (2003). Germ-line alterations in MSR1 gene and prostate cancer risk. *Clin. Cancer Res.* **9(14)**, 5252–5256.
98. Xu, J., Zheng, S. L., Komiya, A., et al. (2002). Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Nat. Genet.* **32**(2), 321–325.
99. Bastian, P. J., Palapattu, G. S., Lin, X., et al. (2005). Preoperative serum DNA GSTP1 CpG island hypermethylation and the risk of early prostate-specific antigen recurrence following radical prostatectomy. *Clin. Cancer Res.* **11(11)**, 4037–4043.
100. Henrique, R. and Jeronimo, C. (2004). Molecular detection of prostate cancer: a role for GSTP1 hypermethylation. *Eur. Urol.* **46(5)**, 660–669; discussion 669.
101. Rizzo, S., Attard G., and Hudson D. L. (2005). Prostate epithelial stem cells. *Cell Prolif.* **38(6)**, 363–374.
102. Wang, S., Garcia, A. J., Wu, M., Lawson D. A., Witte, O. N., and Wu H. (2006). Pten deletion leads to the expansion of a prostatic stem/progenitor cell subpopulation and tumor initiation. *Proc. Natl. Acad. Sci. USA* **103(5)**, 1480–1485.
103. Xin L., Lawson, D. A., and Witte, O. N. (2005). The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. *Proc. Natl. Acad. Sci. USA* **102(19)**, 6942–6947.
104. Garraway, L. A, Lin, D., Signoretti, S., et al. (2003). Intermediate basal cells of the prostate: in vitro and in vivo characterization. *Prostate* **55(3)**, 206–218.
105. Religa, P., Cao, R., Bjorndahl, M., Zhou, Z., Zhu, Z., and Cao Y. (2005). Presence of bone marrow-derived circulating progenitor endothelial cells in the newly formed lymphatic vessels. *Blood* **106(13)**, 4184–4190.
106. Tabatabai, G., Bahr, O., Mohle, R., et al. (2005). Lessons from the bone marrow: how malignant glioma cells attract adult haematopoietic progenitor cells. *Brain* **128(Pt 9)**, 2200–2211.
107. Voermans, C., van Heese, W. P., de Jong, I., Gerritsen, W. R., and van Der Schoot, C. E. (2002). Migratory behavior of leukemic cells from acute myeloid leukemia patients. *Leukemia* **16(4)**, 650–657.

108. Kaplan, R. N., Riba, R. D., Zacharoulis, S., et al. (2005). VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438(7069)**, 820–827.
109. Burger, J. A., Spoo, A., Dwenger, A., Burger, M., and Behringer, D. (2003). CXCR4 chemokine receptors (CD184) and alpha4beta1 integrins mediate spontaneous migration of human CD34+ progenitors and acute myeloid leukaemia cells beneath marrow stromal cells (pseudoemperipolesis). *Br. J. Haematol.* **122(4)**, 579–589.
110. Gazitt, Y. (2004). Homing and mobilization of hematopoietic stem cells and hematopoietic cancer cells are mirror image processes, utilizing similar signaling pathways and occurring concurrently: circulating cancer cells constitute an ideal target for concurrent treatment with chemotherapy and antilineage-specific antibodies. *Leukemia* **18(1)**, 1–10.
111. Uchiyama, H., Barut, B. A., Chauhan, D., Cannistra, S. A., and Anderson, K. C. (1992). Characterization of adhesion molecules on human myeloma cell lines. *Blood* **80(9)**, 2306–2314.
112. Taichman, R. S., Cooper, C., Keller, E. T., Pienta, K. J., Taichman, N. S., and McCauley, L. K. (2002). Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res.* **62(6)**, 1832–1837.
113. Cooper, C. R., Chay, C. H., and Pienta, K. J. (2002). The Role of alpha(v)beta(3) in prostate cancer progression. *Neoplasia* **4(3)**, 191–194.
114. Edlund, M., Miyamoto, T., Sikes, R. A., et al. (2001). Integrin expression and usage by prostate cancer cell lines on laminin substrata. *Cell Growth Differ.* **12(2)**, 99–107.
115. Karhadkar, S. S., Bova, G. S., Abdallah, N., et al. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* **431(7009)**, 707–712.
116. Sanchez, P., Hernandez, A. M., Stecca, B., et al. (2004). Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. *Proc. Natl. Acad. Sci. USA* **101(34)**, 12,561–12,566.
117. Sheng, T., Li, C., Zhang, X., et al. (2004). Activation of the hedgehog pathway in advanced prostate cancer. *Mol. Cancer.* **3**, 29.
118. Edlund, M., Sung, S. Y., and Chung, L. W. (2004). Modulation of prostate cancer growth in bone microenvironments. *J. Cell Biochem.* **91(4)**, 686–705.
119. Hsieh, C. L., Gardner, T. A., Miao, L., Balian, G., and Chung, L. W. (2004). Cotargeting tumor and stroma in a novel chimeric tumor model involving the growth of both human prostate cancer and bone stromal cells. *Cancer Gene Ther.* **11(2)**, 148–155.
120. Feldman, B. J. and Feldman, D. (2001). The development of androgen-independent prostate cancer. *Nat. Rev. Cancer* **1(1)**, 34–45.
121. Bamias, A. and Dimopoulos, M. A. (2005). Thalidomide and immunomodulatory drugs in the treatment of cancer. *Expert Opin. Investig. Drugs* **14(1)**, 45–55.
122. Capitosti, S. M., Hansen, T. P., and Brown, M. L. (2004). Thalidomide analogues demonstrate dual inhibition of both angiogenesis and prostate cancer. *Bioorg. Med. Chem.* **12(2)**, 327–336.
123. Tu, S. M., Kim, J., Pagliaro, L. C., et al. (2005). Therapy tolerance in selected patients with androgen-independent prostate cancer following strontium-89 combined with chemotherapy. *J. Clin. Oncol.* **23(31)**, 7904–7910.
124. Kopetz, E. S., Nelson, J. B., and Carducci, M. A. (2002). Endothelin-1 as a target for therapeutic intervention in prostate cancer. *Invest. New Drugs* **20(2)**, 173–182.
125. Uehara, H., Kim, S. J., Karashima, T., et al. (2003). Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastases. *J. Natl. Cancer Inst.* **95(6)**, 458–470.
126. Wu, J. D., Odman, A, Higgins, L. M., et al. (2005). In vivo effects of the human type I insulin-like growth factor receptor antibody A12 on androgen-dependent and androgen-independent xenograft human prostate tumors. *Clin. Cancer Res.* **11(8)**, 3065–3074.
127. Mantzoros, C. S., Tzonou, A., Signorello, L. B., Stampfer, M., Trichopoulos, D., and Adami, H.O. (1997). Insulin-like growth factor 1 in relation to prostate cancer and benign prostatic hyperplasia. *Br. J. Cancer* **76(9)**, 1115–1118.
128. Hsieh, C. L. and Chung, L. W. (2001). New prospectives of prostate cancer gene therapy: molecular targets and animal models. *Crit. Rev. Eukaryot. Gene Expr.* **11(1–3)**, 77–120.
129. Hsieh, C. L., Yang, L., Miao, L., et al. (2002). A novel targeting modality to enhance adenoviral replication by vitamin D(3) in androgen-independent human prostate cancer cells and tumors. *Cancer Res.* **62(11)**, 3084–3092.

6

Androgen Receptor Function in Prostate Cancer Progression

Helmut Klocker, Iris E. Eder, Barbara Comuzzi, Georg Bartsch, and Zoran Culig

Summary

Despite initially encouraging response to hormone therapy, prostate tumors finally develop a resistant phenotype and progress to a life-threatening disease. Critically involved in the mechanisms of therapy resistance and tumor progression is the androgen receptor, the central mediator of the proliferation and survival effects of androgens, and the target of endocrine therapy. There is mounting evidence that alteration of androgen signaling and function enables tumor cells to survive and adapt to hormone deprivation, resulting in autonomous activation of growth-promoting downstream targets. Changes include increased expression, gain of function mutations, activation of ligand-independent stimulation mechanisms, and altered expression of and interaction with co-modulatory proteins. A profound understanding of the pivotal changes underlying prostate cancer progression will be the key to the development of more efficient hormone therapy and novel treatment strategies for prostate cancer patients.

Key Words: Amplification; androgen ablation; androgen receptor; coactivators; ligand-independent activation; mutations; prostate cancer.

1. INTRODUCTION

The prostate gland is dependent on androgen hormones. It develops and grows under the influence of androgens, and their permanent stimulation is required for maintenance and function in the adult organ. Prostate tumors share this hormone-dependence with nonmalignant prostate tissue, at least in the early stages of tumor development. Since the pioneering work of Huggins, who first described this dependency, this forms the basis for the treatment of inoperable prostate cancer by hormone ablation *(1)*. Withdrawal of androgens and inhibition of androgen signal transduction by anti-androgens stop proliferation and induce programmed cell death (apoptosis) in prostate tumor cells, allowing control of tumor growth until therapy resistance is developing.

The central molecule for mediation of androgen function and the target for endocrine therapy of prostate cancer is the androgen receptor (AR), a ligand-activated transcription factor and member of the superfamily of nuclear receptors. This receptor also is in the center of the mechanism enabling prostate tumor cells to escape therapy. In the last decade of prostate cancer research, several such mechanisms involving AR function have been uncovered. We still do not have a detailed picture of all of the underlying molecular mechanisms, but the emerging picture gives us invaluable clues for improving currently used prostate cancer therapy and developing new treatment concepts. The importance of the AR and AR signaling in prostate cancer progression is illustrated by the recent finding that alterations in this pathway are the only consistent change in gene profiling studies eluci-

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

dating the mechanism of prostate tumor progression *(2)*. Amplifications of the AR gene occur in approximately one-third of tumors escaping endocrine therapy *(3,4)*.

2. AR STRUCTURE AND FUNCTION IN THE PROSTATE

The androgen hormone dihydrotestosterone (DHT) is the principal regulator of prostate development and growth during embryonal development and puberty *(5)*. In the adult prostate, it is required for maintenance of tissue homeostasis and secretory function. The circulating steroid hormone, testosterone, diffuses into the cells and is there converted by the 5-α reductase enzymes to the more potent androgen, DHT *(6)*. DHT and other androgen hormones present in the prostate cells bind to a high-affinity binding protein required for mediating their effects, the AR. It is a member of the superfamily of nuclear receptors, its closest relatives are the progesterone, glucocorticoid, and the other steroid hormone receptors *(7,8)*. Binding of androgen hormones triggers activation of the receptor to a transcription factor that interacts with and regulates the activity of gene promoters containing androgen-responsive elements (AREs). Activation involves a cascade of activation processes and the interaction with other components, such as co-regulatory proteins and proteins of the cellular transcription machinery.

The AR is encoded by a single gene on the long arm of the X chromosome spanning approx 180 kb (*see* gene atlas at: www.dsi.univ-paris5.fr/genatlas/fiche.php?n=5328) and is inherited in an X-linked fashion. The gene shows the typical structure of the nuclear receptor genes, with eight exons encoding the large N-terminal domain, a central DNA-binding domain composed of two zinc finger elements, a hinge region, and the C-terminal ligand-binding domain *(9–11)* (Fig. 1). The AR gene promoter is characterized by a short GC-box and a homopurin stretch, but lacks TATA and CAAT boxes typical for many eukaryotic genes *(12,13)*. Binding sites for the transcription factor SP1 and a cAMP-responsive element were characterized *(14,15)*. Two messenger RNA (mRNA) species are transcribed, the major one is 10 kb and the minor one is 7 kb in size *(13)*. The difference is in the large 3' untranslated region that follows the open-reading frame. The receptor protein has 919 amino acids, however, because of two polymorphic triplet repeats in exon 1, one encoding a polyglutamine (CAG) repeat and the second encoding a polyglycine (GCN) repeat with variable lengths, the individual size can vary *(16,17)*. Short triplet repeat regions seem to be associated with a moderately increased risk to develop prostate cancer *(18)* and extension beyond 40 repeats is the underlying cause of spinal and bulbar muscular atrophy *(19)*. The AR gene is a locus of frequent mutations (http//:www.mcgill.ca/androgendb). Approximately 300 mutations have been identified, most of them in male pseudohermaphroditism patients with various degrees of androgen insensitivity caused by loss of AR function *(20,21)*. Approximately 70 AR mutations have been identified in prostate cancer cell lines and specimens. Many cause the AR to acquire promiscuous properties and are described in Chapter 5.

Inactive AR is associated with heat shock proteins, and the binding of the hormone to the ligand-binding domain initiates a cascade transforming the AR into an active transcription factor. Activation includes dissociation of heat shock proteins, hyperphosphorylation, conformational changes, translocation into the nucleus, dimerization, and association with co-modulatory proteins *(22–25)*. Activated AR then interacts with the cellular transcription machinery regulating transcription of genes through binding to AREs *(26)*. Gene regulation may be positive or negative depending on the promoter and cellular context *(27–29)*.

Activated AR interacts with chromatin through its two central zinc finger motifs that are held in place by four conserved cysteine side chains coordinated to a zinc ion *(30,31)*. This domain is the most highly conserved region among the steroid receptors. Nuclear magnetic resonance and X-ray diffraction analysis revealed that the interaction with the DNA occurs through a helix that fits into the major DNA groove *(30–32)*. The N-terminal domain comprises approximately half of the protein and contains the activation function domain (AF)-1 . It brings about most of the transactivation activity, in contrast to other steroid receptors, the AF2 located in the C-terminus is weak in the AR *(33–35)*.

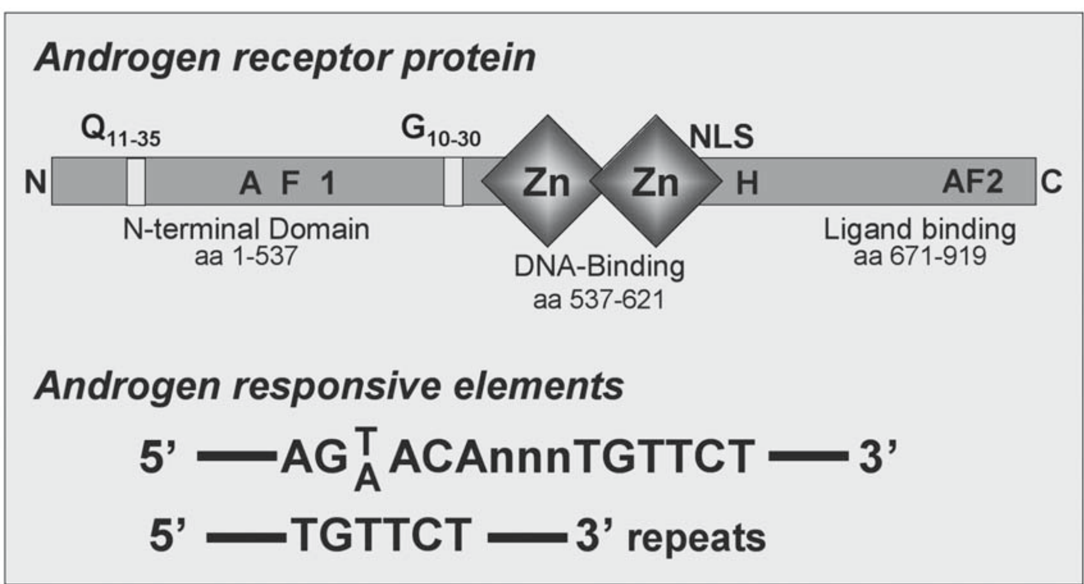


Fig. 1. Androgen receptor (AR) structure. The AR is a ligand-activated transcription factor mediating the effect of androgen hormones. Binding of androgens to the hormone-binding domain in the C-terminus induces a cascade of activation steps resulting in transformation to an active transcription factor. With the central DNA-binding domain built-up of two zinc (Zn) finger motifs, it interacts and binds to AREs in the promoters of genes. Immediately after the second zinc finger, there is a nuclear localization signal (NLS) that is essential for nuclear uptake of the receptor. A short hinge region (H) separates the DNA from the ligand-binding domain at the C-terminus. The large N-terminal transactivation domain contains two polymorphic amino acid repeats encoded by triplet repeats in the AR gene that vary in size among individuals (polyQ and polyG). The N-terminal domain also harbors the main transactivation function, activation function domain (AF)-1. A second transactivation function is localized in the ligand-binding domain, AF2. Both AF units interact with each other and are involved in recruitment of co-modulator proteins and cofactors of the transcription machinery.

The encoding gene is located on the long arm of the X-chromosome, and malfunction is associated with three diseases: syndromes of male sex ambiguity caused by partial or complete loss of AR function; prostate cancer, in which escape from hormone-ablation therapy is associated with AR alterations that result in an autonomous AR activation; and, finally, spinal and bulbar muscular atrophy, which is characterized by late onset and progressive weakening of skeletal muscles caused by an extension of the CAG triplet repeat.

More than 70 mutations have been detected in prostate cancer tissue, xenografts, and cell lines, the vast majority of them missense mutations. Several of the mutations located in the ligand-binding domain were shown to generate promiscuous receptors. Mutations located in the N-terminus of the receptor seem to alter the interaction with co-regulator proteins.

However, for full transactivation, both domains are required, and there is a physical interaction between the AF1 region in the N-terminus and the AF2 region in the C-terminus, which is important for recruitment of some coactivator proteins *(36,37)*.

Between the DNA-binding domain and the ligand-binding domain, the so-called hinge region provides a nuclear translocation signal, the function of which is essential for nuclear localization *(38)*. In addition, this region also provides the interface for interacting proteins, such as filamin A or p21-activated kinase 6 (PAK) *(39,40)*. The ligand-binding domain at the C-terminus of the receptor protein is structured in 12 α-helices and a β-sheet, and has a 3D structure very similar to the ligand-binding domains of other steroid receptors. The orientation of helix 12 differs depending on agonist or antagonist binding. It closes the ligand-binding pocket in case of an agonist and leaves the pocket

open when an antagonist is bond *(41)*. In mutant receptors that are activated by nonandrogenic steroids or anti-androgens, the orientation of helix 12 is the same as after androgen binding, thus, providing a structural explanation for the promiscuous behavior *(42)*.

The ARE consensus element is made up of two imperfect palindrome 6 bp elements separated by a spacer of 3 nucleotides (Fig. 1) *(43)*. This element is not specific for the AR, related steroid receptors such as progesterone and glucocorticoid receptors also bind to and activate transcription through this element. Androgen-specific regulation of these promoters requires additional cofactors acting in a cell- and tissue-specific manner *(44,45)*. In several androgen target genes, the AREs contain direct hexamere repeats of the canonical sequence 5'-TGTTCT-3' *(46–48)*.

3. AUTONOMOUS AR ACTIVATION IN THERAPY-REFRACTORY PROSTATE CANCER

Induction of programmed cell death induced by blockade of AR signaling is the fundamental treatment for non-organ-confined prostate cancer. This can be achieved through androgen withdrawal by surgical or chemical castration or interruption of androgen signal transmission by anti-androgens *(49)*. Often, a combination of androgen withdrawal and receptor inhibition by anti-androgens is also applied to achieve total androgen blockade. Although initially effective in the vast majority of treated patients, essentially all tumors develop resistance after a mean time of approx 2 years. Research into the basics of this progression to a hormone-insensitive tumor state provided mounting evidence in the last decade that changes in AR signaling are crucially involved *(50,51)*. The underlying mechanisms can be grouped into four major categories, increase of AR protein levels, gain of function mutations, ligand-independent receptor activation through signaling crosstalk, and changes in the interaction with transactivation co-modulators. These mechanisms are discussed in Chapters 4–7.

4. INCREASED AR EXPRESSION

Escape from androgen ablation therapy is associated with AR gene amplification in approx 20 to 30% of patients *(52,53)*. Moreover, increased AR protein levels were also found in tumors without gene amplification *(54)*. Amplification and overexpression is the result of a selection process observed after escape from hormone therapy, but only very rarely in untreated tumors. Not surprisingly, increase of the receptor protein seems to enable tumor cells to survive in the condition of androgen deprivation and adapt to it. Upregulation of AR expression is the only gene expression change that is consistently found in xenograft tumor progression models *(2)*. Interestingly, patients with an amplified AR gene showed a better response to second-line androgen ablation treatment, although this did not seem to bring about an advantage with regard to survival *(55)*.

5. AR MUTATIONS

The second mechanism used by prostate cancer cells to escape therapy is mutating the AR. Contrary to AR mutations found in male pseudohermaphroditism patients, which cause partial or complete loss of function, mutations occurring in prostate cancer provide a gain of function. Approximately 80 mutations have been identified *(56)*. In most cases, prostate cancer AR mutations result in exchange of a single amino acid, only few deletions or mutations that introduce premature stop codons or affect noncoding regions of the AR gene have been found. With only few exceptions, AR mutations in prostate cancer are somatic *(57–59)*.

AR mutations have been found in advanced and metastatic tumors as well as in primary, untreated tumor tissue and also in cases of latent carcinoma *(57,60–67)*. The predominant properties of mutant receptors found in prostate tumors are loss of androgen specificity and increased agonistic activation through nonandrogen steroid hormones and antiandrogens (Table 1). The best-studied example of this kind of AR mutation is AR T877A, initially detected in the LNCaP cell line and later also found in a number of prostate tumor specimens. In addition to androgens, the steroid hormones, estradiol

Table 1
Androgen Receptor Mutations in Prostate Cancer[a]

Amino acid position	Mutation	Description	Properties	References
Zinc finger 2	Duplication of exon 3	CWR22 xenograft In-frame insertion of exon 3 Generated during propagation	Decreased AR transcriptional activity Low level of PSA expression Promiscuous activation by DHEA	*(80,167)*
640	Gln→Stop	Metastatic lesion AR T877A mutation in the same tumor	Constitutive activity	*(81)*
646	Ser→Phe	High-grade tumor Short response to endocrine therapy	Increased transactivation of single ARE promoters	*(168)*
670	GluArg	Primary tumor of untreated patient Mutation in the AR hinge region	Promiscuous activation by progesterone, adrenal androgens, and hydroxyflutamide	*(60,169)*
715	Val→Met	Primary tumor of hormone-refractory patient	Promiscuous activation by progesterone, adrenal androgens, DHT metabolites, and hydroxyflutamide	*(170,171)*
726	Arg→Leu	Germline mutation Overrepresented in Finnish PCa patients	Promiscuous activation by estradiol	*(75,76)*
730	Arg→Leu	Primary tumor	Promiscuous activation by DHT metabolites and hydroxyflutamide	*(171,172)*
874	His→Tyr	CWR-22 PCa xenograft derived from a bone metastasis	Promiscuous activation by DHEA, estradiol, progesterone, and hydroxyflutamide	*(65,173–175)*
877	Thr→Ser	Primary tumor	Promiscuous activation by estradiol, progesterone, and hydroxyflutamide	*(65,173)*
877	Thr→Ala	LNCaP cell line derived from lymph node metastasis Several tumor specimens Mutation hot spot in hormone-refractory tumors	Promiscuous activation by estradiol, progesterone, hydroxyflutamide, and pregnenolone	*(64,69,71,74, 176,177)*
877+701	Thr→Ala Leu→His	Double mutation MDA PCa 2b cell line derived from tumor metastasis	Promiscuous activation by cortisol, corticosterone, and C17, C19, and C21 steroids Androgen activation decreased	*(42,77,79,178)*

[a]Only a small fraction of androgen receptor mutations found in prostate cancer have been functionally characterized. This table lists mutations for which information regarding altered function is available. For a complete list of all AR mutations detected in prostate cancer, see the AR database website (www.mcgill.ca/androgendb). AT, ; PSA, prostate-specific antigen; DHEA, dihydroepiandrosterone; ARE, androgen-responsive element; DHT, dihydrotestosterone; PCa, prostate cancer; AR, androgen receptor.

and progesterone, and the anti-androgens, cyproterone acetate, hydroxyflutamide, and nilutamide, bind to and activate this mutant AR and stimulate proliferation of LNCaP cells *(68–73)*. The amino acid site 877 and the region around it seems to be a mutational hot spot in prostate cancers. In patient tissues, a number of mutations have been identified in this region *(74)*.

Only a limited number of the mutant receptors found in prostate tumors have been functionally characterized thus far. In several cases, a promiscuous activity could be attributed to them. Among the activating ligands are estradiol, progesterone, glucocorticoids, adrenal androgens, androgen metabolites, and the anti-androgens used in prostate cancer treatment (Table 1). These mutations are all located in the ligand-binding domain. Among them is also one germ line mutation occurring in the Finnish population (AR R726L) that is overrepresented in Finnish prostate cancer patients *(75)*. This mutant receptor shows increased activation in response to estradiol *(76)*. An interesting mutant receptor was detected in a tumor cell line derived from the metastasis of a prostate cancer patient. It harbors two mutations, the AR T877A LNCaP mutation, plus a mutation at amino acid position 701, L701H *(77)*. It is activated by cortisol and corticosterone *(78,79)*. A mutant AR identified in a cell line derived from the CWR22 xenograft prostate cancer model has a duplication of exon 3, the second zinc finger of the DNA-binding domain *(80)*. This mutation occurred during propagation of the xenograft and was not present in the original tumor. A mutation that does not seem to change hormone specificity but elicits increased transcriptional activity on single ARE promoters was located in the hinge region and identified in a patient with a very short response to endocrine therapy (S646F) *(61)*. Yet another mechanism for AR activation under the condition of endocrine therapy is demonstrated by mutant AR Q640Stop identified in a cancer patient after escape from therapy *(81)*. This mutation generates a truncated receptor lacking the ligand-binding domain that is constitutively active and does not require hormone binding for transcriptional activity.

The importance of AR mutation for progression of prostate cancer was recently confirmed in a transgenic model, the TRAMP mouse *(82)*. In tumors that developed after castration, nine AR mutations were identified, seven in the transactivation domain and two in the ligand-binding domain *(83)*. One of these mutant receptors, E231G, demonstrated increased activity in response to androgen and estradiol in the presence of an AR coactivator. This demonstrates another aberrant function of mutant ARs, alteration of modulatory activity of coactivators and co-repressors.

Han and colleagues used transgenic mice to manipulate the expression level of AR in the prostate and to specifically introduce mutant ARs *(84)*. Whereas expression of a wild-type or a promiscuous (mouse AR T857A corresponding to human A877T) did not cause development of prostate cancer, the mouse AR variant E231G, which alters the AR in a highly conserved motif of the N-terminal transactivation domain involved in co-regulator interaction caused rapid development of precancerous neoplasia that progressed to invasive and metastatic cancer in all animals. This confirms that altered interaction with co-modulatory proteins caused by AR mutations can trigger prostate carcinogenesis and tumor progression.

6. AR COACTIVATORS AND PROSTATE CANCER DEVELOPMENT AND PROGRESSION

Treatment of diverse cell lines with androgens results in variable induction of target genes. The most likely explanation for differential activation of the AR is cell-specific presence of proteins that enhance (coactivators) or diminish (co-repressors) AR activity. The fact that transcriptional activity of steroid receptors decreases after transfection of other steroid receptors led to the assumption that different steroid receptors compete for the same co-regulators. The first AR co-regulatory molecule, androgen receptor associated protein 70 (ARA70), was discovered by a yeast two-hybrid screening approach with a GAL4 AR fusion protein as the bait *(85)*. A similar approach was applied in other studies in which proteins that interact with the AR in a ligand-dependent manner were identified. The effect of coactivators is, in most cases, demonstrated in cotransfection–transactivation assays in which

they further increase ligand-induced AR activity *(86)*. Expression of the AR should not be modified by molecules characterized as coactivators.

Type I coactivators are bridging proteins between the DNA-bound nuclear receptor and the basal transcriptional machinery. Protein inhibitors of activated signal transducers and activators of transcription (STAT) factors (PIAS), whose members exert differential effects on AR activity, belong to this group. AR activation is stimulated by PIAS 1 and 3 but repressed by PIASy *(87)*. β-catenin has a role in cell–cell adhesion by linking the cytoskeleton to adherens junctions, acting as a type I coactivator *(88)*. As an effector of the wingless type MMTV integration site family (Wnt) pathway, β-catenin regulates proliferation, differentiation, and migration *(89)*. Cytoplasmic β-catenin can form a complex with members of the T-cell factor and lymphoid enhancer factor family of transcription factors in the nucleus. Class II coactivators have histone acetyltransferase activity that is required for relaxation of chromatin structure. They stabilize ligand binding and influence translocation of the AR.

Coactivators of the p160 group that interact with several steroid receptors are class II co-regulators *(90)*. Other examples of cofactors that belong to that group are BCl-2 associated athamogene 1L (BAG-1L), steroid receptor coactivators-1 proteins (SRC-1), ARA70, and ARA24 *(90–92)*. AR interacts with general transcription factors such as transcription factor IIF (TFIIF) through its NH_2-terminal. TFIIF domains serve in transcriptional initiation and elongation. It consists of six core subunits and a protein kinase moiety, cdk-activating kinase (CAK), which contains three catalytic subunits. One of these subunits, cyclin dependent kinase 7 (cdk7), interacts with AR.

Because a large number of co-regulatory proteins interact with AR (for a comprehensive list see the AR database, http://www.androgendb.mcgill.ca), it is not easy to assess the impact of a particular coactivator for AR function. This issue may be relevant when discussing new ways to inhibit AR action in prostate cancer. Although there are several means by which downregulation of AR coactivators could be achieved, it should be emphasized that there is a redundancy in their action. This is additionally supported by data showing that targeted disruption of SRC-1 does not cause an androgen-insensitive phenotype *(93)*. In some experimental studies, there was an effect on in vitro growth of prostate cancer cells in which a specific coactivator was knocked down *(94,95)*. However, in vivo confirmation is still missing. Most early studies with AR cofactors were performed by semiquantitative RT-PCR *(96)*. They largely confirmed nearly universal presence of coactivators in both benign and malignant tissue. More recently, improved research work using specific antibodies has allowed detailed studies of their localization and function. In addition, functional studies performed with chromatin immunoprecipitation assays allow assessment of recruitment of coactivators to promoters of target genes in various prostate cancer models *(97)*.

The purpose of this chapter is to familiarize readers with established facts and concepts in coactivator research. Some studies have focused on the role of the p160 coactivator, SRC-1, in prostate cancer. SRC coactivators are composed of NH_2-terminal tandem basic helix–loop–helix, PAS domains, three central LXXLL motifs, and a COOH-terminal glutamine-reach region. The AR interacts with SRC-1 through two different regions, one in the N-terminus (AF1) and the second one in the C-terminus (AF2). In studies carried out in material from patients with endocrine therapy-resistant carcinoma of the prostate, it was found that SRC-1 levels increase *(98)*. Similar findings were obtained in the recurrent CWR22 xenograft *(99)*. Increased levels of SRC-1 in therapy-resistant carcinoma of the prostate facilitate activation of the AR by adrenal androgens and metabolites. However, a study investigating the expression of 16 AR co-regulators, including SRC-1, by real-time PCR and *in situ* hybridization, found diminished expression of SRC-1 mRNA in therapy-refractory carcinomas *(100)*. On the other hand, its overexpression was found in the LuCaP 70 xenograft. Work by Fujimoto and associates suggested an opposite trend toward increased expression of SRC-1 in tumor specimens obtained from patients with poor prognosis *(101)*. These results indicate that more research is needed to investigate expression of SRC-1 and perhaps other cofactors in selected cell subpopulations representing benign and malignant epithelium and adjacent stroma. In addition, regu-

lation of mRNA and protein for most of the cofactors in prostate cancer is not well understood. SRC-1 is a mitogen-activated protein kinase (MAPK) phosphorylation substrate and has a role in ligand-independent activation of the AR by interleukin (IL)-6, a multifunctional cytokine involved in regulation of prostate cancer cell proliferation, apoptosis, and differentiation *(102)*. Similar changes in the expression of another p160 coactivator, transcriptional intermediary factor 2 (TIF-2), were also observed in patients with recurrent prostate cancer *(98)*. Posttranscriptional increase in TIF-2 expression was found in prostate cancer cells in response to epidermal growth factor (EGF) *(103)*. Phosphorylated TIF-2, thus, contributes to the potentiation of ligand-dependent AR activity by EGF. The levels of the p160 coactivator, ras-related C3 botulinum substrate 3 (RAC3), also correlate with prostate tumor grade and stage *(104)*.

Transcriptional integrators p300 and its functional homolog, CREB-binding protein (CBP), are also implicated in regulation of AR activity and cell growth in several ways. Both proteins, which can act either as type I or type II coactivators, were demonstrated to potentiate ligand-dependent activation of the AR *(86,105)*. They could perform enzymatic activities, such as acetylation and phosphorylation, or interact with other coactivators. For p300, there is an experimental evidence showing its involvement in ligand-independent activation by IL-6 *(106)*. Overexpression of p300 was sufficient to overcome lack of activation caused by blockade of the MAPK pathway. A p300 mutant that lacked histone acetyltransferase activity did not show an effect on IL-6-caused activation of the AR. When p300 levels were downregulated by short interfering RNA (siRNA), the ability of IL-6 to induce the expression of prostate-specific antigen (PSA) was abolished. Thus, different experimental approaches were used to demonstrate the role of p300 in the cross talk between IL-6 and AR signaling pathways. Increased expression of p300 in biopsies from patients treated with radical prostatectomy correlated with tumor volume, extraprostatic extension, seminal vesicle involvement, and progression after surgery *(107)*. Proliferation of prostate cancer cells was inhibited by p300 siRNA.

CBP was originally discovered in prostate cancer cells as a factor responsive for opposite effects of androgen and AP-1 *(86)*. Expression of CBP was demonstrated in both benign and malignant prostate tumors *(108)*. CBP may be particularly important for determining the outcome of treatment of cancer cells with anti-androgens. These drugs can exert undesirable agonistic effects because of the presence of AR point mutations or overexpression of specific cofactors. Agonistic properties of hydroxyflutamide were potentiated in transfected DU-145 or LNCaP cells in which CBP was overexpressed *(108)*. This seems to be a selective enhancement of agonistic action because the effect in the presence of bicalutamide was only minor. The levels of *CBP* mRNA and protein in prostate cancer cells LNCaP were downregulated by the synthetic androgen, R1881. This finding implies that, during androgen ablation, the expression of *CBP* (and some other coactivators) may increase, contributing to a hypersensitive reaction of the AR. In most patients who failed endocrine therapy, strong presence of CBP in tumors was detected by immunohistochemistry.

Expression of the AR coactivator, Tat-interacting protein 60 (Tip60), which directly acetylates the AR, is regulated in a similar manner in prostate cancer cells as that of CBP. A high expression of Tip60 was observed in nuclei in prostate cancers that failed to respond to endocrine therapy *(109)*. Upregulation and nuclear accumulation of Tip60 was induced after androgen ablation *(109)*. Tip60 is recruited to the PSA promoter in androgen-dependent prostate cancer cells after androgen treatment *(97)*. However, in androgen-independent sublines, this recruitment occurs even in the absence of androgen. This may be an important mechanism regulating androgen-independent expression of PSA and other AR target genes in prostate cancer. In androgen-depleted conditions, there is also enhanced expression of the F-actin-binding protein, gelsolin, which strongly potentiates AR activation by hydroxyflutamide *(110)*. Another example of a cofactor-supported agonistic effect of hydroxyflutamide is that of ARA55, a protein whose sequence shows a high homology to the mouse transforming growth factor-β-inducible gene, *hic5 (111)*. There is a lower expression of ARA55 in endocrine therapy-resistant prostate carcinoma and in LNCaP or DU-145 cells. The intermediary

molecule in transforming growth factor-β signaling, Smad3, was reported to exert either stimulatory or inhibitory effects on AR signaling depending on cell type *(112,113)*. The effects of estradiol and hydroxyflutamide were potentiated by ARA54, a co-regulatory protein with ubiquitin ligase activity, preferentially with the LNCaP mutated AR *(114)*. Coactivators whose expression increases after androgen ablation should be used in screening tests for novel anti-androgenic drugs. These novel compounds should be used in experimental therapy for prostate cancer only if they do not promote acquisition of agonistic properties of AR blockers.

ARA70, as mentioned, was the first coactivator whose interaction with the AR was investigated. However, it potentiates the effects of respective ligands on activation of progesterone and also the glucocorticoid receptor *(115)*. In prostate cancer, it is involved in AR activation by estradiol, δ-5-androstanediol, and anti-androgens, without showing a preference for hydroxyflutamide, bicalutamide, or cyproterone acetate *(116–118)*. Controversy continues regarding the role of ARA70 in prostate cancer cells. It was shown that LNCaP colony growth was suppressed by ARA70 and that advanced prostate cancers lost its expression *(119)*. These findings suggest that the coactivator has a primarily tumor-suppressing function. These data, however, differ from those of Hu and associates who presented the opposite results *(120)*. It seems that ARA70 is an AR target gene. In the CWR22 xenograft, its expression follows that of several well-defined genes regulated by the AR *(99)*. In addition, downregulation of AR levels by resveratrol leads also to reduction of ARA70 expression *(121)*. In general, studies on ARA70 are not conclusive and more research is needed to better understand its role at different stages of prostate carcinogenesis.

Retinoblastoma and BRCA1 proteins are tumor suppressors that induce AR transcriptional activity *(118,122)*. One could hypothesize that these coactivators modulate the prodifferentiation action of high concentrations of androgens. Regulation of cellular events in prostate cancer by retinoblastoma is, however, limited because prostate cancers present with loss of this tumor suppressor. In contrast, AR activity is also influenced by molecules that drive progression of the cell cycle, such as cyclin E *(123)*. This interaction occurs through the N-terminal region of the AR. Similarly, cdc25, a dual-specific phosphatase that promotes cell cycle progression by activating cyclin-dependent kinases also potentiates AR function and is overexpressed in tumors with a high Gleason score *(124)*. ARA160 is a coactivator that associates with the N-terminal of the AR and cooperates with ARA70 in enhancement of AR activity *(92)*.

7. CONTRIBUTION OF NONSTEROIDAL ACTIVATION OF THE AR TO PROSTATE CANCER PROGRESSION

Most steroid receptors can be activated in a ligand-independent manner or in a synergistic manner by a ligand and a cytokine or other compound that increases intracellular kinase levels. For example, human estrogen receptor-α exhibits ligand-independent activation, whereas progesterone and glucocorticoid receptor activity is regulated in a cooperative manner. This type of activation of the AR is of importance for advanced stage prostate cancer. Because of androgen-ablation therapy, the levels of active androgens in serum are low, whereas AR is expressed at appreciable levels. For the clinical situation, it is particularly important that nonsteroidal activators reduce the threshold needed for maximal stimulation of the receptor. The most important consequence of cross talk between steroid and peptide regulators of prostate growth is, thus, full functionality of the AR despite endocrine therapy. The extent of ligand-independent or synergistic AR activity varies between different cell lines, which express different levels of coactivators required for activation in the absence of ligand. Ligand-independent activation of the AR has been mostly studied in prostate cancer cells in which satisfactory levels of reporter gene activity could be measured after transfection, or in heterologous cell lines devoid of endogenous steroid receptors.

Various compounds that induce AR activation in the absence of ligand cause activation of signaling pathways of MAPK, phosphoinositide 3-kinase (PI3-K), or Akt. Whether the AR is directly phos-

phorylated by p42/p44 MAPK is a matter of debate *(24,125)*. MAPKs are phosphorylated in response to human epidermal growth factor receptor (HER)-2/neu or EGF, and correlation between AR phosphorylation by HER-2/neu and progression of the xenograft LAPC-4 is well-established *(126)*. The ability of nonsteroidal anti-androgens to antagonize induction of AR activity is impaired if MAPKs are increasingly phosphorylated *(127)*. Although some controversies regarding the impact of HER-2/neu expression exist in prostate cancer, it seems that its overexpression is restricted to a subgroup of patients with advanced disease. EGF expression in prostate cancer cell lines correlates with tumor growth and is increased in AR-negative PC-3 and DU-145 cells *(128)*. AR activation levels after treatment with EGF are relatively low in comparison with those induced by androgen *(129)*. Consistent with those findings, HER-2/neu but not EGF increased expression of PSA *(127)*. However, a recent study demonstrated the ability of EGF to increase the levels of human glandular kallikrein, a gene regulated by the AR *(130)*.

EGF increased phosphorylation of Ser650 in the AR, and this finding does not suggest the involvement of MAPK in activation through growth factors *(24)*. The same amino acid is phosphorylated in response to forskolin or phorbol ester, compounds that were also reported to increase AR activity. Taken together with previously discussed results from Gregory and associates, it seems that ligand-independent activation of the AR requires the presence of specific coactivator(s) rather than being a result of phosphorylation by intracellular kinases.

Another pathway that is activated in response to HER-2/neu is that of PI3-K/Akt. AR serine residues 213 and 791 are targets for phosphorylation by Akt *(131)*. Outcome of AR activation by Akt in LNCaP cells depends on a passage number. In low passages, AR activity is suppressed, whereas, in higher passage numbers, Akt is a stimulator of the androgen-responsive pathway *(132)*. The Akt pathway is required for activation of the AR by IL-4, a cytokine that is expressed at higher levels in human prostate cancer *(133)*. This activation is also dependent on the presence of nuclear factor-κB, a transcription factor highly expressed and activated in prostate cancer. Activation of the AR is also observed by IL-8, a proangiogenic cytokine. IL-8 signals through tyrosine kinase Src and focal adhesion kinase, thus, regulating cell growth and migration *(134)*. Stimulatory effects of insulin-like growth factor-I on AR-mediated signal transduction are mediated through β-catenin, whose stability is enhanced by the growth factor *(129,135)*. Insulin-like growth factor-I-mediated enhancement of AR activity was reduced if a dominant-negative construct of β-catenin was used *(135)*. Ligand-induced AR activation is enhanced by caveolin, a principal constituent of caveolae membranes *(136)*. Caveolin is implicated in the survival of cancer cells and is important for prostate tumor metastasis. Androgen-dependent transcription of PSA is potentiated by triiodothyronine *(137)*. Cadmium is identified as a carcinogenic compound in the prostate, promoting growth of LNCaP cells and AR activity *(138)*.

Human AR could also be activated by peptide hormones that induce elevation in intracellular cAMP levels. Their effects are normally studied in cells treated with a nonmetabolizable analog of cAMP. Ligand-independent effects of analogs of cAMP that then increase activity of the protein kinase A pathway were demonstrated in transfected PC-3 cells *(139)*. AR mutants lacking the DNA-binding domain were not activated by forskolin. In organ cultures from human prostate cancer, forskolin, a compound that activates adenylcyclase to synthesize cAMP and stimulates protein kinase A activity, upregulated endogenous PSA expression *(140)*. The ability of anti-androgens to inhibit AR activity induced by cAMP is not impaired. In cells transfected with AR complementary DNA (cDNA) and reporter genes, there is no change in expression of AR by nonsteroidal activators. Various experiments showed that the protein kinase A pathway is also implicated in steroidal activation of the AR *(139)*. The use of a specific inhibitor of this pathway diminished AR activation caused by androgen.

There is a considerable interest in interaction between IL-6 and the AR *(102,141–147)*. IL-6 causes multifunctional effects in target cells, depending on a predominant activation of an indi-

vidual signal transduction cascade. Phosphorylation of Janus kinases (JAK)/STAT, MAPK, and PI3-K is regulated to different extents by IL-6. This is the basis for differential and sometimes contrasting effects on cell number reported in the literature. AR activation by IL-6 is well-documented both in cells transfected with AR cDNA (DU-145) as well as in LNCaP cells in which PSA was induced. IL-6 is a cytokine clearly associated with tumor morbidity and is upregulated in organ-confined prostate cancer *(148)*. High IL-6 levels are measured in sera from patients with metastatic disease. Association between STAT3 and the AR is promoted by IL-6 *(149)*. Conversely, dominant-negative STAT3 decreases AR activation. AR activation by IL-6 was observed also in the LNCaP–IL-6$^+$ subline, in which upregulation of endogenous IL-6 leads to abolition of a negative growth response *(144)*. This subline was developed to study signaling changes in patients with prostate disease caused by chronic exposure to IL-6. Parental LNCaP cells, in which IL-6 induced PSA expression, is the only available prostate cancer cell line that is inhibited by IL-6 *(150)*. The major signaling pathway activated by IL-6 in LNCaP cells is the JAK/STAT pathway. Although STAT3 phosphorylation in LNCaP cells is associated with growth inhibition and neuroendocrine differentiation, STAT3 phosphorylation is elevated in most prostate cancer tissues *(151,152)*. The requirement of MAPK for IL-6-induced AR activity was also reported by several investigators *(107,147)*.

Another IL-6-related cytokine that activates the AR is oncostatin M. This activation was, however, associated with an acquisition of agonistic properties of hydroxyflutamide and inability of bicalutamide to reverse ligand-independent activation *(153)*. This is in contrast to action of these two anti-androgens in the presence of IL-6. Oncostatin M stimulates growth of prostate cancer cells in both a paracrine and autocrine manner *(154)*.

As mentioned, IL-6 regulation of AR activity in LNCaP cells is associated with induction of cellular differentiation *(141)*. The prodifferentiation compound, phenylbutyrate, also induces AR activity and PSA *(155)*. Taken together, these results show that nonsteroidal activation of the AR is not necessarily associated with progressive tumor growth and inhibition of apoptosis in prostate cancer.

8. TARGETING AR IN HORMONE REFRACTORY CANCER

Accumulating knowledge of how prostate cancer escapes therapeutic mechanisms led to a revival of interest in AR inhibition as a therapeutic concept. It became clear that the AR is a central therapeutic target in hormone-refractory tumors, not only in hormone-sensitive tumors. This led to novel target-directed approaches concentrating on developing new methods that enable inhibition of AR function in tumors that escape classic hormone ablation therapy *(156)*. The new strategies reach from blockade of AR function to prevention of AR activation in an androgen-deprived environment and elimination of AR expression using antisense technologies (Table 2).

Blockade of the function of heat shock protein (HSP)-90 by geldanamycin antibiotics interferes with proper AR folding and leads to partial elimination of AR and some other HSP90-dependent proteins. It was demonstrated that these compounds inhibit growth of tumor cells. In vivo models and the first clinical trial were initiated with 17-allylamino-17-demoxygeldanamycin *(157,158)*. Antisense molecules targeting AR mRNA were developed to eliminate AR at the level of the mRNA. Oligonucleotides directed toward the CAG-repeat region in the AR mRNA inhibit prostate cancer cells in vitro as well as in vivo *(159,160)*. For preventing ligand-independent AR activation through cross talk with growth factor and regulatory signaling pathways, small molecule inhibitors, antibodies, antisense oligonucleotides, dominant negative mutants, and decoy peptides have been tested and, in some cases, have been entered into clinical trials (Table 2) *(161–166)*. There is hope that some of these new therapies based on the biology of prostate tumors will soon reach clinical application and improve and broaden treatment options for therapy–refractory prostate cancer.

Table 2
Novel Concepts for Targeting Androgen Receptor in Hormone-Insensitive Tumors

Target	Therapy concept	Molecules	References	Status
HSP90	Inhibition of HSP90 caperon function prevents proper conformation of HSP90 dependent proteins, AR affected among others	Geldanamycin compounds	*(158,179)*	Clinical studies
AR Protein	Downregulation using small antisense nucleic acids	Antisense oligonucleotides and siRNA	*(159,160,180,181)*	Pre-clinical
AR function	Androgen receptor modulators affecting tissue and cell type specific AR function	SARMs	*(182,183)*	Clinical studies
AR protein	Modulation of AR protein levels by natural compounds	Epigallocatechin gallate, genistein, quercetin	*(184–186)*	Pre-clinical
Growth factor pathways	Inhibition of AR activation through AR-growth factor cross talk	GF receptor antibodies, protein kinase inhibitors	*(161,163,166,187)*	Clinical studies
AR transactivation function	Decoy molecules that mimic AR but lack intrinsic activity compete with the endogenous AR,	Peptides and proteins	*(188–190)*	Pre-clinical
	Decoy androgen responsive element	DNA Oligonucleotides		
AR modulators	Inhibition of AR coactivators, stimulation of AR corepressors	Peptides, mall molecule drugs	*(191)*	Pre-clinical

[a]HSP, heat shock protein; AR, androgen receptor; siRNA, short interfering RNA; SARM, specific androgen receptor modulator; GF, growth factor.

REFERENCES

1. Huggins, C. and Hodges, C. V. (1941). Studies on prostatic cancer: the effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res.* **1**, 293–297.
2. Chen, C. D., Welsbie, D. S., Tran, C., et al. (2004). Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* **10**, 33–39.
3. Koivisto, P., Kononen, J., Palmberg, C., et al. (1997). Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res.* **57**, 314–319.
4. Linja, M. J. and Visakorpi, T. (2004). Alterations of androgen receptor in prostate cancer. *J. Steroid Biochem. Mol. Biol.* **92**, 255–264.
5. Hiort, O., Holterhus, P. M., and Nitsche, E. M. (1998). Physiology and pathophysiology of androgen action. *Baillieres Clin. Endocrinol. Metab.* **12**, 115–132.
6. Bartsch, G., Rittmaster, R. S., and Klocker, H. (2002). Dihydrotestosterone and the concept of 5alpha-reductase inhibition in human benign prostatic hyperplasia. *World J. Urol.* **19**, 413–425.
7. Robinson-Rechavi, M., Escriva Garcia, H., and Laudet, V. (2003). The nuclear receptor superfamily. *J. Cell Sci.* **116**, 585–586.
8. McEwan, I. J. (2004). Sex, drugs and gene expression: signalling by members of the nuclear receptor superfamily. *Essays Biochem.* **40**, 1–10.

9. Lubahn, D. B., Joseph, D. R., Sullivan, P. M., Willard, H. F., French, F. S., and Wilson, E. M. (1988). Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* **240**, 327–330.
10. Lubahn, D. B., Brown, T. R., Simental, J. A., et al. (1989). Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity [published erratum appears in *Proc. Natl. Acad. Sci. USA* 1990;**87**:4411]. *Proc. Natl. Acad. Sci. USA* **86**, 9534–9538.
11. Fuller, P. J. (1991). The steroid nuclear receptor superfamily: mechanisms of diversity. *FASEB J.* **5**, 2243–2249.
12. Tilley, W. D., Marcelli, M., and McPhaul, M. J. (1990). Expression of the human androgen receptor gene utilizes a common promoter in diverse human tissues and cell lines. *J. Biol. Chem.* **265**, 13,776–13,781.
13. Faber, P. W., van Rooij, H. C., van der Korput, H. A., et al. (1991). Characterization of the human androgen receptor transcription unit. *J. Biol. Chem.* **266**, 10,743–10,749.
14. Mizokami, A., Yeh, S. Y., and Chang, C. (1994). Identification of 3',5'-cyclic adenosine monophosphate response element and other cis-acting elements in the human androgen receptor gene promoter. *Mol. Endocrinol.* **8**, 77–88.
15. Faber, P. W., van Rooij, H. C., Schipper, H. J., Brinkmann, A. O., and Trapman, J. (1993). Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen receptor promoter. The role of Sp1 *J. Biol. Chem.* **268**, 9296–9301.
16. Chang, B. L., Zheng, S. L., Hawkins, G. A., et al. (2002). Polymorphic GGC repeats in the androgen receptor gene are associated with hereditary and sporadic prostate cancer risk. *Hum. Genet.* **110**, 122–129.
17. Zitzmann, M. and Nieschlag, E. (2003). The CAG repeat polymorphism within the androgen receptor gene and maleness. *Int. J. Androl.* **26**, 76–83.
18. Clark, P. E., Irvine, R. A., and Coetzee, G. A. (2003). The androgen receptor CAG repeat and prostate cancer risk. *Methods Mol. Med.* **81**, 255–266.
19. Walcott, J. L. and Merry, D. E. (2002). Trinucleotide repeat disease. The androgen receptor in spinal and bulbar muscular atrophy. *Vitam. Horm.* **65**, 127–147.
20. Hiort, O. and Holterhus, P. M. (2003). Androgen insensitivity and male infertility. *Int. J. Androl.* **26**, 16–20.
21. McPhaul, M. J. and Griffin, J. E. (1999). Male pseudohermaphroditism caused by mutations of the human androgen receptor. *J. Clin. Endocrinol. Metab.* **84**, 3435–3441.
22. Veldscholte, J., Berrevoets, C. A., Zegers, N. D., van der Kwast, T. H., Grootegoed, J. A., and Mulder, E. (1992). Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* **31**, 7422–7430.
23. Beato, M., Truss, M., and Chavez, S. (1996). Control of transcription by steroid hormones. *Ann. NY Acad. Sci.* **784**, 93–123.
24. Gioeli, D., Ficarro, S. B., Kwiek, J. J., et al. (2002). Androgen receptor phosphorylation: Regulation and identification of the phosphorylation sites. *J. Biol. Chem.* **15**, 15.
25. Kuiper, G. G., De Ruiter, P. E., and Brinkmann, A. O. (1993). Androgen receptor phosphorylation. Ann. NY Acad. Sci. **684**, 224–226.
26. McEwan, I. J. (2000). Gene regulation through chromatin remodelling by members of the nuclear receptor superfamily. *Biochem. Soc. Trans.* **28**, 369–373.
27. Schneikert, J., Peterziel, H., Defossez, P. A., Klocker, H., Launoit, Y., and Cato, A. C. (1996). Androgen receptor-Ets protein interaction is a novel mechanism for steroid hormone-mediated down-modulation of matrix metalloproteinase expression. *J. Biol. Chem.* **271**, 23,907–23,913.
28. Chesire, D. R. and Isaacs, W. B. (2002). Ligand-dependent inhibition of beta-catenin/TCF signaling by androgen receptor. T cell factor. *Oncogene* **21**, 8453–8469.
29. Chipuk, J. E., Cornelius, S. C., Pultz, N. J., et al. (2002). The androgen receptor represses transforming growth factor-beta signaling through interaction with Smad3. *J. Biol. Chem.* **277**, 1240–1248.
30. Freedman, L. P. (1992). Anatomy of the steroid receptor zinc finger region. *Endocr. Rev.* **13**, 129–145.
31. Schwabe, J. W. and Rhodes, D. (1991). Beyond zinc fingers: steroid hormone receptors have a novel structural motif for DNA recognition. *Trends Biochem. Sci.* **16**, 291–296.
32. Schwabe, J. W. R., Neuhaus, D., and Rhodes, D. (1990). Solution structure of the DNA-binding domain of the oestrogen receptor. *Nature* **348**, 458–461.
33. Jenster, K., van der Korput, H. A. G. M., Trapman, J., and Brinkmann, A. O. (1995). Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *J. Biol. Chem.* **270**, 7341–7346.
34. Poukka, H., Karvonen, U., Yoshikawa, N., Tanaka, H., Palvimo, J. J., and Janne, O. A. (2000). The RING finger protein SNURF modulates nuclear trafficking of the androgen receptor. *J. Cell. Sci.* **113**(Pt 17), 2991–3001.
35. Brinkmann, A. O. (2001). Lessons to be learned from the androgen receptor. *Eur. J. Dermatol.* **11**, 301–303.
36. Saitoh, M., Takayanagi, R., Goto, K., et al. (2002). The presence of both the amino- and carboxyl-terminal domains in the AR is essential for the completion of a transcriptionally active form with coactivators and intranuclear compartmentalization common to the steroid hormone receptors: a three-dimensional imaging study. *Mol. Endocrinol.* **16**, 694–706.
37. Thompson, J., Saatcioglu, F., Janne, O. A., and Palvimo, J. J. (2001). Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity. *Mol. Endocrinol.* **15**, 923–935.
38. Jenster, G., Trapman, J., and Brinkmann, A. O. (1993). Nuclear import of the human androgen receptor. *Biochem. J.* **293**, 761–768.

39. Loy, C. J., Sim, K. S., and Yong, E. L. (2003). Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. *Proc. Natl. Acad. Sci. USA* **100**, 4562–4567.
40. Lee, S. R., Ramos, S. M., Ko, A., et al. (2002). AR and ER interaction with a p21-activated kinase (PAK6). *Mol. Endocrinol.* **16**, 85–99.
41. Matias, P. M., Donner, P., Coelho, R., et al. (2000). Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *J. Biol. Chem.* **275**, 26,164–26,171.
42. Matias, P. M., Carrondo, M. A., Coelho, R., et al. (2002). Structural basis for the glucocorticoid response in a mutant human androgen receptor (AR(ccr)) derived from an androgen-independent prostate cancer. *J. Med. Chem.* **45**, 1439–1446.
43. Roche, P. J., Hoare, S. A., and Parker, M. G. (1992). A consensus DNA-Binding site for the androgen receptor. *Mol. Endocrinol.* **6**, 2229–2235.
44. Ning, Y. M. and Robins, D. M. (1999). AML3/CBFalpha1 is required for androgen-specific activation of the enhancer of the mouse sex-limited protein (Slp) gene. *J. Biol. Chem.* **274**, 30,624–30,630.
45. Kang, Z., Janne, O. A., and Palvimo, J. J. (2004). Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. *Mol. Endocrinol.* **18**, 2633–2648.
46. Rennie, P. S., Bruchovsky, N., Leco, K. J., et al. (1993). Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. *Mol. Endocrinol.* **7**, 23–36.
47. Zhou, Z., Corden, J. L., and Brown, T. R. (1997). Identification and characterization of a novel androgen response element composed of a direct repeat. *J. Biol. Chem.* **272**, 8227–8235.
48. Verrijdt, G., Schoenmakers, E., Alen, P., et al. (1999). Androgen specificity of a response unit upstream of the human secretory component gene is mediated by differential receptor binding to an essential androgen response element. *Mol. Endocrinol.* **13**, 1558–1570.
49. Miyamoto, H., Messing, E. M., and Chang, C. (2004). Androgen deprivation therapy for prostate cancer: current status and future prospects. *Prostate* **61**, 332–353.
50. Culig, Z., Klocker, H., Bartsch, G., Steiner, H., and Hobisch, A. (2003). Androgen receptors in prostate cancer. *J. Urol.* **170**, 1363–1369.
51. Eder, I. E., Culig, Z., Putz, T., Nessler-Menardi, C., Bartsch, G., and Klocker, H. (2001). Molecular biology of the androgen receptor: from molecular understanding to the clinic. *Eur. Urol.* **40**, 241–251.
52. Koivisto, P., Kolmer, M., Visakorpi, T., and Kallioniemi, O. P. (1998. Androgen receptor gene and hormonal therapy failure of prostate cancer. *Am. J. Pathol.* **152**, 1–9.
53. Elo, J. P. and Visakorpi, T. (2001). Molecular genetics of prostate cancer. Ann Med 33, 130–141.
54. Linja, M. J., Savinainen, K. J., Saramaki, O. R., Tammela, T. L., Vessella, R. L., and Visakorpi, T. (2001). Amplification and overexpression of androgen receptor gene in hormone- refractory prostate cancer. *Cancer Res.* **61**, 3550–3555.
55. Palmberg, C., Koivisto, P., Kakkola, L., Tammela, T. L., Kallioniemi, O. P., and Visakorpi, T. (2000). Androgen receptor gene amplification at primary progression predicts response to combined androgen blockade as second line therapy for advanced prostate cancer. *J. Urol.* **164**, 1992–1995.
56. Gottlieb, B., Beitel, L. K., Wu, J. H., and Trifiro, M. (2004). The androgen receptor gene mutations database (ARDB): 2004 update. *Hum. Mutat.* **23**, 527–533.
57. Marcelli, M., Ittmann, M., Mariani, S., et al. (2000). Androgen receptor mutations in prostate cancer. *Cancer Res.* **60**, 944–949.
58. Buchanan, G., Greenberg, N. M., Scher, H. I., Harris, J. M., Marshall, V. R., and Tilley, W. D. (2001). Collocation of androgen receptor gene mutations in prostate cancer. *Clin. Cancer Res.* **7**, 1273–1281.
59. Culig, Z., Hobisch, A., Erdel, M., Bartsch, G., and Klocker, H. (2003). Studies on androgen receptor mutations and amplification in human prostate cancer. *Methods Mol. Med.* **81**, 267–275.
60. Tilley, W. D., Buchanan, G., Hickey, T. E., and Bentel, J. M. (1996). Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clin. Cancer Res.* **2**, 277–285.
61. Thompson, J., Hyytinen, E. R., Haapala, K., et al. (2003). Androgen receptor mutations in high-grade prostate cancer before hormonal therapy. *Lab. Invest.* **83**, 1709–1713.
62. Taplin, M. E., Rajeshkumar, B., Halabi, S., et al. (2003). Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. *J. Clin. Oncol.* **21**, 2673–2678.
63. Hyytinen, E. R., Haapala, K., Thompson, J., et al. (2002). Pattern of somatic androgen receptor gene mutations in patients with hormone-refractory prostate cancer. *Lab. Invest.* **82**, 1591–1598.
64. Suzuki, H., Sato, N., Watabe, Y., Masai, M., Seino, S., and Shimazaki, J. (1993). Androgen receptor gene mutations in human prostate cancer. *J. Steroid Biochem. Mol. Biol.* **46**, 759–765.
65. Taplin, M. E., Bubley, G. J., Shuster, T. D., et al. (1995). Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.* **332**, 1393–1398.
66. Taplin, M. E., Bubley, G. J., Ko, Y. J., et al. (1999). Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Res.* **59**, 2511–2515.
67. Takahashi, H., Furusato, M., Allsbrook, W. C., Jr., et al. (1995). Prevalence of androgen receptor gene mutations in latent prostatic carcinomas from Japanese men. *Cancer Res.* **55**, 1621–1624.

68. Veldscholte, J., Ris-Stalpers, C., Kuiper, G. G. J. M., et al. (1990). A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Comm.* **17**, 534–540.
69. Veldscholte, J., Berrevoets, C. A., Ris Stalpers, C., et al. (1992). The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J. Steroid. Biochem. Mol. Biol.* **41**, 665–669.
70. Montgomery, B. T., Young, C. Y., Bilhartz, D. L., et al. (1992). Hormonal regulation of prostate-specific antigen (PSA) glycoprotein in the human prostatic adenocarcinoma cell line, LNCaP. *Prostate* **21**, 63–73.
71. Veldscholte, J., Ris Stalpers, C., Kuiper, G. G., et al. (1990). A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **173**, 534–540.
72. Veldscholte, J., Voorhorst, O. M., Bolt, d. V. J., van, R. H., Trapman, J., and Mulder, E. (1990). Unusual specificity of the androgen receptor in the human prostate tumor cell line LNCaP: high affinity for progestagenic and estrogenic steroids. *Biochim. Biophys. Acta* **1052**, 187–194.
73. Veltscholte, J., Berrevoets, C. A., Zegers, N. D., van der Kvast, T. H., Grootegoed, J. A., and Mulder, E. (1992). Hormone-induced dissociation of the androgen receptor-heat-shock protein complex. Use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* **31**, 7422–7430.
74. Gaddipati, J. P., McLeod, D. G., Heidenberg, H. B., et al. (1994). Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. *Cancer Res.* **54**, 2861–2864.
75. Mononen, N., Syrjakoski, K., Matikainen, M., et al. (2000). Two percent of Finnish prostate cancer patients have a germ-line mutation in the hormone-binding domain of the androgen receptor gene. *Cancer Res.* **60**, 6479–6481.
76. Elo, J. P., Kvist, L., Leinonen, K., et al. (1995). Mutated human androgen receptor gene detected in a prostatic cancer patient is also activated by estradiol. *J. Clin. Endocrinol. Metab.* **80**, 3494–3500.
77. Zhao, X. Y., Boyle, B., Krishnan, A. V., Navone, N. M., Peehl, D. M., and Feldman, D. (1999). Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a. *J. Urol.* **162**, 2192–2199.
78. Zhao, X.-Y., Malloy, P. J., Krishnan, A. V., et al. (2000). Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat. Med.* **6**, 703–706.
79. Krishnan, A. V., Zhao, X. Y., Swami, S., et al. (2002). A glucocorticoid-responsive mutant androgen receptor exhibits unique ligand specificity: therapeutic implications for androgen-independent prostate cancer. *Endocrinology* **143**, 1889–1900.
80. Tepper, C. G., Boucher, D. L., Ryan, P. E., et al. (2002). Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer Res.* **62**, 6606–6614.
81. Ceraline, J., Cruchant, M. D., Erdmann, E., et al. (2004). Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer. *Int. J. Cancer* **108**, 152–157.
82. Gingrich, J. R., Barrios, R. J., Kattan, M. W., Nahm, H. S., Finegold, M. J., and Greenberg, N. M. (1997). Androgen-independent prostate cancer progression in the TRAMP model. *Cancer Res.* **57**, 4687–4691.
83. Han, G., Foster, B. A., Mistry, S., et al. (2001). Hormone status selects for spontaneous somatic androgen receptor variants that demonstrate specific ligand and cofactor dependent activities in autochthonous prostate cancer. *J. Biol. Chem.* **276**, 11,204–11,213.
84. Han, G., Buchanan, G., Ittmann, M., et al. (2005). Mutation of the androgen receptor causes oncogenic transformation of the prostate. *Proc. Natl. Acad. Sci. USA* **102**, 1151–1156.
85. Yeh, S. and Chang, C. (1996). Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proc. Natl. Acad. Sci. USA* **93**, 5517–5521.
86. Fronsdal, K., Engedal, N., Hagsvold, T., and Saatcioglu, F. (1998). CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. *J. Biol. Chem.* **273**, 31,853–31,859.
87. Gross, M., Liu, B., Tan, J., French, F. S., Carey, M., and Shuai, K. (2001). Distinct effects of PIAS proteins on androgen-mediated gene activation in prostate cancer cells. Oncogene **20**, 3880–3887.
88. Yang, F., Li, X., Sharma, M., et al. (2002). Linking beta-catenin to androgen-signaling pathway. *J. Biol. Chem.* **277**, 1336–1344.
89. Verras, M., Brown, J., Li, X., Nusse, R., and Sun, Z. (2004). Wnt3a growth factor induces androgen receptor-mediated transcription and enhances cell growth in human prostate cancer cells. *Cancer Res.* **64**, 8860–8866.
90. Ikonen, T., Palvimo, J. J., and Janne, O. A. (1997). Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. *J. Biol. Chem.* **272**, 29,821–29,828.
91. Froesch, B. A., Takayama, S., and Reed, J. C. (1998). BAG-1L protein enhances androgen receptor function. *J. Biol. Chem.* **273**, 11,660–11,666.
92. Hsiao, P. W. and Chang, C. (1999). Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. *J. Biol. Chem.* **274**, 22,373–22,379.

93. Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J., and O´Malley, B. W. (1998). Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* **279**, 1922–1925.
94. Rahman, M. M., Miyamoto, H., Lardy, H., and Chang, C. (2003). Inactivation of androgen receptor coregulator ARA55 inhibits androgen receptor activity and agonist effect of antiandrogens in prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 5124–5129.
95. Rahman, M. M., Miyamoto, H., Takatera, H., Yeh, S., Altuwaijri, S., and Chang, C. (2003). Reducing the agonist activity of antiandrogens by a dominant-negative androgen receptor coregulator ARA70 in prostate cancer cells. *J. Biol. Chem.* **278**, 19,619–19,626.
96. Nessler-Menardi, C., Jotova, I., Culig, Z., et al. (2000). Expression of androgen receptor coregulatory proteins in prostate cancer and stromal-cell culture models. *Prostate* **45**, 124–131.
97. Halkidou, K., Gnanapragasam, V. J., Mehta, P. B., et al. (2003). Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. Oncogene **22**, 2466–2477.
98. Gregory, C. W., He, B., Johnson, R. T., et al. (2001). A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res.* **61**, 4315–4319.
99. Gregory, C. W., Hamil, K. G., Kim, D., et al. (1998). Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res.* **58**, 5718–5724.
100. Linja, M. J., Porkka, K. P., Kang, Z., et al. (2004). Expression of androgen receptor coregulators in prostate cancer. *Clin. Cancer Res.* **10**, 1032–1040.
101. Fujimoto, N., Mizokami, A., Harada, S., and Matsumoto, T. (2001). Different expression of androgen receptor coactivators in human prostate. *Urology* **58**, 289–294.
102. Ueda, T., Bruchovsky, N., and Sadar, M. D. (2002). Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. *J. Biol. Chem.* **277**, 7076–7085.
103. Gregory, C. W., Fei, X., Ponguta, L. A., et al. (2004). Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. *J. Biol. Chem.* **279**, 7119–7130.
104. Gnanapragasam, V. J., Leung, H. Y., Pulimood, A. S., Neal, D. E., and Robson, C. E. (2001). Expression of RAC3, a steroid hormone receptor co-activator in prostate cancer. *Br. J. Cancer* **85**, 1928–1936.
105. Aarnisalo, P., Palvimo, J. J., and Janne, O. A. (1998). CREB binding protein in androgen receptor-mediated signaling. *Proc. Natl. Acad. Sci. USA* **95**, 2122–2127.
106. Debes, J. D., Schmidt, L. J., Huang, H., and Tindall, D. J. (2002). P300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6. *Cancer Res.* **62**, 5632–5636.
107. Debes, J., Sebo, T. J., Lohse, C. M., Murphy, L. M., Haugen de, A. L., and Tindall, D. J. (2003). p300 in prostate cancer proliferation and progression. *Cancer Res.* **63**, 7638–7640.
108. Comuzzi, B., Lambrinidis, L., Rogatsch, H., et al. (2003). The transcriptional co-activator cAMP response element-binding protein-binding protein is expressed in prostate cancer and enhances androgen- and anti-androgen-induced androgen receptor function. *Am. J. Pathol.* **162**, 233–241.
109. Comuzzi, B., Nemes, C., Schmidt, S., et al. (2004). The androgen receptor co-activator CBP is up-regulated following androgen withdrawal and is highly expressed in advanced prostate cancer. *J. Pathol.* **204**, 159–166.
110. Nishimura, K., Ting, H. J., Harada, Y., et al. (2003). Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. *Cancer Res.* **63**, 4888–4894.
111. Fujimoto, N., Yeh, S., Kang, H. Y., et al. (1999). Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. *J. Biol. Chem.* **274**, 8316–8321.
112. Kang, H. Y., Lin, H. K., Hu, Y. C., Yeh, S., Huang, K. E., and Chang, C. (2001). From transforming growth factor-beta signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **98**, 3018–3023.
113. Hayes, S. A., Zarnegar, M., Sharma, M., et al. (2001). SMAD3 represses androgen receptor-mediated transcription. *Cancer Res.* **61**, 2112–2118.
114. Kang, H. Y., Yeh, S., Fujimoto, N., and Chang, C. (1999). Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J. Biol. Chem.* **274**, 8570–8576.
115. Alen, P., Claessens, F., Schoenemakers, E., et al. (1999). Interaction of the putative androgen receptor-specific coactivator ARA70/ELE1 alpha with multiple steroid receptors and identification of an internally deleted ELE1beta isoform. *Mol. Endocrinol.* **13**, 117–128.
116. Miyamoto, H., Yeh, S., Wilding, G., and Chang, C. (1998). Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. *Proc. Natl. Acad. Sci. USA* **95**, 7379–7384.
117. Miyamoto, H., Yeh, S., Lardy, H., Messing, E., and Chang, C. (1998). Delta 5-androstanediol is a natural hormone with androgenic activity in human prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **95**, 11,083–11,088.
118. Yeh, S., Miyamoto, H., Nishimura, K., et al. (1998). Retinoblastoma, a tumor suppressor is a coactivator for the androgen receptor in human prostate cancer DU-145 cells. *Biochem. Biophys. Res. Commun.* **248**, 361–367.
119. Li, P., Yu, X., Ge, K., Melamed, J., Roeder, R. G., and Wang, Z. (2002). Heterogeneous expression and functions of androgen receptor co-factors in primary prostate cancer. *Am. J. Pathol.* **161**, 1467–1474.

120. Hu, Y. C., Yeh, S., Yeh, S. D., et al. (2004). Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. *J. Biol. Chem.* **279**, 33,438–33,446.
121. Mitchell, S. H., Zhu, W., and Young, C. Y. (1999). Resveratrol inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. *Cancer Res.* **59**, 5892–5895.
122. Park, J. J., Irvine, R. A., Buchanan, G., et al. (2000). Breast cancer susceptibility gene 1 (BRCAI) is coactivator of the androgen receptor. *Cancer Res.* **60**, 5946–5949.
123. Yamamoto, A., Hashimoto, Y., Kohri, K., et al. (2000). Cyclin E as a coactivator of the androgen receptor. *J. Cell. Biol.* **150**, 873–880.
124. Ngan, E. S. W., Hashimoto, Y., Ma, Z.-Q., Tsai, M. J., and Tsai, S. Y. (2002). Overexpression of Cdc25B, an androgen receptor coactivator, in prostate cancer. *Oncogene* **22**, 734–739.
125. Yeh, S., Lin, H.-K., Kang, H.-Y., Thin, T. H., Lin, M.-F., and Chang, C. (1999). From HER2/Neu signal cascade to androgen receptor and its coactivators: A novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **96**, 5458–5463.
126. Craft, N., Shostak, Y., Carey, M., and Sawyers, C. L. (1999). A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat. Med.* **5**, 280–285.
127. Craft, N., Chhor, C., Tran, C., et al. (1999). Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-independent tumors through a two-step process. *Cancer Res.* **59**, 5030–5036.
128. MacDonald, A. and Habib, F. K. (1992). Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. *Br. J. Cancer* **65**, 177–182.
129. Culig, Z., Hobisch, A., Cronauer, M. V., et al. (1994). Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res.* **54**, 5474–5478.
130. Festuccia, C., Gravina, G. L., Angelucci, A., et al. (2005). Additive antitumor effects of the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa), and the nonsteroidal antiandrogen, bicalutamide (Casodex), in prostate cancer cells in vitro. *Int. J. Cancer*, in press.
131. Wen, Y., Hu, M. C., Makino, K., et al. (2000). HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res.* **60**, 6841–6845.
132. Lin, H. K., Hu, Y. C., Yang, L., et al. (2003). Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *J. Biol. Chem.* **278**, 50,902–50,907.
133. Lee, S. O., Lou, W., Hou, M., Onate, S. A., and Gao, A. C. (2003). Interleukin-4 enhances prostate-specific antigen expression by activation of the androgen receptor and Akt pathway. *Oncogene* **22**, 6037–6044.
134. Lee, L. F., Louie, M. C., Desai, S. J., et al. (2004). Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene*, in press.
135. Verras, M. and Sun, Z. (2005). Beta-catenin is inolved in insulin-like growth factor 1-mediated transactivation of the androgen receptor. *Mol. Endocrinol.* **19**, 391–398.
136. Lu, M. L., Schneider, M. C., Zheng, Y., Zhang, X., and Richie, J. P. (2001). Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. *J. Biol. Chem.* **276**, 13,442–13,451.
137. Zhang, S., Hsieh, M. L., Zhu, W., Klee, G. G., Tindall, D. J., and Young, C. Y. (1999). Interactive effects of triiodothyronine and androgens on prostate cell growth and gene expression. *Endocrinology* **140**, 1665–1671.
138. Martin, M. B., Voeller, H. J., Gelmann, E. P., et al. (2002). Role of cadmium in the regulation of AR gene expression and activity. *Endocrinology* **143**, 264–275.
139. Nazareth, L. V. and Weigel, N. L. (1996). Activation of the human androgen receptor through a protein kinase A signaling pathway. *J. Biol. Chem.* **271**, 19900–19907.
140. Nakhla, A. M., Romas, N. A., and Rosner, W. (1997). Estradiol activates the prostate androgen receptor and prostate-specific antigen secretion through the intermediacy of sex hormone- binding globulin. *J. Biol. Chem.* **272**, 6838–6841.
141. Hobisch, A., Eder, I. E., Putz, T., et al. (1998). Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. *Cancer Res.*, in press.
142. Twillie, D. A., Eisenberger, M. A., Carducci, M. A., Hseih, W.-S., Kim, W. Y., and Simons, J. W. (1995). Interleukin-6: a candidate mediator of human prostate cancer morbidity. *Urology* **45**, 542–549.
143. Chen, T., Wang, L. H., and Farrar, W. L. (2000). Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. *Cancer Res.* **60**, 2132–2135.
144. Hobisch, A., Ramoner, R., Fuchs, D., et al. (2001). Prostate cancer cells (LNCaP) generated after long-term interleukin-6 treatment express interleukin-6 and acquire an interleukin-6-partially resistant phenotype. *Clin. Cancer Res.* **7**, 2941–2948.
145. Spiotto, M. T. and Chung, T. D. (2000). STAT3 mediates IL-6-induced neuroendocrine differentiation in prostate cancer cells. *Prostate* **42**, 186–195.
146. Hobisch, A., Eder, I. E., Putz, T., et al. (1998). Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. *Cancer Res.* **58**, 4640–4645.

147. Lin, D. L., Whitney, M. C., Yao, Z., and Keller, E. T. (2001). Interleukin-6 induces androgen responsiveness in prostate cancer cells through up-regulation of androgen receptor expression. *Clin. Cancer Res.* **7**, 1773–1781.
148. Twillie, D. A., Eisenberger, M. A., Carducci, M. A., Hseih, W. S., Kim, W. Y., and Simons, J. W. (1995). Interleukin-6: a candidate mediator of human prostate cancer morbidity. *Urology* **45**, 542–549.
149. Ahmed, S. F., Cheng, A., Dovey, L., et al. (2000). Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. *J. Clin. Endocrinol. Metab.* **85**, 658–665.
150. Degeorges, A., Tatoud, R., Fauvel Lafeve, F., et al. (1996). Stromal cells from human benign prostate hyperplasia produce a growth-inhibitory factor for LNCaP prostate cancer cells, identified as interleukin-6. *Int. J. Cancer* **68**, 207–214.
151. Spiotto, M. T. and Chung, T. D. (2000). STAT3 mediates IL-6-induced growth inhibition in the human prostate cancer cell line LNCaP. *Prostate* **42**, 88–98.
152. Barton, B. E., Karras, J. G., Murphy, T. F., Barton, A., and Huang, H. (2004). Signal transducer and activator of transcription (STAT3) activation in prostate cancer: direct STAT3 inhibition induces apoptosis in prostate cancer lines. *Mol. Cancer Ther.* **3**, 11–20.
153. Godoy-Tundidor, S., Hobisch, A., Pfeil, K., Bartsch, G., and Culig, Z. (2002). Acquisition of agonistic properties of nonsteroidal antiandrogens after treatment with oncostatin M in prostate cancer cells. *Clin. Cancer Res.* **8**, 2356–2361.
154. Mori, S., Murakami-Mori, K., and Bonavida, B. (1999). Oncostatin M (OM) promotes the growth of DU 145 human prostate cancer cells, but not PC-3 or LNCaP, through the signaling of the OM specific receptor. *AntiCancer Res.* **19**, 1011–1015.
155. Sadar, M. D. and Gleave, M. E. (2000). Ligand-independent activation of the androgen receptor by the differentiation agent butyrate in human prostate cancer cells. *Cancer Res.* **60**, 5825–5831.
156. Eder, E. I., Haag, P., Bartsch, G., and Klocker, H. (2005). Targeting the androgen receptor in hormone-refractory prostate cancer—new concepts. *Future Oncol.* **1**, 93–101.
157. Gan, Y., Au, J. L., Lu, J., and Wientjes, M. G. (1998). Antiproliferative and cytotoxic effects of geldanamycin, cytochalasin E, suramin and thiacetazone in human prostate xenograft tumor histocultures. *Pharm. Res.* **15**, 1760–1766.
158. Solit, D. B., Zheng, F. F., Drobnjak, M., et al. (2002). 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clin. Cancer Res.* **8**, 986–993.
159. Eder, I. E., Hoffmann, J., Rogatsch, H., et al. (2002). Inhibition of LNCaP prostate tumor growth in vivo by an antisense oligonucleotide directed against the human androgen receptor. *Cancer Gene Ther.* **9**, 117–125.
160. Eder, I. E., Culig, Z., Ramoner, R., et al. (2000). Inhibition of LncaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. *Cancer Gene Ther.* **7**, 997–1007.
161. Morris, M. J., Reuter, V. E., Kelly, W. K., et al. (2002). HER-2 profiling and targeting in prostate carcinoma. *Cancer* **94**, 980–986.
162. Tortora, G., Bianco, R., Damiano, V., et al. (2000). Oral antisense that targets protein kinase A cooperates with taxol and inhibits tumor growth, angiogenesis, and growth factor production. *Clin. Cancer Res.* **6**, 2506–2512.
163. Grzmil, M., Hemmerlein, B., Thelen, P., Schweyer, S., and Burfeind, P. (2004). Blockade of the type I IGF receptor expression in human prostate cancer cells inhibits proliferation and invasion, up-regulates IGF binding protein-3, and suppresses MMP-2 expression. *J. Pathol.* **202**, 50–59.
164. Tolcher, A. W., Reyno, L., Venner, P. M., et al. (2002). A randomized phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. *Clin. Cancer Res.* **8**, 2530–2535.
165. Agrawal, S., Kandimalla, E. R., Yu, D., et al. (2002). GEM 231, a second-generation antisense agent complementary to protein kinase A RIalpha subunit, potentiates antitumor activity of irinotecan in human colon, pancreas, prostate and lung cancer xenografts. *Int. J. Oncol.* **21**, 65–72.
166. Smith, P. C. and Keller, E. T. (2001). Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. *Prostate* **48**, 47–53.
167. Tan, J., Sharief, Y., Hamil, K. G., et al. (1997). Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. *Mol. Endorinol.* **11**, 450–459.
168. Thompson, J., Hyytinen, E. R., Haapala, K., et al. (2003). Androgen receptor mutations in high-grade prostate cancer before hormonal therapy. *Lab. Invest.* **83**, 1709–1713.
169. Buchanan, G., Yang, M., Harris, J. M., et al. (2001). Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function. *Mol. Endocrinol.* **15**, 46–56.
170. Culig, Z., Hobisch, A., Cronauer, M. V., et al. (1993). Mutant androgen receptor detected in an advanced-stage of prostatic carcinoma is activated by adrenal androgens and progesterone. *Mol. Endocrinol.* **7**, 1541–1550.
171. Peterziel, H., Culig, Z., Stober, J., et al. (1995). Mutant androgen receptors in prostatic tumors distinguish between amino-acid-sequence requirements for transactivation and ligand binding. *Int J Cancer* **63**, 544–550.
172. Newmark, J. R., Hardy, D. O., Tonb, D. C., et al. (1992). Androgen receptor gene mutations in human prostate cancer. *Proc. Natl. Acad. Sci. USA* **89**, 6319–6323.

173. Bubley, G., Fenton, M., Fertig, A., et al. (1996). The hormonal sensitivity of androgen receptor (AR) mutations derived from human tumors [abstract]. *Cancer Res.* **37**, 1680A.
174. Tan, J., Sharief, J., Hamil, K. G., et al. (1996). Altered ligand specificity of a mutant androgen receptor (AR) in the androgen dependent human prostate cancer cenograft, CWR-22: comparison with the LNCaP mutant AR [abstract]. *J. Urol.* **155**, 340A.
175. Shao, T. C., Li, H., Eid, W., Ittmann, M., Unni, E., and Cunningham, G. R. (2003). In vivo preservation of steroid specificity in CWR22 xenografts having a mutated androgen receptor. *Prostate* **57**, 1–7.
176. Suzuki, H., Akakura, K., Komiya, A., Aida, S., Akimoto, S., and Shimazaki, J. (1996). Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome. *Prostate* **29**, 153–158.
177. Grigoryev, D. N., Long, B. J., Njar, V. C., and Brodie, A. H. (2000). Pregnenolone stimulates LNCaP prostate cancer cell growth via the mutated androgen receptor. *J. Steroid Biochem. Mol. Biol.* **75**, 1–10.
178. Zhao, X. Y., Malloy, P. J., Krishnan, A. V., et al. (2000). Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat. Med.* **6**, 703–706.
179. Enmon, R., Yang, W. H., Ballangrud, A. M., et al. (2003). Combination treatment with 17-N-allylamino-17-demethoxy geldanamycin and acute irradiation produces supra-additive growth suppression in human prostate carcinoma spheroids. *Cancer Res.* **63**, 8393–8399.
180. Eder, E. I., Haag, P., Bartsch, G., and Klocker, H. (2004). Gene therapy strategies in prostate cancer. *Current Gene Therapy* **5**, 1–10, 2005.
181. Ko, Y. J., Devi, G. R., London, C. A., et al. (2004). Androgen receptor down-regulation in prostate cancer with phosphorodiamidate morpholino antisense oligomers. *J. Urol.* **172**, 1140–1144, .
182. Yin, D., Gao, W., Kearbey, J. D., et al. (2003). Pharmacodynamics of selective androgen receptor modulators. *J. Pharmacol. Exp. Ther.* **304**, 1334–1340.
183. Marhefka, C. A., Gao, W., Chung, K., et al. (2004). Design, synthesis, and biological characterization of metabolically stable selective androgen receptor modulators. *J. Med. Chem.* **47**, 993–998.
184. Ren, F., Zhang, S., Mitchell, S. H., Butler, R., and Young, C. Y. (2000). Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. *Oncogene* **19**, 1924–1932.
185. Bektic, J., Berger, A. P., Pfeil, K., Dobler, G., Bartsch, G., and Klocker, H. (2004). Androgen receptor regulation by physiological concentrations of the isoflavonoid genistein in androgen-dependent LNCaP cells is mediated by estrogen receptor beta. *Eur. Urol.* **45**, 245–251.
186. Xing, N., Chen, Y., Mitchell, S. H., and Young, C. Y. (2001). Quercetin inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. *Carcinogenesis* **22**, 409–414.
187. Bianco, R., Caputo, R., Damiano, V., et al. (2004). Combined targeting of epidermal growth factor receptor and MDM2 by gefitinib and antisense MDM2 cooperatively inhibit hormone-independent prostate cancer. *Clin. Cancer Res.* 10, 4858–4864.
188. Minamiguchi, K., Kawada, M., Ohba, S., Takamoto, K., and Ishizuka, M. (2004). Ectopic expression of the amino-terminal peptide of androgen receptor leads to androgen receptor dysfunction and inhibition of androgen receptor-mediated prostate cancer growth. *Mol. Cell Endocrinol.* **214**, 175–187.
189. Bramlett, K. S., Dits, N. F., Sui, X., Jorge, M. C., Zhu, X., and Jenster, G. (2001). Repression of androgen-regulated gene expression by dominant negative androgen receptors. *Mol. Cell Endocrinol.* **183**, 19–28.
190. Kuratsukuri, K., Sugimura, K., Harimoto, K., Kawashima, H., and Kishimoto, T. (1999). "Decoy" of androgen-responsive element induces apoptosis in LNCaP cells. *Prostate* **41**, 121–126.
191. Ko, Y. J. and Balk, S. P. (2004). Targeting steroid hormone receptor pathways in the treatment of hormone dependent cancers. Curr. Pharm. Biotechnol. **5**, 459–470.

7
Hedgehog Signaling in Development and Cancer

Wade Bushman

Summary

The Hedgehog (Hh) signaling pathway, first identified in *Drosophila*, is a conserved signaling pathway that plays critically important roles in the development of a variety of structures in the growing embryo. Recently, Hh signaling has been implicated as an important growth stimulus in cancers of several organs, including the prostate, where Hh signaling plays an important role in during embryonic development. In this chapter, we review the role of Hh signaling in prostate development and emerging data on its role in prostate cancer growth and progression. The studies to date show a broad consensus that Hh pathway activation can significantly accelerate tumor growth. Work with established tumor cell lines and xenograft tumors clearly shows that tumor growth can be accelerated by both paracrine and signaling mechanisms. Striking inhibition of xenograft tumor growth by chemical inhibition of Hh signaling suggests that prostate cancer may be one of several tumor types (including pancreatic cancer, small cell lung cancer, medulloblastoma, and basal cell carcinoma) in which inhibitors of Hh signaling may offer a new and potentially potent treatment opportunity.

Key Words: Cyclopamine; Gli; paracrine; prostate; Sonic Hedgehog.

1. INTRODUCTION

Hedgehog (Hh) was first identified as an important signaling molecule in *Drosophila*. The basic paradigm of this system is conserved in vertebrates and plays critically important roles in the development of a variety of structures in the growing embryo, including the central nervous system, limbs, skin, lung, gut, and genitourinary tract structures. Investigation of Hh signaling has been paralleled from the very beginning by discovery of mutated Hh pathway components playing an etiological role in a variety of cancers, including basal cell carcinoma of the skin, rhabdomyosarcoma, glioblastoma, and medulloblastoma. In fact, the transactivator, Gli1, was originally identified as an oncogene in glioblastoma, a common childhood brain tumor *(1)*. Inactivating mutations in one allele of the Hh receptor, patched (Ptc)-1, was found in 10% of medulloblastomas, another brain tumor *(2)* and loss-of-function mutations in Ptc1 were implicated as the etiology of Gorlin/nevoid basal cell carcinoma syndrome *(3)*. The intertwined paths of discovery in development and cancer continue. Recently, Hh signaling has been implicated as an important growth stimulus in cancers of several organs, including the prostate, where Sonic Hedgehog (Shh) plays an important role in during embryonic development. We will review here the Hh signaling pathway, its role in prostate development, and emerging data on its role in prostate cancer growth and progression.

2. HH SIGNALING: AN OVERVIEW

Shh, Indian Hh (*Ihh*), and Desert Hh (*Dhh*) are the three mammalian homologs to the *Drosophila Hh* gene. *Shh* is the most widely and abundantly expressed ligand in the vertebrate embryo, expressed in the developing brain and spinal cord and at the epithelial–mesenchymal interface in many devel-

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

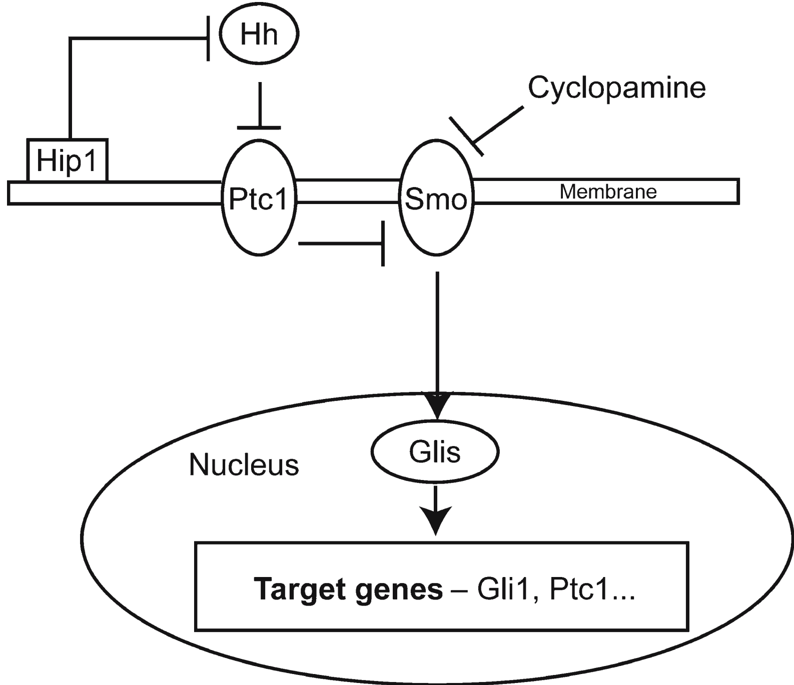


Fig. 1. Schematic of Hedgehog (Hh) signaling mechanism. Hip, Hh interacting protein; Ptc, Patched; Smo, smoothened.

oping organs. It is a potent inducer of morphogenesis and is essential for development of diverse structures in the vertebrate embryo, including the limb, neural tissues, lung, craniofacial skeleton, hair, tooth, gastrointestinal tract, and pituitary (reviewed in ref. *4*). *Shh* encodes a secreted glycopeptide. Shh peptide binds to a specific receptor (*Ptc*) on the target cell surface and acts through an intracellular signal transduction pathway involving the Gli family of transcription factors to modulate transcription of specific genes in the target cell (Fig. 1). *Gli1* and *Ptc* expression are conserved targets of Hh activation and are, therefore, widely used as indicators of Hh signaling activity.

All Hh ligands undergo autoprocessing and lipid modification. The *Shh* gene product is a 46-kDa glycoprotein that undergoes autolytic cleavage to produce 19-kDa and 27-kDa fragments representing the amino terminus or carboxy terminus, respectively. All known biological activity is present in the 19-kDa fragment. The 19-kDa peptide is modified by a cholesterol adduct at the C-terminal end structure and a palmityl adduct at its N-terminal end. These lipid modifications favor association of Shh with the cell membrane and regulate diffusion and long-range activity *(5)*. Secretion and diffusion of Shh seems to be additionally regulated by Dispatched, a transporter-like protein, and extracellular sulfate proteoglycans *(6)*.

The signal transduction mechanism for Hh signaling is subject to several different modes of regulation. One level of regulation occurs at the cell membrane. The Ptc receptor is a transmembrane protein that represses Hh pathway activity through an interaction with a second transmembrane protein, Smoothened (Smo). A second Ptc protein, Ptc2, is often present on the secreting cell and could play a role in autocrine signaling or in sequestering Hh ligand. Binding of Hh ligand to Ptc disrupts this interaction and leads to derepression of pathway activity. *Ptc* is a conserved target of Hh signaling, and induction of *Ptc* expression by Hh creates a negative feedback loop that reasserts repression of signaling at the level of the membrane. A second conserved Hh target is Hh interacting protein (Hip1), an extracellular peptide that competes with Ptc for binding to Hh ligand. Increased *Hip*

expression in response to Hh signaling provides a second negative feedback loop controlling signaling activity at the level of the membrane *(4,7)*.

An additional layer of regulation exists in the differential contributions of several *Gli* genes to regulating transcription of the *Hh* target genes. The Gli family of transcriptional regulators includes three Gli proteins, Gli1, Gli2, and Gli3, which share a highly conserved zinc finger DNA-binding domain. Gli1 is a pure transcriptional activator of Hh target genes. It binds to a 9-base pair recognition sequence in the promoter region and interacts with the transcription regulatory complex to activate gene transcription *(8–10)*. Gli2 provides redundancy in the transcriptional activating functions of Gli1 *(9,11)*. Gli3 is thought to function primarily as a transcriptional repressor that balances and refines transcriptional activation by Gli1 and Gli2 *(8,11–14)*.

The three mammalian Gli genes are homologs of the *Drosophila* gene, *Ci*. *Ci* encodes a zinc finger protein that both positively and negatively regulates the transcription of Hh target genes, and the presence or absence of Hh ligand influences the state and activity of Ci. In the absence of ligand, Ci is cleaved to a repressor protein that inhibits transcription of Hh target genes. Binding of Hh ligand blocks Ci proteolysis and facilitates entry of the full-length activator form of the protein to the nucleus, where it activates expression of *Hh* target genes. The three vertebrate *Gli* genes seem to have divided some of the distinct features of Ci function. Similar to Ci, Gli3 undergoes protein kinase A (PKA)-dependent proteolytic cleavage, generating an N-terminal protein that acts as a repressor of *Hh* target genes. Hh stimulation blocks proteolytic cleavage and results in the accumulation of full-length Gli3 *(5,6)*. Gli3 functions primarily as a repressor of gene expression and Gli3 loss of function is often functionally equated to overactivity of the Hh pathway. Whether Gli3 can also act as a transactivator of gene expression remains unresolved. Gli2 clearly functions as a transactivator of *Hh* target genes *(7,11,12)* and is critically important for mediating the Hh signal during development. *Gli2*-null mice die prenatally and exhibit neural tube defects, including a complete loss of the floor plate and a reduction in V3 interneurons *(11,12)*. Whether Gli2 also plays a role in repressing *Hh* target genes remains unresolved. Proteolytic cleavage of Gli2 has been observed in certain model systems, but it is independent of Hh signaling and has not been observed in any developmental context. Gli1 is an *Hh* target gene activator that induces expression of *Hh* target genes if overexpressed in cell and tissue culture *(6,10,14)*. Upregulation of *Gli1* is a reliable marker of Hh signaling, and overexpression of *Gli1* is a consistent hallmark of cancers with aberrant Hh pathway activation. Interestingly, despite the dependence of normal development on intact Hh signaling, transgenic mice lacking the zinc finger DNA-binding domain of *Gli1* are viable and fertile *(15)*. This apparently reflects functional redundancy between Gli1 and Gli2 in *Hh* target gene transactivation. Gli1, similar to Ci, contains a zinc finger domain, but does not seem to undergo proteolytic cleavage and there is no evidence for repressor activity. A comprehensive understanding of how the three vertebrate Gli proteins work independently and coordinately to positively and negatively regulate the Hh signal has remained elusive. A recent study using a battery of embryonic fibroblasts from *Gli* mutant mice has provided new insight into the individual and cooperative roles of the three *Gli* genes in regulating the expression of *Hh* target genes.

A third domain of Hh pathway regulation is in the cytosol. Most of what is known comes from studies in *Drosophila*, in which Ci activity depends on a complex network of regulatory elements that is modulated by Hh stimulation. Fused and Suppressor of Fused are proteins that exert positive and negative effects on Hh signal transduction by regulating the location and activity of the Gli proteins *(15)*.

The protein Costal-2 (Cos2) promotes the formation of the repressor form of Ci, possibly by anchoring Ci to microtubules, where it is phosphorylated and cleaved. Hh binding promotes stabilization and accumulation of the Smo protein, which recruits Cos2 away from Ci. This blocks Ci proteolysis and allows the nuclear importation of full-length Ci *(6)*. We have recently shown that the induction of Ptc1 by both Gli2 and Gli3 was blocked by cyclopamine, a chemical inhibitor of Hh signaling that acts directly on Smo *(16)*. This suggests that in vertebrates, Hh signaling acts through

Smo to regulate the activity of both Gli2 and Gli3. Whether a vertebrate Cos2 acts on Gli2 and Gli3 in vertebrates in the same way as it acts on Ci in *Drosophila* is unknown. Proteolytic cleavage of Ci requires phosphorylation of specific serine–threonine residues by PKA *(17)*. Forskolin, an activator of adenylate cyclase, inhibits both Gli2- and Gli3-mediated *Hh* target gene induction. Interestingly, both Gli3 and Gli2 have PKA site clusters C-terminal to the zinc finger *(12)*. PKA-dependent phosphorylation and subsequent cleavage generating a Gli3 repressor form have been described. Wang et al. found that activation of PKA by forskolin resulted in phosphorylation of Gli2 but did not generate a cleavage product *(12)*. These findings suggest that regulation by PKA is a conserved mechanism of upstream regulation for Gli2 and Gli3.

3. HH SIGNALING IN PROSTATE DEVELOPMENT

Shh is the most abundantly expressed Hh ligand in the developing mouse prostate *(18)*. *Ihh* is also expressed but at comparatively low levels. *Dhh* expression has not been observed. *Shh* gene expression in the urogenital sinus (UGS) increases before the initiation of ductal budding at embryonic day 17.5 (E17.5). Expression is most abundant during the period of ductal budding in late gestation. *Shh* expression gradually diminishes through the first 10 days after birth, a period characterized by continued bud formation and by outgrowth and branching of newly formed ducts. *Shh* gene expression declines progressively, and, by 30 days postnatal, has approached the low level of expression seen in the adult. This decrease parallels the completion of the ductal branching process. A schematic illustration of how *Shh* expression fits in the timeline of key morphogenetic events in prostate development is presented in Fig. 2.

Shh expression in the human fetal prostate was demonstrated by semiquantitative RT-PCR on RNA prepared from snap-frozen fetal prostate tissues (age 15.5 and 18 weeks). Expression was present at both fetal time points, with relatively greater expression at the earlier time point. Immunostaining for Shh peptide was performed on prostatic tissue sections from seven human male fetuses aged 9.5 to 20 weeks of gestation.

Staining was weak at 9.5 weeks but intense at 11.5, 13, and 16 weeks. The increase in Shh expression was found to coincide with the onset of ductal budding and outgrowth, and particularly robust expression was noted in newly formed prostatic buds *(19)*. Staining diminished at 18 and 20 weeks, in agreement with results obtained by RT-PCR, and staining was absent at 34 weeks. Thus, Shh protein expression in the human prostate is most abundant during the early phase of prostate development when ductal budding occurs (10–17 weeks) and is downregulated before birth. In contrast to the mouse, in which *Shh* expression remains low in the adult, robust *Shh* expression is present in both normal adult prostate and benign prostatic hyperplasia (BPH) *(20)*.

Localization of *Shh* expression during prostate development is correlated with the process of ductal morphogenesis. In the late gestation embryo (E15), *Shh* is diffusely expressed in the epithelium of the prostatic portion of the UGS. Just before the onset of ductal budding, *Shh* expression is upregulated and, at the onset of bud formation (E17), expression condenses at the epithelial evaginations that form the nascent ducts. *Shh* expression in the UGS is not dependent on testosterone, but the redistribution that occurs coincident with ductal budding does occur in response to androgen stimulation *(21)*. As ductal budding proceeds, *Shh* expression becomes restricted to the developing buds and, by P1, *Shh* expression is strictly localized to the primary ducts of the developing prostate. Expression is strongest in the advancing distal regions of the ducts and diminishes in the already formed proximal duct segments.

Shh expression during prostate development seems to act primarily in a paracrine fashion. During budding, the genes for the Shh receptor *Ptc1* and the transcriptional activator *Gli1*, both induced targets of Hh signaling, are highly localized in the mesenchyme of the UGS immediately surrounding the nascent buds. Gli2 and Gli3 are also expressed primarily in the UGS mesenchyme (Fig. 3). As *Shh* expression becomes localized to the apical regions of elongating ducts, the expression for *Ptc1*

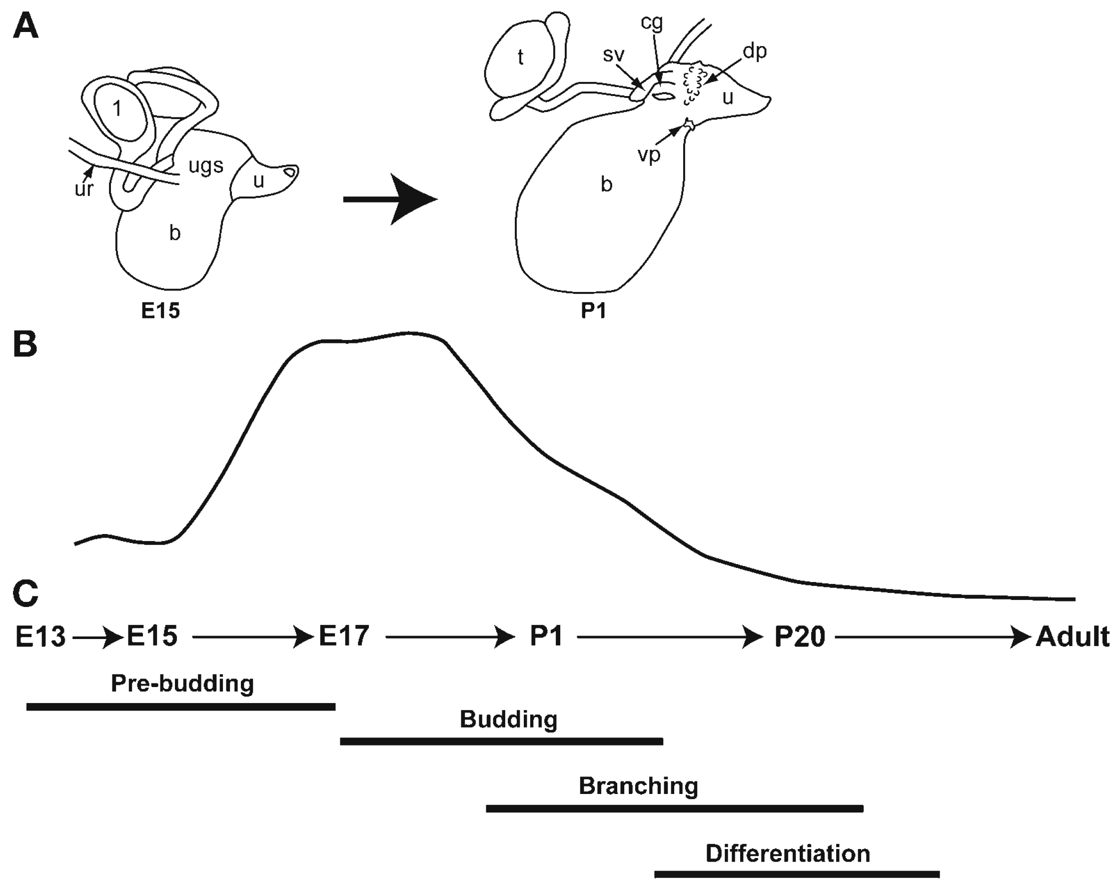


Fig. 2. (A) The mouse lower urogenital tract at embryonic day 15 (E15) and at birth (P1) showing the outgrowth of ductal buds in the region of the coagulating gland (cg), dorsal prostate (dp), and ventral prostate (vp). **(B)** The expression profile of Sonic Hedgehog (*Shh*) during mouse prostate morphogenesis. Expression increases before budding at E17.5, remains abundant during late gestation and through birth, and then gradually diminishes after birth to very low levels in the adult. **(C)** Key events of prostate development, including epithelial budding, ductal branching, and ductal differentiation. T, testis; ur, ureter; b, bladder; u, urethra; sv, seminal vesicle; ugs, urogenital sinus.

seems to be strongest in the mesenchyme surrounding the distal duct segments *(21,22)*. Expression of the three *Gli* genes in the ductal mesenchyme also exhibits a proximodistal gradient *(22)*. This asymmetric distribution of elements of the Shh–Gli pathway during ductal morphogenesis may signal the early establishment of a proximodistal heterogeneity in the morphology and function of the adult prostatic ducts *(23)*. Although most studies support a primarily paracrine mechanism of Shh action, the presence of low *Ptc1*, *Gli1*, and *Gli3* in the UGS epithelium leaves open the possibility of some autocrine signaling activity *(21,22)*.

In vitro studies have confirmed the paracrine mechanism of action. Exogenous Shh peptide exerts an inductive effect on both *Ptc* and *Gli1* gene expression (known downstream targets of the pathway) in isolated mouse male E14 UGS, and this effect is direct and inhibited by cyclopamine, a specific and potent chemical inhibitor of Hh action. The target genes of Hh signaling in the prostate are not yet described, but work in other systems has identified targets that may be conserved. Hepatocyte nuclear factor-3β is a well-established target of Gli1 regulation *(10)*. Using a cell-based assay, Yoon

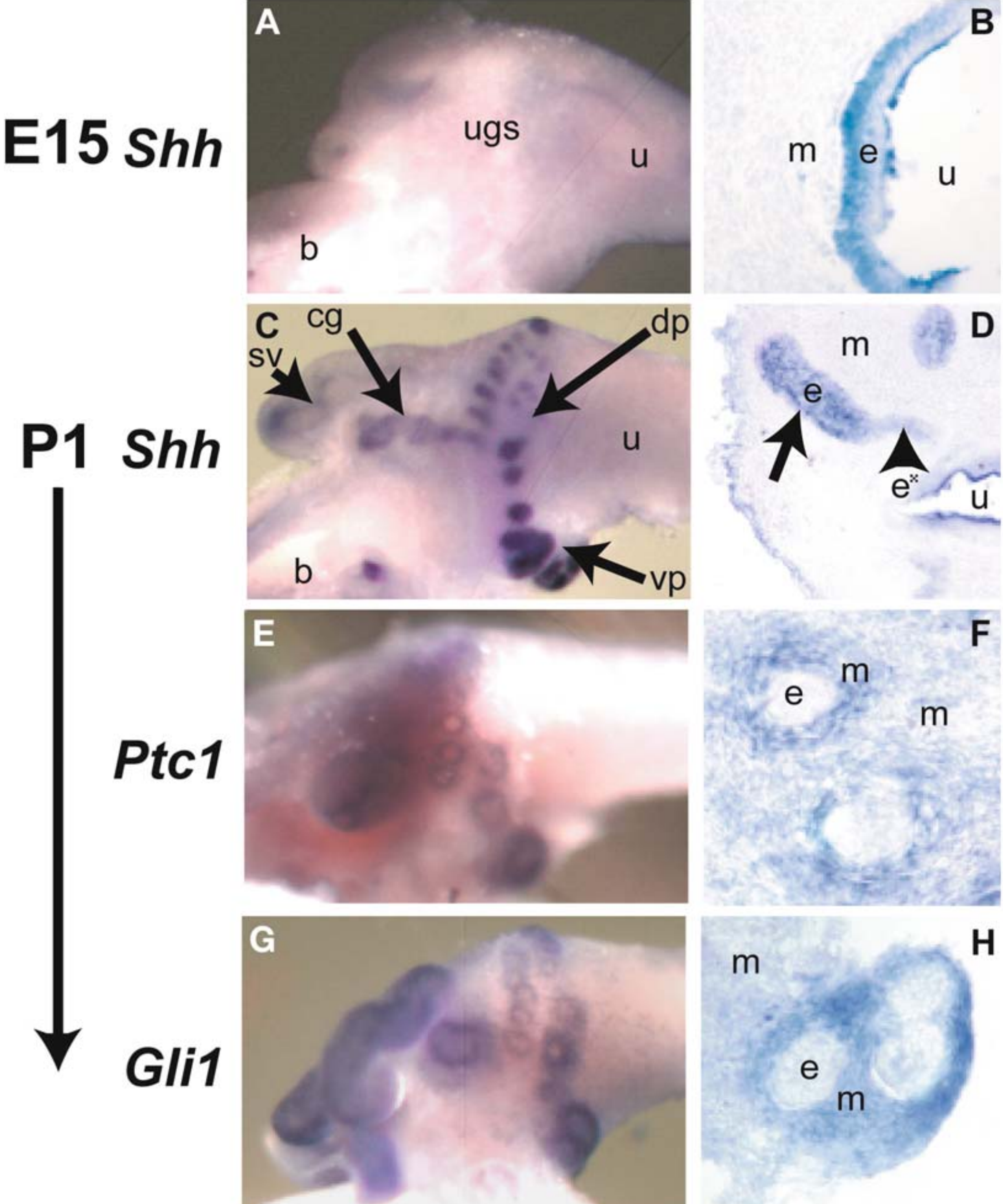


Fig. 3. Whole-mount *in situ* hybridization for Sonic Hedgehog (*Shh*), patched (*Ptc*)-1 and *Gli1* expression in the developing mouse prostate. **(A)** In the whole-mount E15 mouse specimen, little expression is seen. **(B)** Sectioning reveals uniform *Shh* expression in epithelium (e) lining the lumen of the urethra (u). **(C)** At P1, *Shh* expression is focused to the nascent buds of the dorsal prostate (dp), coagulating gland (cg), and ventral prostate (vp). **(D)** Apparent concentration of *Shh* expression is exhibited in the epithelium (e) of the distal duct (*long arrow*) relative to the proximal duct (*short arrow*). Note the diminished expression in the epithelium (e*) of the urethra (u). No *Shh* expression is detected in the mesenchyme (m) at any stage of prostate development. Expression of *Ptc1* **(E)** and *Gli1* **(G)** surround the prostatic buds, and expression of both genes is more concentrated in the mesenchyme immediately surrounding the epithelium source of the Shh ligand **(F,H)**. B, bladder; sv, seminal vesicle. (Photographs reprinted with permission from ref. *21*).

et al. *(24)* identified approx 30 targets of Gli-1, including cyclin D2, osteopontin, insulin-like growth factor binding protein 6, mitogen-activated protein kinase 6c, and plakoglobin. One of these, insulin-like growth factor binding protein 6, has recently been shown to be a Shh-regulated gene in the fetal mouse prostate *(16)*.

Functional studies of Hh signaling in prostate development have used antibody blockade, chemical inhibition, and genetic loss of function models. These studies have yielded somewhat conflicting data on the requirement of Shh for normal prostate development. A polyclonal antibody to Shh seemed to block prostate development in a subcapsular renal graft model *(18)*. However, the UGS of the *Shh*-null transgenic mouse exhibited budding morphogenesis in organ culture and prostatic glandular morphogenesis when grafted under the renal capsule of an adult male host mouse *(25)*. This apparent discrepancy could be explained if Ihh, which is also expressed in the UGS, provides functional redundancy for Shh. A polyclonal antibody would likely block the action of both Shh and Ihh, whereas the *Shh*-null would manifest a selective loss of Shh function.

The compound cyclopamine is a steroidal alkaloid derived from the subalpine flower *Veratrum californicum*. Cyclopamine is a specific a chemical inhibitor of Hh signaling that acts by interfering with Shh signal transduction by the transmembrane protein, Smo, and blocks signaling by all Hh ligands. Cyclopamine treatment of cultured UGS tissues has produced a variety of observations that may be a function of the stage in prostate development when signaling is disrupted. Cyclopamine blockade initiated in the prebud E14 mouse UGS inhibits epithelial cell proliferation and seems to decrease the total number of discrete prostatic buds *(21)*. Together with the effect of Shh antibody blockade on prostate development, these data may suggest an early requirement for Hh signaling in prostate development and budding morphogenesis. Cyclopamine blockade later in development, in E16.5 mouse UGS or the neonate rat ventral prostate, produces apparently opposite effects. Epithelial cell proliferation is increased, prostate growth is enhanced, and the number of ducts is either increased or not significantly affected *(25,26)*. Exogenous Shh produces the opposite effects, inhibiting cell proliferation and decreasing the number of prostatic ducts *(25,26)*. Collectively, these observations suggest a shift in the role for Shh signaling in prostate morphogenesis: from promoting bud formation and outgrowth via increased epithelial cell proliferation to inhibiting cell proliferation and branching morphogenesis in the postnatal prostate.

Given this inhibitory action of Shh signaling on epithelial proliferation and ductal branching during postnatal prostate development, the decline in *Shh* expression after birth can be viewed as permissive for branching morphogenesis. Shh upregulates the expression of transforming growth factor-β1 and activin A. These factors are both expressed in prostatic mesenchyme and inhibit prostate branching morphogenesis *(26–28)*. Bone morphogenetic factor (Bmp) 4 is another member of the transforming growth factor family that inhibits branching and is expressed in the prostatic mesenchyme *(29)*. Whether *Bmp4* is a direct target of Shh signaling in the prostate remains to be resolved *(22, 26)*. Shh was shown to downregulate the expression of mesenchymal fibroblast growth factor (Fgf)-10 in rat ventral prostate, and exogenous Fgf-10 was shown to reverse Shh-mediated inhibition of prostate growth and branching. A model for ductal branching that involves the interaction of Shh, FGF-10, and Bmp4 was recently proposed *(22)*.

Two studies suggest that Shh may influence differentiation of ductal epithelium into basal and luminal cells, but these studies have yielded conflicting observations. One study showed that exogenous Shh increased the proportion of epithelial cells that did not express CK14 and p63. This was interpreted as showing that Shh increased luminal cell differentiation. Cyclopamine was shown to have the opposite effect *(26)*. The other study found that cyclopamine accelerated both ductal canalization and epithelial cell differentiation *(30)*. The apparent discrepancy between these two studies could result from unique features of the experimental design, which differentially highlight particular aspects of the complex actions of Shh during ductal development. Clearly, a better understanding of the role of Shh signaling in the process of differentiation is of considerable interest.

4. HH SIGNALING IN ANDROGEN-INDUCED REGROWTH

Shh expression and pathway activity is very low in the normal adult mouse prostate. To examine the role of Hh signaling in prostate regeneration, Karhadkar et al. *(31)* tested the effect of pathway blockade on androgen-induced regrowth of the castrate ventral prostate. Both Hh-neutralizing antibody and chemical inhibition of Hh signaling abolished prostate regeneration in this model. This finding suggested that Shh signaling might be involved in the recruitment and/or renewal of progenitor cell populations.

An emerging paradigm for cancer development holds that malignant transformation occurs in stem cells or progenitor cells that have an unlimited proliferation potential. It is postulated that injury and inflammation activate proliferation of stem cells as part of the repair process. In the presence of inflammation, these proliferating progenitor cells are exposed to oncogenic forces, such as oxidative stress, that may induce genetic or epigenetic changes that lead to persistent stem cell activation. Depending on the tissue type, either Hh or Wnt signaling seem to figure prominently in the process of stem cell activation, and tumor development is postulated to involve persistent and autonomous activation of Hh or Wnt signaling that drives tumor growth.

Shh has recently been postulated to regulate stem cell recruitment in tissues where it plays a key role in regulating growth during development, including the central nervous system, retina, bile duct, bone, lung, and prostate *(32)*. Evidence for such a role has recently been obtained in studies of a model of lung injury and repair *(33,34)*. Given the clear-cut role of Shh in prostate development and the recently identified role of chronic inflammation in prostate carcinogenesis, it is reasonable to speculate that Hh signaling could play a central role in the development and progression of prostate cancer.

5. HH SIGNALING IN HUMAN PROSTATE CANCER

Analysis of specimens from young adult men, from men with BPH, and from men undergoing radical prostatectomy for prostate cancer revealed *Shh* expression in all three groups *(20)*. The level of Shh expression and Gli1 activation in these tissues, as determined by quantitative RT-PCR, was surprisingly robust-comparable to the level of expression in the fetal brain. This was an unexpected observation that contrasts with the low level of expression in the adult mouse prostate. Whether this reflects a persistent generalized growth activity in the postpubertal human prostate or is associated with multifocal processes of inflammation and hyperplasia in the human prostate remains to be determined. Several studies have examined the expression of Shh in localized prostate cancer and in metastatic prostate cancer *(20,31,35,36)*. They have yielded a picture of variably increased Shh expression in localized tumors exerting a combination of autocrine and paracrine signaling activity and showing dramatically increased pathway activation in metastatic disease.

Among the tissues studied by Fan et al. *(20)*, there was a trend toward higher *Shh* expression in the cancer specimens as a group and a mean level of expression nearly 10-fold higher than that observed in the nontumor specimens. Karhadkar et al. *(31)* performed PCR analysis of 12 localized tumors and found substantial *Shh* expression in all of them. They also detected the presence of lower level *Ihh* expression. Sheng et al. *(36)* performed immunostaining for Shh peptide and found protein expression in more than half the tumors they examined. Sanchez et al. *(35)* found Shh expression in both normal prostate tissue and prostate cancer, but found more intense staining in 33% of the tumors. These studies all agree that *Shh* expression is a common feature of localized prostate cancer and suggest that very high expression is present in a subset of tumors. However, they also indicate that the range of expression in these tumors is quite broad and occurs against a background of robust and variable expression in the normal and hyperplastic prostate. Consequently, the specificity of *Shh* overexpression for cancerous tissue is an important but as yet unresolved issue. Fan et al. *(20)* compared expression by RT-PCR in 11 histologically confirmed tumors and benign tissues from the same gland and found no significant difference in *Shh* expression between the cancer specimens and zone

homologous benign tissue. This suggests that the robust level of Shh expression in localized prostate cancer is generally mirrored by equally strong expression in histologically benign tissue from the same gland. On the other hand, Sanchez et al. *(35)* showed a variable (0- to 9.8-fold) increase in *Shh* expression measured by RT-PCR in six matched specimens. This observation suggests that tumor-specific increased Shh expression may occur in a subset of prostate cancers. On the basis of immunostaining, Sheng et al. *(36)* suggested that Shh overexpression may occur in a subset of high Gleason grade tumors. Interestingly, our unpublished studies revealed very high levels of Shh expression in locally advanced tumors that required transurethral resection for outlet obstruction. This is consistent with the notion that Shh overexpression may be associated with increased tumor progression and/or growth.

The activity of Hh signaling pathway in normal and tumor tissue depends both on the expression of the Hh ligands and on the responsiveness of the target cell. Therefore, the true activity of the pathway is best measured by the expression of target genes *Gli1* and *Ptc* that are reliably induced by Hh ligand. Studies of Shh expression in benign prostate tissue and localized cancer generally suggest a correlation between Shh expression and Gli1 expression, but two studies suggest that changes in pathway regulation in tumor cells may result in disproportionate pathway activation for the level of ligand. Fan et al. *(20)* examined *Gli1* expression by real time RT-PCR in adult normal, BPH, and cancer specimens and found expression in all tissues examined. In addition, they found an extremely tight correlation of *Shh* and *Gli1* expression in all of these tissues, indicating a generally tight linkage between expression of Shh ligand and pathway activation. They also observed a tight correlation between *Shh* expression and the levels of *Gli2* and *Gli3* expression. Sanchez et al. *(35)* observed both *Gli1* and *Ptc* expression in all six matched normal and tumor tissues they examined. Increased Shh expression in specimens of prostate cancer was generally accompanied by increased expression of *Ptc1*, *Gli1*, *Gli2*, and *Gli3*. In contrast to these studies, Karhadkar et al. *(31)* did not observe either *Ptc* or *Gli1* expression in 12 specimens of normal prostate tissue. These authors observed substantial *Ptc* and *Gli1* expression in only 3 of 12 localized tumors, but found abundant *Ptc* and *Gli1* expression in their survey of 15 metastatic prostate cancers. This was attributed to enhanced pathway responsiveness caused by increased *SMO* expression in the metastatic lesions. Sheng et al. *(36)* presented evidence that Hh pathway activation may also occur through mutation in the regulatory protein, Suppressor of Fused. Collectively, the studies to date suggest that Shh expression and pathway activation in benign tissues is tightly correlated, but that tumor tissue is characterized by variable degrees of increased Shh expression and increased pathway responsiveness that may correlate with tumor grade, growth, and metastasis.

6. HH SIGNALING AND TUMOR GROWTH

Whereas Shh signaling in prostate development is primarily a paracrine signaling from the epithelium to the stroma, both paracrine and autocrine signaling have been identified in prostate cancer and may each contribute to tumor growth (Fig. 4). Localization of *Shh* and *Gli1* expression in normal prostate tissue and in specimens of localized prostate cancer by radioactive *in situ* hybridization showed *Shh* expression by the prostatic epithelium and *Gli1* expression in the adjacent stroma *(20)*. *Ptc* expression was also assayed by this technique and showed expression in both the epithelium and adjacent stroma (C. Pepicelli, personal communication). The presence of *Ptc* expression in both tissue layers contrasts with the specific localization of *Gli1* expression to the stroma. This is consistent with the fact that *Ptc* expression, although upregulated by Hh signaling, occurs independent of Hh signaling, whereas *Gli1* expression is nearly completely dependent on Hh pathway activation. These data suggest that paracrine signaling is the predominant mechanism in the normal prostate and in localized tumors. However, *in situ* hybridization using digoxigenin-labeled *Shh*, *Gli1*, and *Ptc* probes gave conflicting results, with expression of all three genes colocalizing in both normal and tumor epithelium. These studies suggest that variable degrees of autocrine signaling also occur.

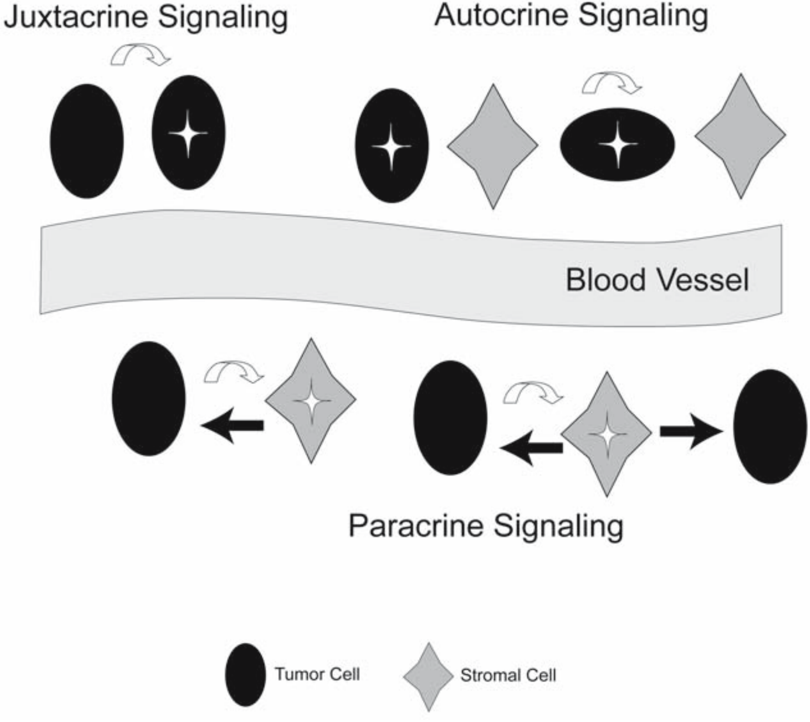


Fig. 4. Schematic of potential Sonic Hedgehog (Shh) signaling interactions in prostate cancer involving (*black*) tumor and (*grey*) stromal cells. Hedgehog ligand is indicated by curved arrow. Pathway activation is indicated by star. (Top) Pathway activation in tumor cells may occur by ligand-dependent (*curved arrow*) mechanisms or ligand-independent (mutational) activation. (Bottom) Paracrine signaling may involve ligand-dependent reciprocal interaction between individual tumor and stromal cells (left) or a generalized response of stromal cells to Sonic Hedgehog ligand (right). The stromal response could involve secreted paracrine factors, accelerated angiogenesis, or stromal mediated effects on the extracellular matrix.

To examine the functional consequence of paracrine Shh signaling in prostate tumors, Fan et al. *(20)* examined the effect of Shh overexpression in LNCaP xenografts. The LNCaP cell line has extremely low endogenous Shh expression. It is also unresponsive to Shh ligand. When exposed to exogenous Shh, LNCaP cells exhibit no induction of *Ptc* or *Gli1*, and LNCaP cells overexpressing *Shh* grow at the same rate as the parent cell line. When xenografts were made with Shh-overexpressing LNCaP cells, there was no autocrine stimulation of the Hh pathway in the LNCaP cells, but there was paracrine activation of *Gli1* and *Ptc1* in the xenograft stroma. Tumors overexpressing Shh grew significantly faster than control tumors, and this effect on tumor growth was caused by enhanced proliferation of LNCaP cells. This clearly showed that paracrine activation of stromal signals by Shh expressed from the tumor cells indirectly stimulates tumor cell proliferation.

LNCaP and PC3 cells both express Shh, Ptc, and Gli1 *(31,35)* Cyclopamine was found to inhibit proliferation of LNCaP cells in culture *(35,36)*. Cyclopamine treatment inhibited Gli1 expression in LNCaP cells, arguing strongly that the effect is pathway specific, but the unresponsiveness of LNCaP cells to exogenous Shh *(35)* suggests that cyclopamine is acting to inhibit ligand-independent pathway activation. Cyclopamine also was found to inhibit proliferation of PC3 cells in culture *(31,35,36)*. Karhadkar et al. *(31)* found that a Shh-blocking antibody also inhibited PC3 proliferation in culture, but Sanchez et al. *(35)* found that PC3 proliferation was unaffected by either a Shh-blocking antibody

or recombinant Shh. This discrepancy highlights the uncertainty in the relative contributions of ligand-dependent and ligand-independent pathway activation to PC3 proliferation.

The potential of chemical blockade of Hh signaling to inhibit tumor growth was examined by measuring the effect of cyclopamine administration on xenograft tumor growth *(31)*. Both PC3 and 22RV1 tumors showed a dose-dependent inhibition of tumor growth and complete and sustained regression at the highest dose tested. The specificity of this effect was confirmed by showing that the xenografts made with tumor cells overexpressing Gli1 were resistant to the anti-tumor effect of cyclopamine. Additional experiments performed with rodent tumor cell lines showed that cyclopamine could inhibit the growth and metastasis of the aggressive AT6.3 cell line and that Gli1 overexpression conferred a highly aggressive and metastatic phenotype to the normally less aggressive AT2.1 cell line.

In summary, studies to date show a broad consensus that Hh pathway activation can significantly accelerate tumor growth. Work with established tumor cell lines and xenograft tumors clearly shows that tumor growth can be accelerated by paracrine signaling *(20)* as well as by autocrine pathway activation by both ligand-dependent *(31)* and ligand-independent mechanisms *(31,35)*. A striking inhibition of xenograft tumor growth was achieved by chemical inhibition of Hh signaling, suggesting that prostate cancer may be one of several tumor types (including pancreatic cancer, small cell lung cancer, medulloblastoma, and basal cell carcinoma) in which inhibitors of Hh signaling may offer a new and potentially potent treatment opportunity.

REFERENCES

1. Kinzler, K. W., Bigner, S. H., Bigner, D. D., et al. (1987). Identification of an amplified, highly expressed gene in a human glioma. *Science* **236(4797)**, 70–73.
2. Raffel, C., Jenkins, R. B., Frederick, L., et al. (1997). Sporadic medulloblastomas contain PTCH mutations *Cancer Res.* **57(5)**, 842–845.
3. Johnson, R. L., Rothman, A. L., Xie, J., et al. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome *Science* **272**(5268), 1668–1671.
4. Ingham, P. W., and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev*. **15(23)**, 3059–3087.
5. Feng, J., White, B., Tyurina, O. V., et al. (2004). Synergistic and antagonistic roles of the Sonic hedgehog N- and C-terminal lipids. *Development* **131(17)**, 4357–4370.
6. Lum, L. and Beachy, P. A. (2004). The Hedgehog response network: sensors, switches, and routers. *Science* **304(5678)**, 1755–1759.
7. McMahon, A. P., Ingham, P. W., and Tabin, C. J. (2003). Developmental roles and clinical significance of hedgehog signaling. *Curr. Top. Dev. Biol*. **53**, 1–114.
8. Aza-Blanc, P., Lin, H. Y., Ruiz i Altaba, A. and Kornberg, T. B. (2000). Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. *Development* **3**, 301.
9. Buttitta, L., Mo, R., Hui, C. C., and Fan, C. M. (2003). Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. *Development* **130**, 6233–6243.
10. Sasaki, H., Hui, C., Nakafuku, M., and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313–1322.
11. Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* **126**, 3915–3924.
12. Wang, B., Fallon, J. F., and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423–434.
13. Bai, C. B., Stephen, D., and Joyner, A. L. (2004). All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. *Dev. Cell* **6**, 103–115.
14. Lei, Q., Zelman, A. K., Kuang, E., Li, S., and Matise, M. P. (2004). Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord. *Development* **131**, 3593–3604.
15. Murone, M., Luoh, S. M., Stone, D., et al. (2002). Gli regulation by the opposing activities of fused and suppressor of fused. *Nat. Cell Biol*. **2**(5), 310–312.
16. Lipinski, R. J., Cook, C. H., Barnett, D. H., et al. (2005) Sonic hedgehog signaling regulates the expression of insulin-like growth factor binding protein-6 during fetal prostate development. *Dev. Dyn.* **233**, 829–836.

17. Chen, J. K., Taipale, J., Cooper, M. K., and Beachy, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743–2748.
18. Podlasek, C. A., Barnett, D. H., Clemens, J. Q., et al. (1999). Prostate development requires sonic hedgehog expressed by the urogenital sinus epithelium. *Dev. Biol.* **209**, 28–39.
19. Barnett, D. H., Huang, H. Y., Wu, X. R., et al. (2002). The human prostate expresses sonic hedgehog during fetal development. *J. Urol.* **168**, 2206–2210.
20. Fan, L., Pepicelli, C. V., Dibble, C. C., et al. (2004) Hedgehog signaling promotes prostate xenograft tumor growth. *Endocrinology* **145**, 3961–3970.
21. Lamm, M. L. G., Catbagan, W. S., Laciak, R. J., et al. (2002). Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. *Dev. Biol.* **249**, 349–366.
22. Pu, Y., Huang, L., Prins, G. (2004). Sonic hedgehog-patched Gli signaling in the developing rat prostate gland: lobe-specific suppression by neonatal estrogens reduces ductal growth and branching. *Dev. Biol.* **273**, 257–275.
23. Lee, C. (1997). Biology of the prostate ductal system. In: Prostate: Clinical and Basic Aspects. (Nez R., ed.). CRC Press, Boca Raton, FL, pp. 53–71.
24. Yoon, J. W., Kita, Y., Frank, D. J. et al. (2002). Gene expression profiling leads to identification of Gli1 binding elements in target genes and a role for multiple downstream pathways in Gli1 induced cell transformation. *J. Biol. Chem.* **277**(7), 5548–5555.
25. Berman, D. M., Desai, N., Wang, X., et al. (2004). Roles for hedgehog signaling in androgen production and prostate ductal morphogenesis. *Dev. Biol.* **267**, 387–398.
26. Wang, B., Shou, J., Ross, S., et al. (2003). Inhibition of epithelial ductal branching in the prostate by sonic hedgehog is indirectly mediated by stromal cells. *J. Biol. Chem.* **278**, 18,506–18,513.
27. Cunha, G. R., Alarid, E. T., Turner, T., et al. (1992). Normal and abnormal development of the male urogenital tract: role of androgens, mesenchymal–epithelial interactions, and growth factors. *J. Androl.* **13**, 465–475.
28. Cancilla, B., Jarred, R. A., Wang, H., et al. (2001). Regulation of prostate branching morphogenesis by activin A and follistatin. *Dev. Biol.* **237**, 145–158.
29. Lamm, M. L. G., Podlasek, C. A., Barnett, D. H., et al. (2001). Mesenchymal factor bone morphogenetic protein 4 restricts ductal budding and branching morphogenesis in the developing prostate. *Dev. Biol.* **232**, 301–314.
30 Freestone, S. H., Marker, P., Grace, O. C., et al. (2003). Sonic hedgehog regulates prostatic growth and epithelial differentiation. *Dev. Biol.* **264**, 352–362.
31. Karhadkar, S. S., Bova, G. S., Abdallah, N. et al. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature 431, 707–712.
32. Beachy, P. A., Karhadkar, S. S., and Berman, D. M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature* **432**, 324–331.
33. Stewart, G. A., Hoyne, G. F., Ahmad, S. A., et al. (2003). Expression of the developmental sonic hedgehog (Shh) signaling pathway is up-regulated in chronic lung fibrosis and the Shh receptor patched I is present in circulating T lymphocytes. *J. Pathol.* **199**, 488–495.
34. Watkins, D. N., Berman, D. M., Burkholder, S. G., Wang, B., Beachy, P. A., Baylin, S. B. (2003). Hedgehog signaling within airway epithelial progenitors and in small-cell lung cancer. *Nature* **422(6929)**, 313–317.
35. Sanchez, P., Hernandez, A. M., Stecca, B., et al. (2004). Inhibition of prostate cancer proliferation by interference with sonic hedgehog-Gli1 signaling. *Dev. Biol.* **101(34)**, 12,561–12,566.
36. Sheng T., Li C., Zhang X., et al. (2004). Activation of the hedgehog pathway in advanced prostate cancer. *Mol. Cancer* **3**(1), 29.

8

Cholesterol, Cell Signaling, and Prostate Cancer

Mohini Lutchman, Keith R. Solomon, and Michael R. Freeman

Summary

Cholesterol, a sterolic lipid, accumulates in solid tumors. Biochemical mechanisms essential to cholesterol metabolism are altered with age and with the transition to the malignant state. In cell membranes, cholesterol is a mediator of the liquid-ordered state, a biophysical condition that provokes sequestration within discrete membrane microdomains of certain signaling proteins and other lipids. Cholesterol-enriched membrane domains, commonly referred to as lipid rafts, serve as platforms for signal transduction mechanisms that mediate cell growth, survival, and other processes relevant to cancer. This review summarizes the established links between cholesterol and prostate cancer, with a focus on how accumulation of cholesterol within the lipid raft component of the plasma membrane may promote progression to hormone refractory disease. Large-scale characterization of proteins that localize to cholesterol-rich domains may help unravel signaling networks and lead to identification of novel mediators of disease progression.

Key Words: Cholesterol; lipid rafts; prostate cancer progression; signal transduction.

1. OVERVIEW

Cells maintain normal structure and function by responding appropriately to cues from the surrounding milieu. Extracellular stimuli are transduced from the surface through the plasma membrane by a complex series of interactions between ligands, their receptors, and intracellular signaling partners (e.g., kinases and G proteins). It has recently become clear that cholesterol-enriched membrane microdomains, generally referred to as "lipid rafts," which exist within the lipid bilayer of all mammalian cells, play an important role in signaling from the cell surface to the various subcellular compartments. Advances in biophysical and microscopic techniques have demonstrated that cholesterol-rich rafts can be altered in response to extracellular or intracellular stimuli. This suggests that raft dynamics could play a role in aberrant and pathological cellular processes, including oncogenesis and metastasis.

Recent molecular and cell biological studies have linked signal transduction mechanisms implicated in tumor growth and aggressiveness to cholesterol-rich rafts. These findings seem to be consistent with epidemiological evidence suggesting that the modern Western diet, which contains substantial levels of cholesterol and other fatty substances, can promote prostate cancer (PCa) progression. Consistent with this idea, prolonged inhibition of the cholesterol synthesis pathway by pharmacological intervention in men has recently been associated with dramatically reduced PCa incidence.

In this chapter, we discuss the possibility that membrane cholesterol can promote PCa progression by a mechanism that involves dysregulation of raft–resident signaling complexes. Such changes, if they occur during oncogenic transformation and progression, are likely to affect multiple processes,

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

such as tumor cell cytokinesis and cell survival mechanisms. As we will show, this hypothesis provides new avenues for mechanistic and translational studies of PCa.

2. EPIDEMIOLOGY AND DIET IN PROSTATE CANCER PROGRESSION

PCa remains a major public health concern because of the high morbidity, mortality, and economic costs associated with the detection, treatment, and palliation of the disease. Our understanding of PCa in mechanistic terms is still poor, partly because of the lack of definitive mechanistic information from linkage studies *(1)*. PCa progression rates are associated with environmental factors, most likely diet, suggesting that a better knowledge of the role of specific dietary factors in cancer risk and progression may provide new insight into origins of the disease.

Studies of migrants have provided evidence that environmental factors, particularly nutrition and diet, may have a substantial effect on the progression rate of PCa. For example, a 30-fold differential exists between nations with the lowest PCa mortality rates (China and Japan) and those with the highest rates (United States, Western and Northern Europe, and Australia) *(2)*. Migrant populations from countries with a low incidence of clinical PCa tend to acquire the incident rate of the host country *(2)*. First-generation Asian-Americans demonstrate a three- to five-fold greater risk of PCa compared with their native counterparts in Japan and China, even though they experience rates of disease approximately one-third to one-half those of white Americans *(3)*. PCa incidence rates have recently risen in Asian countries that have undergone Westernization *(4–6)*. These data suggest that environmental agents may be important in converting microfoci of latent cancer to more aggressive disease *(5–7)*.

Since the 1980s, reports have linked increased risk of aggressive PCa to the consumption of animal products and/or fatty food. Recently, Fleshner et al. *(8)* critically appraised 33 published case–control and cohort studies examining the possible relationship between dietary fat and PCa. Eight studies suggested a statistically significant association between intakes of fatty foods and PCa risk, with several demonstrating a significant association for dairy products and meat *(8)*. The basis for the apparent association between fat intake and increased PCa risk is unknown, but it could be related to a variety of factors, including circulating cholesterol or androgen levels, intake of fat-soluble pesticides, and/or increased production of reactive oxygen species *(8–10)*. Although the evidence remains inconclusive, the cumulative weight of these studies suggests the existence of a link between dietary fat and PCa progression.

3. LINKING CHOLESTEROL TO PCA PROGRESSION

Cells in the prostate, as in other tissues, synthesize cholesterol endogenously via the mevalonate pathway. Cellular cholesterol also derives from absorption from circulating lipoproteins *(11)*. Consequently, control of cellular cholesterol content is a balance between metabolic processes intrinsic to the cell and the regulation of cholesterol distribution by the organism. Cholesterol content of cell membranes is determined by a complex set of processes, including synthetic pathways in the endoplasmic reticulum, transfer from lipoproteins to the exoplasmic leaflet, receptor-mediated internalization, intracellular transport mechanisms, and efflux from the cell via secretion of lipoprotein complexes.

Extensive evidence indicates that this complex homeostatic mechanism breaks down in aging tissues and in cancer. Accumulation of cholesterol has been described in a variety of tumor types *(12–15)*. Cholesterol content has also been reported to be altered in normal tissue surrounding malignancies *(16)*. Early reports of histological observations described substantial (two-fold) increases in cholesterol content in benign prostatic hyperplasia compared with normal prostate *(17)*. These studies concluded that a relationship existed between cholesterol accumulation in tissues and cellular hyperplasia, consistent with even older findings reported for nonprostatic tumors *(18)*. Studies of human and animal tissues have also described increases in cholesterol content of prostatic secretions that correlate with disease, age, or the presence of malignancy *(12,19)*.

In general, epidemiological studies have not found an association between circulating cholesterol levels, whether or not linked to diet, and cancer risk (reviewed in ref. *20*). However, several studies have reported statistically significant positive correlations between cholesterol intake and cancer risk *(21–23)*, implying that prolonged consumption of cholesterol-rich foods might promote cancer in select tissues.

Epidemiological and animal studies have revealed an association between dietary fat and the incidence of breast cancer, which is similar to PCa in its sex hormone dependence and origin from alveolar–ductal epithelium *(24)*. Cholesterol increases in breast tumor tissues have been attributed to multiple mechanisms, including increased absorption from the circulation *(24–26)*, loss of feedback regulation through downregulation of low-density lipoprotein receptors *(26)*, and upregulation of components of the mevalonate pathway, particularly HMG-CoA reductase *(26)*.

In the 1960s, Schaffner and colleagues provided the first evidence that lowering cholesterol levels systemically might alter prostate cell growth and/or survival *(19)*. These investigators demonstrated that prostate regression was selectively induced in dogs and rodents by oral administration of hypocholesteremic agents, such as the polyene macrolide candicidin *(19)* (reviewed in ref. *20*). Candicidin and structurally similar agents, such as amphotericin B, filipin, and nystatin, bind to cholesterol and closely related sterols *(27)*, thereby lowering circulating cholesterol by inhibiting its absorption through the gut *(28)*. Several human trials of oral candicidin for benign prostatic hyperplasia in the 1970s reported symptomatic improvement *(29)* with no alteration in hormonal status *(29,30)*, indicating that changes in the prostate were likely not the result of suppression of androgen production or use. These early studies suggest the intriguing possibility that lowering circulating cholesterol levels could pharmacologically modulate hyperplastic prostate growth and, potentially, neoplasia.

Recent studies using molecular approaches also point toward a potential relationship between cholesterol and PCa. Pathological studies of prostate tumor samples demonstrated an association between caveolin (Cav)-1, a cholesterol-binding and transport protein, and PCa aggressiveness. In these reports, the expression level of the Cav-1 protein was shown to correlate positively with higher Gleason grade *(32)*. Consistent with these findings, intercross of Cav-1-null (–/–) mice with the transgenic mouse PCa model (TRAMP), in which prostate adenocarcinoma originates from tissue-specific expression of the SV40 T antigen, resulted in significant reductions in tumor volume and fewer metastases to distant sites *(33)*. Cav-1 is an integral membrane protein that, in addition to binding cholesterol, binds to and regulates many signaling proteins, including the classic androgen and estrogen receptors *(34,35)* and the Akt (protein kinase B) pro-survival kinase *(36)*. Taken together, these studies provide evidence that Cav-1 upregulation may provide a survival advantage to PCa cells and, thereby, promote disease progression *(33)*.

Studies performed by our group also suggest that cholesterol-dependent signaling events are important for the progression of PCa. In published reports, we demonstrated that androgen-responsive, Cav-negative, human PCa cells (LNCaP) are stimulated to undergo apoptosis when membrane cholesterol is dispersed by treatment with filipin, a cholesterol-binding drug *(37)*. This treatment was shown to alter Akt signaling, an effect that was attenuated by replenishing the membrane with cholesterol. Akt, an important mediator of cell survival and proliferation in many cell types, is upregulated in as many as 50% of advanced PCas because of frequent inactivation of PTEN, a lipid phosphatase that negatively regulates this pathway *(38)*. In contrast to prostate tumor cells, normal prostate epithelial cells do not undergo apoptosis in response to cholesterol-binding drugs or cholesterol synthesis inhibition *(39)*. Our findings are consistent with earlier reports that cancer cells, but not normal cells, undergo apoptosis in response to treatment with cholesterol-binding drugs *(40)*. They also suggest that some prostate tumor cells may become dependent on ("addicted to") cholesterol-regulated survival mechanisms.

In light of these observations, it is interesting that some studies have reported an inverse correlation between cancer incidence and cholesterol levels for certain neoplasms *(41)*. Evidence suggests that this negative relationship is likely attributable in many cases to the hypocholesteremic effects of

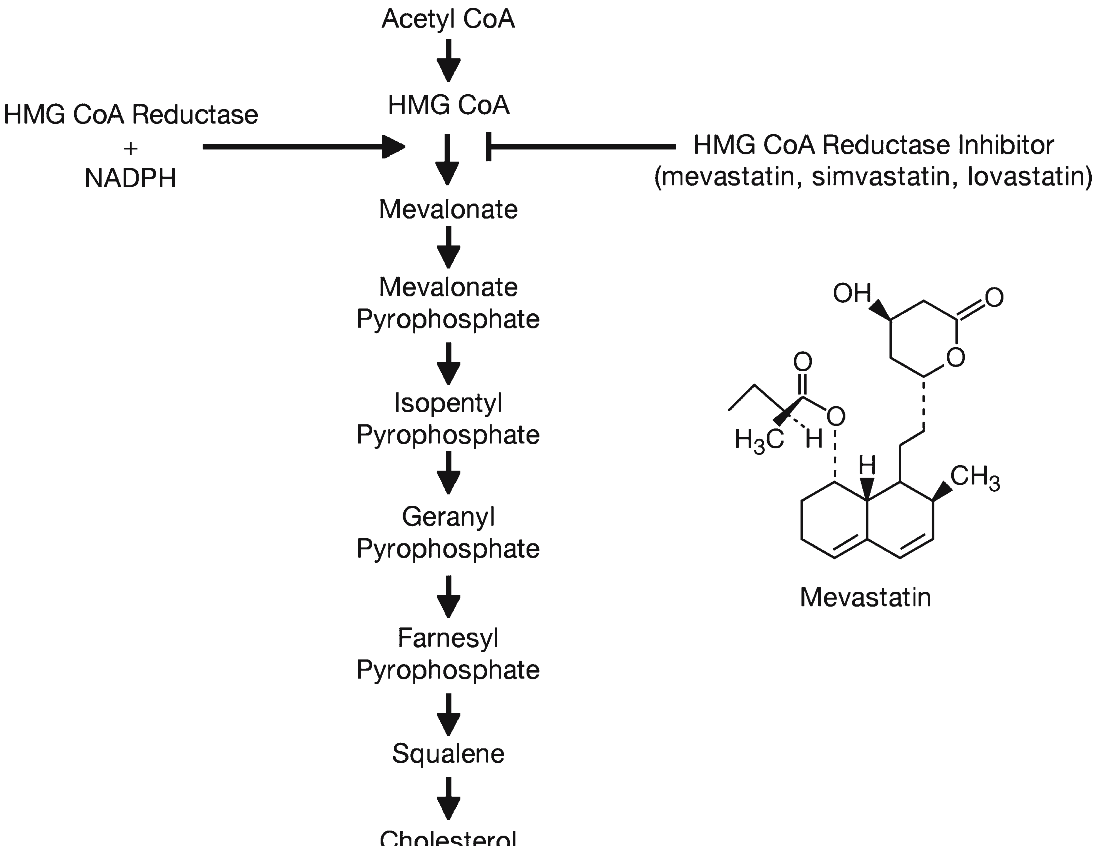


Fig. 1. The mevalonate pathway.

preexisting cancer *(42,43)*. Although the potential for occult cancer to lower circulating cholesterol has not been conclusively established *(44)*, it is clear that frank cancer is associated with lower cholesterol in human patients *(45–47)*.

4. INHIBITION OF CHOLESTEROL SYNTHESIS AND CANCER THERAPY

HMG-CoA reductase catalyzes the rate-limiting step of cholesterol biosynthesis (Fig. 1). HMG-CoA reductase inhibitors are cholesterol-lowering drugs used in the treatment of lipid disorders, especially hypercholesterolemia. The "statin" family of HMG-CoA reductase inhibitors includes simvastatin, lovastatin, atorvastatin, fluvastatin, cerivastatin, and pravastatin. Recent clinical data from studies on cardiovascular disease and cancer chemoprevention suggest that prolonged statin therapy may reduce the risk of prostate and other cancers. Results of a large-scale study of patients taking statins compared with other cardioprotective agents *(48)* showed a 20% reduction in total cancer incidence with statin treatment, with prostate and kidney cancers showing the largest reductions. Interestingly, patients who terminated statin therapy returned to a baseline level of risk within 6 months. Another randomized, placebo-controlled study of cause-specific mortality rates in patients on long-term simvastatin therapy (up to 8 years) reported fewer deaths from cancer in the simvastatin group in comparison with placebo, although the difference was not considered significant *(49)*.

Some studies have reported more modest effects of statins. A population-based cohort study by Friis et al. showed only a slightly reduced incidence of all cancers with statin therapy *(50)*. In a recent

nested case–control study that addressed the potential risks of HMG-CoA reductase inhibitor therapy with respect to cancer incidence, nearly all cancers examined were either unaffected or increased with statin therapy *(51)*. However, in this last report, PCa incidence did decline in the HMG-CoA reductase inhibitor group.

Additional studies on the effect of statins on cancer are currently in progress at other centers. At approximately the same time that this chapter was submitted for publication, a large prospective cohort study of 34,438 men (ages 44–79 yr) administered statin drugs between 1998 and 2000 was presented in abstract form at a national cancer meeting *(52)*. This study concluded that the risk of PCa was approximately half in the statin-users group compared with the nonuse group. Further, PCa risk decreased with increasing duration of use, and risk of metastatic disease was inversely correlated with statin use *(52)*.

Anticancer efficacy of statins in comparison with other cholesterol-lowering therapies may be caused by the ability of statins to reduce cholesterol in peripheral tissues (including the prostate) in addition to the liver. The prostate may be a relatively privileged site for cholesterol-targeting therapy because it synthesizes cholesterol at a high rate, possibly even higher than the liver *(53)*. In general, tumor cells are more sensitive to the growth inhibitory effects of statins in comparison with normal cells *(54)*, an attractive feature when considering the possibility of their potential use in cancer therapy or chemoprevention *(39)*.

General conclusions about the anticancer effectiveness of the statins await further study. One potential problem with interpreting existing studies is that different statins exhibit significantly different activity profiles against tumor cells *(54)*. This difference may account for conflicting reports on the efficacy of statin cancer therapy *(55)*. Although more work needs to be done, the present evidence supports the conclusion that inhibiting HMG-CoA reductase pharmacologically may have clinical benefit in the chemoprevention—and, in some instances, the treatment—of PCa.

5. PLEIOTROPIC EFFECTS OF CHOLESTEROL SYNTHESIS INHIBITION

HMG-CoA reductase inhibitors have been demonstrated to exert potent anticancer effects in cell culture and animal models. In vitro studies on cell lines have shown that statins can inhibit tumor cell growth *(56)* by inducing cell cycle arrest or apoptosis *(57–59)*. These activities have been observed in breast and leukemia culture models and during treatment of xenotransplanted EJ3-derived colorectal tumors in mice *(60,61)*. Several studies of lovastatin's effects on human lymphoma, glioma, melanoma, and NIH3T3 cells have shown reduced invasiveness in Matrigel assays correlating with a reduction in matrix metalloproteinase activity *(62,63)*. In monocytes, statin treatment leads to decreased matrix metalloproteinase-9 and plasminogen activator receptor levels *(64)*. In addition, fluvastatin has been shown to block invasiveness of breast, pancreatic, and colon cell lines, whereas mevastatin has been shown to inhibit cell cycle progression in PC-3 human PCa cells by inhibiting cdk2 phosphorylation *(65,66)*.

In addition to the evidence from cell culture studies, data from in vivo studies indicate that some statin drugs can affect tumor growth *(66)*, angiogenesis *(67)*, and metastasis *(66,68)*. Although reports of antitumor effects predominate in the literature and statins have been shown, under some circumstances, to be antiangiogenic, they have also been shown to elicit angiogenic effects *(67,69–73)*. Interestingly, the proangiogenic effects of statins are mediated by nitric oxide and seem to occur largely in cells with low Cav expression *(74)*. This is a potential explanation for the dissimilar effects that have been observed in different cell backgrounds.

HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, an early step in the cholesterol synthesis pathway (Fig. 1). This metabolic step affects the formation downstream of various metabolic intermediates and synthetic end products, including isoprenoids, geranylgeranyl pyrophosphate (GGPP), farnesyl pyrophosphate (FPP), heme A, and dolichols *(75)*. To what extent do antitumor effects of statins originate from effects on the biosynthesis of these substances?

Products of the mevalonate pathway, such as isopentenyl adenosine and adenine, have been implicated in important cellular functions, such as the initiation of DNA synthesis in the S phase of the cell cycle *(76)*. GGPP and FPP are essential for (iso)prenylation and activation of a variety of intracellular proteins, such as the GTPases, Ras, Rho, Rab, Ral, and Rap; heterotrimeric G protein subunits; nuclear lamins; transducins; and rhodopsin kinase *(77)*. A number of prenylated proteins, particularly the small GTPases, have important biological functions in regulating cell proliferation, differentiation, migration, and apoptosis *(78,79)*. Many studies have concluded that inhibition of protein prenylation may have profound effects on signaling pathways, including the Ras/mitogen-activated protein kinase, the Rac/stress kinase, and the Rab-dependent endocytic pathways *(80–82)*.

Farnesylated Ras proteins are targeted to the membrane and become associated with receptors that generate mitogenic signals in response to growth factor stimulation. Rho family members (Rho, Rac1, and cdc42) can be both farnesylated and geranylgeranylated, a modification that facilitates signal transduction from membrane receptors. Statin treatment was reported to lead to the accumulation of inactive Ras and Rho in the cytoplasm *(77)*, an effect that was explained by inhibition of posttranslational modification of these proteins. Consistent with this, in a study of the activated T-cell receptor, inhibition of protein prenylation by simvastatin resulted in dramatic impairment in the pathways regulated by Ras, Rho, and Rac *(83)*. Treatment with exogenous FPP and GGPP completely reversed the inhibition of mitogen-activated protein kinase pathway signaling by simvastatin.

Alternatively, other reports suggest that farnesylated Ras may not be critical for some effects of statins; rather, other modifications could play an important role. In one such study, De Clue and colleagues demonstrated that NIH3T3 cells transformed by myristoylated (membrane-bound) Ras were susceptible to lovastatin-induced growth inhibition in a similar manner to wild-type Ras transfected cells; however, statin effects occurred regardless of the farnesylation status of Ras *(84)*. Agarwal et al. provided evidence that it is the geranylated status of Ras that might be responsible for statin-induced morphological changes and apoptosis *(85)*. However, their studies also suggested that farnesylated or geranylgeranylated RhoB could both suppress tumor growth as well as play a role in inactivation of RhoA *(85)*. Based on these findings, it was suggested that an imbalance between relative amounts of RhoA and RhoB might be a potential mechanism for lovastatin-induced apoptosis *(85)*. Inhibitors of geranylgeranylation of Rho proteins have been used to demonstrate antitumor effects in mouse models and cultured tumor cells *(86,87)*.

Although the above studies are informative, their conclusions remain speculative, because in no single study were all of the following factors considered:

1. Cholesterol level before and after FPP and GGPP.
2. The prenylation status of the proteins.
3. The importance of other lipid modifications, for instance, palmitoylation.
4. The ability of cholesterol to reverse inhibitory effects of statins.

Inhibition of cholesterol synthesis and reduction of protein prenylation may result in overlapping effects. Although inhibition of prenylation may affect the ability of proteins to localize appropriately to the membrane, reduction of membrane cholesterol levels may also alter the localization and association of small GTPases to the membrane, resulting in demonstrable biological effects *(88)*.

Recent molecular and cell biological studies of membrane domain organization may partly explain some of the puzzling observations of statin effects on cells. Lipid modification (palmitoylation) of Ras family proteins has been shown to underlie differential localization to specific lipid microdomains and to modulate signal output *(89)*. In addition, the subcellular distribution of H-and N-Ras is a function of the constitutive de-/re-acylation cycle that controls rapid changes in localization from the plasma membrane to the Golgi in response to growth factors *(89)*. Approximately 20% of *PTEN*-null prostate tumors show activation of Ras signaling pathways *(90)*. Thus, further study of aberrant subcellular localization of mutant Ras proteins in the *PTEN*-null background may provide a better understanding of the mechanistic basis of the cellular effects of statins.

6. CHOLESTEROL-ENRICHED MICRODOMAINS

Cholesterol makes up almost one-third of the plasma membrane lipids. The effect of cholesterol on membrane organization is a feature of its unique chemistry, where the combination of the stiff, fused ring system and small head group affects cholesterol packing with other lipids and proteins *(11,92)*. Cholesterol orders the fluid bilayer by restricting the number of gauche conformations that acyl side chains of phospholipids can assume. In mammalian cells, cholesterol content is one of the key regulators of membrane properties, and its concentration in cell membranes is tightly regulated, even as the external availability of cholesterol varies widely.

The plasma membrane of eukaryotic cells is sufficiently complex and heterogeneous to permit structural and functional compartmentalization. These compartments include multiple types of microdomains with specialized functions, such as focal adhesions, tight and adherens junctions, and clathrin-coated pits. Studies of the biophysical properties of plasma membranes, of the behavior of glycosylphosphatidylinositol (GPI)-anchored proteins in membranes, of membrane organelles termed caveolae, and of intracellular transport processes provide evidence for the existence of a distinct type of membrane microdomain generally referred to as the lipid raft *(93)*. Lipid rafts, also sometimes referred to as detergent-resistant membranes, are microdomains that contain high concentrations of cholesterol and fatty acids with long saturated acyl chains (e.g., sphingolipids), relative to the majority of the plasma membrane. The acyl chain composition of the lipids in the membrane is a major determinant of lipid segregation into rafts, with cholesterol providing an order to the fluid lipid bilayer. At high concentrations, the tight packing of these components causes rafts to exist in a liquid-ordered phase, in contrast to the liquid-disordered state of the wider membrane *(93,94)*. Lipid rafts are insoluble in cold nonionic detergents such as Triton-X 100 and, consequently, can be isolated from other subcellular elements using biochemical methods.

Many studies indicate that lipid rafts, as discrete domains within the "lipid sea," serve as privileged sites for certain types of cell signaling, signal pathway cross talk, and signal amplification. In the lipid raft model, lipid-modified integral membrane proteins segregate in a directed manner into membrane regions rich in cholesterol and sphingolipids, where they perform specialized signaling functions that may not occur at other subcellular locations *(93)*. Although the means by which proteins localize to rafts is still not fully understood, several mechanisms involve posttranslational modification. Targeting mechanisms include the attachment of a GPI-anchor, dual acylation (Src family of nonreceptor tyrosine kinases and heterotrimeric G protein subunits) *(95)* and linkage to cholesterol (Hedgehog [Hh]) *(96)*. In contrast, some prenylated proteins (e.g., Rap1 and Rab5) may be excluded from rafts *(96–98)*. The range of signaling molecules identified in rafts in various cell types include the endothelin receptor, thrombin receptors, multiple growth factor receptors, ion channels and pumps, the inositol 1,4,5-trisphosphate receptor, phosphoinositide 3-kinase (PI3-K), and protein kinase C isoforms *(98–103)*.

Peripheral membrane proteins, which exist on the cytoplasmic face of the plasma membrane, can also associate with raft domains through charge–charge interactions between the proteins and the head group motif of membrane lipids, much the same way as they associate with nonraft membranes *(93)*. The partitioning of signaling proteins, such as Src family kinases, to raft domains is dependent on hydrophobic interactions of the acyl chain in the lipid anchor with the membrane interior, as well as electrostatic interactions between the acidic phospholipid head groups of the membrane and basic amino acids in the lipid-binding sites of peripheral membrane proteins *(92,104)*.

7. STRUCTURE AND FUNCTION OF LIPID RAFTS

Early evidence for the existence of cholesterol-rich membrane domains was based on observations of GPI-anchored proteins on lymphocytes and brush border membranes of the kidney and gut *(105–107)*. These early studies described GPI-anchored proteins as being lipid-anchored and "detergent-insoluble," yet capable of delivering signals when crosslinked. It was subsequently

demonstrated that GPI-anchored proteins coimmunoprecipitated with both Src family kinases *(108,109)* and heterotrimeric G proteins *(110)*. The detergent-insoluble structures were shown to be cholesterol- and sphingolipid-rich subdomains.

Lipid rafts are thought to be abundant at the plasma membrane in most cells but are also found in biosynthetic and endocytic pathways *(94)*. The formation of lipid rafts begins with the synthesis of cholesterol in the endoplasmic reticulum and is completed in the Golgi, where, after assembly, the rafts traffic out toward the plasma membrane *(94)*. Lipid rafts can recycle either at the cell surface or via endosomes to the Golgi *(111)*.

At least two types of lipid rafts exist: morphologically flat rafts (sometimes termed G domains) and invaginated domains called caveolae (little caves). Caveolae, which are abundant in adipocytes, myocytes, osteoblasts, and endothelia, are the most studied form of lipid raft and are identifiable in electron micrographs as flask-like invaginations of the plasma membrane. Caveolae sequester sphingolipids, cholesterol, and Cav proteins and appear under the electron microscope as striated, 50- to 100-nm invaginated vesicles *(112)*. Caveolae also exist as intracellular vesicles and have been implicated in endocytosis *(113)* and transcytosis *(103)* of albumin and other proteins across the endothelial cell membrane, although some of these functions remain controversial *(112)*. Caveolae likely serve a variety of functions related to the transport of molecules to and from the plasma membrane and between cellular compartments, and also seem to be involved in compartmentalization of signaling pathways. In early myocyte development, newly formed T-tubules are composed of interconnected caveolae-like elements and caveolar structural proteins (e.g., Cav-3), which specifically associate with T-tubules during this process *(114)*.

Cavs are structural proteins important for the formation of the invaginated caveolar architecture. Cavs have also been shown to regulate the activities of many signaling molecules and to be involved in cholesterol transport. Mice genetically null for each of the three Cav isoforms (Cav-1, -2, and -3) have been created and display relatively mild phenotypes, even though knockout of both Cav-1 and -3 leads to total ablation of an entire class of intracellular organelle (caveolae) in many tissues. This somewhat curious observation suggests the possibility that flat rafts may be able to replace caveolae functionally *(115,116)*.

Cav-1, as we already pointed out, was identified in the 1990s as a marker of aggressive PCa *(31)*. The protein is secreted by PCa cells and can be detected in the serum of PCa patients *(117)*. Studies by Li et al. *(118)* have shown that Cav-1 maintains the Akt kinase in an activated state in PCa cells by inhibiting the serine/threonine protein phosphatases PP1 and PP2A *(36)*. Cav-1 is also capable of mediating translocation of phosphorylated AR to the nucleus *(36)*. Overexpression of Cav-1 in androgen-responsive LNCaP PCa cells, which contain a mutant PTEN that leads to constitutive Akt activation, provides a survival advantage by further increasing Akt activity *(118)*. These data suggest that upregulation of Cav-1 provides the tumor an advantage in surviving androgen ablation *(36,119–121)*. Consistent with these findings, ablation of Cav-1 in the TRAMP transgenic PCa mouse model by intercross with Cav-1 (–/–) animals did not affect the appearance of minimally invasive PCa, but its absence prevented progression to metastatic, invasive cancer *(33)*. Despite the evidence for Cav-1 involvement in PCa progression mechanisms, Cav-1 is not required for sustained tumorigenesis because some tumorigenic cells (e.g., LNCaP) are Cav-1-negative *(122)*.

Flat lipid rafts do not contain Cav proteins and, thus, do not form membrane vesicles identifiable by electron microscopy *(123,124)*. Flat rafts have been difficult to visualize as discrete domains by microscopic techniques. Evidence for the existence of these transient structures has been inferred from study of biochemical extracts, model membranes, and advanced microscopic methods, such as fluorescence energy transfer. In 1997, Brown and London *(125)* used cold detergent extraction of cells to demonstrate that lipid anchored proteins could enter cholesterol- and sphingolipid-rich fractions, supporting the idea that detergent-resistant domains might serve as platforms for signal transduction *(125)*. Varma et al. used fluorescence energy transfer measurements of fluorescent folate analogs to demonstrate that folate interacts with its receptors within lipid raft domains in living cells

(126). Clustering of rafts has been observed by electron microscopy during IgE signaling through the FcεR1 receptor *(127)*. Pralle et al. *(128)* used photonic force microscopy to measure the size of lipid rafts in living cells, whereas others used biochemical crosslinking of GPI-anchored proteins in the proximity of rafts *(128,129)*. Crosslinking of cells treated with antibodies or ligands to raft-resident proteins results in the production of 50-nm patches, each containing approx 3500 sphingolipid molecules, that collectively have been estimated to comprise approx 10 to 15% of the exterior plasma membrane surface *(93,128)*.

Model membranes have been used to determine the behavior of sphingolipids, cholesterol, and glycerolipids, and to infer how they interact to form rafts in native cell membranes. Much of the current conception of the physical nature of raft microdomains originates from this modeling approach. For example, studies of lipid monolayers have demonstrated how mixtures of phospholipids, sphingolipids, and cholesterol, in ratios approximating the composition of detergent-insoluble membranes isolated from cells, can evolve into definable structures *(92,130)*. These studies shed light on the manner in which lipid bilayer leaflets can be coupled during signal transduction, and on the dynamic behavior and minimum size of cholesterol-rich membrane complexes *(92,130)*.

Experiments with native cell membranes have demonstrated that caveolae and flat rafts can be isolated by biochemical extraction because they resist solubilization by cold nonionic detergents, such as Triton X-100 and Nonidet P-40 *(92,125)*. This process yields aggregated detergent-resistant membranes that sediment at low density in sucrose gradients and are enriched in raft-resident proteins, cholesterol, sphingolipids, and long chain fatty acids *(123)*.

8. LIPID RAFTS AND PROSTATE CANCER PROGRESSION: RECEPTOR-MEDIATED SIGNALING

Many examples of signaling mechanisms involving lipid rafts can be found in the literature, and some of these are listed in Table 1. For the purposes of this discussion, we briefly discuss two of these, the Sonic Hh (Shh) and ErbB/human epidermal growth factor receptor (HER) receptor tyrosine kinase pathways, both of which have been linked to PCa progression.

Shh is a well-studied homolog of the *Drosophila melanogaster* segment polarity protein, Hh. In vertebrates, a family of Hh paracrine factors has been shown to be important for embryonic patterning of many organs, including the neural tube, axial skeleton, limbs and lungs *(131)*. In addition to its influence on development, Hh pathway signaling has been implicated in cancers of the brain, lung, stomach, pancreas, and prostate *(131–134)*. Shh is a secreted protein that acts on target cells to increase transcription of target genes, including members of the Wnt and transforming growth factor-β family and the Shh receptor, Patched (Ptc) *(131)*. Studies of *Drosophila* embryos have demonstrated that the active fragment of Hh contains a cholesterol moiety and is associated with lipid rafts *(96,135,136)*. Shh is also modified posttranslationally by addition of a cholesterol moiety *(135,136)*. Localization of Shh to rafts seems to be important for its function, yet it is not sufficient, because replacement of the cholesterol anchor with a GPI-anchor prevents release of Shh from its membrane location and inhibits activation of subsequent downstream signaling cascades *(96,137)*. This illustrates that the cholesterol modification of Shh is an important determinant of its signaling capacity and, consequently, an element of accurate morphogenetic tissue patterning *(137)*.

Recent reports have implicated Shh signaling in PCa growth and progression. Linkage studies suggest that Shh target genes, the Shh transducer Smoothened (Smo), and Suppressor of Fused, a negative regulator of Shh, are localized to candidate PCa chromosomal loci *(138,139)*. Shh signaling has been shown to be a feature of prostate development, and sporadic tumors depend on activation of the Shh–Gli pathway for proliferation *(140)*. Increased expression of Shh target proteins, Ptc and Hh interacting protein, have also been detected in advanced or metastatic PCa *(134,141,142)*. The proliferation of cells from primary prostate tumors can be inhibited by an antibody to Shh and inhibition of Hh signaling in PCa cell lines, by either cyclopamine (a Smo inhibitor) or inhibition of Gli by RNA interference, resulting in decreased cell proliferation *(142)*. Metastatic cell lines are no longer

Table 1
Signaling Molecules Associated with Lipid Rafts

Signaling protein	Lipid raft residence or association	Function	Reference
FCεR1	Raft resident and clustering on receptor crosslinking	Src-like tyrosine kinase activation and downstream histamine release	*(127,163)*
TCR	"	Phosphorylation of ITAM by Lyn and Fyn, phosphorylates LAT linking TCR to many signaling pathways	*(129,164)*
B-cell receptor	"	Tyrosine phosphorylation and activation of Erk	*(165,166)*
EGF receptor	Raft and endosome associated	Autophosphorylation and signaling cascade, such as Erk	*(167,168)*
PDGF receptor	"	"	*(169,170)*
Insulin receptor	"	Tyrosine phosphorylation of Src homology proteins	*(171)*
Ephrin B1 receptor	Raft association on ligand activation	Bidirectional cell contact signaling	*(172,173)*
Neurotrophin receptor	Raft association	Intracellular calcium signaling	*(174,175)*
GDNF	Raft associated on GDNF stimulation	Binding to Ret in rafts for signaling	*(176)*
Hedgehog	Cholesterol modification for raft association	Restricts long-range signaling during tissue patterning	*(137)*
H-Ras	Raft association	Raf activation and Erk and MAP signaling	*(177,178)*
Integrins	Raft association	Raft regulation for domain-specific signaling events in anchorage-dependent cells	*(88,179)*
eNos	Myristolyation and palmitoylation for raft association	Inhibition of adenyl cyclase inhibition	*(180)*

[a]TCR, T-cell receptor; ", same as above; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; GDNF, glial cell-derived neurotrophic factor; MAP, mitogen-activated protein; eNos, endothelial nitric oxide synthase, Erk, extracellular signal-related kinase.

sensitive to antibody inhibition of Shh, but apparently activate the Shh signaling pathway independently of Shh *(134)*.

The studies of Karpen et al. have demonstrated that Ptc and Cav-1 colocalize in caveolae/lipid rafts and it seems that the localization of Ptc to lipid rafts serves to recruit Smo to these signaling platforms *(143)*. Ptc shares similarities to the Neimann–Pick type C protein (NPC-1), which is mutated in Neimann–Pick Type C disease, a fatal neurovascular disorder characterized by the accumulation of low-density lipoprotein-derived cholesterol in lysosomes. The results of Carstea et al. *(144)* suggest that NPC-1 is essential for intracellular cholesterol transport via the endocytic pathway *(144)*. This suggests that Ptc, in addition to being important for Shh ligand binding, may also regulate the trafficking of cholesterol between the Golgi and the membrane *(143)*. Thus, association of the Shh receptor complex with Cav-1 may occur in the Golgi, leading to trafficking to the exocytic pathway *(143)*. In addition, Cav-1 may negatively regulate Smo signaling by maintaining the Ptc–Smo complex in a latent state that is relieved by Shh binding and release of Smo from the complex *(143)*.

Similar to Shh, the epidermal growth factor (EGF) receptor (EGFR/ErbB1/HER1) and some components of its signaling pathways are altered in many malignancies, including PCa *(145)*. Early evidence of potential EGFR involvement in PCa was provided by Culig et al., when they demonstrated that EGF is capable of inducing transcriptional activity from the AR in an androgen-independent manner *(146)*. Other studies have shown that overexpression of the ErbB2/HER2 kinase in LNCaP cells and derivative sublines enhanced AR function as well as hormone-independent tumor growth *(147,148)*. Studies by Mellinghoff et al. analyzed cross talk between ErbB family kinases and AR function using a dual EGFR/HER2 inhibitor *(149)*. These workers demonstrated ErbB kinase signals are required for optimal AR function at low androgen concentrations *(149)*. Surprisingly, however, the signal to the AR was transmitted from HER2/HER3 heterodimers rather than from the EGFR. These findings may be relevant to the poor response seen thus far in PCa patients treated with the selective EGFR inhibitor, gefitinib (Iressa).

Lipid rafts mediate EGFR-activated signals to downstream kinases, such as Akt, that are important for survival of PCa cells. Zhuang et al. used Cav-1-negative LNCaP cells to assess the extent to which cell survival signaling was cholesterol-dependent in a *PTEN*-null background *(37)*. Previous studies had shown that EGFR signaling to the PI3-K/Akt pathway elicits a cell survival effect in LNCaP and other *PTEN*-null tumor cells *(150,151)*. Zhuang et al. demonstrated that disruption of lipid rafts by cholesterol-binding compounds inhibited the prosurvival effects of EGFR activation, inhibited Akt signaling, and stimulated apoptosis. These effects were reversed by replenishing cell membranes with cholesterol and, because of the absence of Cav proteins, were attributed to flat—as opposed to caveolar—lipid rafts.

9. LIPID RAFTS AND PROSTATE CANCER PROGRESSION: ACTIN POLYMERIZATION

Metastasis requires the migration of malignant cells out of the tumor as well as invasive entry at distant sites. Both of these processes are dependent on the motile behavior of the tumor cells. Cancer cell motility begins with the extension of cell protrusions in the direction of cell movement *(152)*, a process that requires actin polymerization at the leading edge *(153)*. Thus, regulators of actin cytoskeleton rearrangement are important effectors of tumor cell invasion. Studies from our laboratories demonstrate that lipid rafts mediate mitogenic signals through lipid rafts *(154)* that can result in actin rearrangement and the formation of lamellopodia (Fig. 2). Recently, a study by Papayannopoulos et al. *(155)* has shown that a potent actin nucleator *(156)*, known as the neuronal Wiskott–Aldrich syndrome protein (N-WASP), is regulated through a lipid raft-mediated signaling process. N-WASP localizes to lamellipodia of invasive tumor cells *(157)*, where it plays a role in the formation of invadopodia, an invasive extension of the cytoplasm that is induced by EGFR signaling *(157)*. PIP2, a lipid signaling molecule, switches N-WASP from an inhibited to an active state that can initiate *de novo* polymerization of actin filaments *(155)*, an essential process for tumor cell proliferation and motility.

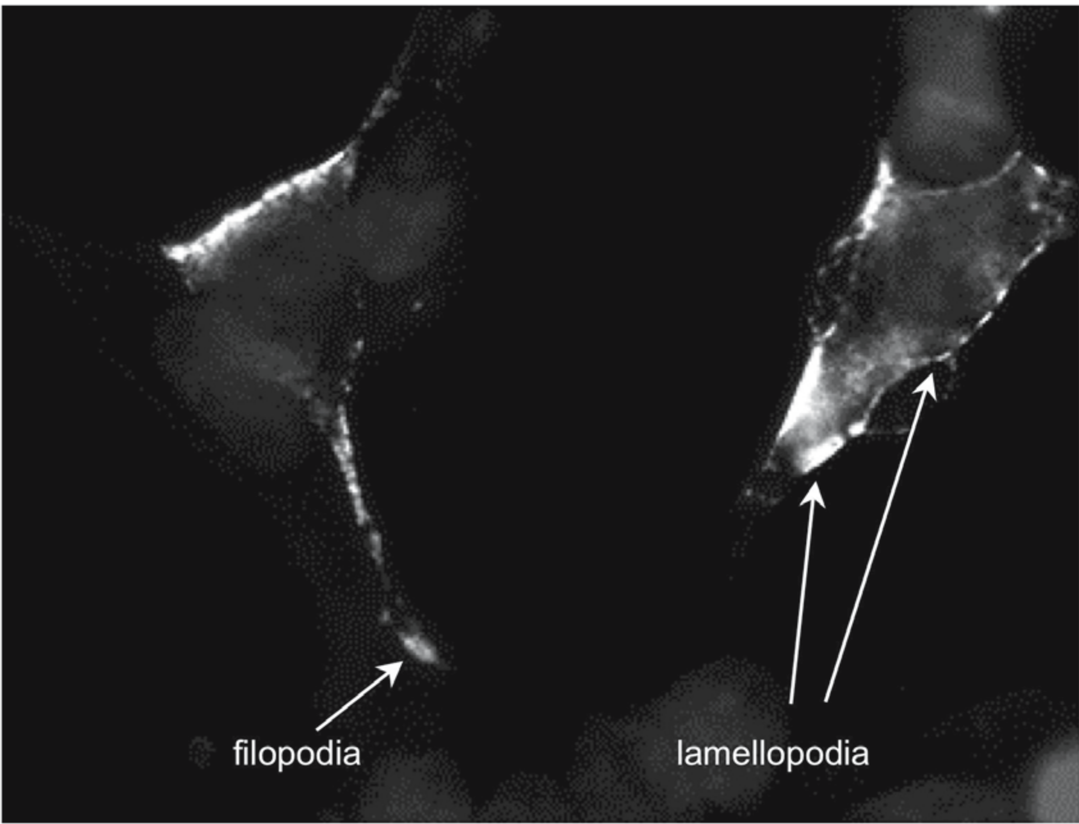


Fig. 2. Lipid rafts and filopodia and lamellipodia formation. LNCaP cells labeled with Alexa-Cholera toxin B subunit, which labels a raft-resident ganglioside (GM1), demonstrate that cell extensions contain lipid rafts.

In addition to its role in the motile phenotype of tumor cells, certain actin-associated proteins may also link actin nucleation events in focal adhesions to microtubule assembly and cell division. One example is the Diaphanous formin homolog, mDia2, which is known to be involved in the assembly and polarization of actin filaments *(158)*. Palazzo et al. *(159)* showed that integrin-mediated activation of focal adhesion kinase at the leading edge of motile fibroblasts is required for microtubule stabilization via a Rho–mDia2 signaling complex localized in lipid rafts. Rho is a small GTPase involved in the endosomal recruitment of the serine/threonine kinase PRK1, EGFR, and the PI3-K effector, PDK1 *(160)*. The involvement of RhoB in endosomal recruitment implicates this GTPase in the control of intracellular trafficking, a function of RhoB that has already been demonstrated for Akt *(161)*. Other actin nucleators may also be involved in the Rho-mediated signaling pathways, as Sandilands et al. *(162)* recently demonstrated for the Scar1 regulator of actin polymerization that is present in Src- and RhoB-associated endosomes. This study suggested that RhoB recruits Src to endosomes, which leads to actin nucleation events initiated by Scar1 *(162)*. Our own unpublished work suggests that a novel formin family member can bind Src and that the resultant complex may traffic between the perinuclear region and lipid rafts via RhoB-associated endosomes in EGF-stimulated LNCaP cells. This trafficking event correlates with changes in cellular shape and motility that are related to PCa tumor progression (M. Lutchman and M. Freeman, unpublished observations). Such studies may provide a new approach to studying the mechanism by which Src regulates cell adhesion, invasion, and motility in PCa.

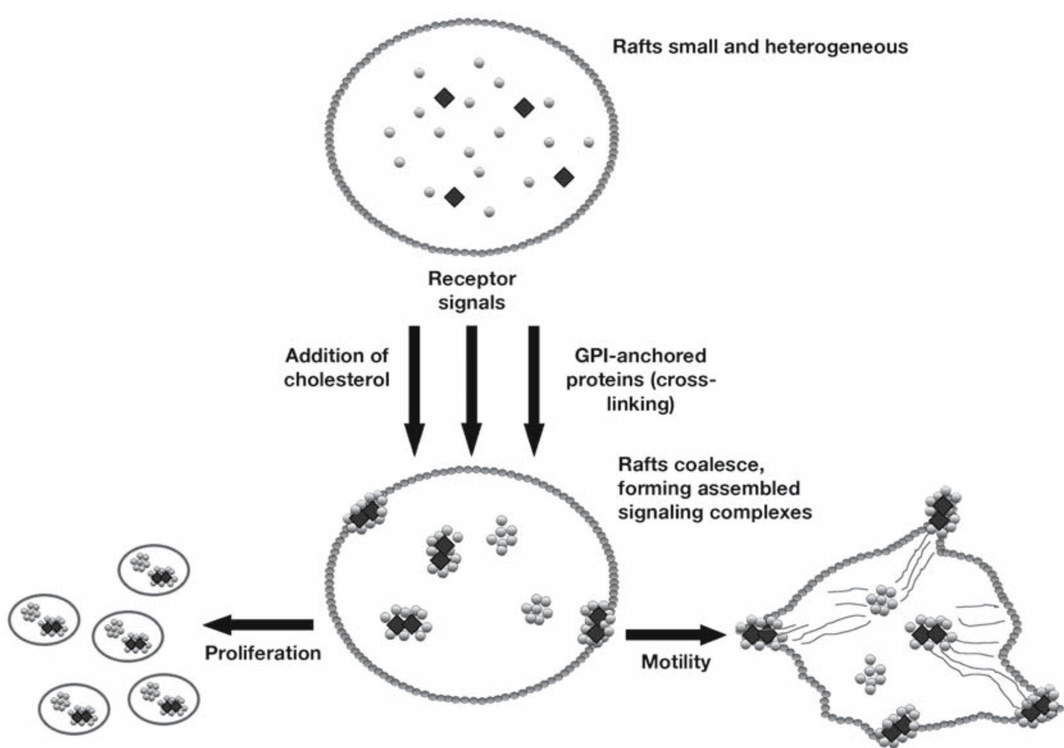


Fig. 3. Raft dynamics and cellular responses. Excess cholesterol, receptor signaling, and crosslinking of GPI-anchored proteins may promote the coalescence of lipid rafts, as well as protein and lipid trafficking into and out of rafts, leading to alterations in cell proliferation, survival, and motility.

10. CONCLUSIONS

We have summarized a large literature that points to a role for cholesterol in PCa progression. The evidence for this conclusion arises from epidemiology studies suggesting that a Western diet can promote, and long-term treatment with cholesterol-lowering drugs can inhibit, PCa growth. These findings in humans are supported by an array of molecular biological studies that point to a role for membrane cholesterol in cell signaling mechanisms relevant to PCa progression. Much of the data in the literature can be interpreted through the paradigm of the lipid raft model (diagrammed in Fig. 3), in which cholesterol-rich domains serve to process biochemical signals for tumor cell survival, proliferation, and migration. This model also accounts for the descriptive findings discussed here that suggest that malignant growth and cancer progression coincide with local tissue alterations in fat metabolism, and in particular, accumulation of cholesterol in tumor cell membranes. Further study of cholesterol-enriched microdomains in the context of cell signaling mechanisms will lead to new avenues for therapeutic intervention in PCa and other malignancies.

REFERENCES

1. Schaid, D. J. and Chang, B. L. (2005). Description of the international consortium for prostate cancer genetics, and failure to replicate linkage of hereditary prostate cancer to 20q13. *Prostate* **63**, 276–290.
2. Cook, L. S., Goldoft, M., Schwartz, S. M., and Weiss, N. S. (1999). Incidence of adenocarcinoma of the prostate in Asian immigrants to the United States and their descendants. *J. Urol.* **161**, 152–155.
3. Shimizu, H., Ross, R. K., Bernstein, L., Yatani, R., Henderson, B. E., and Mack, T. M. (1991). Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br. J. Cancer* **63**, 963–966.

4. Pu, Y. S., Chiang, H. S., Lin, C. C., Huang, C. Y., Huang, K. H., and Chen, J. (2004). Changing trends of prostate cancer in Asia. *Aging Male* **7**, 120–132.
5. Yatani, R., Shiraishi, T., Nakakuki, K., et al. (1988). Trends in frequency of latent prostate carcinoma in Japan from 1965–1979 to 1982–1986. *J. Natl. Cancer Inst.* **80**, 683–687.
6. Weisburger, J. H. (1997). Dietary fat and risk of chronic disease: mechanistic insights from experimental studies. *J. Am. Diet Assoc.* **97**, S16–S23.
7. Pienta, K. J. (1994). The epidemiology of prostate cancer: clues for chemoprevention. *In Vivo* **8**, 419–422.
8. Fleshner, N., Bagnell, P. S., Klotz, L., and Venkateswaran, V. (2004). Dietary fat and prostate cancer. *J. Urol.* **171**, S19–S24.
9. Harvei, S., Bjerve, K. S., Tretli, S., Jellum, E., Robsahm, T. E., and Vatten, L. (1997). Prediagnostic level of fatty acids in serum phospholipids: omega-3 and omega-6 fatty acids and the risk of prostate cancer. *Int. J. Cancer* **71**, 545–551.
10. Fleshner, N. E. and Kucuk, O. (2001). Antioxidant dietary supplements: Rationale and current status as chemopreventive agents for prostate cancer. *Urology* **57**, 90–94.
11. Simons, K. and Vaz, W. L. (2004). Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 269–295.
12. Dessi, S., Batetta, B., Anchisi, C., et al. (1992). Cholesterol metabolism during the growth of a rat ascites hepatoma (Yoshida AH-130). *Br. J. Cancer* **66**, 787–793.
13. Dessi, S., Batetta, B., Pulisci, D., et al. (1994). Cholesterol content in tumor tissues is inversely associated with high-density lipoprotein cholesterol in serum in patients with gastrointestinal cancer. *Cancer* **73**, 253–258.
14. Kolanjiappan, K., Ramachandran, C. R., and Manoharan, S. (2003). Biochemical changes in tumor tissues of oral cancer patients. *Clin. Biochem.* **36**, 61–65.
15. Rudling, M. and Collins, V. P. (1996). Low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA levels are coordinately reduced in human renal cell carcinoma. *Biochim. Biophys. Acta* **1299**, 75–79.
16. Nygren, C., von Holst, H., Mansson, J. E., and Fredman, P. (1997). Increased levels of cholesterol esters in glioma tissue and surrounding areas of human brain. *Br. J. Neurosurg.* **11**, 216–220.
17. Swyer, G. (1942). The cholesterol content of normal and enlarged prostates. *Cancer Res* **2**, 372–375.
18. White, R. M. (1909). What do Statin trials tell us? *Clin. Cardiol.* **24**, 1113–1117.
19. Schaffner, C. P. and Gordon, H. W. (1968). The hypocholesterolemic activity of orally administered polyene macrolides. *Proc. Natl. Acad. Sci. USA* **61**, 36–41.
20. Freeman, M. R. and Solomon, K. R. (2004). Cholesterol and prostate cancer. *J. Cell Biochem.* **91**, 54–69.
21. De Stefani, E., Mendilaharsu, M., Deneo-Pellegrini, H., and Ronco, A. (1997). Influence of dietary levels of fat, cholesterol, and calcium on colorectal cancer. *Nutr. Cancer* **29**, 83–89.
22. Horn-Ross, P. L., Morrow, M., and Ljung, B. M. (1997). Diet and the risk of salivary gland cancer. *Am. J. Epidemiol.* **146**, 171–176.
23. Jarvinen, R., Knekt, P., Seppanen, R., and Teppo, L. (1997). Diet and breast cancer risk in a cohort of Finnish women. *Cancer Lett.* **114**, 251–253.
24. Graziani, S. R., Igreja, F. A., Hegg, R., et al. (2002). Uptake of a cholesterol-rich emulsion by breast cancer. *Gynecol. Oncol.* **85**, 493–497.
25. Tatidis, L., Masquelier, M., and Vitols, S. (2002). Elevated uptake of low density lipoprotein by drug resistant human leukemic cell lines. *Biochem. Pharmacol.* **63**, 2169–2180.
26. Caruso, M. G., Notarnicola, M., Cavallini, A., and Di Leo, A. (2002). 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity and low-density lipoprotein receptor expression in diffuse-type and intestinal-type human gastric cancer. *J. Gastroenterol.* **37**, 504–508.
27. Charbonneau, C., Fournier, I., Dufresne, S., Barwicz, J., and Tancrede, P. (2001). The interactions of amphotericin B with various sterols in relation to its possible use in anticancer therapy. *Biophys. Chem.* **91**, 125–133.
28. Gordon, H. W., and Schaffner, C. P. (1968). The effect of polyene macrolides on the prostate gland and canine prostatic hyperplasia. *Proc. Natl. Acad. Sci. USA* **60**, 1201–1208.
29. Keshin, J. G. (1973). Effect of candicidin on the human benign hypertrophied prostate gland. *Int. Surg.* **58**, 116–122.
30. Sporer, A., Cohen, S., Kamat, M. H., and Seebode, J. J. (1975). Candicidin: physiologic effect on prostate. *Urology* **6**, 298–304.
31. Yang, G., Truong, L. D., Wheeler, T. M., and Thompson, T. C. (1999). Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. *Cancer Res.* **59**, 5719–5723.
32. Yang, G., Truong, L. D., Timme, T. L., et al. (1998). Elevated expression of caveolin is associated with prostate and breast cancer. *Clin. Cancer Res.* **4**, 1873–1880.
33. Williams, T. M., Hassan, G. S., Li, J., et al. (2005). Caveolin-1 promotes tumor progression in an autochthonous mouse model of prostate cancer: Genetic ablation of Cav-1 delays advanced prostate tumor development in TRAMP mice. *J. Biol. Chem.* **280**, 25,134–25,145
34. Schlegel, A., Wang, C., Katzenellenbogen, B. S., Pestell, R. G., and Lisanti, M. P. (1999). Caveolin-1 potentiates estrogen receptor alpha (ERalpha) signaling. caveolin-1 drives ligand-independent nuclear translocation and activation of ERalpha. *J. Biol. Chem.* **274**, 33,551–33,556.

35. Schlegel, A. and Lisanti, M. P. (2001). The caveolin triad: caveolae biogenesis, cholesterol trafficking, and signal transduction. *Cytokine Growth Factor Rev.* **12**, 41–51.
36. Li, L., Ren, C. H., Tahir, S. A., Ren, C., and Thompson, T. C. (2003). Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. *Mol. Cell Biol.* **23**, 9389–9404.
37. Zhuang, L., Lin, J., Lu, M. L., Solomon, K. R., and Freeman, M. R. (2002). Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. *Cancer Res.* **62**, 2227–2231.
38. Paez, J. and Sellers, W. R. (2003). PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. *Cancer Treat Res.* **115**, 145–167.
39. Zhuang, L., Kim, J., Adam, R. M., Solomon, K. R., and Freeman, M. R. (2005). Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. *J. Clin. Invest.* **115**, 959–968.
40. Fisher, P. B., Goldstein, N. I., Bonner, D. P., Mechlinski, W., Bryson, V., and Schaffner, C. P. (1975). Toxicity of amphotericin B and its methyl ester toward normal and tumor cell lines. *Cancer Res.* **35**, 1996–1999.
41. Kaplan, S., Novikov, I., and Modan, B. (1997). Nutritional factors in the etiology of brain tumors: potential role of nitrosamines, fat, and cholesterol. *Am. J. Epidemiol.* **146**, 832–841.
42. Knekt, P., Reunanen, A., Aromaa, A., Heliovaara, M., Hakulinen, T., and Hakama, M. (1988). Serum cholesterol and risk of cancer in a cohort of 39,000 men and women. *J. Clin. Epidemiol.* **41**, 519–530.
43. Wald, N. J., Thompson, S. G., Law, M. R., Densem, J. W., and Bailey, A. (1989). Serum cholesterol and subsequent risk of cancer: results from the BUPA study. *Br. J. Cancer* **59**, 936–938.
44. Vatten, L. J. and Foss, O. P. (1990). Total serum cholesterol and triglycerides and risk of breast cancer: a prospective study of 24,329 Norwegian women. *Cancer Res.* **50**, 2341–2346.
45. Eichholzer, M., Stahelin, H. B., Gutzwiller, F., Ludin, E., and Bernasconi, F. (2000). Association of low plasma cholesterol with mortality for cancer at various sites in men: 17-y follow-up of the prospective Basel study. *Am. J. Clin. Nutr.* **71**, 569–574.
46. Fiorenza, A. M., Branchi, A., and Sommariva, D. (2000). Serum lipoprotein profile in patients with cancer. A comparison with non-cancer subjects. *Int. J. Clin. Lab. Res.* **30**, 141–145.
47. Umeki, S. (1993). Decreases in serum cholesterol levels in advanced lung cancer. *Respiration* **60**, 178–181.
48. Graaf, M. R., Beiderbeck, A. B., Egberts, A. C., Richel, D. J., and Guchelaar, H. J. (2004). The risk of cancer in users of statins. *J. Clin. Oncol.* **22**, 2388–2394.
49. Pedersen, T. R., Wilhelmsen, L., Faergeman, O., et al. (2000). Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering. *Am. J. Cardiol.* **86**, 257–262.
50. Friis, S., Poulsen, A. H., Johnsen, S. P., et al. (2005). Cancer risk among statin users: A population-based cohort study. *Int. J. Cancer* **114**, 643–647.
51. Blais, L., Desgagne, A., and LeLorier, J. (2000). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and the risk of cancer: a nested case–control study. *Arch. Intern. Med.* **160**, 2363–2368.
52. Platz, E. A., Leitzmann, M.F., Visvanathan, K., et al. (2005). Cholesterol-lowering drugs including statins and risk of prostate cancer in a large prospective cohort study. *AACR Abstract 4374*.
53. Schaffner, C. P., Brill, D. R., Singhal, A. K., Bonner, D. P., Goldstein, N. I., and Wang, G. M. (1981). Absence of cholesterogenesis regulation in the liver and prostate of the BIO 87.20 hamster. *Lipids* **16**, 835–840.
54. Wong, W. W., Tan, M. M., Xia, Z., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. (2001). Cerivastatin triggers tumor-specific apoptosis with higher efficacy than lovastatin. *Clin. Cancer Res.* **7**, 2067–2075.
55. Coogan, P. F., Rosenberg, L., Palmer, J. R., Strom, B. L., Zauber, A. G., and Shapiro, S. (2002). Statin use and the risk of breast and prostate cancer. *Epidemiology* **13**, 262–267.
56. Jakobisiak, M., Bruno, S., Skierski, J. S., and Darzynkiewicz, Z. (1991). Cell cycle-specific effects of lovastatin. *Proc. Natl. Acad. Sci. USA* **88**, 3628–3632.
57. Macaulay, R. J., Wang, W., Dimitroulakos, J., Becker, L. E., and Yeger, H. (1999). Lovastatin-induced apoptosis of human medulloblastoma cell lines in vitro. *J. Neurooncol.* **42**, 1–11.
58. Rao, S., Porter, D. C., Chen, X., Herliczek, T., Lowe, M., and Keyomarsi, K. (1999). Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. *Proc. Natl. Acad. Sci. USA* **96**, 7797–7802.
59. Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A. B. (1991). Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. *Cancer Res.* **51**, 3602–3609.
60. Sebti, S. M., Tkalcevic, G. T., and Jani, J. P. (1991). Lovastatin, a cholesterol biosynthesis inhibitor, inhibits the growth of human H-ras oncogene transformed cells in nude mice. *Cancer Commun.* **3**, 141–147.
61. Iishi, H., Tatsuta, M., Baba, M., et al. (2000). ras p21 Isoprenylation inhibition induces flat colon tumors in Wistar rats. *Dis. Colon Rectum* **43**, 70–75.
62. Matar, P., Rozados, V. R., Roggero, E. A., and Scharovsky, O. G. (1998). Lovastatin inhibits tumor growth and metastasis development of a rat fibrosarcoma. *Cancer Biother. Radiopharm.* **13**, 387–393.
63. Prasanna, P., Thibault, A., Liu, L., and Samid, D. (1996). Lipid metabolism as a target for brain cancer therapy: synergistic activity of lovastatin and sodium phenylacetate against human glioma cells. *J. Neurochem.* **66**, 710–716.

64. Ganne, F., Vasse, M., Beaudeux, J. L., et al. (2000). Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits urokinase/urokinase-receptor expression and MMP-9 secretion by peripheral blood monocytes—a possible protective mechanism against atherothrombosis. *Thromb. Haemost.* **84**, 680–688.
65. Ukomadu, C. and Dutta, A. (2003). Inhibition of cdk2 activating phosphorylation by mevastatin. *J. Biol. Chem.* **278**, 4840–4846.
66. Alonso, D. F., Farina, H. G., Skilton, G., Gabri, M. R., De Lorenzo, M. S., and Gomez, D. E. (1998). Reduction of mouse mammary tumor formation and metastasis by lovastatin, an inhibitor of the mevalonate pathway of cholesterol synthesis. *Breast Cancer Res. Treat.* **50**, 83–93.
67. Park, H. J., Kong, D., Iruela-Arispe, L., Begley, U., Tang, D., and Galper, J. B. (2002). 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. *Circ. Res.* **91**, 143–150.
68. Farina, H. G., Bublik, D. R., Alonso, D. F., and Gomez, D. E. (2002). Lovastatin alters cytoskeleton organization and inhibits experimental metastasis of mammary carcinoma cells. *Clin. Exp. Metastasis* **19**, 551–559.
69. Vincent, L., Soria, C., Mirshahi, F., et al. (2002). Cerivastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme a reductase, inhibits endothelial cell proliferation induced by angiogenic factors in vitro and angiogenesis in in vivo models. *Arterioscler. Thromb. Vasc. Biol.* **22**, 623–629.
70. Feleszko, W., Balkowiec, E. Z., Sieberth, E., et al. (1999). Lovastatin and tumor necrosis factor-alpha exhibit potentiated antitumor effects against Ha-ras-transformed murine tumor via inhibition of tumor-induced angiogenesis. *Int. J. Cancer* **81**, 560–567.
71. Weis, M., Heeschen, C., Glassford, A. J., and Cooke, J. P. (2002). Statins have biphasic effects on angiogenesis. *Circulation* **105**, 739–745.
72. Kureishi, Y., Luo, Z., Shiojima, I., et al. (2000). The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat. Med.* **6**, 1004–1010.
73. Urbich, C., Dernbach, E., Zeiher, A. M., and Dimmeler, S. (2002). Double-edged role of statins in angiogenesis signaling. *Circ. Res.* **90**, 737–744.
74. Brouet, A., DeWever, J., Martinive, P., et al. (2005). Antitumor effects of in vivo caveolin gene delivery are associated with the inhibition of the proangiogenic and vasodilatory effects of nitric oxide. *Faseb J.* **19**, 602–604.
75. Koga, T., Shimada, Y., Kuroda, M., Tsujita, Y., Hasegawa, K., and Yamazaki, M. (1990). Tissue-selective inhibition of cholesterol synthesis in vivo by pravastatin sodium, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. *Biochim. Biophys. Acta* **1045**, 115–120.
76. Siperstein, M. D. (1984). Role of cholesterogenesis and isoprenoid synthesis in DNA replication and cell growth. *J. Lipid Res.* **25**, 1462–1468.
77. Van Aelst, L. and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev.* **11**, 2295–2322.
78. Adamson, P., Marshall, C. J., Hall, A., and Tilbrook, P. A. (1992). Post-translational modifications of p21rho proteins. *J. Biol. Chem.* **267**, 20,033–20,038.
79. Armstrong, S. A., Hannah, V. C., Goldstein, J. L., and Brown, M. S. (1995). CAAX geranylgeranyl transferase transfers farnesyl as efficiently as geranylgeranyl to RhoB. *J. Biol. Chem.* **270**, 7864–7868.
80. Weitz-Schmidt, G. (2002). Statins as anti-inflammatory agents. *Trends Pharmacol. Sci.* **23**, 482–486.
81. Blanco-Colio, L. M., Tunon, J., Martin-Ventura, J. L., and Egido, J. (2003). Anti-inflammatory and immunomodulatory effects of statins. *Kidney Int.* **63**, 12–23.
82. Wong, W. W., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. (2002). HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. *Leukemia* **16**, 508–519.
83. Ghittoni, R., Patrussi, L., Pirozzi, K., et al. (2005). Simvastatin inhibits T-cell activation by selectively impairing the function of Ras superfamily GTPases. *Faseb J.* **19**, 605–607
84. DeClue, J. E., Vass, W. C., Papageorge, A. G., Lowy, D. R., and Willumsen, B. M. (1991). Inhibition of cell growth by lovastatin is independent of ras function. *Cancer Res.* **51**, 712–717.
85. Agarwal, B., Halmos, B., Feoktistov, A. S., et al. (2002). Mechanism of lovastatin-induced apoptosis in intestinal epithelial cells. *Carcinogenesis* **23**, 521–528.
86. Pollack, I. F., Bredel, M., Erff, M., Hamilton, A. D., and Sebti, S. M. (1999). Inhibition of Ras and related guanosine triphosphate-dependent proteins as a therapeutic strategy for blocking malignant glioma growth: II—preclinical studies in a nude mouse model. *Neurosurgery* **45**, 1208–1214; discussion 1214–1215.
87. Sun, J., Qian, Y., Hamilton, A. D., and Sebti, S. M. (1998). Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-Ras prenylation but each alone is sufficient to suppress human tumor growth in nude mouse xenografts. *Oncogene* **16**, 1467–1473.
88. del Pozo, M. A., Alderson, N. B., Kiosses, W. B., Chiang, H. H., Anderson, R. G., and Schwartz, M. A. (2004). Integrins regulate Rac targeting by internalization of membrane domains. *Science* **303**, 839–842.
89. Rocks, O., Peyker, A., Kahms, M., et al. (2005). An acylation cycle regulates localization and activity of palmitoylated ras isoforms. *Science*. **307**, 1746–1752.
90. Roy, S., Luetterforst, R., Harding, A., et al. (1999). Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains. *Nat. Cell Biol.* **1**, 98–105.

91. Yeagle, P. L., Alderfer, J. L., and Albert, A. D. (1995). Structure of the carboxy-terminal domain of bovine rhodopsin. *Nat. Struct. Biol.* **2**, 832–834.
92. Mukherjee, S. and Maxfield, F. R. (2004). Membrane domains. *Annu. Rev. Cell Dev. Biol.* **20**, 839–866.
93. Simons, K. and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39.
94. Simons, K. and Ikonen, E. (1997). Functional rafts in cell membranes. *Nature* **387**, 569–572.
95. Moffett, S., Brown, D. A., and Linder, M. E. (2000). Lipid-dependent targeting of G proteins into rafts. *J. Biol. Chem.* **275**, 2191–2198.
96. Rietveld, A., Neutz, S., Simons, K., and Eaton, S. (1999). Association of sterol- and glycosylphosphatidylinositol-linked proteins with Drosophila raft lipid microdomains. *J. Biol. Chem.* **274**, 12,049–12,054.
97. Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002). Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* **296**, 913–916.
98. Bi, K., Tanaka, Y., Coudronniere, N., et al. (2001). Antigen-induced translocation of PKC-theta to membrane rafts is required for T cell activation. *Nat. Immunol.* **2**, 556–563.
99. Chun, M., Liyanage, U. K., Lisanti, M. P., and Lodish, H. F. (1994). Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc. Natl. Acad. Sci. USA* **91**, 11,728–11,732.
100. Liu, J., Oh, P., Horner, T., Rogers, R. A., and Schnitzer, J. E. (1997a). Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains. *J. Biol. Chem.* **272**, 7211–7222.
101. Liu, P., Ying, Y., and Anderson, R. G. (1997b). Platelet-derived growth factor activates mitogen-activated protein kinase in isolated caveolae. *Proc. Natl. Acad. Sci. USA* **94**, 13,666–13,670.
102. Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997). Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. *J. Biol. Chem.* **272**, 30,429–30,438.
103. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell. Biol.* **127**, 1217–1232.
104. Mukherjee, A., Arnaud, L., and Cooper, J. A. (2003). Lipid-dependent recruitment of neuronal Src to lipid rafts in the brain. *J. Biol. Chem.* **278**, 40,806–40,814.
105. Hooper, N. M., Low, M. G., and Turner, A. J. (1987). Renal dipeptidase is one of the membrane proteins released by phosphatidylinositol-specific phospholipase C. *Biochem. J.* **244**, 465–469.
106. Hooper, N. M. and Turner, A. J. (1988). Ectoenzymes of the kidney microvillar membrane. Differential solubilization by detergents can predict a glycosyl-phosphatidylinositol membrane anchor. *Biochem. J.* **250**, 865–869.
107. Hooper, N. M. and Turner, A. J. (1988). Ectoenzymes of the kidney microvillar membrane. Aminopeptidase P is anchored by a glycosyl-phosphatidylinositol moiety. *FEBS Lett.* **229**, 340–344.
108. Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994). Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae. *J. Cell Biol.* **126**, 353–363.
109. Stefanova, I., Horejsi, V., Ansotegui, I. J., Knapp, W., and Stockinger, H. (1991). GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* **254**, 1016–1019.
110. Solomon, K. R., Mallory, M. A., and Finberg, R. W. (1998). Determination of the non-ionic detergent insolubility and phosphoprotein associations of glycosylphosphatidylinositol-anchored proteins expressed on T cells. *Biochem. J.* **334**(Pt 2), 325–333.
111. Brown, D. A. and London, E. (1998). Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111–136.
112. van Deurs, B., Roepstorff, K., Hommelgaard, A. M., and Sandvig, K. (2003). Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol.* **13**, 92–100.
113. Parton, R. G. (1996). Caveolae and caveolins. *Curr. Opin. Cell Biol.* **8**, 542–548.
114. Parton, R. G., Way, M., Zorzi, N., and Stang, E. (1997). Caveolin-3 associates with developing T-tubules during muscle differentiation. *J. Cell Biol.* **136**, 137–154.
115. Galbiati, F., Volonte, D., Liu, J., et al. (2001). Caveolin-1 expression negatively regulates cell cycle progression by inducing G(0)/G(1) arrest via a p53/p21(WAF1/Cip1)-dependent mechanism. *Mol. Biol. Cell* **12**, 2229–2244.
116. Razani, B. and Lisanti, M. P. (2001). Two distinct caveolin-1 domains mediate the functional interaction of caveolin-1 with protein kinase A. *Am. J. Physiol. Cell Physiol.* **281**, C1241–1250.
117. Tahir, S. A., Ren, C., Timme, T. L., et al. (2003). Development of an immunoassay for serum caveolin-1: a novel biomarker for prostate cancer. *Clin. Cancer Res.* **9**, 3653–3659.
118. Li, P., Lee, H., Guo, S., Unterman, T. G., Jenster, G., and Bai, W. (2003). AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. *Mol. Cell Biol.* **23**, 104–118.
119. Mouraviev, V., Li, L., Tahir, S. A., et al. (2002). The role of caveolin-1 in androgen insensitive prostate cancer. *J. Urol.* **168**, 1589–1596.
120. Li, L., Yang, G., Ebara, S., et al. (2001). Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells. *Cancer Res.* **61**, 4386–4392.

121. Li, L., Ittmann, M. M., Ayala, G., et al. (2005). The emerging role of the PI3-K-Akt pathway in prostate cancer progression. *Prostate Cancer Prostatic Dis*. **8**, 108–118.
122. Kim, J., Adam, R. M., Solomon, K. R., and Freeman, M. R. (2004). Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. *Endocrinology* **145**, 613–619.
123. Simons, K. and van Meer, G. (1988). Lipid sorting in epithelial cells. *Biochemistry* **27**, 6197–6202.
124. Simons, K. and Wandinger-Ness, A. (1990). Polarized sorting in epithelia. *Cell* **62**, 207–210.
125. Brown, D. A. and London, E. (1997). Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem. Biophys. Res. Commun*. **240**, 1–7.
126. Varma, R. and Mayor, S. (1998). GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* **394**, 798–801.
127. Sheets, E. D., Holowka, D., and Baird, B. (1999). Membrane organization in immunoglobulin E receptor signaling. *Curr. Opin. Chem. Biol*. **3**, 95–99.
128. Pralle, A. and Florin, E. L. (2002). Cellular membranes studied by photonic force microscopy. *Methods Cell Biol*. **68**, 193–212.
129. Janes, P. W., Ley, S. C., Magee, A. I., and Kabouridis, P. S. (2000). The role of lipid rafts in T cell antigen receptor (TCR) signalling. *Semin. Immunol*. **12**, 23–34.
130. Edidin, M. (2003). The state of lipid rafts: from model membranes to cells. *Annu. Rev. Biophys. Biomol. Struct*. **32**, 257–283.
131. Ruiz i Altaba, A., Sanchez, P., and Dahmane, N. (2002). Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat. Rev. Cancer* **2**, 361–372.
132. Pasca di Magliano, M. and Hebrok, M. (2003). Hedgehog signalling in cancer formation and maintenance. *Nat. Rev. Cancer* **3**, 903–911.
133. Karhadkar, S. S., Bova, G. S., Abdallah, N., et al. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* **431**, 707–712.
134. Sanchez, P., Hernandez, A. M., Stecca, B., et al. (2004). Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. *Proc. Natl. Acad. Sci. USA* **101**, 12,561–12,566.
135. Peters, C., Wolf, A., Wagner, M., Kuhlmann, J., and Waldmann, H. (2004). The cholesterol membrane anchor of the Hedgehog protein confers stable membrane association to lipid-modified proteins. *Proc. Natl. Acad. Sci. USA* **101**, 8531–8536.
136. Porter, J. A., Ekker, S. C., Park, W. J., et al. (1996). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21–34.
137. Incardona, J. P. and Eaton, S. (2000). Cholesterol in signal transduction. *Curr. Opin. Cell Biol*. **12**, 193–203.
138. Easton, D. F., Schaid, D. J., Whittemore, A. S., and Isaacs, W. J. (2003). Where are the prostate cancer genes?—A summary of eight genome wide searches. *Prostate* **57**, 261–269.
139. Xu, J., Gillanders, E. M., Isaacs, S. D., et al. (2003). Genome-wide scan for prostate cancer susceptibility genes in the Johns Hopkins hereditary prostate cancer families. *Prostate* **57**, 320–325.
140. Dahmane, N., Sanchez, P., Gitton, Y., et al. (2001). The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. *Development* **128**, 5201–5212.
141. Fan, L., Pepicelli, C. V., Dibble, C. C., et al. (2004). Hedgehog signaling promotes prostate xenograft tumor growth. *Endocrinology* **145**, 3961–3970.
142. Sheng, T., Li, C., Zhang, X., Chi, S., et al. (2004). Activation of the hedgehog pathway in advanced prostate cancer. *Mol. Cancer* **3**, 29.
143. Karpen, H. E., Bukowski, J. T., Hughes, T., Gratton, J. P., Sessa, W. C., and Gailani, M. R. (2001). The sonic hedgehog receptor patched associates with caveolin-1 in cholesterol-rich microdomains of the plasma membrane. *J. Biol. Chem.* **276**, 19,503–19,511.
144. Carstea, E. D., Morris, J. A., Coleman, K. G., et al. (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* **277**, 228–231.
145. Freeman, M. R. (2004). HER2/HER3 heterodimers in prostate cancer: Whither HER1/EGFR? *Cancer Cell* **6**, 427–428.
146. Culig, Z., Hobisch, A., Cronauer, M. V., et al. (1994). Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res*. **54**, 5474–5478.
147. Craft, N., Chhor, C., Tran, C., et al. (1999). Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res*. **59**, 5030–5036.
148. Yeh, S., Lin, H. K., Kang, H. Y., Thin, T. H., Lin, M. F., and Chang, C. (1999). From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **96**, 5458–5463.
149. Mellinghoff, I. K., Tran, C., and Sawyers, C. L. (2002). Growth inhibitory effects of the dual ErbB1/ErbB2 tyrosine kinase inhibitor PKI-166 on human prostate cancer xenografts. *Cancer Res*. **62**, 5254–5259.
150. Okano, J., Gaslightwala, I., Birnbaum, M. J., Rustgi, A. K., and Nakagawa, H. (2000). Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J. Biol. Chem.* **275**, 30,934–30,942.

151. Lin, J., Adam, R. M., Santiestevan, E., and Freeman, M. R. (1999). The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. *Cancer Res.* **59**, 2891–2897.
152. Friedl, P. and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer* **3**, 362–374.
153. Pollard, T. D. and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112**, 453–465.
154. MacLellan, D. L., Steen, H., Adam, R.M., et al. (2005). A quantitative proteomic analysis of growth factor-induced compositional changes in lipid rafts of human smooth muscle cells. *Proteomics*. **5**, 4733–4742.
155. Papayannopoulos, V., Co, C., Prehoda, K. E., Snapper, S., Taunton, J., and Lim, W. A. (2005). A polybasic motif allows N-WASP to act as a sensor of PIP(2) density. *Mol. Cell* **17**, 181–191.
156. Schafer, D. A. (2002). Coupling actin dynamics and membrane dynamics during endocytosis. *Curr. Opin. Cell Biol.* **14**, 76–81.
157. Yamaguchi, H., Lorenz, M., Kempiak, S., et al. (2005). Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. *J. Cell Biol.* **168**, 441–452.
158. Evangelista, M., Zigmond, S., and Boone, C. (2003). Formins: signaling effectors for assembly and polarization of actin filaments. *J. Cell Sci.* **116**, 2603–2611.
159. Palazzo, A. F., Eng, C. H., Schlaepfer, D. D., Marcantonio, E. E., and Gundersen, G. G. (2004). Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. *Science* **303**, 836–839.
160. Flynn, P., Mellor, H., Casamassima, A., and Parker, P. J. (2000). Rho GTPase control of protein kinase C-related protein kinase activation by 3-phosphoinositide-dependent protein kinase. *J. Biol. Chem.* 275, 11,064–11,070.
161. Adini, I., Rabinovitz, I., Sun, J. F., Prendergast, G. C., and Benjamin, L. E. (2003). RhoB controls Akt trafficking and stage-specific survival of endothelial cells during vascular development. *Genes Dev.* **17**, 2721–2732.
162. Sandilands, E., Cans, C., Fincham, V. J., et al. (2004). RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. *Dev. Cell* **7**, 855–869.
163. Conner, E. R. and Saini, S. S. (2005). The immunoglobulin e receptor: expression and regulation. *Curr. Allergy Asthma Rep.* **5**, 191–196.
164. He, H. T., Lellouch, A., and Marguet, D. (2005). Lipid rafts and the initiation of T cell receptor signaling. *Semin. Immunol.* **17**, 23–33.
165. Cheng, P. C., Dykstra, M. L., Mitchell, R. N., and Pierce, S. K. (1999). A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* **190**, 1549–1560.
166. Janas, E., Priest, R., and Malhotra, R. (2005). Functional role of lipid rafts in CD20 activity? *Biochem. Soc. Symp.* 165–175.
167. Waugh, M. G., Lawson, D., and Hsuan, J. J. (1999). Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains. *Biochem. J.* **337**(Pt 3), 591–597.
168. Puri, C., Tosoni, D., Comai, R., et al. (2005). Relationships between EGFR signaling-competent and endocytosis-competent membrane microdomains. *Mol. Biol. Cell.* **16**, 2704–2718.
169. Heldin, C. H., Ostman, A., and Ronnstrand, L. (1998). Signal transduction via platelet-derived growth factor receptors. *Biochim. Biophys. Acta.* **1378**, F79–F113.
170. Pike, L. J. (2003). Lipid rafts: bringing order to chaos. *J. Lipid Res.* **44**, 655–667.
171. Mastick, C. C., Brady, M. J., Printen, J. A., Ribon, V., and Saltiel, A. R. (1998). Spatial determinants of specificity in insulin action. *Mol. Cell Biochem.* **182**, 65–71.
172. Gauthier, L. R. and Robbins, S. M. (2003). Ephrin signaling: One raft to rule them all? One raft to sort them? One raft to spread their call and in signaling bind them? *Life Sci.* **74**, 207–216.
173. Bruckner, K., Pablo Labrador, J., Scheiffele, P., Herb, A., Seeburg, P. H., and Klein, R. (1999). EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains. *Neuron* **22**, 511–524.
174. Yu, W., Guo, W., and Feng, L. (2004). Segregation of Nogo66 receptors into lipid rafts in rat brain and inhibition of Nogo66 signaling by cholesterol depletion. *FEBS Lett.* **577**, 87–92.
175. Bilderback, T. R., Gazula, V. R., Lisanti, M. P., and Dobrowsky, R. T. (1999). Caveolin interacts with Trk A and p75(NTR) and regulates neurotrophin signaling pathways. *J. Biol. Chem.* **274**, 257–263.
176. Tansey, M. G., Baloh, R. H., Milbrandt, J., and Johnson, E. M., Jr. (2000). GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron* **25**, 611–623.
177. Prior, I. A., Muncke, C., Parton, R. G., and Hancock, J. F. (2003). Direct visualization of Ras proteins in spatially distinct cell surface microdomains. *J. Cell Biol.* **160**, 165–170.
178. Roy, S., Wyse, B., and Hancock, J. F. (2002). H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. *Mol. Cell Biol.* **22**, 5128–5140.
179. Wary, K. K., Mariotti, A., Zurzolo, C., and Giancotti, F. G. (1998). A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell* **94**, 625–634.
180. Shaul, P. W., Smart, E. J., Robinson, L. J., et al. (1996). Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* **271**, 6518–6522.

9

Gene Therapy for Advanced Prostate Cancer

Juan Antonio Jiménez, Xiong Li, Thomas A. Gardner, and Chinghai Kao

Summary

Despite recent advances in early detection and conventional therapies, prostate cancer continues to be the second-leading cause of cancer mortality in American men. Advanced prostate cancer progresses, metastasizes, and becomes resistant to treatment. Clinical evaluation of gene therapy for prostate cancer has demonstrated its safety, and early success warrants further study. In combination with conventional therapies, which are limited by treatment-related morbidity and toxicity, molecular therapy for advanced prostate cancer shows great promise. In this chapter, we review the current status of gene therapy approaches for prostate cancer, which include corrective gene therapy, oncolytic viral therapy, cytotoxic gene therapy, and immunotherapy.

Key Words: AAV; Ad5/35; adenovirus; gene therapy; GVAX; HSV-TK; oncolytic viral therapy; osteocalcin; prostate cancer vaccine; PSES; TRAIL.

1. INTRODUCTION

It is estimated that, in 2005, prostate cancer will account for the most new cancer diagnoses aside from skin cancer, affecting 232,090 men in the United States, and will be the second most common cause of cancer deaths, with 30,350 fatalities *(1)*. Current therapies for men presenting with localized prostate cancer include radical prostatectomy, cryoablation therapy, external beam radiation, and brachytherapy. However, 25% of these men will experience biochemical disease recurrence within 10 years of treatment *(2,3)*. Up to 10% of patients will be diagnosed with metastatic disease at initial presentation *(1)*. In men with locally advanced and distant metastatic prostate cancer, current treatment approaches are merely palliative. In these patients, androgen ablation therapy slows the dissemination of the disease, but, once the cancer changes its androgen status, tumors become refractory to hormonal treatment. Suppression of serum prostate-specific antigen (PSA) levels is observed in only 85% of cases, with PSA rebounds occurring in 12 to 24 months *(4)*. Results from phase III clinical studies have recently suggested a role for docetaxel in the treatment of androgen-independent prostate cancer, demonstrating a 2-month survival advantage in addition to palliation *(5,6)*. Unfortunately, dose-limiting toxicities associated with such chemotherapies limit the amount of the drug that can be delivered to the tumor, allowing the cancer to survive and fail therapy. Because of the limitations of current treatment modalities, there is an urgent need to develop novel therapies to target both organ-confined and metastatic prostate cancer.

Significant progress in basic medical science research has lead to an understanding of the molecular events underlying the development and progression of prostate cancer. Preclinical studies have translated into strategies for the molecular therapy of hormone-refractory prostate cancer. Advances in molecular and cellular biology have lead to the discovery of novel therapeutic genes and improve-

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

Table 1
Characteristics of Vector Systems in Gene Therapy[a]

	Adenovirus	Adeno-associated virus	Retrovirus	Vaccinia virus	Nonviral (liposome)
Maximum insert size	10–35 kb	2.4/4.8 kb	8 kb	>30 kb	? Limit
In vivo gene delivery High	High	Low	High	Low	
Gene expression	Transient	Stable	Stable	Transient	Transient
Cell cycle dependent No	No	Yes	No	No	
Genome integration	No	Site-specific	Random	No	No
Immunoreactive	Yes/no	No	No	Yes	No

[a]This table lists the key attributes of the vectors used previously in gene therapy clinical trials for prostate cancer.

ment of vector systems for the delivery of those genes. Prostate-specific targeting of molecular therapies is now possible because of the identification and characterization of prostate-specific sequences. Our laboratory has focused much of its efforts on the development of chimeric tissue-specific promoters, such as prostate-specific enhancing sequence (PSES), and the application of well-characterized promoters, such as osteocalcin (OC), in the development of molecular therapy for androgen-independent primary and metastatic prostate cancer, which has translated to several clinical trials. In this chapter, we review the current status of gene therapy for advanced prostate cancer.

2. GENE DELIVERY VECTORS

The antitumor effect of gene therapy in prostate cancer is achieved when a therapeutic gene, under the control of a promoter, is delivered directly to a target cell by a vector. The ideal vector would be specific for prostate cancer cells and would have a high rate of transgene delivery. Furthermore, a vector should be inexpensive to produce and administer, nontoxic to the patient, and nonmutagenic. Two categories of vectors are used for human gene therapy, viral and nonviral; however, most prostate cancer clinical trials involve the use of viral vectors. The currently available vectors for gene delivery are listed in Table 1. Much research is underway to improve the specificity, transduction efficiency, and safety of gene therapy vectors.

The route of administration of the therapeutic agent depends by and large on the vector used. At the present time, most prostate cancer gene therapy trials involve the intralesional administration of the vector, which is very suitable because of the ability to visualize the prostate using transrectal ultrasound and its convenient transrectal or transperineal access. Ultimately, the desired route of administration is systemic delivery via intravenous injection of the vector. This approach would seek and destroy all foci of cancer, regardless of their location; however, limiting factors include vector half-life, hematological inactivation of the vector and the infection of nontarget organs. Recent improvements in prostate-specific promoter systems and viral targeting has allowed this approach to be used in human prostate cancer clinical trials.

2.1. *Adenovirus*

The most commonly used viral vector in human gene therapy clinical trials is adenovirus (Ad), a non-enveloped, icosahedral virus containing a double-stranded linear DNA genome, 36 kb in size. This nonintegrating virus has a wide safety profile and is advantageous over many vectors in part because of its ability to carry a large insert and infect any cell, regardless of its cell cycle status. In addition, its genome is easily manipulated in the laboratory, and it is readily produced in high titers with relatively minimal expense. Early generation adenoviral vectors induced strong innate and adaptive immune responses, thereby limiting their gene delivery potential *(7)*. Furthermore, it has been

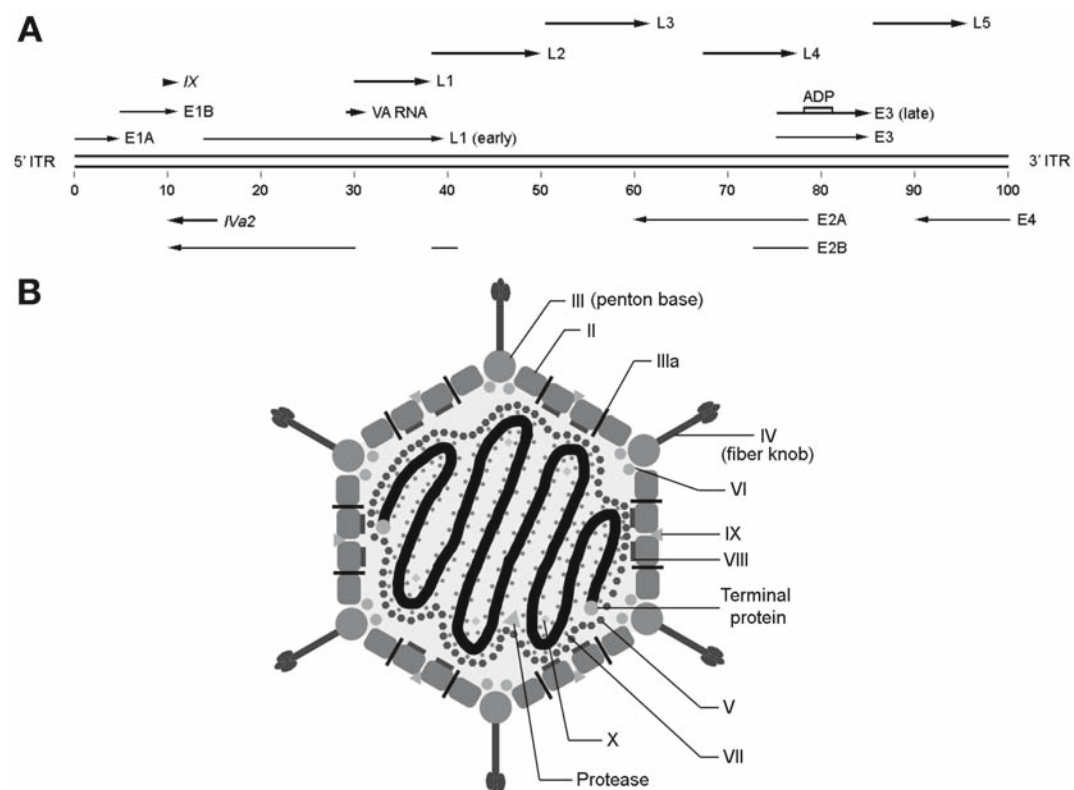


Fig. 1. Genome organization and structure of adenovirus. The transcription map of adenovirus **(A)** depicts the early transcription units with thin arrows, late transcripts in heavy arrows, and delayed mRNAs in *italics*. Encoded within the *E3* region and under the control of the major late promoter is a late transcript, which includes adenoviral death protein (ADP). Map units are indicated by the hashes. Products of the late transcripts contribute to the structure of adenovirus **(B)**. Proteins II, III, IIIa, IV, VI, VIII, and IX form the viral capsid. Proteins V, VII, X, and terminal protein (TP) associate with the adenoviral genome and form the viral core. Protease is responsible for the maturation of proteins IIIa, VI, VII, VIII, and TP. VA, virus associated; ITR, inverted terminal repeat.

shown that nearly all humans have developed humoral immunity to Ad because of previous exposure; however, only 55% of the detected immunoglobulins are neutralizing antibodies *(8)*. Recently, adenoviral vectors have been modified to reduce their immunogenicity, reinstating hope in their clinical usefulness *(9)*.

The adenoviral genome, as depicted in Fig. 1A, is flanked on both ends by short inverted terminal repeats (ITRs), which contain identical origins of replication. Near one end is a short packaging signal (ψ), which is required for proper association of the viral genome with the capsid proteins *(10)*. Its transcription units are divided into two groups, early and late genes. The viral genome carries six early units (*E1A*, *E1B*, *E2*, *E3* , *E4* , and *E5*), two delayed early units (*IVa2* and *IX*), and two late units, one that is processed into five messenger RNAs (mRNAs) (*L1–L5*) and one in the *E3* region that is controlled by the major late promoter. In addition, either one or two copies of virus-associated (*VA*) genes are encoded and transcribed by RNA polymerase III. As shown in Fig. 1A, the transcription of adenoviral genes occurs from both strands and uses alternative splicing and multiple poly(A) sequences.

E1A is the first sequence transcribed in the adenoviral genome. Controlled by a constitutively active promoter, the *E1A* transcription unit encodes up to five polypeptides, of which only two, E1A 12S and E1A 13S, have known functions. E1A proteins transactivate the transcription of other adenoviral genes *(11)*. Expression of *E1A* is critical to the virus. In fact, deletion of the *E1* region of the genome results in a replication-deficient Ad *(12)*. E1A proteins also stimulate viral DNA synthesis by preventing G_1 arrest and advancing the host cell into S-phase. This is accomplished by binding the tumor suppressor retino blastoma (pRB) and releasing the associated transcription factor E2F *(13)*, antagonizing the cyclin-dependent kinase inhibitory protein, $p27_{kip1}$ *(14)*, or inhibiting the transactivation of *p53* by p300/CBP *(15)*. Furthermore, E1A proteins induce apoptosis of infected cells through p53-dependent and p53-independent pathways *(15)*, both of which are blocked through the action of E1B proteins *(16)*. Acting in concert, E1B and E4 proteins shut down host cell protein synthesis by blocking the cytoplasmic accumulation of cellular mRNAs, while stabilizing and exporting viral mRNAs from the nucleus to the cytoplasm *(17,18)*. Although Ad is not responsible for human malignancies, the products of the *E1A*, *E1B*, and *E4* genes transform cells in vitro *(19)*; however, Ad5, the adenoviral serotype used in prostate cancer gene therapy belongs to a non-oncogenic subgroup. Viral DNA synthesis occurs as *E2* gene products accumulate. The *E2* region encodes a DNA polymerase that is essential for viral DNA replication *(20)* and the terminal protein which is covalently bound to the 5' ends of the viral chromosome and serves as a primer for DNA synthesis *(21)*. The expression of *E3* is not essential for viral replication; however, it protects virally infected cells from lysis by cytotoxic T-lymphocytes (CTLs) by downregulating the expression of major histocompatibility complex (MHC) class I antigen *(22)* and Fas receptor *(23)* on the infected cell surface. Further protection from the body's antiviral defense system is provided by *VA* RNA, which forms a hairpin–loop structure and inhibits the activation of interferon-induced RNA-dependent protein kinase *(24)*.

Late gene products include 10 structural proteins, of which, 7 (II, III, IIIa, IV, VI, VIII, and IX) form the capsid and three (V, VII, and μ) are involved with the DNA-containing core. Figure 1B depicts the structure of the Ad particle. The most abundant protein on the capsid surface is the trimeric hexon (II), whose assembly requires the assistance of the L4 100-kDa scaffold protein *(25)*. Neutralizing antibodies to the capsid are formed against surface loops of the hexon structure. Polypeptides IIIa, VI, VIII, and IX stabilize the hexon capsid structure and form a bridge with the adjacent core proteins. The penton base (III) forms at the 12 vertices of the capsid structure, with a trimeric fiber and distal knob (IV) projecting from each base. Together, polypeptides III and IV form the penton complex and mediate host cell binding. Initially, the knob domain of polypeptide IV binds to its cellular receptor, the coxsackie and Ad receptor (CAR) *(26)*, followed by binding of an arg–gly–asp (RGD) motif in the penton to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the cell surface, stimulating internalization *(27)*. The *E3* region encodes one late protein called Ad death protein, which is controlled by the major late promoter and is responsible for cell lysis and release of viral progeny *(28)*. Recently, vectors have been constructed that overexpress Ad death protein, leading to enhanced viral spread and oncolysis *(29)*.

Although prostate cancer cells upregulate CAR expression *(30)*, tumors vary in adenoviral susceptibility. For this reason, efforts have been made to retarget the virus, thereby enhancing its ability to infect prostate cells. Furthermore, the detargeting of Ad has narrowed its expansive tropism, allowing for safer systemic delivery without infection of nontarget tissues, such as liver and respiratory epithelium. Early studies in adenoviral retargeting used bispecific antibodies that crosslinked the virus to alternative cellular receptors *(31,32)*. This has been applied to prostate cancer recently, with a bifunctional antibody to the adenoviral fiber knob and prostate-specific membrane antigen (PSMA) *(33)*. Perhaps a more clinically feasible approach to prostate-specific retargeting of Ad is the genetic modification of the fiber knob. Cell-binding peptides have been displayed on the carboxy terminus of the fiber knob *(34)*; however, this approach is unfavorable because of size constraints and structural

hindrance. Larger peptides can be incorporated into the HI loop of the fiber knob without structural consequences *(35)*. Lupold et al. identified two candidates for prostate-targeting peptides by phage display library that bind to PSMA *(36)*; however, there exist no reports of its use for the transductional targeting of Ad. A final approach to the detargeting of Ad was devised by Shayakhmetov et al., in which the Ad5 fiber was replaced by the short-shafted Ad35 fiber *(37)*. This chimeric Ad5/35 uses CD46 as its cellular receptor *(38)*, which is upregulated on the surface of many cancer cells. Most importantly, systemic administration of these vectors does not result in hepatic infection, and Ad5/35 has demonstrated prostate cancer tropism *(39)*. In combination with transcriptional targeting, the transductional detargeting and retargeting of adenoviral vectors will increase the safety and efficacy of systemically delivered molecular therapies for prostate cancer.

2.1.2. Adeno-Associated Virus

Adeno-associated virus (AAV), a member of the parvovirus family, is a small single-stranded DNA virus that is dependent on a helper virus, such as Ad, for replication. AAV is an attractive vector for gene therapy because it elicits nearly no immune response, is known to cause no disease in humans, and integrates stably and site specifically in a region on chromosome 19 *(40)*. The genome consists of two ITRs, which encode a packaging signal and the origin of replication, and two genes, *rep* and *cap*. *Cap* encodes the viral capsid proteins whereas *rep* encodes four products of alternative splicing, Rep40, Rep52, Rep68, and Rep78, of which only Rep68 or Rep78 are necessary for replication and site-specific integration *(41)*. Recombinant AAV vectors are made by deleting *rep* and *cap* and inserting approx 4.7 kb of therapeutic DNA; however, it has been observed that deletion of *rep* results in nonspecific integration of the virus *(42)*. Nonetheless, the majority of AAV vectors used today are *rep*-deleted. Recombinant AAV is produced in HEK293 cells by co-transfecting a plasmid containing the therapeutic DNA cloned between two ITRs and a plasmid containing the *rep* and *cap* genes. Subsequently, the cells are infected with Ad or transfected with a plasmid containing the adenoviral genes required for AAV replication *(43)*. Recently, methods have been devised to produce higher AAV titers, necessary for large-scale production for gene therapy applications *(44)*. After infection, the rate-limiting step in gene expression seems to be the synthesis of the second strand *(45)*. This has been overcome by the recent development of self-complementary, double-stranded AAV vectors that carry half the insert size of the single-stranded virus *(46)*. Similar to Ad, peptides can be inserted into the AAV capsid to retarget the vector specifically to receptors on the surface of prostate cancer cells *(47)*. To achieve site-specific integration of a transgene with the targeting capability of an adenoviral vector, Recchia et al. developed a hybrid Ad/AAV vector that carries a drug-inducible *rep* expression cassette and a transgene cassette flanked by two AAV ITRs. Site-specific integration of the ITR-flanked transgene cassette was observed in a *rep*-dependent fashion *(48)*.

3. PROSTATE-SPECIFIC PROMOTERS

Recently, much effort has been made to develop tissue-specific delivery systems that eliminate the threat of harm to the patient. Several studies have demonstrated the importance of tissue-specific vectors, revealing systemic toxicity with the administration of high doses of nonspecific vectors *(49,50)*. Viral vectors with broad tropisms can transduce any cell in the body, provided that the cell expresses the correct receptor specific to the virus. Through the use of prostate-specific promoters and enhancers, the expression of the therapeutic gene can be limited to cells that contain the appropriate activators and transcription factors. Several prostate-specific genes have been identified and well-characterized. Recently, chimeric promoters in which repressor elements have been deleted, activating mutations incorporated or enhancer sequences combined, have achieved greater prostate specificity and stronger transcriptional activity. No promoter is prostate cancer-specific; therefore, all prostate cells are susceptible to the delivered therapeutic gene. This however, is not significant, because the prostate is a nonvital organ in the postreproductive population.

3.1. PSA Promoter

PSA is released into the bloodstream when prostatic basement membrane is compromised, such as occurs in prostate cancer and, therefore, is used as a sensitive serum marker for the diagnosis and progression of prostate cancer *(51)*. PSA, a serine protease, is encoded by the human Kallikrein 3 (*hK3*) gene *(52)*. PSA expression is androgen receptor (AR)-dependent, and its transcript levels are significantly reduced in the absence of androgen *(53)*. AR regulates *PSA* expression by binding to a 440-base pair (bp) androgen-responsive enhancer core (AREc) in the upstream 5' flanking region of the *PSA* gene *(54,55)*. In vitro experiments have confirmed the tissue specificity and androgen dependency of this promoter *(56)*. In addition, this promoter has been used in multiple gene therapy studies *(57,58)*. Although this promoter confers high tissue specificity, its usefulness in men undergoing androgen ablation therapy is limited. To circumvent this problem, Gotoh et al. characterized the long (5837-bp) PSA promoter as less dependent on androgen and, therefore, more active than the short (631-bp) PSA promoter in the absence of androgen *(57)*. Two *cis*-acting elements within the long PSA promoter, a 440-bp AREc and a 150-bp pN/H androgen-independent positive regulator, are responsible for this androgen-independent activity. A chimeric promoter with threefold higher activity than the native PSA promoter has been produced by juxtaposing both elements *(59)*. Further attempts to enhance the activity while retaining specificity of the PSA promoter in Ad vectors include duplication of the AREc, which led to a 20-fold increase *(60)*, or tandem duplication of the PSA promoter, which led to a 50-fold increase above basal promoter activity *(61)*.

3.2. PSMA Promoter

PSMA was discovered by Horoszewicz et al. by a monoclonal antibody produced in mice immunized with cell membranes from LNCaP cells *(62)*. PSMA is a type II integral membrane glycoprotein with folate hydrolase *(63)*, *N*-acetylated α-linked acidic dipeptidase *(64)*, and glutamate carboxypeptidase *(65)* activities. PSMA is expressed predominantly in prostate tissue and tumor neovasculature, with low levels detected in the gastrointestinal tract, salivary glands, kidney, and brain *(66)*. Its expression is elevated higher in prostate cancer than in benign hyperplasia or normal prostate *(67)*. In addition, serum PSMA levels are highest in patients with metastatic disease, suggesting enhanced PSMA expression as prostate cancer progresses *(68)*. Unlike *PSA*, *PSMA* expression is upregulated under androgen-depleted conditions *(69)*. A 1.2-kb PSMA promoter has been identified with high promoter activity *(70)*; however, significant leaky activity in PSMA-negative cells limits its clinical usefulness as a gene therapy promoter. Recently, the PSMA enhancer (PSME) was discovered within the third intron of the *PSMA* gene, *FOLH1 (71)*. Lee et al. have demonstrated PSMA activity mediated by NFATc1 cooperatively binding at the AP-3 site within PSME *(72)*. PSME has been used to transcriptionally target suicide *(73)* and oncolytic *(74)* gene therapies for prostate cancer under low androgen levels.

3.3. Prostate-Specific Enhancing Sequence

To achieve the highest transcriptional activity with strong prostate specificity, Lee et al. developed a novel chimeric promoter, PSES, under the hypothesis that AREc and PSME could function synergistically in any androgen environment. Through deletion and linker scan mutagenesis, the main prostate-specific enhancer activity of the PSA AREc and PSME were located in a 189-bp region called AREc3 and a 331-bp region called PSME(del2), respectively. PSES was developed by combining both AREc3 and PSME(del2) and placing AREc3 upstream from PSME(del2). AREc3 contains six GATA transcription factor-binding sites and three AR-binding sites leading to high enhancer activity once surrounding silencer regions were deleted. PSME(del2) contains eight AP-1 and three AP-3 binding sites acting as positive regulators in the absence of androgen, and a downstream deletion of an *Alu* transcription-silencing repeat. PSES showed significantly stronger transcriptional activity than either AREc3 or PSME(del2) alone in the presence or absence of andro-

gen. Furthermore, PSES demonstrated fivefold higher activity than universal promoter RSV and activity equal to CMV promoter. In vitro studies revealed that PSES is active in several PSA- and PSMA-positive prostate cancer cell lines, but not in PSA- and PSMA-negative prostate cells or nonprostate cell lines *(75)*. A replication-competent Ad was created using the bidirectional PSES promoter to control both *E1a* and *E4* adenoviral early genes *(76)*. Because of its small size, high level of tissue specificity, and strong promoter activity regardless of androgen status, PSES is an ideal promoter for use in prostate cancer gene therapy.

3.4. OC Promoter

OC is a highly conserved bone γ-carboxyglutamic acid protein that has been shown to be transcriptionally regulated by 1,25-dihydroxyvitamin D_3 *(77)*. This noncollagenous bone protein constitutes 1 to 2% of the total protein in bone, and its expression is limited to differentiated osteoblasts and osteotropic tumors, especially primary and metastatic prostate cancer *(78)*. The osteoblastic nature of osseous prostate cancer metastases is well-characterized *(79)*, and the mechanism is thought to be via its osteomimetic properties, specifically, its ability to express bone-related proteins such as OC *(80)*. The human OC promoter contains numerous regulatory elements, including a vitamin D-responsive element, making it inducible by vitamin D_3 administration *(81,82)*, a glucocorticoid response element, an AP-1 binding site *(83)*, and an AML-1 binding site, which has been shown to be responsible for 75% of *OC* expression *(84)*. The OC promoter retained its tissue-specificity in a recombinant OC promoter-driven herpes simplex virus (HSV)-thymidine kinase (TK)-expressing adenoviral vector. Ko et al. developed a gene therapy for osteosarcoma in which coadministration of Ad-OC-TK and acyclovir resulted in osteoblast-specific cell toxicity *(85)*. A similar strategy was developed for the intralesional injection of Ad-OC-TK to osseous prostate cancer metastases followed by administration of valacyclovir (VAL). In phase I clinical trials, this therapy induced apoptosis in every lesion treated, without serious adverse effects to the patients *(86,87)*.

3.5. Human Telomerase Promoter

Telomeres are tandem repeat structures found at the termini of chromosomes that maintain chromosomal integrity by preventing DNA rearrangements, degradation, and end-to-end fusions. In most normal somatic cells, the telomeric cap is shortened with each cycle of DNA replication and cell division. When telomeres shorten to a critical length, cells progress toward irreversible arrest of growth and cellular senescence *(88)*. In contrast, tumor cells have evolved a means to prevent telomere shortening through the activation of the catalytic component of human telomerase reverse transcriptase (hTERT) *(89)*. The hTERT promoter region has been cloned and characterized, and contains a high GC content. Unlike most promoters, it does not contain TATA or CAAT boxes *(90)*. Importantly, the hTERT promoter is active in most cancer cells, including prostate cancer cells *(91)* and inactive in most normal cells, thereby providing a unique approach to target cancer cells. Promising results have been reported using the hTERT promoter to deliver tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) *(92)* and Bax *(93)*, inducers of apoptosis, to prostate cancer cells. Recently, the hTERT promoter was used to control adenoviral genes *E1a* and *E1b* to control the replication of an oncolytic Ad in a tumor-specific manner. This virus replicated efficiently in and killed a broad spectrum of cancer cells without harming normal human cells lacking telomerase activity *(94)*. Clinical use of this promoter may be limited, however, to local intralesional gene therapy, because systemic delivery of an hTERT virus could have toxic effects on normal proliferating cells and stem cells in which telomerase is active.

4. PROSTATE CANCER GENE THERAPY APPROACH

The ideal therapy for hormone-refractory prostate cancer is one that kills cancer cells, but spares normal cells. This therapy would have limited toxicities, increase the survival, and enhance the qual-

ity of life of men afflicted with prostate cancer. Because of the success of preclinical and clinical studies, gene therapy promises to overcome these challenges. Although locally advanced prostate cancer can cause much suffering, mortality is usually secondary to the inability to inhibit metastatic spread. Lack of conventional therapies for locally advanced and metastatic prostate cancer makes these patients excellent candidates. Of course, as with most therapies, the ability to treat men with low-volume disease should enhance the success of the therapy. This is a critical point for the therapeutic approaches discussed in the proceeding sections.

With further research into the genetic determinants of prostate cancer progression, a protective approach could be used to decrease the morbidity and mortality associated with conditions such as benign prostatic hyperplasia and prostatic intraepithelial neoplasia. Current studies have demonstrated a prophylactic effect against the development of prostate cancer with conventional therapies for benign prostatic hyperplasia *(95)*. As prostate-specific targeting and tumor-killing efficacy continue to evolve, the laboratory vision of a molecular prostatectomy may soon translate into the urology clinic. Furthermore, because the postfertility prostate is a nonessential organ and the side effects from chemotherapy, radiation, and surgical intervention are so great, molecular ablation has vast appeal.

During the past decade, four categories of gene therapy approaches for prostate cancer have emerged, corrective gene therapy, oncolytic viral therapy, cytotoxic gene therapy, and immunotherapy. Each molecular therapy has a strong foundation of preclinical data, allowing for the approval of several clinical studies. Currently, 72 gene transfer protocols registered with the Office of Biotechnology Activities (OBA) are targeted against prostate cancer. This accounts for 11% of all cancer gene therapy protocols listed to date *(96)*. Table 2 summarizes the approaches as they appear in clinical trials registered with the OBA and the Recombinant DNA Advisory Committee (RAC).

4.1. Corrective Gene Therapy

This approach repairs inherited or acquired genetic defects that give the cancer a survival advantage, such as those affecting tumor suppressors or growth-promoting oncogenes. These mutations affect the regulation of the cell growth cycle and are among the multiple mutations that occur in the pathogenesis and progression of prostate cancer. Correction of a single genetic insult may not be sufficient to change the cell phenotype. Nevertheless, in vivo correction of single gene defects has shown success in several preclinical and clinical studies.

4.1.1. p53

The tumor suppressor, p53, is referred to as the molecular gatekeeper, protecting the integrity of the genome *(97)*. When cellular DNA damage occurs, wild-type p53 is activated and stimulates the expression of growth arrest and DNA damage (*GADD*)-45 and the CDK inhibitor, *p21*. p21 inhibits the CDK–cyclin D complex required to phosphorylate Rb, thereby halting the cell at the G_1/S checkpoint to allow for DNA repair. If GADD45-mediated DNA repair is unsuccessful, p53 activates bax, which mediates apoptosis *(98)*. p53 mutations occur in approximately one-third of early prostate cancers *(99)*, and this increases in patients with advanced and metastatic disease *(100)*. Replacement of wild-type *p53* with recombinant adenoviral vectors (Ad-p53) resulted in growth inhibition and induction of apoptosis in prostate cancer both in vitro *(101,102)* and in vivo *(102,103)*. In addition, intratumoral administration of Ad-p53 has been shown to slow the progression of prostate cancer to metastatic disease *(104)*. Hernandez et al. described a natural variant of p53, p53(R172L), in which an arginine-to-leucine mutation at codon 172 confers stronger protection against malignancy. When introduced into a transgenic adenocarcinoma mouse prostate model, higher levels of bax were detected in addition to a lower incidence and reduced rate of prostate cancer growth *(105)*. Perhaps the most powerful use of *p53* replacement is in combination with conventional therapies. Ad-p53 has been shown to sensitize prostate cancer cells in vitro and in vivo to DNA-damaging drugs, such as cisplatin, doxorubicin, 5-fluorouracil, methotrexate, and etoposide *(106)*. Similarly, correction of

Table 2
Current Approaches for Prostate Cancer Gene Therapy Clinical Trials[a]

Strategy	Vector	Transgene
Corrective	Adenovirus	p16
		p53
	Retrovirus	c-myc antisense
Cytoreductive		
(Suicide)	Adenovirus	CD/HSV-TK
		HSV-TK
		NIS
		TRAIL
(Oncolytic)	Adenovirus	OC promoter
		PSA promoter
Immunotherapy	AAV	GM-CSF
	Adenovirus	IL-12
		Inf-β
		MUC-1/CD40L
		p501
		PSA
	Liposome	hTERT/Ig heavy chain
		IL-2
		Prostatic acid phosphatase
		PSA
		PSMA
	Retrovirus	α(1,3) galactosyltransferase
		GM-CSF
	RNA	hTERT
		PSA
		Tumor RNA
	Vaccinia	MUC-1/IL-2
		PSA
		PSA/B7.1
		PSA/B7.1/ICAM-1/LFA-3
		PSA/B7.1/ICAM-1/LFA-3/GM-CSF

[a]This list includes all clinical trials listed on the Office of Biologic Activities (OBA) protocol list as of December 06, 2004 *(96)*. CD, cytosine deaminase; HSV, herpes simplex virus; TK, thymidine kinase; NIS, sodium iodide symporter; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; OC, osteocalcin; PSA, prostate-specific antigen; AAV, adeno-associated virus; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; Inf, interferon; MUC, mucin; hTERT, human telomerase reverse transcriptase; PSMA, prostate-specific membrane antigen; ICAM, intracellular adhesion molecule; LFA, leukocyte function-associated; Ig, immunoglobulin.

p53 mutations with adenoviral vectors sensitized radio-resistant prostate cancer cells to radiation in vitro *(107,108)*. Cowen et al. described an additive effect when orthotopic LNCaP mouse xenografts were treated with both Ad-p53 and radiation therapy *(109)*. Phase I/II clinical trials are ongoing to determine the safety of such therapies *(110,111)*.

4.1.2. Murine Double Minute Clone 2

A key regulator of p53 is the oncoprotein murine double minute (mdm) clone 2 (mdm2). It binds to the native tetrameric form of p53 to inactivate its transcriptional function *(112)* or to promote the ubiquitination *(113)* and degradation *(114)* of p53. Mdm2 primarily binds p53 in the N-terminal transactivation domain, overlapping the p300- and TAF-binding sites *(115)*. Overexpression of *mdm2* in prostate cancer is associated with tumors that are more aggressive, increased cell proliferation, and larger tumor volumes *(116)*. Zhang et al. developed second-generation antisense oligonucleotides against *mdm2* to target the degradation of *mdm2* mRNA. This led to decreased proliferation, increased apoptosis, and sensitization to paclitaxel in LNCaP, PC-3, and DU-145 cell lines in addition to chemosensitization and growth suppression in LNCAP and PC-3 xenografts *(117)*. As expected, antisense *mdm2* sensitizes prostate cancer cell lines and xenografts to radiation therapy *(118,119)*. Interestingly, the delivery of antisense *mdm2* to LNCaP cells restored the apoptotic response to androgen deprivation *(120)*. The addition of antisense *mdm2* to existing *p53* corrective gene therapy may enhance the antitumor effect and further sensitize prostate cancer to conventional therapies.

4.1.3. Phosphatase and Tensin Homolog

PTEN is a tumor suppressor with plasma membrane lipid phosphatase activity. Its primary function is to remove the 3' phosphate on PIP3 in the phosphatidylinositol signaling cascade. After growth factor stimulation, phosphoinositide 3-kinase (PI3-K) phosphorylates PIP2, generating PIP3, which transmits growth and survival signals. By regenerating PIP2, PTEN reverses the PI3-K signal *(121)*. Loss of PTEN occurs in 20% of prostate tumors and is associated with higher Gleason score and advanced-stage cancer *(122)*. PTEN loss also leads to altered cell cycle regulation *(123)*, increased cellular invasion *(124)*, enhanced cell survival *(125)*, and stimulation of angiogenesis *(126)*. Davies et al. demonstrated the inhibition of cell cycle progression in PC-3 cells infected with Ad-PTEN. Intratumoral injection of Ad-PTEN into orthotopic PC-3 tumors did not result in tumor growth inhibition; however, it suppressed metastasis formation *(127)*. In *Bcl-2*-overexpressing PC-3 and LNCaP prostate cancer cell lines, Ad-PTEN sensitized cells to radiation therapy and induced a G_2/M cell cycle arrest *(128)*. Similarly, Ad-PTEN sensitized PC-3 and DU-145 cells to doxorubicin treatment *(129)*.

4.1.4. p16

Similar to p53, tumor suppressor p16 is a negative regulator of the cell growth cycle that prevents the phosphorylation of Rb by sequestering CDK4. Underphosphorylated Rb arrests the cell at G_1, and loss of normal p16 function is common in prostate cancer *(98)*. Small homozygous deletions have been identified as the major mechanism of inactivation of *p16*, which occurs in 40% of primary prostate cancers and 71% of advanced androgen-independent prostate cancers *(130–132)*. Replacement of *p16* with adenoviral vectors suppressed cell growth and induced senescence in several prostate cancer cell lines, including LNCaP, C4-2, DU-145, PPC-1, and PC-3 *(133–135)*. Furthermore, intratumoral injection of Ad-RSV-p16 suppressed the growth of PPC-1 tumor xenografts in experimental animal models while prolonging survival *(133)*.

4.2. Oncolytic Viral Therapy

Safety concerns of nonspecific viral replication in immune-compromised cancer patients limited the early clinical trials to the use of replication-deficient adenoviral vectors; however, previous studies have shown that the replication of Ad within a cell is sufficient to kill prostate cancer cells *(136)*. By placing adenoviral early genes under the control of prostate-specific promoters, viral replication can be limited to prostate cells. This strategy allows the viral vector to propagate from a limited number of infected cells to the whole tumor mass, overcoming the problem of inadequate in vivo infectivity. This local viral amplification limits the number of injections required.

4.2.1. ONYX-015

The first tumor-specific oncolytic Ad, named ONYX-015, was developed by Bischoff et al. to replicate specifically in *p53*-deficient cells, a mutation common to several cancers. Tumor specificity was achieved by deleting the adenoviral *E1B-55K* gene, whose gene product binds to and inactivates p53, enabling infected cells to enter S-phase and promote viral replication. Theoretically, normal cells would not be permissive to ONYX-015 replication. Originally, this virus was reported to demonstrate mutant p53-dependent replication *(137)*, effective tumor regression after intratumoral or systemic injection in several nude mouse xenograft models including colon, cervical, and laryngeal carcinomas *(138,139)*, and augmentation of tumor killing with coadministration of chemotherapeutic agents, such as cisplatin and 5-fluorouracil *(138)*. To date, ONYX-015 has been tested in 16 phase I and II clinical trials for multiple malignancies, including head and neck carcinoma, pancreatic cancer, ovarian cancer, colorectal cancer, hepatobiliary cancer, gastric cancer, and gliomas. In these trials, no dose-limiting toxicities were observed; however, only modest-to-no objective responses were achieved *(140–146)*. Although the virus has been shown clinically not to destroy normal tissues *(147)*, several groups have reported that ONYX-015 replication is independent of *p53* status *(148–150)*. Recently, O'Shea et al. reported that the tumor-specific replication of ONYX-015 is caused by differential late viral mRNA export in malignant cells rather than the cell's *p53* status *(151)*. Despite the conflicting reports, ONYX-015 seems to favor replication in malignant cells over normal cells. The clinical usefulness of ONYX-015 for prostate cancer has not been tested, but prostate cancer gene therapy vectors with similar *E1B-55K* deletions have been developed *(152)*.

4.2.2. Calydon Virus 706 and 787

The first prostate-specific replication-competent Ad (PSRCA) was developed by Rodriguez et al. Calydon virus (CV706) was engineered by placing the adenoviral *E1A* gene under the control of the minimal PSA promoter and enhancer sequences. In vitro, *E1A* expression was limited to PSA-positive LNCaP cells. Furthermore, in vivo analysis demonstrated the powerful antitumor efficacy of the virus against LNCaP mouse xenografts *(153)*. When combined with radiation therapy, a significant synergistic effect was demonstrated both in vitro and in vivo in LNCaP xenograft models *(154)*. CV706 was the first PSRCA tested in a human gene therapy clinical trial. The virus was found to be safe, as the maximum tolerable dose was not reached, and a drop in serum PSA greater than 50% was demonstrated in patients treated with the highest doses of CV706 *(154)*. Yu et al. developed CV787, a PSRCA with higher killing efficiency than CV706. This virus retains the entire adenoviral *E3* region, which enhances the virus' oncolytic effect. In addition, two promoters drive the expression adenoviral early genes. *E1A* is under the control of the prostate-specific rat probasin promoter, and *E1B* is controlled by the human PSA promoter/enhancer. This virus replicates as efficiently as wild-type Ad and eliminated LNCaP xenograft tumors via tail vein injection *(155)*. Further in vivo analysis revealed a synergistic enhancement of CV787 with both radiation therapy *(156)* and chemotherapeutic agents such as paclitaxel or docetaxel *(157)*. Results from ongoing CV787 clinical studies are pending. Although CV706 and CV787 promise success in combination with conventional therapies, the androgen-dependency of the promoter systems used may limit their success.

4.2.3. Ad-OC-E1A and Ad-hOC-E1

Matsubara et al. developed a PSRCA using the mouse OC promoter to restrict the expression of *E1A* to prostate epithelia and its supporting bone stroma in osseous metastases of prostate cancer. This virus, named Ad-OC-E1A, seems to be more effective than a *PSA*-controlled virus at killing a broader spectrum of prostate cancer cells, including LNCaP, C4-2, and ARCaP (PSA-positive), as well as PC-3 and DU-145 (PSA-negative). Intratumoral injection of Ad-OC-E1A was effective at obliterating subcutaneous androgen-independent PC-3 athymic mouse xenograft models. In addition, intraosseous C4-2 prostate cancer xenografts responded very well to the systemic administration of

Ad-OC-E1A. One hundred percent of the treated mice responded with a drop in serum PSA below detectable levels. At the conclusion of the study, 40% of the treated mice were cured of prostate cancer, as no PSA rebound or prostate cancer cells in the skeleton were detected *(158)*. To improve on this virus, Hsieh et al. developed a second PSRCA, Ad-hOC-E1, containing a single bidirectional human *OC* promoter to control the expression of both *E1A* and *E1B (159)*. Previous studies have shown that controlling the expression of the early gene *E1B* in addition to *E1A* results in better viral replication control *(160)*. Under the control of this vitamin D-responsive element-containing promoter, Ad-hOC-E1 replication is induced 10-fold higher than wild-type viral replication, and cytotoxicity is enhanced by the administration of vitamin D *(159)*. Although still controversial *(161)*, some preclinical studies indicate that vitamin D has an antiproliferative effect on androgen-independent prostate cancer *(162,163)*. In preclinical studies, administration of vitamin D_3 in nude mice with subcutaneous DU-145 xenografts demonstrated a therapeutic effect; however, the systemic administration of Ad-hOC-E1 in combination with vitamin D showed marked repression of the tumors, indicating the potential for clinical use *(159)*. The previously outlined preclinical findings have translated into a phase I clinical trial of OC-driven oncolytic adenoviral intratumoral therapy for androgen-independent prostate cancer.

4.2.4. Ad-E4-PSES-E1A

In previous gene therapy approaches, the control of viral replication has been achieved by tightly controlling both *E1A* and *E1B* adenoviral genes under prostate-specific promoters. Recent studies have also demonstrated that tight control of viral replication can be achieved by placing *E4* in addition to *E1A* under the control of two separate or duplicate tissue-specific promoters *(164,165)*. Because of the difficulty of finding two active and tightly regulated promoters for the prostate and because promoter duplication might induce recombination, Li et al. developed a novel strategy to control *E1A* and *E4* genes under a single promoter. This is also the first report of the use of PSES to drive the replication of a PSRCA. The virus, Ad-E4-PSES-E1A, also contains the gene encoding green fluorescent protein (GFP) driven by CMV promoter for the purpose of in vivo viral tracking. In vitro, the replication and cell-killing abilities of this virus were similar to that of wild-type Ad in PSA/PSMA-positive cells. As expected, replication of Ad-E4-PSES-E1A was severely impaired in PSA/PSMA-negative cells. Ad-E4-PSES-E1A effectively suppressed the growth of androgen-independent CWR22rv mouse xenograft tumors *(76)*. The oncolytic properties of this virus could be augmented by the replacement of CMV-*GFP* with a therapeutic gene and prostate-specific promoter.

4.2.5. Control of the Host Immune Response

It will become critical to temporarily suppress the host's immune system or enhance the killing activity of the virus so that it can eliminate tumors within a shorter period of time, allowing it to escape attack from the host immune system. Early clinical trials administered immunosuppressants such as corticosteroids at the time of injection; however, shutdown of the entire immune system could be problematic during infection with replication-competent Ads. For this reason, genes encoding immunosuppressive molecules could be delivered via a PSRCA directly to the local tumor environment. Among the immune regulators, transforming growth factor (TGF)-β and Fas-ligand (Fas-L) are likely the best candidates for incorporation into a PSRCA. There are five members reported in the TGF-β family; three of them (TGF-β1, TGF-β2, and TGF-β3) are expressed in mammals. These three isoforms share a high degree of sequence homology in the mature domain, and have similar actions on cells in tissue culture. Suppression of the immune response includes the inhibition of T- and B-cell proliferation, the downregulation of natural killer cell activity and CTL response, and the regulation of macrophage activation *(166)*. TGF-β is a mediator of immune suppression that allows tumors to escape from immune surveillance, and its use has been explored to suppress the inflammatory and alloreactive immune responses in liver transplantation *(167)*. Besides TGF-β, Fas-L is another immune modulator that has been explored for use in kidney transplant patients to suppress alloreactive lymphocytes *(168)*. Fas-L, also known as CD95 or APO-1, is a membrane-

bound protein of the TNF family, and it is expressed in several cell types, including tumors, T cells, and B cells. Cells expressing the Fas receptor undergo apoptosis when they encounter Fas-L *(169)*. Besides their immune modulatory function, TGF-β and Fas-L are also strong growth inhibitors and induce apoptosis in a variety of cancers, including prostate cancer *(170)*. Therefore, incorporating TGF-β or Fas-L into a PSRCA could potentially enhance the tumoricidal activity of the vector and blanket the tumor site from the immune system.

4.2.6. Overcoming Current Limitations

Through the use of intravital imaging, Li et al. mapped the replication and spread of a PSRCA. Three days after Ad-E4-PSES-E1A injection, a burst in GFP expression was observed, indicating rapid replication and viral spreading, which decreased 1 week after injection. At 2 weeks, tumor growth and cell killing reached equilibrium. After this, tumor growth exceeded the killing rate. A subsequent rebound in GFP expression was observed, indicating renewed viral amplification. Although immunohistochemistry analysis revealed persistent viral infection, the xenograft tumors did not completely respond to the viral therapy *(76)*. Other studies have also described limited viral spread and lack of tumor response despite the high oncolytic activity of a virus *(171)*. One explanation for this is that conditions within the established tumor become unfavorable to support viral replication. As tumor lysis occurs, the tumor environment becomes highly necrotic and hypoxic, unlike the well-oxygenated tissues Ad normally infects. The fact that virus particles accumulate around blood vessels within a tumor provides further supporting evidence *(172)*. Shen et al. found that levels of E1A protein but not mRNA are reduced in Ad-infected cells under hypoxic conditions *(173)*. The significance of this finding is great because transcription of adenoviral genes, and, therefore, adenoviral replication, is dependent on E1A. Further work in this area is needed to improve the stability of E1A in hypoxic conditions. Perhaps the E1A from another Ad serotype is more stable under hypoxia and can be used to replace Ad5 E1A.

A second hypothesis to explain the poor tumor response to oncolytic therapy also involves the hypoxic environment created within the tumor. Such a condition would induce angiogenesis, and the resulting neovasculature would then support new tumor growth. Eventually, this growth can overcome the viral replication. If this is a significant factor in lowering the efficacy of oncolytic viral therapy, then the use of an antiangiogenic factor as adjuvant therapy should enhance the tumor response to oncolytic therapy. Recently, Jin et al. demonstrated the combined effectiveness of Ad-hOC-E1 and Ad-Flk1-Fc, a vector delivering the secreted form of the antiangiogenic factor, Flk-1, also known as vascular endothelium growth factor (VEGF) receptor 2. Alone, Ad-hOC-E1 and Ad-Flk1-Fc reduced C4-2 mouse xenograft models by 60% or 40%, respectively. When co-infected with both viruses, average tumor size reduction was 90% in 8 weeks, and 30% of the tumors completely regressed by week 3 *(174)*. Liu et al. combined antiangiogenesis with an *E1B-55K*-deleted oncolytic virus expressing the apoptosis inducer, TRAIL. In this study, Ad-k5, a replication-deficient Ad delivering the fifth kringle domain of plasminogen, which inhibits angiogenesis by regulating the intrinsic angiogenic factor pathway, was co-injected with ZD55-hTRAIL into colorectal carcinoma mouse xenografts. This combined therapy resulted in the complete eradication of all treated tumors *(175)*. Because the coadministration of multiple adenoviral vectors can result in reduced gene transduction efficiency, PSRCAs should be engineered to deliver the antiangionic factor directly. Another alternative is to administer soluble antiangiogenic factors intravenously, such as Avastin, a humanized monoclonal antibody against VEGF approved for the use in metastatic colorectal cancer patients *(176)*.

4.3. Cytotoxic Gene Therapy

This approach to the molecular therapy of prostate cancer results in the killing of cancer cells by delivery and expression of prodrug enzyme genes, such as *HSV-TK* and apoptosis-inducing genes, such as *TRAIL*. To ensure the safety of such a therapy, the use of prostate-specific promoters is crucial to protect nontarget tissues from the expression of such deadly gene products. Furthermore,

the use of PSRCA vectors to delivery the cytotoxic genes overcomes the need for multiple doses of the virus because of limited viral transduction and poor transgene expression.

4.3.1. HSV-TK

In this approach, prostate cancer is virally transduced with HSV-TK followed by systemic administration of any one of several antiherpetic agents, such as ganciclovir (GCV), acyclovir, or VAL. These nucleoside analogs are phosphorylated specifically by HSV-TK, allowing incorporation of the activated analogs into cellular DNA during DNA replication, resulting in chain termination and, ultimately, cell death. The safety of this therapy resides in the fact that only the cells receiving HSV-TK are capable of converting the nontoxic prodrug into an active metabolite. Fortunately, however, the toxic effect is not limited to the cells in which the prodrug is activated. Through a bystander effect mediated by intercellular gap junctions and phagocytosis of debris from dying cells, the activated drug is delivered to neighboring cells *(177)*, resulting in an amplification of the cytotoxic effect throughout the entire tumor site.

Previously, Eastham et al. demonstrated the sensitivity of human prostate cancer cells PC-3 and DU-145 to GCV cytotoxicity after the in vitro transduction of the cells with HSV-TK using a recombinant replication-deficient adenoviral vector *(178)*. Similar results were obtained in vivo in murine subcutaneous xenograft prostate cancer models after the intralesional injection of Ad-RSV-TK and Ad-CMV-TK *(179,180)*. Intratumoral injection of the vector is required if universal promoters, such as RSV or CMV, are used to drive the expression of HSV-TK. Herman et al. developed the initial HSV-TK clinical trial in which a replication-deficient Ad carrying HSV-TK driven by RSV promoter was injected intralesionally, followed by administration of GCV in men with locally recurrent prostate cancer 1 or more years after definitive external beam radiotherapy. This trial demonstrated the tumoricidal activity of this combination therapy, as evidenced by sustained decreases in serum PSA. Unfortunately, several of the patients experienced self-limiting toxicities and one patient experienced moderate but reversible hepatic dysfunction and thrombocytopenia *(50)*. To circumvent such toxicities, Gotoh et al. developed a replication-deficient adenoviral vector that controlled HSV-TK expression by the PSA promoter *(57)*.

The first transcriptionally targeted *HSV-TK* gene therapy to be translated into a clinical protocol was developed by Koeneman, et al. to test the hypothesis that the OC promoter could regulate HSV-TK expression specifically within prostate cancer cells and the supportive stroma of a metastasis *(86)*. Kubo et al. performed the phase I clinical trial in which two postsurgical local recurrences and nine metastatic lesions (five osseous and four lymph nodes) were injected with replication-defective Ad-OC-TK vector followed by the administration of oral VAL. All patients tolerated this therapy with no severe adverse effects. Of the 11 men, local cancer cell death was observed in 7 patients; however, the treated lesions of all 11 men showed histological changes as a result of the treatment. One patient demonstrated regression and stabilization of the treated lesion for up to 317 days post-treatment without additional treatments *(87)*. Clinical results with this virus are encouraging; however, HSV-TK as adjuvant to radiation therapy may prove to be more successful.

To improve the efficacy of HSV-TK gene therapy, Freytag et al. developed a novel three-legged approach to gene therapy for prostate cancer in which HSV-TK was fused with cytosine deaminase (*CD*) complementary DNA (cDNA) and delivered within an oncolytic virus, followed by treatment with external beam radiation therapy *(152)*. In this approach, CD, an enzyme found only in bacteria and certain yeast, converts the prodrug 5-fluorocytosine to a highly toxic nucleoside analog, 5-fluorouracil, conferring an additive effect to HSV-TK gene therapy. This virus, Ad5-CD/TK*rep,* is based on the oncolytic ONYX-015 viral backbone. In the initial phase I study, this virus showed moderate efficacy as demonstrated by decreases in serum PSA values for several patients and complete tumor destruction in 2 of 16 patients 1 year after treatment *(181)*. In murine orthotopic C4-2 tumors, Ad5-CD/TK*rep* was shown to be an effective adjuvant to radiation therapy *(182)*. This efficacy was tested in a phase I study that combined Ad5-CD/TK*rep* prodrug therapy with radiation therapy. All patients

experienced a decrease in serum PSA, with a mean PSA half-life of 0.6 months, vs 2 months for patients receiving Ad5-CD/TK*rep* without radiation therapy or 2.4 months for patients receiving radiation therapy alone. Furthermore, up to 9 months after treatment, 5 out of 10 patients maintained PSA levels of at most 0.5 ng/mL *(183)*.

4.3.2. TNF-Related Apoptosis-Inducing Ligand

TRAIL, also known as Apo-2 ligand, is a member of the TNF family and has been shown to preferentially kill tumor cells over normal cells. Originally discovered because of its similarity to Fas-L, TRAIL is a 32-kDa type II transmembrane protein, whose C-terminal extracellular domain (amino acids 114–281) is homologous with other members of the TNF family *(184,185)*. TRAIL induces apoptosis by binding to the death domain-containing receptors, DR4 and DR5; however, the death signal is not transduced via the adaptor molecule Fas-associated death domain (FADD). Instead, the death protease FADD-like IL-1B-converting enzyme (FLICE2) is thought to be engaged, which cleaves the initiating caspase 8 to begin the caspase cascade *(186)*.

The selectivity of TRAIL for cancer cells over normal cells makes it a prime candidate for anticancer therapy. TRAIL expression has been detected in several normal human tissues, which suggests that TRAIL is not toxic to those cells in vivo *(187)*. In essence, these cells are protected from the apoptotic effects of TRAIL by the antagonistic decoy receptors, DcR1 and DcR2, which lack functional intracellular domains and are found on the surface membrane of TRAIL-resistant cells *(186)*. Many prostate cancer cell lines, including ALVA-31, DU-145, and PC-3, are extremely sensitive to TRAIL and undergo apoptosis when exposed; however, other cell lines, such as LNCaP, are highly resistant *(188)*. This resistance has been shown to be reversed by infection of those cells with Ad *(189)*, treatment of the cells with radiation therapy *(190)*, or simultaneous administration of chemotherapeutic agents, such as paclitaxel, vincristine, vinblastine, etoposide, doxorubicin, or camptothecin *(191)*. For these reasons, TRAIL is a promising cytotoxic gene product for the molecular therapy of prostate cancer; however, Jo et al. recently demonstrated sensitivity of TRAIL in cultured human hepatocytes to TRAIL *(192)*. Recombinant forms of TRAIL with reduced hepatotoxicity are being investigated *(193)* in addition to monoclonal antibodies that antagonize the TRAIL receptor in hepatocytes *(194)*. Furthermore, Hao et al. demonstrated antitumor effect with systemic delivery of soluble TRAIL without toxicity to human hepatocytes in human xenograft mouse livers *(195)*. The controversial hepatotoxicity has been proposed to be an artifact of the recombinant protein preparation. Kaliberov et al. developed a replication-deficient Ad, called AdFlt-TRAIL, targeted to prostate cancer cells and the surrounding endothelium. Membrane-bound TRAIL expression was controlled by the Flt-1 promoter, also known as VEGF receptor-1, and killed both prostate cancer and endothelial cells. AdFlt-TRAIL effectively suppressed the growth of DU-145 xenografts when intralesional injection was combined with radiation therapy *(196)*. Recently, Vanoosten et al. developed a novel strategy to sensitize prostate cancer cell lines to the cytotoxic effect of TRAIL after infection with replication-deficient Ad5-TRAIL. This was achieved by treating ALVA-31 and DU-145 cells with depsipeptide, a histone deacetylase inhibitor that was shown to increase CAR expression, enhance transgene expression, and increase DR4 and DR5 levels in membrane lipid rafts *(197)*.

4.4. Immunotherapy

Prostate cancer, like most cancers, has developed mechanisms to evade the host immune system. Such mechanisms include the downregulation of class I MHC molecules on the tumor cell surface *(198)* as well as the downregulation of the co-stimulatory B7 molecules *(199)*. These means of evasion result in decreased presentation of tumor antigens to CTLs. The goal of immunotherapy is to enhance the host immune response to prostate cancer cells. Current approaches involve *ex vivo* gene therapy of autologous or allogeneic tumor cells and subsequent vaccination with the irradiated cells now expressing cytokines such as interleukin (IL)-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), *ex vivo* gene transfer of PSA cDNAs, such as PSA, PSMA, and prostatic acid

phosphatase, into autologous dendritic cells (DCs), and in vivo intratumoral gene transfer of cytokine genes.

4.4.1. Prostate Cancer Vaccines

The host immune system is capable of recognizing and eliminating malignant cells; however, the ability of tumor cells to evade immune surveillance and the inefficiency of the body's antitumor response allows prostate cancer to persist and progress. To develop a tumor vaccine, prostate cancer cells are harvested from the patient during radical prostatectomy, transfected with cytokines that upregulate cell surface antigens, such as B7 or MHC class I and II, expanded in culture, and irradiated to kill the cells. After infusion of the autologous vaccine cells, the enhanced immunogenicity of the cells aids the immune system to mount a local or systemic immune response to the cancer *(200,201)*. The first prostate vaccine clinical trial was developed by Simons et al. In this study, eight men with metastatic prostate cancer were administered autologous, GM-CSF-transduced, irradiated cancer vaccines. Side effects were limited to pruritis, erythema, and swelling at the site of vaccination. Both B- and T-cell responses were observed in seven of the eight patients, whereas T-cell responses were observed in only two of the eight patients before vaccination *(202)*. A subsequent clinical trial demonstrated no therapeutic value of systemic administration of recombinant GM-CSF, thereby suggesting the importance of local secretion of GM-CSF by the cancer vaccines *(203)*. Clearly, a limitation of this approach is the harvesting and *ex vivo* manipulation of prostate cancer cells. To overcome this, Simons et al. developed an allogeneic tumor vaccine, GVAX, from GM-CSF-transduced irradiated PC-3 and LNCaP prostate cancer cell lines. In a phase II clinical trial, the vaccines were well-tolerated, and no dose-limiting toxicities were observed. Two years after treatment, the survival rate of patients receiving low booster doses was 41%, compared with 70% in patients who received higher booster doses *(204)*. A phase I/II clinical trial evaluated the safety of GVAX reengineered to secrete higher levels of GM-CSF *(205)*. Phase III clinical studies of this cancer vaccine are underway.

4.4.2. DC Immunotherapy

A second approach to prostate cancer vaccination involves the use of DCs, the most potent antigen-presenting cell of the immune system. DCs produce a strong systemic T-cell response by presenting tumor antigens on both MHC class I and II molecules *(206)*. In this vaccination strategy, autologous DCs are collected from the patient by leukophoresis, expanded in culture, challenged with a prostate-specific antigen, such as PSA, prostatic acid phosphatase, or PSMA, by either peptide pulsing or transfection with antigen cDNA, and reintroduced into the patient. Murphy et al. demonstrated the safety of HLA-A2-specific PSMA peptide-pulsed DC immunotherapy in a phase I clinical trial in which an enhanced cellular immune response was observed in all HLA-A2-positive patients; however, only 7 out of 51 patients experienced a decrease in serum PSA of at least 50% *(207,208)*. In a phase II clinical trial, GM-CSF was administered as a systemic adjuvant in patients receiving PSMA-pulsed autologous DC vaccines. Nineteen out of 62 patients were identified as partial or complete responders. Of the responders, 58% seemed to have durable responses *(203,209)*.

4.4.3. In Vivo Immunotherapy

A third approach to immunotherapy for prostate cancer is the delivery of cytokines to the local tumor environment. IL-2 is a potent activator of $CD4^+$ and $CD8^+$ T cells as well as natural killer cells, and its effect on prostate cancer is caused by its ability to expand T-cell populations with antitumor activity. Tumors that respond to IL-2 therapy demonstrate an infiltration of lymphocytes. In mouse PC-3 orthotopic tumor models, tumor growth was suppressed by 94% after systemic injection of IL-2 *(210)*. Belldegrun et al. developed a gene therapy protocol in which 24 men with locally advanced prostate cancer were injected intraprostatically with a DNA-liposome complex encoding the IL-2 gene. This therapy was well-tolerated. Immunohistochemical analysis of the tumor site demonstrated T-cell infiltration, and serum PSA responses were observed in 16 of the men on day 1 and

14 of the 24 men on day 8 *(211)*. Trudel et al. developed a phase I clinical trial to evaluate the safety of intraprostatic injection of adenoviral vectors encoding the IL-2 gene. Twelve men were injected with AdIL-2 4 weeks before prostatectomy. No dose-limiting toxicities were observed. On pathological evaluation, all patients experienced a local inflammatory response with an infiltrate of $CD3^+/CD8^+$ cells. At low viral doses, five out of five patients experienced a mean decrease in PSA of 33%, whereas patients receiving the highest viral doses experienced transient elevations in PSA levels before returning to baseline *(212)*.

6. CONCLUSION

The safety and efficacy of gene therapy for prostate cancer has been demonstrated in several preclinical and clinical trials. In recent years, interest in this field has expanded and will continue to expand. Results from clinical trials indicate that gene therapy alone may not cure prostate cancer; however, in combination with conventional therapies, gene therapy promises to fill the therapeutic void left by chemotherapy and androgen ablation therapy. It is conceivable that such adjuvant therapies will result in fewer undesired side effects and an enhancement of the quality of life of the patient. Because of its convenient administration through ultrasound-guided transrectal injection, it is also conceivable that gene therapy, applied in an outpatient clinic, may someday augment radical prostatectomy and radiation therapy to treat early stage disease. More exciting, however, is the potential for systemic delivery of a PSRCA to eliminate micrometastases before they become clinically detectable. Factors impeding the widespread use of gene therapy for prostate cancer include slow clinical translation of laboratory research, funding of expensive clinical trials, and misconceptions and fear of gene therapy from the general public. As the number of successful gene therapy trials increase, these factors will diminish.

REFERENCES

1. Jemal, A., Murray, T., Ward, E., et al. (2005). Cancer statistics, 2005. *CA Cancer J. Clin.* **55**, 10–30.
2. Coen, J. J., Zietman, A. L., Thakral, H., and Shipley, W. U. (2002). Radical radiation for localized prostate cancer: local persistence of disease results in a late wave of metastases. *J. Clin. Oncol.* **20**, 3199–3205.
3. Han, M., Partin, A. W., Pound, C. R., Epstein, J. I., and Walsh, P. C. (2001). Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience. *Urol. Clin. North. Am.* **28**, 555–565.
4. Chodak, G. W., Keane, T., and Klotz, L. (2002). Critical evaluation of hormonal therapy for carcinoma of the prostate. *Urology* **60**, 201–208.
5. Petrylak, D. P., Tangen, C. M., Hussain, M. H., et al. (2004). Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N. Engl. J. Med.* **351**, 1513–1520.
6. Tannock, I. F., de Wit, R., Berry, W. R., et al. (2004). Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N. Engl. J. Med.* **351**, 1502–1512.
7. Worgall, S., Wolff, G., Falck-Pedersen, E., and Crystal, R. G. (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum. Gene Ther.* **8**, 37–44.
8. Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R., and Wilson, J. (1999). Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.* **6**, 1574–1583.
9. Schiedner, G., Morral, N., Parks, R. J., et al. (1998). Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat. Genet.* **18**, 180–183.
10. Hearing, P., Samulski, R. J., Wishart, W. L., and Shenk, T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* **61**, 2555–2558.
11. Nevins, J. R. (1981). Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell* **26**, 213–220.
12. Rich, D. P., Couture, L. A., Cardoza, L. M., et al. (1993). Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis. *Hum. Gene Ther.* **4**, 461–476.
13. Bagchi, S., Raychaudhuri, P., and Nevins, J. R. (1990). Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. *Cell* **62**, 659–669.
14. Mal, A., Poon, R. Y., Howe, P. H., Toyoshima, H., Hunter, T., and Harter, M. L. (1996). Inactivation of p27Kip1 by the viral E1A oncoprotein in TGFbeta-treated cells. *Nature* **380**, 262–265.
15. Somasundaram, K. and El-Deiry, W. S. (1997). Inhibition of p53-mediated transactivation and cell cycle arrest by E1A through its p300/CBP-interacting region. *Oncogene* **14**, 1047–1057.

16. Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., and White, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA* **89**, 7742–7746.
17. Halbert, D. N., Cutt, J. R., and Shenk, T. (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J. Virol.* **56**, 250–257.
18. Pilder, S., Moore, M., Logan, J., and Shenk, T. (1986). The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell Biol.* **6**, 470–476.
19. Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H., and Dobner, T. (1997). The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. *Proc. Natl. Acad. Sci. USA* **94**, 1206–1211.
20. Field, J., Gronostajski, R. M., and Hurwitz, J. (1984). Properties of the adenovirus DNA polymerase. *J. Biol. Chem.* **259**, 9487–9495.
21. Challberg, M. D., Desiderio, S. V., and Kelly, T. J., Jr. (1980). Adenovirus DNA replication in vitro: characterization of a protein covalently linked to nascent DNA strands. *Proc. Natl. Acad. Sci. USA* **77**, 5105–5109.
22. Burgert, H. G., Maryanski, J. L., and Kvist, S. (1987). "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proc. Natl. Acad. Sci. USA* **84**, 1356–1360.
23. Elsing, A. and Burgert, H. G. (1998). The adenovirus E3/10.4K-14.5K proteins down-modulate the apoptosis receptor Fas/Apo-1 by inducing its internalization. *Proc. Natl. Acad. Sci. USA* **95**, 10,072–10,077.
24. Mathews, M. B. and Shenk, T. (1991). Adenovirus virus-associated RNA and translation control. *J. Virol.* **65**, 5657–5662.
25. Leibowitz, J. and Horwitz, M. S. (1975). Synthesis and assembly of adenovirus polypeptides. III. Reversible inhibition of hexon assembly in adenovirus type 5 temperature-sensitive mutants. *Virology* **66**, 10–24.
26. Bergelson, J. M., Cunningham, J. A., Droguett, G., et al. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320–1323.
27. Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**, 309–319.
28. Tollefson, A. E., Scaria, A., Hermiston, T. W., Ryerse, J. S., Wold, L. J., and Wold, W. S. (1996). The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J. Virol.* **70**, 2296–2306.
29. Doronin, K., Toth, K., Kuppuswamy, M., Ward, P., Tollefson, A. E., and Wold, W. S. (2000). Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *J. Virol.* **74**, 6147–6155.
30. Rauen, K. A., Sudilovsky, D., Le, J. L., et al. (2002). Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. *Cancer Res.* **62**, 3812–3818.
31. Douglas, J. T., Rogers, B. E., Rosenfeld, M. E., Michael, S. I., Feng, M., and Curiel, D. T. (1996). Targeted gene delivery by tropism-modified adenoviral vectors. *Nat. Biotechnol.* **14**, 1574–1578.
32. Wickham, T. J., Segal, D. M., Roelvink, P. W., et al. (1996). Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol.* **70**, 6831–6838.
33. Kraaij, R., van Rijswijk, A. L., Oomen, M. H., Haisma, H. J., and Bangma, C. H. (2005). Prostate specific membrane antigen (PSMA) is a tissue-specific target for adenoviral transduction of prostate cancer in vitro. *Prostate* **62**, 253–259.
34. Wickham, T. J., Roelvink, P. W., Brough, D. E., and Kovesdi, I. (1996). Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. *Nat. Biotechnol.* **14**, 1570–1573.
35. Krasnykh, V., Dmitriev, I., Mikheeva, G., Miller, C. R., Belousova, N., and Curiel, D. T. (1998). Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J. Virol.* **72**, 1844–1852.
36. Lupold, S. E. and Rodriguez, R. (2004). Disulfide-constrained peptides that bind to the extracellular portion of the prostate-specific membrane antigen. *Mol. Cancer Ther.* **3**, 597–603.
37. Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., and Lieber, A. (2000). Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. *J. Virol.* **74**, 2567–2583.
38. Gaggar, A., Shayakhmetov, D. M., and Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. *Nat. Med.* **9**, 1408–1412.
39. Sova, P., Ren, X. W., Ni, S., et al. (2004). A tumor-targeted and conditionally replicating oncolytic adenovirus vector expressing TRAIL for treatment of liver metastases. *Mol. Ther.* **9**, 496–509.
40. Kotin, R. M., Siniscalco, M., Samulski, R. J., et al. (1990). Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **87**, 2211–2215.
41. Linden, R. M., Ward, P., Giraud, C., Winocour, E., and Berns, K. I. (1996). Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **93**, 11,288–11,294.
42. Kearns, W. G., Afione, S. A., Fulmer, S. B., et al. (1996). Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. *Gene Ther.* **3**, 748–755.
43. Rolling, F. and Samulski, R. J. (1995). AAV as a viral vector for human gene therapy. Generation of recombinant virus. *Mol. Biotechnol.* **3**, 9–15.
44. Urabe, M., Ding, C., and Kotin, R. M. (2002). Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum. Gene Ther.* **13**, 1935–1943.

45. Ferrari, F. K., Samulski, T., Shenk, T., and Samulski, R. J. (1996). Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol* **70**, 3227–3234.
46. McCarty, D. M., Monahan, P. E., and Samulski, R. J. (2001). Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther.* **8**, 1248–1254.
47. Warrington, K. H., Jr., Gorbatyuk, O. S., Harrison, J. K., Opie, S. R., Zolotukhin, S., and Muzyczka, N. (2004). Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. *J. Virol.* **78**, 6595–6609.
48. Recchia, A., Perani, L., Sartori, D., Olgiati, C., and Mavilio, F. (2004). Site-specific integration of functional transgenes into the human genome by adeno/AAV hybrid vectors. *Mol. Ther.* **10**, 660–670.
49. Brand, K., Arnold, W., Bartels, T., et al. (1997). Liver-associated toxicity of the HSV-tk/GCV approach and adenoviral vectors. *Cancer Gene Ther.* **4**, 9–16.
50. Herman, J. R., Adler, H. L., Aguilar-Cordova, E., et al. (1999). In situ gene therapy for adenocarcinoma of the prostate: a phase I clinical trial. *Hum. Gene Ther.* **10**, 1239–1249.
51. Stamey, T. A., Yang, N., Hay, A. R., McNeal, J. E., Freiha, F. S., and Redwine, E. (1987). Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N. Engl. J. Med.* **317**, 909–916.
52. Riegman, P. H., Vlietstra, R. J., van der Korput, J. A., Romijn, J. C., and Trapman, J. (1989). Characterization of the prostate-specific antigen gene: a novel human kallikrein-like gene. *Biochem. Biophys. Res. Commun.* **159**, 95–102.
53. Cleutjens, K. B., van der Korput, H. A., van Eekelen, C. C., van Rooij, H. C., Faber, P. W., and Trapman, J. (1997). An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol. Endocrinol.* **11**, 148–161.
54. Pang, S., Dannull, J., Kaboo, R., et al. (1997). Identification of a positive regulatory element responsible for tissue-specific expression of prostate-specific antigen. *Cancer Res.* **57**, 495–499.
55. Schuur, E. R., Henderson, G. A., Kmetec, L. A., Miller, J. D., Lamparski, H. G., and Henderson, D. R. (1996). Prostate-specific antigen expression is regulated by an upstream enhancer. *J. Biol. Chem.* **271**, 7043–7051.
56. Riegman, P. H., Vlietstra, R. J., van der Korput, J. A., Brinkmann, A. O., and Trapman, J. (1991). The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Mol. Endocrinol.* **5**, 1921–1930.
57. Gotoh, A., Ko, S. C., Shirakawa, T., et al. (1998). Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J. Urol.* **160**, 220–229.
58. Lu, Y., Carraher, J., Zhang, Y., et al. (1999). Delivery of adenoviral vectors to the prostate for gene therapy. *Cancer Gene Ther.* **6**, 64–72.
59. Yeung, F., Li, X., Ellett, J., Trapman, J., Kao, C., and Chung, L. W. (2000). Regions of prostate-specific antigen (PSA) promoter confer androgen-independent expression of PSA in prostate cancer cells. *J. Biol. Chem.* **275**, 40,846–40,855.
60. Wu, L., Matherly, J., Smallwood, A., et al. (2001). Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors. *Gene Ther.* **8**, 1416–1426.
61. Latham, J. P., Searle, P. F., Mautner, V., and James, N. D. (2000). Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. *Cancer Res.* **60**, 334–341.
62. Horoszewicz, J. S., Kawinski, E., and Murphy, G. P. (1987). Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res.* **7**, 927–935.
63. Pinto, J. T., Suffoletto, B. P., Berzin, T. M., et al. (1996). Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.* **2**, 1445–1451.
64. Carter, R. E., Feldman, A. R., and Coyle, J. T. (1996). Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc. Natl. Acad. Sci. USA* **93**, 749–753.
65. Pangalos, M. N., Neefs, J. M., Somers, M., et al. (1999). Isolation and expression of novel human glutamate carboxypeptidases with N-acetylated alpha-linked acidic dipeptidase and dipeptidyl peptidase IV activity. *J. Biol. Chem.* **274**, 8470–8483.
66. Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D., and Cordon-Cardo, C. (1997). Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res.* **3**, 81–85.
67. Xiao, Z., Adam, B. L., Cazares, L. H., et al. (2001). Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. *Cancer Res.* **61**, 6029–6033.
68. Sweat, S. D., Pacelli, A., Murphy, G. P., and Bostwick, D. G. (1998). Prostate-specific membrane antigen expression is greatest in prostate adenocarcinoma and lymph node metastases. *Urology* **52**, 637–640.
69. Wright, G. L., Jr., Grob, B. M., Haley, C., et al. (1996). Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology* **48**, 326–334.
70. O'Keefe, D. S., Su, S. L., Bacich, D. J., et al. (1998). Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim. Biophys. Acta* **1443**, 113–127.
71. Watt, F., Martorana, A., Brookes, D. E., et al. (2001). A tissue-specific enhancer of the prostate-specific membrane antigen gene, FOLH1. *Genomics* **73**, 243–254.
72. Lee, S. J., Lee, K., Yang, X., et al. (2003). NFATc1 with AP-3 site binding specificity mediates gene expression of prostate-specific-membrane-antigen. *J. Mol. Biol.* **330**, 749–760.

73. Uchida, A., O'Keefe, D. S., Bacich, D. J., Molloy, P. L., and Heston, W. D. (2001). In vivo suicide gene therapy model using a newly discovered prostate-specific membrane antigen promoter/enhancer: a potential alternative approach to androgen deprivation therapy. *Urology* **58**, 132–139.
74. Lee, S. J., Zhang, Y., Lee, S. D., et al. (2004). Targeting prostate cancer with conditionally replicative adenovirus using PSMA enhancer. *Mol. Ther.* **10**, 1051–1058.
75. Lee, S. J., Kim, H. S., Yu, R., et al. (2002). Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Mol. Ther.* **6**, 415–421.
76. Li, X., Zhang, Y. P., Kim, H. S., et al. (2005). Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer. *Cancer Res.* **65**, 1941–1951.
77. Pan, L. C. and Price, P. A. (1984). The effect of transcriptional inhibitors on the bone gamma-carboxyglutamic acid protein response to 1,25-dihydroxyvitamin D3 in osteosarcoma cells. *J. Biol. Chem.* **259**, 5844–5847.
78. Jung, C., Ou, Y. C., Yeung, F., Frierson, H. F., Jr., and Kao, C. (2001). Osteocalcin is incompletely spliced in non-osseous tissues. *Gene* **271**, 143–150.
79. Wu, T. T., Sikes, R. A., Cui, Q., et al. (1998). Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *Int. J. Cancer* **77**, 887–894.
80. Koeneman, K. S., Yeung, F., and Chung, L. W. (1999). Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* **39**, 246–261.
81. Bortell, R., Owen, T. A., Bidwell, J. P., et al. (1992). Vitamin D-responsive protein-DNA interactions at multiple promoter regulatory elements that contribute to the level of rat osteocalcin gene expression. *Proc. Natl. Acad. Sci. USA* **89**, 6119–6123.
82. Lian, J. B., Stein, G. S., Stein, J. L., and van Wijnen, A. J. (1999). Regulated expression of the bone-specific osteocalcin gene by vitamins and hormones. *Vitam. Horm.* **55**, 443–509.
83. Banerjee, C., Stein, J. L., Van Wijnen, A. J., Frenkel, B., Lian, J. B., and Stein, G. S. (1996). Transforming growth factor-beta 1 responsiveness of the rat osteocalcin gene is mediated by an activator protein-1 binding site. *Endocrinology* **137**, 1991–2000.
84. Banerjee, C., Hiebert, S. W., Stein, J. L., Lian, J. B., and Stein, G. S. (1996). An AML-1 consensus sequence binds an osteoblast-specific complex and transcriptionally activates the osteocalcin gene. *Proc. Natl. Acad. Sci. USA* **93**, 4968–4973.
85. Ko, S. C., Cheon, J., Kao, C., et al. (1996). Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res.* **56**, 4614–4619.
86. Koeneman, K. S., Kao, C., Ko, S. C., et al. (2000). Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. *World J. Urol.* **18**, 102–110.
87. Kubo, H., Gardner, T. A., Wada, Y., et al. (2003). Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum. Gene Ther.* **14**, 227–241.
88. Chiu, C. P. and Harley, C. B. (1997). Replicative senescence and cell immortality: the role of telomeres and telomerase. *Proc. Soc. Exp. Biol. Med.* **214**, 99–106.
89. Kim, N. W., Piatyszek, M. A., Prowse, K. R., et al. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015.
90. Horikawa, I., Cable, P. L., Afshari, C., and Barrett, J. C. (1999). Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer Res.* **59**, 826–830.
91. Sommerfeld, H. J., Meeker, A. K., Piatyszek, M. A., Bova, G. S., Shay, J. W., and Coffey, D. S. (1996). Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res.* **56**, 218–222.
92. Lin, T., Huang, X., Gu, J., et al. (2002). Long-term tumor-free survival from treatment with the GFP-TRAIL fusion gene expressed from the hTERT promoter in breast cancer cells. *Oncogene* **21**, 8020–8028.
93. Gu, J., Andreeff, M., Roth, J. A., and Fang, B. (2002). hTERT promoter induces tumor-specific Bax gene expression and cell killing in syngenic mouse tumor model and prevents systemic toxicity. *Gene Ther.* **9**, 30–37.
94. Kawashima, T., Kagawa, S., Kobayashi, N., et al. (2004). Telomerase-specific replication-selective virotherapy for human cancer. *Clin. Cancer Res.* **10**, 285–292.
95. Thompson, I. M., Goodman, P. J., Tangen, C. M., et al. (2003). The influence of finasteride on the development of prostate cancer. *N. Engl. J. Med.* **349**, 215–224.
96. Office of Biotechnology Activities' Recombinant DNA and Gene Transfer Web Page, http://www4.od.nih.gov/oba/rdna.htm.
97. Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323–331.
98. Sherr, C. J. (1996). Cancer cell cycles. *Science* **274**, 1672–1677.
99. Downing, S. R., Russell, P. J., and Jackson, P. (2003). Alterations of p53 are common in early stage prostate cancer. *Can. J. Urol.* **10**, 1924–1933.
100. Eastham, J. A., Stapleton, A. M., Gousse, A. E., et al. (1995). Association of p53 mutations with metastatic prostate cancer. *Clin. Cancer Res.* **1**, 1111–1118.

101. Yang, C., Cirielli, C., Capogrossi, M. C., and Passaniti, A. (1995). Adenovirus-mediated wild-type p53 expression induces apoptosis and suppresses tumorigenesis of prostatic tumor cells. *Cancer Res.* **55**, 4210–4213.
102. Eastham, J. A., Hall, S. J., Sehgal, I., et al. (1995). In vivo gene therapy with p53 or p21 adenovirus for prostate cancer. *Cancer Res.* **55**, 5151–5155.
103. Ko, S. C., Gotoh, A., Thalmann, G. N., et al. (1996). Molecular therapy with recombinant p53 adenovirus in an androgen-independent, metastatic human prostate cancer model. *Hum. Gene Ther.* **7**, 1683–1691.
104. Eastham, J. A., Grafton, W., Martin, C. M., and Williams, B. J. (2000). Suppression of primary tumor growth and the progression to metastasis with p53 adenovirus in human prostate cancer. *J. Urol.* **164**, 814–819.
105. Hernandez, I., Maddison, L. A., Wei, Y., et al. (2003). Prostate-specific expression of p53(R172L) differentially regulates p21, Bax, and mdm2 to inhibit prostate cancer progression and prolong survival. *Mol. Cancer Res.* **1**, 1036–1047.
106. Gurnani, M., Lipari, P., Dell, J., Shi, B., and Nielsen, L. L. (1999). Adenovirus-mediated p53 gene therapy has greater efficacy when combined with chemotherapy against human head and neck, ovarian, prostate, and breast cancer. *Cancer Chemother. Pharmacol.* **44**, 143–151.
107. Colletier, P. J., Ashoori, F., Cowen, D., et al. (2000). Adenoviral-mediated p53 transgene expression sensitizes both wild-type and null p53 prostate cancer cells in vitro to radiation. *Int. J. Radiat. Oncol. Biol. Phys.* **48**, 1507–1512.
108. Sasaki, R., Shirakawa, T., Zhang, Z. J., et al. (2001). Additional gene therapy with Ad5CMV-p53 enhanced the efficacy of radiotherapy in human prostate cancer cells. *Int. J. Radiat. Oncol. Biol. Phys.* **51**, 1336–1345.
109. Cowen, D., Salem, N., Ashoori, F., et al. (2000). Prostate cancer radiosensitization in vivo with adenovirus-mediated p53 gene therapy. *Clin. Cancer Res.* **6**, 4402–4408.
110. Pantuck, A. J., Zisman, A., and Belldegrun, A. S. (2000). Gene therapy for prostate cancer at the University of California, Los Angeles: preliminary results and future directions. *World J. Urol.* **18**, 143–147.
111. Sweeney, P. and Pisters, L. L. (2000). Ad5CMVp53 gene therapy for locally advanced prostate cancer—where do we stand? *World J. Urol.* **18**, 121–124.
112. Maki, C. G. (1999). Oligomerization is required for p53 to be efficiently ubiquitinated by MDM2. *J. Biol. Chem.* **274**, 16,531–16,535.
113. Fuchs, S. Y., Adler, V., Buschmann, T., Wu, X., and Ronai, Z. (1998). Mdm2 association with p53 targets its ubiquitination. *Oncogene* **17**, 2543–2547.
114. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature* **387**, 296–299.
115. Kussie, P. H., Gorina, S., Marechal, V., et al. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **274**, 948–953.
116. Leite, K. R., Franco, M. F., Srougi, M., et al. (2001). Abnormal expression of MDM2 in prostate carcinoma. *Mod. Pathol.* **14**, 428–436.
117. Zhang, Z., Li, M., Wang, H., Agrawal, S., and Zhang, R. (2003). Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proc. Natl. Acad. Sci. USA* **100**, 11,636–11,641.
118. Wang, H., Oliver, P., Zhang, Z., Agrawal, S., and Zhang, R. (2003). Chemosensitization and radiosensitization of human cancer by antisense anti-MDM2 oligonucleotides: in vitro and in vivo activities and mechanisms. *Ann. NY Acad. Sci.* **1002**, 217–235.
119. Zhang, Z., Wang, H., Prasad, G., et al. (2004). Radiosensitization by antisense anti-MDM2 mixed-backbone oligonucleotide in in vitro and in vivo human cancer models. *Clin. Cancer Res.* **10**, 1263–1273.
120. Mu, Z., Hachem, P., Agrawal, S., and Pollack, A. (2004). Antisense MDM2 oligonucleotides restore the apoptotic response of prostate cancer cells to androgen deprivation. *Prostate* **60**, 187–196.
121. Maehama, T. and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 13,375–13,378.
122. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. R. (1999). Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res.* **59**, 4291–4296.
123. Burgering, B. M. and Kops, G. J. (2002). Cell cycle and death control: long live Forkheads. *Trends Biochem. Sci.* **27**, 352–360.
124. Kotelevets, L., van Hengel, J., Bruyneel, E., Mareel, M., van Roy, F., and Chastre, E. (2001). The lipid phosphatase activity of PTEN is critical for stabilizing intercellular junctions and reverting invasiveness. *J. Cell Biol.* **155**, 1129–1135.
125. Stambolic, V., Suzuki, A., de la Pompa, J. L., et al. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29–39.
126. Zundel, W., Schindler, C., Haas-Kogan, D., et al. (2000). Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev.* **14**, 391–396.
127. Davies, M. A., Kim, S. J., Parikh, N. U., Dong, Z., Bucana, C. D., and Gallick, G. E. (2002). Adenoviral-mediated expression of MMAC/PTEN inhibits proliferation and metastasis of human prostate cancer cells. *Clin. Cancer Res.* **8**, 1904–1914.
128. Rosser, C. J., Tanaka, M., Pisters, L. L., et al. (2004). Adenoviral-mediated PTEN transgene expression sensitizes Bcl-2-expressing prostate cancer cells to radiation. *Cancer Gene Ther.* **11**, 273–279.

129. Tanaka, M., Rosser, C. J., and Grossman, H. B. (2005). PTEN gene therapy induces growth inhibition and increases efficacy of chemotherapy in prostate cancer. *Cancer Detect Prev*. **29**, 170–174.
130. Jarrard, D. F., Bova, G. S., Ewing, C. M., et al. (1997). Deletional, mutational, and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer. *Genes Chromosomes Cancer* **19**, 90–96.
131. Liggett, W. H., Jr. and Sidransky, D. (1998). Role of the p16 tumor suppressor gene in cancer. *J. Clin. Oncol*. **16**, 1197–1206.
132. Isaacs, W. B. (1995). Molecular genetics of prostate cancer. *Cancer Surv*. **25**, 357–379.
133. Steiner, M. S., Zhang, Y., Farooq, F., Lerner, J., Wang, Y., and Lu, Y. (2000). Adenoviral vector containing wild-type p16 suppresses prostate cancer growth and prolongs survival by inducing cell senescence. *Cancer Gene Ther*. **7**, 360–372.
134. Allay, J. A., Steiner, M. S., Zhang, Y., Reed, C. P., Cockroft, J., and Lu, Y. (2000). Adenovirus p16 gene therapy for prostate cancer. *World J. Urol*. **18**, 111–120.
135. Gotoh, A., Kao, C., Ko, S. C., Hamada, K., Liu, T. J., and Chung, L. W. (1997). Cytotoxic effects of recombinant adenovirus p53 and cell cycle regulator genes (p21 WAF1/CIP1 and p16CDKN4) in human prostate cancers. *J. Urol*. **158**, 636–641.
136. Deng, J., Xia, W., and Hung, M. C. (1998). Adenovirus 5 E1A-mediated tumor suppression associated with E1A-mediated apoptosis in vivo. *Oncogene* **17**, 2167–2175.
137. Bischoff, J. R., Kirn, D. H., Williams, A., et al. (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **274**, 373–376.
138. Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D. D., and Kirn, D. H. (1997). ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat. Med*. **3**, 639–645.
139. Heise, C. C., Williams, A. M., Xue, S., Propst, M., and Kirn, D. H. (1999). Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy. *Cancer Res*. **59**, 2623–2628.
140. Ganly, I., Kirn, D., Eckhardt, G., et al. (2000). A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin. Cancer Res*. **6**, 798–806.
141. Nemunaitis, J., Khuri, F., Ganly, I., et al. (2001). Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J. Clin. Oncol*. **19**, 289–298.
142. Khuri, F. R., Nemunaitis, J., Ganly, I., et al. (2000). A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat. Med*. **6**, 879–885.
143. Hecht, J. R., Bedford, R., Abbruzzese, J. L., et al. (2003). A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin. Cancer Res*. **9**, 555–561.
144. Morley, S., MacDonald, G., Kirn, D., Kaye, S., Brown, R., and Soutar, D. (2004). The dl1520 virus is found preferentially in tumor tissue after direct intratumoral injection in oral carcinoma. *Clin. Cancer Res*. **10**, 4357–4362.
145. Reid, T. R., Freeman, S., Post, L., McCormick, F., and Sze, D. Y. (2005). Effects of Onyx-015 among metastatic colorectal cancer patients that have failed prior treatment with 5-FU/leucovorin. *Cancer Gene Ther*. **12**, 673–681
146. Mulvihill, S., Warren, R., Venook, A., et al. (2001). Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. *Gene Ther*. **8**, 308–315.
147. Nemunaitis, J., Ganly, I., Khuri, F., et al. (2000). Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. *Cancer Res*. **60**, 6359–6366.
148. Goodrum, F. D. and Ornelles, D. A. (1998). p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. *J. Virol*. **72**, 9479–9490.
149. Rothmann, T., Hengstermann, A., Whitaker, N. J., Scheffner, M., and zur Hausen, H. (1998). Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J. Virol*. **72**, 9470–9478.
150. Edwards, S. J., Dix, B. R., Myers, C. J., et al. (2002). Evidence that replication of the antitumor adenovirus ONYX-015 is not controlled by the p53 and p14(ARF) tumor suppressor genes. *J. Virol*. **76**, 12,483–12,490.
151. O'Shea, C. C., Johnson, L., Bagus, B., et al. (2004). Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell* **6**, 611–623.
152. Freytag, S. O., Rogulski, K. R., Paielli, D. L., Gilbert, J. D., and Kim, J. H. (1998). A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum. Gene Ther*. **9**, 1323–1333.
153. Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., and Henderson, D. R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res*. **57**, 2559–2563.
154. DeWeese, T. L., van der Poel, H., Li, S., et al. (2001). A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res*. **61**, 7464–7472.

155. Yu, D. C., Chen, Y., Seng, M., Dilley, J., and Henderson, D. R. (1999). The addition of adenovirus type 5 region E3 enables calydon virus 787 to eliminate distant prostate tumor xenografts. *Cancer Res.* **59**, 4200–4203.
156. Dilley, J., Reddy, S., Ko, D., et al. (2005). Oncolytic adenovirus CG7870 in combination with radiation demonstrates synergistic enhancements of antitumor efficacy without loss of specificity. *Cancer Gene Ther.* **12**, 715–722
157. Yu, D. C., Chen, Y., Dilley, J., et al. (2001). Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res.* **61**, 517–525.
158. Matsubara, S., Wada, Y., Gardner, T. A., et al. (2001). A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res.* **61**, 6012–6019.
159. Hsieh, C. L., Yang, L., Miao, L., et al. (2002). A novel targeting modality to enhance adenoviral replication by vitamin D(3) in androgen-independent human prostate cancer cells and tumors. *Cancer Res.* **62**, 3084–3092.
160. Yu, D. C., Sakamoto, G. T., and Henderson, D. R. (1999). Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res.* **59**, 1498–1504.
161. Konety, B. R., Johnson, C. S., Trump, D. L., and Getzenberg, R. H. (1999). Vitamin D in the prevention and treatment of prostate cancer. *Semin. Urol. Oncol.* **17**, 77–84.
162. Getzenberg, R. H., Light, B. W., Lapco, P. E., et al. (1997). Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system. *Urology* **50**, 999–1006.
163. Zhao, X. Y. and Feldman, D. (2001). The role of vitamin D in prostate cancer. *Steroids* **66**, 293–300.
164. Doronin, K., Kuppuswamy, M., Toth, K., et al. (2001). Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. *J. Virol.* **75**, 3314–3324.
165. Banerjee, N. S., Rivera, A. A., Wang, M., et al. (2004). Analyses of melanoma-targeted oncolytic adenoviruses with tyrosinase enhancer/promoter-driven E1A, E4, or both in submerged cells and organotypic cultures. *Mol. Cancer Ther.* **3**, 437–449.
166. Huang, X. and Lee, C. (2003). From TGF-beta to cancer therapy. *Curr. Drug Targets* **4**, 243–250.
167. Narumoto, K., Saibara, T., Maeda, T., et al. (2000). Transforming growth factor-beta 1 derived from biliary epithelial cells may attenuate alloantigen-specific immune responses. *Transpl. Int.* **13**, 21–27.
168. Ke, B., Coito, A. J., Kato, H., et al. (2000). Fas ligand gene transfer prolongs rat renal allograft survival and down-regulates anti-apoptotic Bag-1 in parallel with enhanced Th2-type cytokine expression. *Transplantation* **69**, 1690–1694.
169. Winoto, A. (1997). Cell death in the regulation of immune responses. *Curr. Opin. Immunol.* **9**, 365–370.
170. Norris, J. S., Hyer, M. L., Voelkel-Johnson, C., Lowe, S. L., Rubinchik, S., and Dong, J. Y. (2001). The use of Fas Ligand, TRAIL and Bax in gene therapy of prostate cancer. *Curr. Gene Ther.* **1**, 123–136.
171. Harrison, D., Sauthoff, H., Heitner, S., Jagirdar, J., Rom, W. N., and Hay, J. G. (2001). Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved—deletion of the viral E1b-19-kD gene increases the viral oncolytic effect. *Hum. Gene Ther.* **12**, 1323–1332.
172. Sauthoff, H., Hu, J., Maca, C., et al. (2003). Intratumoral spread of wild-type adenovirus is limited after local injection of human xenograft tumors: virus persists and spreads systemically at late time points. *Hum. Gene Ther.* **14**, 425–433.
173. Shen, B. H. and Hermiston, T. W. (2005). Effect of hypoxia on Ad5 infection, transgene expression and replication. *Gene Ther.* **12**, 902–910.
174. Jin, F., Xie, Z., Kuo, C. J., Chung, L. W., and Hsieh, C. L. (2005). Cotargeting tumor and tumor endothelium effectively inhibits the growth of human prostate cancer in adenovirus-mediated antiangiogenesis and oncolysis combination therapy. *Cancer Gene Ther.* **12**, 257–267.
175. Liu, X. Y., Qiu, S. B., Zou, W. G., et al. (2005). Effective gene-virotherapy for complete eradication of tumor mediated by the combination of hTRAIL (TNFSF10) and plasminogen k5. *Mol. Ther.* **11**, 531–541.
176. Culy, C. (2005). Bevacizumab: antiangiogenic cancer therapy. *Drugs Today (Barc)* **41**, 23–26.
177. Mesnil, M., Piccoli, C., Tiraby, G., Willecke, K., and Yamasaki, H. (1996). Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc. Natl. Acad. Sci. USA* **93**, 1831–1835.
178. Eastham, J. A., Chen, S. H., Sehgal, I., et al. (1996). Prostate cancer gene therapy: herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. *Hum. Gene Ther.* **7**, 515–523.
179. Cheon, J., Kim, H. K., Moon, D. G., Yoon, D. K., Cho, J. H., and Koh, S. K. (2000). Adenovirus-mediated suicide-gene therapy using the herpes simplex virus thymidine kinase gene in cell and animal models of human prostate cancer: changes in tumour cell proliferative activity. *BJU Int.* **85**, 759–766.
180. Hall, S. J., Mutchnik, S. E., Chen, S. H., Woo, S. L., and Thompson, T. C. (1997). Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. *Int. J. Cancer* **70**, 183–187.
181. Freytag, S. O., Khil, M., Stricker, H., et al. (2002). Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res.* **62**, 4968–4976.

182. Freytag, S. O., Paielli, D., Wing, M., et al. (2002). Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model. *Int. J. Radiat. Oncol. Biol. Phys.* **54**, 873–885.
183. Freytag, S. O., Stricker, H., Pegg, J., et al. (2003). Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res.* **63**, 7497–7506.
184. Wiley, S. R., Schooley, K., Smolak, P. J., et al. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3**, 673–682.
185. Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., and Ashkenazi, A. (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J. Biol. Chem.* **271**, 12,687–12,690.
186. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. (1997). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**, 815–818.
187. Pan, G., O'Rourke, K., Chinnaiyan, A. M., et al. (1997). The receptor for the cytotoxic ligand TRAIL. *Science* **276**, 111–113.
188. Nesterov, A., Lu, X., Johnson, M., Miller, G. J., Ivashchenko, Y., and Kraft, A. S. (2001). Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. *J. Biol. Chem.* **276**, 10,767–10,774.
189. Voelkel-Johnson, C., King, D. L., and Norris, J. S. (2002). Resistance of prostate cancer cells to soluble TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) can be overcome by doxorubicin or adenoviral delivery of full-length TRAIL. *Cancer Gene Ther.* **9**, 164–172.
190. Shankar, S., Singh, T. R., and Srivastava, R. K. (2004). Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer in vitro and in vivo: Intracellular mechanisms. *Prostate* **61**, 35–49.
191. Shankar, S., Chen, X., and Srivastava, R. K. (2005). Effects of sequential treatments with chemotherapeutic drugs followed by TRAIL on prostate cancer in vitro and in vivo. *Prostate* **62**, 165–186.
192. Jo, M., Kim, T. H., Seol, D. W., et al. (2000). Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat. Med.* **6**, 564–567.
193. Lawrence, D., Shahrokh, Z., Marsters, S., et al. (2001). Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat. Med.* **7**, 383–385.
194. Mori, E., Thomas, M., Motoki, K., et al. (2004). Human normal hepatocytes are susceptible to apoptosis signal mediated by both TRAIL-R1 and TRAIL-R2. *Cell Death Differ.* **11**, 203–207.
195. Hao, C., Song, J. H., Hsi, B., et al. (2004). TRAIL inhibits tumor growth but is nontoxic to human hepatocytes in chimeric mice. *Cancer Res.* **64**, 8502–8506.
196. Kaliberov, S. A., Kaliberova, L. N., Stockard, C. R., Grizzle, W. E., and Buchsbaum, D. J. (2004). Adenovirus-mediated FLT1-targeted proapoptotic gene therapy of human prostate cancer. *Mol. Ther.* **10**, 1059–1070.
197. Vanoosten, R. L., Moore, J. M., Ludwig, A. T., and Griffith, T. S. (2005). Depsipeptide (FR901228) enhances the cytotoxic activity of TRAIL by redistributing TRAIL receptor to membrane lipid rafts. *Mol. Ther.* **11**, 542–552.
198. Bander, N. H., Yao, D., Liu, H., et al. (1997). MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma. *Prostate* **33**, 233–239.
199. Kwon, E. D., Hurwitz, A. A., Foster, B. A., et al. (1997). Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc. Natl. Acad. Sci. USA* **94**, 8099–8103.
200. Sanda, M. G., Ayyagari, S. R., Jaffee, E. M., et al. (1994). Demonstration of a rational strategy for human prostate cancer gene therapy. *J. Urol.* **151**, 622–628.
201. Vieweg, J., Rosenthal, F. M., Bannerji, R., et al. (1994). Immunotherapy of prostate cancer in the Dunning rat model: use of cytokine gene modified tumor vaccines. *Cancer Res.* **54**, 1760–1765.
202. Simons, J. W., Mikhak, B., Chang, J. F., et al. (1999). Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. *Cancer Res.* **59**, 5160–5168.
203. Simmons, S. J., Tjoa, B. A., Rogers, M., et al. (1999). GM-CSF as a systemic adjuvant in a phase II prostate cancer vaccine trial. *Prostate* **39**, 291–297.
204. Simons, J. W., Nelson, W., Nemunaitis, J., et al. (2002). Phase II trials of a GM-CSF gene-transduced prostate cancer cell vaccine (GVAX) in hormone refractory prostate cancer. *Proc. Am. Soc. Clin. Oncol.* **21**, 729A.
205. Simons, J. W., Higano, C., Corman, J., et al. (2003). A phase I/II study of high dose allogeneic GM-CSF gene-transduced prostate cancer cell line vaccine in patients with metastatic hormone-refractory prostate cancer. *Proc. Am. Soc. Clin. Oncol.* **22**, 166A.
206. Tjoa, B., Boynton, A., Kenny, G., Ragde, H., Misrock, S. L., and Murphy, G. (1996). Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. *Prostate* **28**, 65–69.
207. Murphy, G., Tjoa, B., Ragde, H., Kenny, G., and Boynton, A. (1996). Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *Prostate* **29**, 371–380.
208. Tjoa, B. A., Erickson, S. J., Bowes, V. A., et al. (1997). Follow-up evaluation of prostate cancer patients infused with autologous dendritic cells pulsed with PSMA peptides. *Prostate* **32**, 272–278.

209. Tjoa, B. A., Simmons, S. J., Elgamal, A., et al. (1999). Follow-up evaluation of a phase II prostate cancer vaccine trial. *Prostate* **40**, 125–129.
210. Triest, J. A., Grignon, D. J., Cher, M. L., et al. (1998). Systemic interleukin 2 therapy for human prostate tumors in a nude mouse model. *Clin. Cancer Res.* **4**, 2009–2014.
211. Belldegrun, A., Tso, C. L., Zisman, A., et al. (2001). Interleukin 2 gene therapy for prostate cancer: phase I clinical trial and basic biology. *Hum. Gene Ther.* **12**, 883–892.
212. Trudel, S., Trachtenberg, J., Toi, A., et al. (2003). A phase I trial of adenovector-mediated delivery of interleukin-2 (AdIL-2) in high-risk localized prostate cancer. *Cancer Gene Ther.* **10**, 755–763.

10

Role of Transforming Growth Factor-β in Immunotherapy of Prostate Cancer

Chung Lee, Ali Shah, Victoria Liu, Irwin Park, Larry Wong, Xuemei Huang, Lijun Huang, Tim Fermin, Tom Jang, Som Kundu, Vivian Zhou, Qiang Zhang, and Norm Smith

Summary

In this chapter, we introduce a novel concept for an effective treatment of advanced prostate cancer, using our understanding of transforming growth factor (TGF)-β signaling. TGF-β plays an important role in prostate cancer development and progression. Two characteristic features of TGF-β signaling in aggressive prostate cancer are a reduced sensitivity to TGF-β and an overproduction of TGF-β. A reduced sensitivity to TGF-β removes the inhibitory effect of TGF-β and provides a growth advantage for the cancer cells. An overproduction of TGF-β endows cancer cells the ability to metastasize, to enhance angiogenesis, and to evade host's immune surveillance program, leading to tumor progression and metastasis. TGF-β is a potent tumor-induced immunosuppression. A therapeutic strategy rending host immune cells insensitive to TGF-β should offer an effective approach to eradicate advanced prostate cancer.

Key Words: Adoptive transfer; cytolytic T cells; immunotherapy; TGF-β signaling.

1. INTRODUCTION

Recent advances in the area of cancer gene therapy and immunotherapy have been encouraging. The development of the targeted therapeutic strategy has saved many lives. Despite these promises, the overall survival rate for cancer patients has been disappointing *(1–3)*. In this review, we present an intriguing biology of transforming growth factor (TGF)-β, which offers hope for our fight against cancer.

2. BASIC BIOLOGY OF TGF-β

Historically, TGF-β was recognized as a growth factor for murine sarcoma virus-transformed rat kidney fibroblasts, because it promoted soft agar anchorage-independent grow *(4)*. This original understanding of TGF-β has been modified. Today, we recognize that TGF-β is a multifunctional growth factor, which regulates a wide array of events in physiology and pathology.

2.1. The TGF-β Superfamily

TGF-β is the prototypic member of a superfamily with more than 30 members. Some of the members include inhibin, bone morphogenic proteins, and Mullerian-inhibiting substance. The TGF-β subfamily contains five members (TGF-β1, -β2, -β3, -β4, and -β5). TGF-β4 and -β5 have been identified only in chicken and *Xenopus*, respectively *(5–7)*. TGF-β1, -β2, and -β3 have been identified in mammals *(8,9)*. Although TGF-β is a pleiotropic growth factor, it is mainly a growth inhibitor to

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

most cell types *(10,11)*. This family of growth factors regulates pivotal biological functions, including cell proliferation, differentiation, apoptosis, migration, and extracellular matrix production *(12,13)*.

2.2. Biochemistry of Mammalian TGF-β

TGF-β is synthesized as the C-terminal domain of a precursor form that is cleaved before secretion from the cell *(8,14,15)*. However, the TGF-β pro-peptide, which is referred to as the latency-associated peptide, is noncovalently bound to TGF-β after secretion and cannot bind to receptors *(16)*. Most cell types secrete TGF-β in this biologically inert form. A third component of the latent TGF-β complex is a large secretory glycol-protein known as latent TGF-β-binding protein, which is disulfide-linked to latency-associated peptide. Latent TGF-β-binding protein is implicated in TGF-β secretion, storage in the extracellular matrix, and eventual activation *(17)*.

2.3. TGF-β Receptors

TGF-β exerts its biological effect through its receptors. In animal cells, three types of TGF-β receptors (TβR) have been reported as type I, II, and III receptors *(18)*. TβR-III is a 200- to 400-kDa proteoglycan, with chondroitin sulfate and heparan sulfate chains linked to a 110- to 130-kDa core protein *(19)*. TβR-III has no direct role in TGF-β signal transduction, because it lacks the signaling motif in the cytoplasmic domain *(20)*. It may function as a storage protein that regulates bioavailability of the ligand to target cells *(21–23)*. TβR-I and TβR-II are directly involved in TGF-β signaling, for these receptors contain serine/threonine kinases *(24)*. Each of the receptors possesses an extracellular region, a single transmembrane domain, and cytoplasmic signaling domain, which contain a serine/threonine kinase domain. Current understanding is that TβR-II binds TGF-β first and then recruits TβR-I. Signaling can only occur as a heteromeric complex *(25)*. Because of the knowledge that both TβR-I and TβR-II are required for TGF-β signaling, a loss of expression or functioning of either one of the receptors will lead to TGF-β insensitivity, which is common in cancer cells. In prostate cancer, it is well-established that a loss of TGF-β receptors has been associated with high Gleason grade *(26)* and with a reduced survival period *(27)*.

2.4. Role of Smads in TGF-β Signaling

TGF-β and related factors use a simple mechanism to transmit signals. Binding of the ligand causes the assembly of a receptor complex that phosphorylates proteins of the Smad family, which consists of closely related proteins that bind DNA and recruit transcriptional co-activators or corepressors. Phosphorylation causes Smads to move into the nucleus, where they assemble complexes that control gene expression. Therefore, Smads are a signal mediator and transfer signal from cytoplasmic into specific nucleus target genes *(15,28,29)*. Receptor-regulated Smad (R-Smad) requires TGF-β-induced phosphorylation to assemble transcription regulatory complexes with partner Smads (co-Smads). An R-Smad can move into the nucleus by itself but, to be accessible to membrane receptors, R-Smads are tethered in the cytoplasm by proteins such as Smad anchor for receptor activation (SARA). The type I receptor is kept inactive by a wedge-shaped glycine-serine (GS) region, which presses against the kinase domain, dislocating its catalytic center *(28–30)*. In the ligand-induced complex, the type II receptor phosphorylates the GS domain and activates the type I receptor, which catalyzes R-Smad phosphorylation. Phosphorylation decreases the affinity of R-Smads for SARA and increases their affinity for co-Smads. The resulting Smad complex is free to move into the nucleus and functions as transcriptional co-activators or corepressors. Smads can contact DNA, but effective binding to particular gene regulatory sites is enabled by specific DNA-binding cofactors. R-Smads that move into the nucleus may return to the cytoplasm, but the ubiquitylation and proteasome-dependent degradation in the nucleus provide a way to terminate TGF-β responses *(28,29,31)*. Smad-2 and Smad-4 are frequently mutated in particular tumor subsets, suggesting that they may act as tumor suppressors. In addition, several oncogenic proteins can interact and inhibit the function of Smad proteins *(8,12,28,29,32)*.

3. TGF-β SIGNALING IN NORMAL PHYSIOLOGY

Inhibition of cell proliferation is one of the TGF-β actions in epithelial, endothelial, hematopoietic, neural, and certain types of mesenchymal cells. Escape from this inhibition is a hallmark of many cancer cells. TGF-β is effective at inhibiting cell cycle progression during G1. In most cases, this growth arrest effect is reversible, but in some cases, it is associated with cell apoptosis or cell death *(28,29)*. Two classes of anti-proliferative gene responses are involved in TGF-β growth arrest: downregulation of c-*myc* and expression of inhibitors to cdks. The Myc family members (Myc, N-Myc, and L-Myc) are known to deregulate cell growth by promoting continuous, mitogen-independent, cell cycle progression *(33–37)*. The second class is cdk-inhibitors, which include the induction of p15 and p21 and downregulation of cdc 25A. Most cells that are growth inhibited by TGF-β have different combinations of cdk-inhibitory responses.

C-Myc antagonizes TGF-β signaling by acting as a repressor of cdk-inhibitory responses. Downregulation of c-Myc is, thus, necessary for TGF-β-induced cell cycle arrest *(28,29,38)*. In addition to causing reversible cell cycle arrest in some cell types, TGF-β can induce programmed cell death in others. In fact, apoptosis induced by TGF-β family members is an essential component of the proper development of various tissues and organs *(39,40)*. TGF-β-induced apoptosis and the selective elimination of pre-neoplastic cells may also be involved in the tumor suppression mediated by TGF-β *(41)*. Just as loss of TGF-β-mediated growth arrest might predispose a cell to cancer, loss of TGF-β-mediated apoptosis may permit selective accumulation of premalignant cells *(28,29)*.

4. TGF-β SIGNALING IN PROSTATE CANCER CELLS

Two important mutational events seem to be associated with malignant transformation. They are the loss of the sensitivity to the inhibitory effect of TGF-β and the acquisition of the ability to express an increased level of TGF-β. The following discussion expands this notion.

4.1. Downregulation of TGF-β Sensitivity in Prostate Cancer Cells

A reduced sensitivity to TGF-β removes the inhibitory effect of TGF-β and is associated with high-grade prostate cancer. Results from many studies have clearly indicated that benign prostate epithelial cells are exquisitely sensitive to TGF-β, because they express high levels of TGF-β receptors *(42)*. As the prostate epithelial cells progress through malignant transformation, they gradually reduce their sensitivity to TGF-β, as indicated by a reduced expression of TGF-β receptors, impacting on the survival rate of prostate cancer patients *(27,42)*.

Insensitivity to TGF-β can take place at various points in the TGF-β signaling pathways *(43)*. Genetic mutations resulting in loss of function in TGF-β receptors are rare, except in colon cancer *(44)*. Transcriptional inactivation through mutation or epigenetic alterations for TβR-I and TβR-II has been reported *(45–48)*. Results of a recent study indicated that promoter methylation in both TβR-I and TβR-II is at least a major factor responsible for TGF-β insensitivity in prostate cancer cells *(49)*.

4.2. Overproduction of TGF-β by Prostate Cancer Cells

The literature is replete with the information that TGF-β is overproduced in cancer cells *(50–52)*. Prostate cancer cells are no exception *(53–56)*. The overproduction of TGF-β by cancer cells has a multitude of adverse consequences. TGF-β can promote extracellular matrix production, induce angiogenesis, and inhibit host immune function. The biological consequence of these activities is enhanced tumorigenicity *(5,57)*. The overexpression of TGF-β from cancer cells alters the host–tumor interaction, which consequently facilitates tumor growth. TGF-β can inhibit the host immune system.

TGF-β seems to play a significant role in facilitating MATLyLu rat prostate tumor growth in syngeneic hosts. The MATLyLu cell line has a similar biochemical and histological profile to that of late-stage human prostate cancer. It is either not immunogenic or only weakly immunogenic *(58)*.

The classic studies by Barrack *(57)* and Steiner and Barrack *(59)* demonstrated that overproduction of TGF-β1 in MATLyLu cells was growth inhibitory in vitro but growth stimulatory in vivo. Results of a recent study *(60)* showed that MATLyLu cells transfected with a TGF-β1 antisense expression vector had reduced TGF-β1 production. These transfected cells proliferated in vitro at a much greater rate than wild-type MATLyLu cells. However, they either failed to form tumors or grew smaller tumors than did the wild-type cells. These findings indicate that the excess TGF-β produced by MATLyLu cells is responsible for the aggressive phenotype of this rat prostate cancer.

The high levels of TGF-β produced by cancer cells have an inhibitory effect on surrounding cells, including the host immune cells *(61)*. Although the exact mechanism remains to be defined, TGF-β is a potent tumor-induced immunosuppressor *(62,67–71)*. As a result, high levels of tumor-derived TGF-β have been implicated as being responsible for tumor escape from immune surveillance *(72)*.

5. HOST IMMUNITY IN THE PRESENCE OF TUMOR-DERIVED TGF-β

5.1. Impaired Immune Function in the Presence of Tumor Derived TGF-β

A common property of cancer cells, including prostate cancer cells, is that they overproduce TGF-β. Because TGF-β is an inhibitory growth factor, theoretically, it should be able to inhibit tumor growth. However, tumor cells have acquired the ability by becoming insensitive to TGF-β. Our early studies have shown that prostate cancer cases, especially with high Gleason grades, have lost the expression of at least one or both TGF-β receptors *(42)*. Furthermore, the loss of TGF-β receptors has an impact on survival of prostate cancer patients *(26)*. This property provides a mechanism for cancer cells to escape autocrine inhibition by TGF-β; but, at the same time, the high levels of TGF-β produced by these cells are highly immunosuppressive.

5.2. Removal of TGF-β From Tumor Cells Renders Them Susceptible to the Immune Surveillance Program

Theoretically, if one can remove TGF-β from the cancer cells, their growth should be inhibited by the host immune system. This was confirmed by our study *(60)*. MATLyLu rat prostate cancer cells are extremely aggressive and they produce high levels of TGF-β. When MATLyLu cells were genetically engineered to reduce the statement of TGF-β1, they failed to develop tumors in syngeneic hosts (Copenhagen rats), but tumors still developed in immunodeficient hosts (nude rats). Complete eradication of rat glioma tumors was noted when an antisense TGF-β construct was introduced into tumor cells *ex vivo* and then locally reintroduced into the tumor-bearing host *(73)*. These proof-of-principle studies demonstrated that TGF-β produced by tumor cells was a potent immunosuppressant.

6. TGF-β IN IMMUNE HOMEOSTASIS

TGF-β produced by tumor cells may diminish the effectiveness of antitumor T-cell immune responses *(63–65)*. The main function of TGF-β is to inhibit the growth and activities of T cells. It is viewed as an "anti-cytokine" because, in addition to its action on T cells, it can inhibit many functions of macrophages, B cells, neutrophils, and natural killer cells by counteracting the action of other activating factors. Although it is a negative regulator of the immune response, it stimulates wound healing through the synthesis of collagen. TGF-β is produced by many types of cells, including T cells, B cells, and macrophages. The role of TGF-β is to suppress the immune response when it is no longer needed after an infection and to promote the healing process *(66)*.

6.1. TGF-β is a Potent Immunosuppressant

TGF-β has been implicated in tumor-induced immunosuppression *(67)*. One of the reasons that an overstatement of TGF-β in cancer cells promotes tumor growth in vivo can be attributed to the fact that TGF-β is a potent immunosuppressant *(68,69)*. TGF-β is a powerful inhibitor of T- and B-cell proliferation. Therefore, an excess of TGF-β can inhibit host immune responses. The immunosup-

pressive role of TGF-β can be best demonstrated in TGF-β1-knockout mice. These animals are unable to survive beyond 21 days of age because of a severe widespread inflammatory reaction. These animals could survive longer if they were treated with antibodies to major histocompatibility complex (MHC) antigens or were rendered athymic *(74)*.

6.2. Nonimmune Host Cells in TGF-β-Induced Immunosuppression

Tumor-induced immunosuppression is a key mechanism by which tumors can evade host immunosurveillance. Aside from a direct inhibitory effect of TGF-β on immune cells, TGF-β can act on nonimmune cells and can contribute to the immunosuppressive effect in the host. Interactions between thymic stromal cells and immune cells are the basis for T-cell selection and have an impact on final T-cell repertoire *(75)*. For example, TGF-β expressed from thymic stromal cells regulates the differentiation of $CD4^+/CD8^+$ double-positive stages *(76)*. Another example is the inhibitory effect of TGF-β on the production of interleukin (IL)-7 by nonlymphoid stromal cells, which are important for the development of B cells *(77)*.

6.3. TGF-β in Tumor Immunology

TGF-β is the principal immunosuppressive component derived from tumor cells *(67,78)*. Modification of highly immunogenic C3H tumors with a TGF-β expression vector allowed for growth and escape from immunosurveillance in vivo despite an apparent lack of downregulation of MHC class I or tumor-specific antigen *(79)*. Neutralization of TGF-β results in abrogation of MCF-7 tumors *(80)*. A complete eradication of rat glioma tumors was noted when an antisense TGF-β construct was introduced into tumor cells *ex vivo* and then locally reintroduced into the tumor-bearing host *(73)*. A similar approach was reported with the Dunning rat prostate tumor MATLyLu *(60)*. In a mouse thymoma model, tumor cells engineered to secrete a soluble TβR-II resulted in a suppression of tumorigenicity *(72)*. These reports support the idea that TGF-β production by tumor cells inhibits immunosurveillance and that elimination of TGF-β from tumor cells enhances host immune response.

7. TGF-β-BASED IMMUNOTHERAPY FOR CANCER

7.1. TGF-β Insensitivity in Autoimmune-Like Disease

For the sake of simplicity, we divide the etiology of autoimmune-like disease into thymic origin and/or peripheral tissue origin *(76,81,82)*. Many reports implicated TGF-β in the pathogenesis of autoimmune-like disease. Systemic administration of TGF-β suppressed the symptoms of experimental encephalomyelitis, whereas antibodies to TGF-β enhanced the disease *(82,83)*. Mice null for TGF-β1 developed autoimmune-like syndrome, including enhanced expression of MHC class I and II antigens, circulating systemic lupus erythema-tosus (SLE)-like IgG antibodies to nuclear antigens, pathogenic glomerular IgG deposits, and progressive infiltration of lymphocytes into multiple organs *(84,85)*. Development of autoimmunity is normally resulted in selection processes in the thymus or through mechanisms that maintain tolerance in peripheral tissues. In the thymus, negative selection takes place at the $CD4^+/CD8^+$ double-positive stage *(86)*. Because TGF-β regulates the maturation of these double-positive cells, it is likely that, in the absence of TGF-β action, double-positive cells are generated rapidly for their appropriate elimination *(76,87)*. Mechanisms of maintenance of peripheral tolerance can include a balance of reactive and suppressor (or regulatory) T cells *(88)*. TGF-β, again, plays a major role in this process *(70,75,89)*. However, mechanisms of TGF-β insensitive immune cells in autoimmunity remain undefined.

7.2. Transplant of TGF-β-Insensitive Bone Marrow Leads to Elimination of Tumor Cells

In this section, we describe the development of a murine model of TGF-β insensitivity limited to the hematopoietic tissue of adult C57BL/6 mice. Bone marrow cells from donor mice were rendered insensitive to TGF-β via retroviral expression of the dominant-negative TβR-II (*TβRIIDN*) gene and

were transplanted into irradiated recipient mice before tumor challenge. When tumor cells (mouse B16 melanoma cells or transgenic adenocarcinoma mouse prostate [TRAMP] cancer cells) were injected into these animals, tumor cells were eliminated *(71,90)*. These results suggest that a gene therapy approach to inducing TGF-β insensitivity in immune cells may be a viable anticancer strategy.

7.3. Adoptive Transfer of Tumor-Reactive TGF-β-Insensitive CD8+ T Cells Results in Eradication of Mouse Prostate Tumor in Syngeneic Hosts

Although the above approach described in 7.2 resulted in tumor elimination, recipient animals treated by this approach eventually developed widespread multiorgan inflammatory conditions, suggestive of a manifestation of autoimmune disease. An approach that can lead to tumor eradication and avoid the development of autoimmune disease will be ideal for an effective and safe cancer treatment. The use of adoptive transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells into tumor-bearing mice has accomplished this objective *(56)*. This study is briefly described next.

TRAMP-C2 cells were used as the mouse model of prostate cancer. Donor C57BL/6 mice were primed with irradiated TRAMP-C2 cells by subcutaneous inoculation (5×10^6 cells) every 10 days. At the end of three to five vaccinations, $CD8^+$ T cells were isolated from the spleen, expanded *ex vivo* in the presence of irradiated wide type splenocytes and extracts of TRAMP-C2 cells, and supplemented with IL-2 and anti-CD3 every 3 days. These $CD8^+$ T cells were rendered TGF-β-insensitive by transfecting with TβRIIDN. In vitro cytotoxic assay revealed that these tumor-reactive and TGF-β-insensitive $CD8^+$ T cells had a 25-fold specific tumor killing activity relative to unprimed naïve $CD8^+$ cells. To determine the in vivo antitumor activity, recipient mice were challenged with intravenous injection of 5×10^5 TRAMP-C2 cells for 3, 7, or 21 days followed by adoptive transfer of tumor reactive and TGF-β insensitive $CD8^+$ cells (2×10^6 cells). Tumor metastasized to the lung was eliminated in the group receiving tumor-reactive and TGF-β-insensitive $CD8^+$ T cells. Infiltration of tumor-reactive TGF-β-insensitive $CD8^+$ T cells into tumor parenchyma was evident, whereas naïve $CD8^+$ T cells or non-engineered tumor-reactive control $CD8^+$ T cells were unable to enter tumor tissues. When transferred into tumor-bearing hosts, these $CD8^+$ T cells were maintained at a constant 2% in the spleen and caused an elevation of circulating IL-2 and interferon-γ. Interestingly, when transferred into tumor-free hosts (2×10^6 cells), they showed a steady rate of decay. These results suggest that adoptive therapy of tumor-reactive and TGF-β-insensitive $CD8^+$ T cells may warrant consideration for cancer therapy.

7.4. Adapting a TGF-β-Based Protection Strategy to Enhance Antitumor Immunity

Another example of TGF-β insensitivity in immune cells resulting in tumor elimination was the model of Epstein–Barr virus-specific cytotoxic T lymphocytes (CTLs), which were transduced with a retrovirus vector expressing TβRIIDN, HATGF-βRII Δcyt, for the treatment of Epstein–Barr virus-positive Hodgkin disease. HATGF-βRII Δcyt-transducted CTLs were resistant to the antiproliferative and anticytotoxic effects of exogenous TGF-β. Additionally, these transduced CTLs continued to secrete cytokines in response to antigenic stimulation. Long-term expression of HATGF-βRII Δcyt did not affect CTL function, phenotype, or growth characteristics. Tumor-specific CTLs expressing HATGF-βRII Δcyt should have a selective functional and survival advantage over unmodified CTLs in the presence of TGF-β-secreting tumors *(91)*.

9. CONCLUSION

It is encouraging that there is a possibility of achieving a successful new strategy to effectively treat cancer using tumor-reactive, TGF-β-insensitive immune cells in gene therapy. The host immune system offers a natural defense program against cancer, but this natural immunosurveillance is

rendered ineffective by an overproduction of TGF-β derived from the tumor cells. In the past, many attempts have been made to boost the host immune system as a cure for cancer. Unfortunately, these efforts met with little success, possibly because of inadequate recognition of the powerful effect of tumor-derived TGF-β on immunosuppression. The present discussion illustrates that TGF-β signaling plays a key role in regulating our immune system. Rendering the host immune cells insensitive to TGF-β in a gene therapy program offers hope as a potential anticancer therapy. In the near future, the development of a TGF-β-based therapeutic strategy for the treatment of cancer will be an important research focus.

REFERENCES

1. Blattman, J. N. and Greenberg, P. D. (2004). Cancer immunotherapy: A treatment for the masses. *Science* **305**, 200–205.
2. Yannelli, J. R., Hyatt, C., McConnell, S., et al. (1996). Growth of tumor-infiltrating lymphocytes from human solid cancers: summary of a 5-year experience. *Int. J. Cancer* **65**, 413–421.
3. Tjoa, B. A., Lodge, P. A., Salgaller, M. L., Boynton, A. L., and Murphy, G. P. (1999). Dendritic cell-based immunotherapy for prostate cancer. *CA Cancer J. Clin.* **49**, 117–128.
4. DeLarco, J. E. and Todaro, G. J. (1978). Growth factors from murine sarcoma virus-transformed cells. *Proc. Nat. Acad. Sci. USA* **75**, 4001–4005.
5. Lee, C., Sintich, S. M., Mathews, E. P., and Shah, A. H. (1999). Transforming growth factor-β in benign and malignant prostate. *Prostate* **39**, 285–290.
6. Jakowlew, S. B., Dillard, P. J., Kondaiah, P., Sporn, M. B., and Roberts, A. B. (1988). Complementary deoxyribonucleic acid cloning of a novel transforming growth factor-beta messenger ribonucleic acid from chick embryo chondrocytes. *Mol. Endocrinol.* **2**, 747–755.
7. Kondaiah, P., Sands, M. J., Smith, J. M., et al. (1990). Identification of a novel transforming growth factor beta (TGF-beta5) mRNA in *Xenopus laevis*. *J. Biol. Chem.* **265**, 1089–1093.
8. Derynck R., Jarret, J. A., Chen, E. Y., et al. (1985). Human transforming growth factor-beta complementary DNA sequences and expression in normal and transformed cells. *Nature* **316**, 701–705.
9. Madison, L., Webb, N. R., Rose, T. M., et al. (1988). Transforming Growth Factor-beta2: cDNA cloning and sequence analysis. *DNA* **7**, 1–8.
10. Sutkowski, D. M., Fong, C.-J., Sensibar, J. A., et al. (1992). Interaction of epidermal growth factor and transforming growth factor-β1 in human prostatic epithelial cell in culture. *Prostate* **21**, 133–143.
11. Wilding, G. (1991). Response of prostate cancer cells to peptide growth factor: Transforming Growth Factor-beta. *Cancer Surv.* **11**, 147–163.
12. Dijke, P. T., Goumans, M. J., Itoh, F., and Itoh, S. (2002). Regulation of Cell Proleferation by Smad Proteins. *J. Cell Physiol.* **191**, 1–16.
13. Robert, A. B. and Sporn, M. B. (1990). In: Peptide Growth Factors and Their Receptors, Part I, Vol. 95 (Sporn, M. B., Roberts, A. B., eds.). Berlin: Springer-Verlag, pp. 419–472.
14. Gentry, L. E., Liobin, M. N., Purchio, A. F., and Marquardt, H. (1988). Type 1 Transforming Growth Factor-beta: amplified expression and secretion of mature and precursor polypeptides in Chinese hamster ovary. *Mol. Cell Biol.,* **8**, 4162–4168.
15. Massagué, J. (1998). TGF-beta signal transduction. *Ann. Rev. Cell Biol.* **67**, 753–791.
16. Gentry, L. E., Webb, N. R., Lim, G. J., Brunner, A. M., and Ranchalis, J. E. (1987). Molecular events in the processing of recombinant typel pre-pro- transforming Growth Factor-beta to mature polypeptide. *Mol. Cell Biol.* **7**, 3418–3427.
17. Miyazono, K., Ichijo, H., and Heldin, C. H. (1993). Growth Factor-beta: latent forms, binding proteins and receptor. *Transforming Growth Factor* **8**, 11–22.
18. Cheifetz, S., Hernandez, H., Laiho, M., Ten Dijke, P., Iwata, K. K., and Massagué, J. (1990). Distinct Transforming Growth Factor-beta receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *J. Biol. Chem.* **165**, 20,533–20,538.
19. Cheifetz, S., Ling, N., Guilemin, R., and Massague, J. (1988). A surface component on GH3 pituitary cells that recognizes Transforming Growth Factor-beta, actin and inhibitin. *J. Biol. Chem.* **263**, 17,225–17,228.
20. Lopez-Casillas, F., Chiefetz, S., Doody, J., Andres, J. L., Lane, W. S., and Massagué, J. (1991). Structure and expression the membrane proteoglycan betalycana component. *Cell* **67**, 785–795.
21. Lopez-Casillas, F., Wrana, J. L., and Massagué, J. (1993). Betaglycan presents ligand to the TGF signaling receptor. *Cell* **73**, 1435–1444.
22. Rodriguez, C., Chen, F., Weinberg, R. A., and Lodish, H. F. (1995). Cooperative binding of Transforming Growth Factor-beta2 to the TβR-I and II TGF-beta receptors. *J. Biol. Chem.* **270**, 15,919–15,922.
23. Sankar, M., Mahooti-Brooks, N., Centrella, M., McCarthy, T. H., and Madri, J. A. (1995). Expression of Transforming Growth Factor TβR-III receptor in vascular endothelial cells increase their responsiveness to transforming growth Factor 2. *J. Biol. Chem.* **270**, 2769–2775.

24. Kingsley, D. M. (1994). The TGF-β Superfamily: new members, new receptors, and new genetic tests of function in different organisms (review). *Genes Dev.* **8**, 133–146.
25. Wrana, J. L., Attisan, L., Wieser, R., Ventura, F., and Massagué, J. (1994). Mechanism of activation of the TGF-beta receptor. *Nature* **370**, 341–347.
26. Kim, W. S., Park, C., Jung, Y. S., et al. (1999). Reduced transforming growth factor-beta type II receptor (TGF-beta RII) expression in adenocarcinoma of the lung. *Anticancer Res.* **19**, 301–306.
27. Kim, I. Y., Ahn, H. J., Lang, S., et al. (1998). Loss of expression of transforming growth factor-β receotpors is associated with poor prognosis in prostate cancer patients. *Clin. Cancer Res.* **4**, 1625–1630.
28. Massagué, J. (2000). How cells read TGF-beta signals. *Nat. Rev. Mol. Cell Biol.* **1**, 169–178.
29. Massagué, J. (2000). TGF-beta signal in Growth Control, Cancer, and Heritalble Disorders. *Cell* **103**, 295–309.
30. Huse, M., Chen, Y. G., Massagué, J., and Kuriyan, J. (1999). Crystal structure of the cytoplasmic domain of the type I TGF-β receptor in complex with FKBP. *Cell* **96**, 425–436.
31. Lo, R. S. and Massagué, J. (1999). Ubiquitin-dependent degradation of TGF-β activated SMAD2. *Nat. Cell Biol.* **1**, 472–478.
32. De Caestecker, M. P., Piek, E., and Roberts, A. B. J. (2000). The role of TGF-β signaling in cancer. *Natl. Cancer Inst.,* **92**, 1388–1402.
33. Eilers, M., Schirm, S., and Bishop, J. M. (1991). The MYC protein activates transcription of the $-prothymosin gene *EMBO J.* **10**, 133–141.
34. Askew, D. S., Ashmun, R. A., Simmons, B. C., and Cleveland, J. L. (1991). Competitive expression in an IL-3 dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6**, 1915–1922.
35. Evan, G. I., Wyllie, A. H., Gilbert, C. S., et al. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119–128.
36. Henriksson, M. and Lüscher, B. (1996). Proteins of the myc network: essential regulators of cell growth and differentiation. *Cancer Res.* **68**, 109–182.
37. Lemaitre, J. M., Buckle, R. S., and Mechali, M. (1996). c-Myc in the control of cell proliferation and embryonic development. *Adv. Cancer Res.* **70**, 96–144.
38. Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of GI-phase progression. *Genes Dev.* **13**, 1501–1512.
39. Graham, A., Koentges, G., and Lumsden, A. (1996). Neural crest apoptosis and the establishment of cranofacial pattern: an honorable death. *Mol. Cell Neurosci.* **8**, 76–83.
40. Nguyen, A. V. and Pollard, J. W. (2000). Tansforming growth factor β 3 induces cell death during the first stage of mammary gland involution. *Devel.* **127**, 3107–3118.
41. Gold, L. L. (1999). The role for transforming growth factor β (TGF-β) in human cancer. *Crit. Rev. Oncog.* **10**, 303–360.
42. Kim, I. Y., Ahn, H. J., Zelner, D. J., et al. (1996). Loss of expression of Transforming Growth Factor-beta receptors type I and type II correlates with tumor grade in human prostate cancer tissues. *Clin. Cancer Res.* **2**, 1255–1261.
43. Prime, S. S., Davies, M., Pring, M., and Paterson, I. C. (2004). The role of TGF-beta in epithelial malignancy and its relevance to the pathogenesis of oral cancer (part II). *Crit. Rev. Oral. Biol. Med.* **15,** 337–347.
44. Markowitz, S., Wang, J., Myehoff, L., et al. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* **268,** 1336–1338.
45. Kang, S. H., Bang, Y. J., Im, Y. H., et al. (1999). Transcriptional repression of transforming growth factor beta tpe I receptor gene by DNA methylation results in the development of TGF-β resistance in gastric cancer. *Oncogene* **18,** 7280–7296.
46. Kim, S. J., Im, Y. H., Markowitz, S. D., and Bang, Y. J. (2000). Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev.* **11,** 159–168.
47. Osada, H., Tatematsu, Y., Masuda, A., et al. (2001). Heterogeneous transforming growth factor (TGF)-beta unresponsiveness and loss of TGF-beta receptor type II expression caused by histone deacetylation in lung cancer cell lines. *Cancer Res.* **61,** 8331–8339.
48. Seijo, E. R., Song, H., Lynch, M. A., et al. (2001). Identification of genetic alterations in the TGF-beta type II receptor gene promoter. *Mutat. Res.* **483,** 19–26.
49. Zhang, Q., Rubenstein, J. N., Jang, T. L., et al. (2005). Insensitivity to transforming growth factor-β signaling is resulted from promoter methylation of cognate receptors in human prostate cancer cells. *Mol. Endo.* **19**, 2390–2399.
50. Chen, T. C., Hinton, D. R., Yong, V. W., and Hofman, F. M. (1997). TGF-beta2 and soluble p55 TNFR modulate VCAM-1 expression in glioma cells and brain derived endothelial cells. *J. Neuroimmunol.* **73**, 155–161.
51. Xu, X. L., Brodie, S. G, Yang, X., et al. (2000). Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. *J. Virol.* **19**, 1868–1874.
52. Abou-Shady, M., Bear, H. U., Friess, H., et al. (1999). Transforming growth factor betas and their signaling receptors in Human hepatocellular carcinoma. *Am. J. Surg.* **177**, 209–215.
53. Steiner, M. S., Zhou, Z. Z., Tonb, D. C., and Barrack, E. R. (1994). Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology* **135,** 2240–2247.

54. Perry, K. T., Anthony, C. T., and Steiner, M. S. (1997). Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in normal and malignant human prostate. *Prostate* **33,** 133–140.
55. Wikstrom, P., Bergh, A., and Damber, J. E. (2000). Metastasis, Immunosuppression, transforming growth factor-beta 1 and Prostate Cancer (Review). *Scand. J. Urol. Nephrol.* **34,** 85–94.
56. Zhang, Q., Yang, X., Pins, M., et al. (2005). Adoptive transfer of tumor reactive TGF-β insensitive CD8+ T cells: Eradication of autologous mouse prostate cancer. *Cancer Res.* **65,** 1761–1769.
57. Barrack, E.R. (1997). An experimental model simulating local recurrence and pelvic lymph node metastasis following orthotopic induction of prostate cancer. *Prostate* **31**, 61–70.
58. Vieweg, J., Heston, W. D., Gilboa, E., and Fair, W. R. (1994). TGF-β on prostate cancer: a growth inhibitor that can enhance tumorigenicity. *Prostate* **24**, 291–298.
59. Steiner, M. S. and Barrack, E. R. (1992). Transforming growth factor-beta 1 overproduction in prostate cancer: effects on growth in vivo and in vitro. *Mol. Endocrinol.* **6**, 15–25.
60. Matthews, E., Yang, T., Janulis, L., et al. (2000). Down regulation of TGF-β1 production restores immunogenicity in prostate cancer cells. *Brit. J. Cancer* **83**, 519–525.
61. de Visser, K. E. and Kast, M. W. (1999). Effects of TGF-β on the immune system: implications for cancer immunotherapy. *Leukimia* **13,** 1188–1199.
62. Kao, J. Y., Gong, Y., Chen, C. M., Zheng, Q. D., and Chen, J. J. (2003). Tumor-derived TGF-β reduces the efficacy of dendritic cell/tumor fusion vaccine. *J. Immunol.* **170,** 3806–3811.
63. Hsieh, C. L., Chen, D. S., and Hwang, L. H. (2000). Tumor-induced immunosuppression: a barrier to immunotherapy of large tumor by cytokine-secreting tumor vaccine. *Hum. Gene Ther.* **11**, 681–692.
64. Scarpa, S., Coppa, A., and Ragano-Caracciolo, M. (1996). Transforming Growth Factor beta regulates differentiation and prolfieration of human neuroblastoma. *Exp. Cell Res.* **229**,147–154.
65. Poppema, S., Potters, M., Visser, L., and Van Den Berg, A. M. (1998). Immune escape mechanisms in Hodhkin's disease. *Ann. Oncol.* **9**(Suppl. 5), S21–S24.
66. Levinson, W. and Jawetz, E. (1998). *Medical Microbiology and Immunology*, Chapter 58. pp. 360–361.
67. Wojtowicz-Praga, S. (1997). Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. *J. Immunother.* **20**, 165–177.
68. Letterio, J. J. and Roberts, A. B. (1998). Regulation of immune responses by TGF-β. *Ann. Rev. Immunol.* **13**, 51–69.
69. Fortunel, N. O., Hatzfeld, A., and Hatzfeld, J. (2000). Transforming growth factor-β: pleiotropic role in the regulation of hematopoiesis. *Blood* **96**, 2022–2036.
70. Gorelik, L. and Flavell, R. A. (2000). Transforming growth factor-1 null mutationin mice causes excessive inflammatory response and early death. *Immunity.* **12**, 171–181.
71. Shah, A. H., Tabayoyong, W. B., Kimm S. Y., Kim, S. J., Parijs, L. V., and Lee, C. (2002). Selection of the T cell repertoire. *J. Immunol.* **169,** 3485–3491.
72. Won, J., Hongtae, K., Eun, J. P., Hong, Y., Kim, S. J., and Yun, Y. (1999). Influence of TGF-b on murine thymoctyes development in fetal thymus organ culture. *Cancer Res.* **9**, 1273–1277.
73. Fakhrai H., Dorigo O., Shawler D., et al. (1996). TGF-β down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. *Proc. Natl. Acad. Sci. USA* **93**, 2909–2914.
74. Kulkarni, A. B., Huh, C. G., Becker, D., et al. (1993). The host-tumor immune conflict: from immunosuppression to resistance and destruction. *Proc. Natl. Acad. Sci. USA* **90**,770–774.
75. Sebzda, E., Mariathasan, S., Ohteki, T., Jones, R., Bachmann, M. F., and Ohashi, P.S. (1999). A highly immunogenic tumor transfected with a murine transforming growth factor type β1 cDNA escapes immune surveillance. *Ann. Rev. Immunol.* **17**, 829–874.
76. Plum, J., De Smedt, M., Leclercq, G., and Vandekerckhove, B. (1995). Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *J. Immunol.* **154**, 5789–5798.
77. Tang, J., Nuccie, B. L., Ritterman, I., Liesveld, J. L., Abboud, C. N., and Ryan, D. H. (1997). Eradication of established intracranial rat gliomas by transforming growth factor b antisense gene therapy. *J. Immunol.* **159**, 117–125.
78. Chouaib, S., Asselin-Paturel, C., Mami-Chouaib, F., Caignard, A., and Blay, J. Y. (1997). Tumorigenicity of mouse thymona is suppressed by soluble type II transforming growth factor b receptor therapy. *Immunol. Today* **18**, 493–497.
79. Torre-Amione, G., Beauchamp, R. D., Koeppen, H., et al. (1990). Peripheral selection of the T cell repertoire. *Proc. Natl. Acad. Sci. USA.* **87**, 1486–1490.
80. Arteaga, C. L., Carty-Dugger, T., Moses, H., Hurd, S., and Pietenpol, J. (1993). Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-soecific triggering. *Cell Growth Differ.* **4,** 1647–1651.
81. Rocha, B. and von Boehmer, H. (1991). Involvement of endogenous tumor necrosis factor alpha and transforming growth factor beta during induction of collagen type II arthritis in mice. *Science* **251**, 1225–1228.
82. Miller, A., Lider, O., Roberts, A. B., Sporn, M. B., and Weiner, H. L. (1992). Transforming growth factor beta-1 (TGF-β1) controls statement of major histocompatibility genes in the post-natal mouse: Aberrant histocompatibility antigen statement in the pathogenesis of the TGF-β1 null mouse phenotype. *Proc. Natl. Acad. Sci. USA*, **89**, 421–425.

83. Thorbecke, G. J., Shah, R., Leu, C. H., Kuruvilla, A. P., Hardison, A. M., and Palladino, M. A. (1992). SLE-like autoantibodies and Sjogren's syndrome-like lymphoproliferation in TGF-β knockout mice. *Proc. Natl. Acad. Sci. USA.* **89**, 7375–7379.
84. Geiser, A. G., Letterio, J. J., Kulkarni, A. B., Karlsson, S., Roberts, A. B., and Sporn, M. B. (1993). T-cell apoptosis detected *in situ* during positive and negative selection in the thymus. *Proc. Natl. Acad. Sci. USA.* **90**, 9944–9948.
85. Dong, H., Geiser, A. G., Letterio, J. J., et al. (1995). Early progression of thymocytes along the CD4/CD8 development pathway is regulated by a subset of thymic epithelial cells statement transforming growth factor beta. *J. Immunol.* **155**, 3205–3212.
86. Surh, C. D. and Sprent, J. (1994). Animal models of autoimmunity and their relevance to human diseases. *Nature* **372**, 100–103.
87. Takahama, Y., Letterio, J. J., Suzuki, H., Farr, A. G., and Singer, A. (1994). Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *J. Exp. Med.* **179**, 1495–1506.
88. Sakaguchi, S. (2000). *Curr. Opin. Immunol.* **12**, 684–690.
89. Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A., and Weiner, H. L. (1994). Reconsitituion of lethally irradiated mice with TGF-β insensitive bone marrow leads to myeloid expansion and inflammatory disease. *Science* **265**, 1237–1231.
90. Shah, A. H., Tabayoyong, W. B., Kundu, S. D., et al. (2002). Suppression of tumor metastasis by blockade of TGF-β signaling in bone marrow cells through a retroviral mediated gene therapy in mice. *Cancer Res.* **62**, 7135–7138.
91. Bollard, C. M., Rossig, C. M., Calonge, J., et al. (2002). Adapting A TGF-β-related tumor protection strategy to enhance anti-tumor immunity. *Blood* **99**, 3179–3187.

11

Biology and Therapeutic Basis of Prostate Cancer Bone Metastasis

Evan T. Keller

Summary

Prostate cancer (CaP) is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the United States *(1)*. The most common site of CaP metastasis is the bone with skeletal metastases identified at autopsy in up to 90% of patients dying from CaP. Understanding how tumor interacts with bone may lead toward identifying therapies to prevent or diminish the consequences of bone metastases. CaP forms a mixture of osteolytic (excess bone resorption) and osteoblastic (excess bone production) bone metastases. The osteolytic component of bone metastases is caused, in part, through cancer-mediated activation of the receptor of nuclear factor-κ B ligand (RANKL) pathway. Blocking RANKL has been shown to successfully inhibit establishment or progression of CaP bone metastases in animal models. Other factors that are important in the development of CaP-induced osteolytic lesions include interleukin (IL)-6, parathyroid hormone-related protein (PTHrP), and matrix metalloproteinases (MMPs). The mechanism through which CaP induces osteoblastic lesions is less clear; however, CaP produces a variety of factors that may promote an overall osteoblastic phenotype, including an inhibitor of RANKL, osteoprotegerin (OPG); a vascular-active agent, endothelin (ET)-1; and transforming growth factor (TGF)-β. Several methods to target CaP bone metastases are being explored in clinics, including bisphosphonates, inhibitors of RANKL and ET-1. To effectively treat CaP bone metastases, targeting both the osteolytic and osteoblastic components simultaneously may be important.

Key Words: Bone metastases; endothelin-1; ET-1; IL-6; interleukin-6; OPG; osteoprotegerin; prostate cancer; parathyroid hormone-related protein; PTHrP; RANKL; receptor of nuclear factor-κ B.

1. INTRODUCTION

Prostate cancer (CaP) is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the United States *(1)*. The most common site of CaP metastasis is the bone, with skeletal metastases identified at autopsy in up to 90% of patients dying from CaP *(2–5)*. Skeletal metastasis in CaP patients result in significant complications that diminish the quality of life in affected patients. These complications include bone pain, impaired mobility, pathological fracture, spinal cord compression, and symptomatic hypercalcemia *(6–8)*. Despite advances in the diagnosis and management of CaP, advanced disease with skeletal metastasis remains incurable. Current therapeutic modalities are mostly palliative, and include hormonal therapy, pharmacological management of bone pain, radiotherapy for pain and spinal cord compression *(9)*, various chemotherapy regimens, and the use of bisphosphonates to inhibit osteoclast activity *(10)*. Despite the severe complications of CaP skeletal metastasis, there has not been much advancement in the therapeutic arena to prevent or diminish these lesions. It is critical that a solid understanding of the pathophysiology of CaP skeletal metastatic process is developed to provide the basis for creating strategies to prevent or diminish their occurrence and associated complications.

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

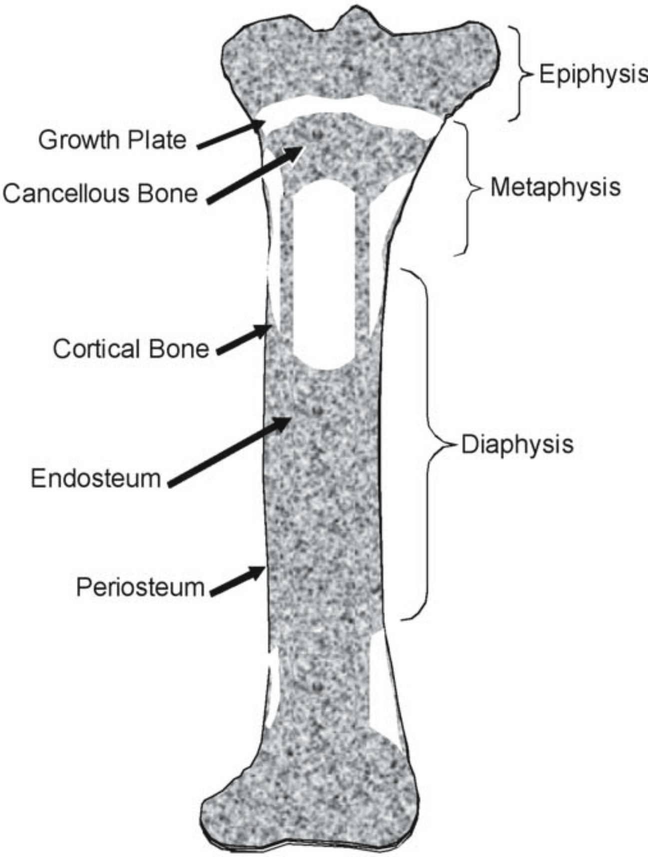


Fig. 1. Anatomy of bone. Longitudinal section of a long bone.

2. BONE BIOLOGY

The skeleton serves several important functions, including a structural framework for the body, a location for hematopoiesis, a calcium storage site that can respond to systemic needs for calcium, and an anchor for muscle attachments. The skeleton is divided into the axial skeleton, consisting of the skull, vertebral column, ribs, and sternum, and the appendicular skeleton, consisting of the limbs. The skeleton constantly remodels dynamically in response to stress placed on it *(11,12)*. A typical bone consists of both a cortical (also known as compact bone) component and a trabecular (also known as spongy or cancellous bone) component (Fig. 1). Cortical bone is made of concentric layers of bone that forms a hard outer shell and is typically found in the shafts of appendicular bone. Trabecular bone consists of struts of bone found within the concentric bone shell and is found through the meataphyses and epiphyses of bones.

Bone is a very active substance that is composed of mineralized and nonmineralized components *(13–15)*. Collagen is the main protein constituent of the unmineralized extracellular matrix (also known as osteoid) and is considered to provide the framework for mineralization. Other proteins that contribute to the osteoid include osteopontin, bone sialoprotein, osteonectin, and alkaline phosphatase *(16,17)*. Bone mineral consists of primarily calcium and phosphate that forms an organized crystalline structure called hydroxyapatite. When bone is initially formed, the collagen fibers are laid down in a disorganized interwoven fashion forming what is termed woven bone *(18)*. As bone matures, the collagen fibrils become lined up in a parallel fashion forming lamellar bone *(19,20)*. Woven bone is

not as strong in terms of biomechanical properties as lamellar bone *(21)*. As bone becomes more mineralized, its strength increases up to a point, but further mineralization beyond an optimal level reduces bone elasticity, leading to the inability of the bone to respond to certain stresses, which then results in fragile bone that is predisposed to fracture *(22–25)*.

Many cells contribute to formation of bone and regulation of bone remodeling, including neurons *(26,27)*, endothelial cells *(28,29)*, and myoblasts *(30)*. However, the key cells that are involved in bone production and remodeling are the osteoblasts and osteoclasts. Osteoblasts are the cells that lay down collagen matrix and promote mineralization. Once the osteoblast surrounds itself with mineral, its appearance changes, including development of neuron-like tendrils, and it is called an osteocyte *(31)*. Osteoblast production requires specific transcription factors. RUNX2 (also called Cbfa1 and OSF2), a member of the runt/Cbfa family of transcription factors, was first identified as the nuclear protein binding to an osteoblast-specific *cis*-acting element activating the expression of osteocalcin *(32)*. RUNX2 was shown to regulate the expression of all of the major genes expressed by osteoblasts and to be a key regulator of osteoblast differentiation in vivo *(33)*. In mice deficient of RUNX2, only an unmineralized cartilage framework of the skeleton is present. In addition to RUNX2, osterix is another osteoblast transcription factor that has been shown to be critical for bone formation *(34,35)*. Absence of osterix results in the inability of mice to form bone *(34)*.

In apposition to the osteoblasts, are the osteoclasts, which are multinucleated cells responsible for bone resorption that both dissolve the mineralized hydroxyapatite and degrade nonmineralized bone matrix *(36)*. Osteoclasts are derived from the colony-forming unit–granulocyte macrophage (CFU-GM) hematopoietic precursor cells. The CFU-GM undergoes a defined progression of maturation steps that ultimately result in fusion of the precursor cells into mature osteoclasts. Several factors promote osteoclastogenesis, including growth factors and cytokines. Both colony-stimulating factor-1 and interleukin (IL)-1 and IL-6 expand the osteoclast precursor pool. Tumor necrosis factor (TNF)-α promotes conversion of the promonocyte to a committed osteoclast precursor *(37)*.

Although several factors promote osteoclastogenesis, one factor that is required for production of mature osteoclasts is receptor activator of nuclear factor-κ B (NF-κB) ligand (RANKL). A member of the tumor necrosis factor family, RANKL is initially expressed by bone marrow stromal cells, osteoblasts, and activated T cells. RANKL is most commonly a membrane-anchored molecule; however, a small fraction of RANKL is released through proteolytic cleavage from the cell surface as a soluble 245-amino acid homotrimeric molecule *(38)*. Both soluble and membrane-bound RANKL promote osteoclast formation and activation by binding to receptor activator of NF-κB (RANK) on the osteoclast precursor membrane *(38–42)* that has the characteristics of a monocyte *(43)*. RANKL binding to RANK induces NF-κB and Fos activation *(44,45)*. Several lines of evidence demonstrate the importance of RANKL in osteoclastogenesis. For example, RANKL has been shown to induce osteoclastogenesis in vitro from CFU-GM *(46)*. Mice that are genetically engineered to overexpress RANKL or RANK are severely osteoporotic *(47)*. Additionally, mice that have had their *RANKL (48)* or *RANK (49)* gene deleted have no osteoclasts and are osteopetrotic.

In addition to RANKL and RANK, another key modulator of osteoclastogenesis is osteoprotegerin (OPG) (also known as osteoclastogenesis inhibitory factor) *(50,51)*. OPG serves as a decoy receptor that binds RANKL and, thus, blocks its ability to bind to RANK and induce osteoclastogenesis. In contrast to RANKL and RANK, whose expression is mainly restricted at low levels to the skeletal and immune systems, OPG is expressed in a variety of tissues, such as liver, lung, heart, kidney, stomach, intestines, skin, and calvaria in mice; and lung, heart, kidney, and placenta in human *(50,52–57)*. In bone, OPG is mainly produced by osteoblastic lineage cells, and its expression increases as the cells become more differentiated *(55,58,59)*. Several factors, including 1,25-dihydroxyvitamin D3, IL-1β, TNF-α, and bone morphogenetic protein (BMP)-2 induce *OPG* messenger RNA (mRNA) expression in human osteoblast cell lines *(55)*. Administration of recombinant OPG to normal rodents resulted in increased bone mass *(50,53)* and completely prevented ovariectomy-induced bone loss without apparent adverse skeletal and extraskeletal side effects *(50)*. Additionally, a single subcuta-

neous injection of OPG is effective in rapidly and profoundly reducing bone turnover for a sustained period in women *(60)*. In fact, based on this activity, the balance ratio of RANKL to OPG seems to be very important in controlling the overall activity (i.e., lysis vs no lysis) that will be observed *(47,59,61,62)*.

When initiating bone resorption, osteoclasts become polarized, and three distinct membrane domains appear: a ruffled border, a sealing zone, and a functional secretory domain *(36)*. The actin cytoskeleton forms an attachment ring at the sealing zone that anchors the osteoclast to the bone matrix. The ruffled border appears inside the sealing zone and vesicle transport to the ruffled border delivers hydrochloric acid and proteases to an area between the ruffled border and the bone surface called the resorption lacuna *(36)*. In this extracellular compartment, crystalline hydroxyapatite is dissolved by acid, and a mixture of proteases degrades the organic matrix. The degradation products of collagen and other matrix components are endocytosed, transported through the cell, and exocytosed through a functional secretory domain *(36,63,64)*.

Proteases that are important mediators of osteoclastic activity include cathepsin K and metalloproteinases. Cathepsin K can cleave bone proteins such as type I collagen, osteopontin, and osteonectin *(65)*. Overexpression of cathepsin K in the mouse results in accelerated bone turnover *(66)*; whereas knockout of cathepsin K results in retarded bone matrix degradation and osteopetrosis *(67)*. Several novel classes of cathepsin K inhibitors have been designed and may provide novel therapeutic agents to target bone resorption *(68,69)*. In addition to the proteases, acid is secreted from osteoclasts to resorb the mineralized matrix. Acid is believed to be secreted through vacuolar H(+)-ATPase-dependent pumps present on the osteoclasts ruffled membranes *(70)*. Several hormones regulate acid secretion, including parathyroid hormone (PTH), which increases acid secretion and calcitonin, which decreases acid secretion. Carbonic anhydrase II seems to be an important mediator of acid production because acetazolamide, a carbonic anhydrase inhibitor-based diuretic, can block bone resorption *(71)*. Another diuretic, indapamide, increased osteoblast proliferation and decreased bone resorption, at least in part, by decreasing osteoclast differentiation via a direct effect on hematopoietic precursors in vitro *(72)*.

2.1. Bone Remodeling

Bone remodeling is the cyclical replacement of old bone by new bone. Remodeling serves to maintain bones mechanical stability and allow it to perform its metabolic actions. In each cycle, a defined volume of bone is removed by osteoclastic resorption and subsequently replaced by osteoblastic formation at the same location (Fig. 2). Remodeling is carried out by elongated structures known as basic multicellular units (BMU; sometimes called bone metabolic units) that travel through or across the surface of bone. In the human, each BMU lasts about 6 months, with continued sequential recruitment of new osteoclasts and osteoblasts *(73–76)*. The BMU is initiated by osteoclasts resorbing old bone followed by osteoblasts synthesizing new bone in the resorption lacunae (areas resorbed by osteoclasts) *(77–79)*.

3. OSTEOBLASTIC BONE METASTASES

There are many challenges to determining the mechanisms that contribute to the selective development of CaP in bone *(80–82)*. These include mechanisms of homing and attachment to bone and invasion into bone. However, once in the bone, CaP tumors have pathobiology that seems to be somewhat unique to cancer skeletal metastases. Specifically, CaP skeletal metastases are most often radiographically characterized as osteoblastic (i.e., increased mineral density at the site of the lesion) as opposed to osteolytic. Other tumors, such as breast cancer, can form osteoblastic lesions; however, these occur less frequently *(83,84)*. Despite the radiographic osteoblastic appearance, it is clear from histological evidence that CaP metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions, although osteoblastic lesions predominate *(85–88)*. Recent evidence shows that osteo-

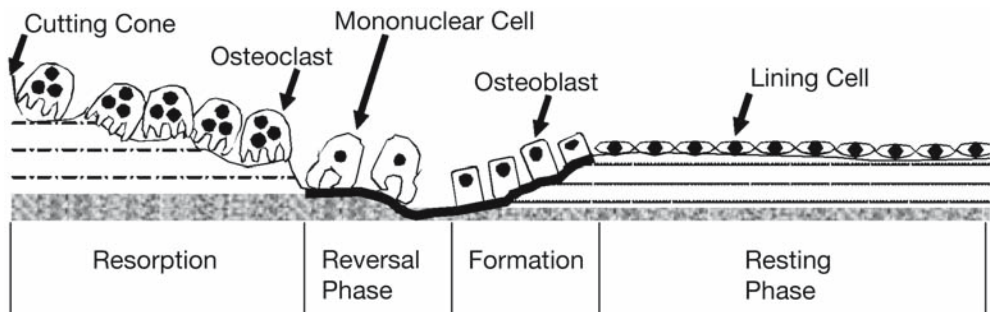


Fig. 2. Bone remodeling. Osteoclast initiation of bone resorption creating a leading edge, termed the cutting cone. Mononuclear cells behind the osteoclasts create a cement line that delimits the area of resorption. This is termed the reversal phase, which is then followed by osteoblasts that create new bone in the formation zone, followed by a resting phase in which the osteoblasts appear as flattened cells, termed lining cells.

blastic metastases form on trabecular bone at sites of previous osteoclastic resorption, and that such resorption may be required for subsequent osteoblastic bone formation *(89,90)*. These findings suggest that CaP induces bone production through an overall increase in bone remodeling, which, in the nonpathological state, is a balance between osteoclast resorption of bone, followed by osteoblast-mediated replacement of resorbed bone (reviewed in refs. *91–93*). In the case of CaP, it seems the induction of osteoblast-mediated mineralization outweighs the increase in osteoclast resorption, resulting in an overall formation of osteoblastic lesions. The osteoblastic lesions result in overall weakening of the bone because of the production of woven bone and hypermineralization *(22,81,94)*. Thus, the combination of underlying osteolysis and production of weak bone leads to a predisposition to fracture. The mechanisms through which CaP cells promote bone mineralization remain poorly understood.

CaP cells produce a variety of factors that have direct or indirect osteogenic properties (Table 1) (reviewed in refs. *95–97*). Some of these factors, such as BMP *(98–100)* and endothelin (ET)-1 *(101)* may directly stimulate differentiation of osteoblast precursors to mature mineral-producing osteoblasts. Other factors, such as PTH-related protein (PTHrP) may work through inhibition of osteoblast apoptosis *(102,103)*. Additionally, there are proteins that may work indirectly to enhance bone production, such as the serine proteases, prostate-specific antigen (PSA), and urinary plasminogen activator, which can activate latent forms of osteogenic proteins, such as transforming growth factor (TGF)-β *(104,105)*. Finally, some molecules, such as OPG *(50,106,107)* and ET-1 (in a dual role with its osteoblast-stimulating activity) *(108)* can enhance osteosclerosis through inhibiting osteoclastogenesis. Despite this gamut of putative mediators of CaP-induced osteosclerosis, we are unaware of in vivo studies that unequivocally demonstrate their role in this process. Other tumor types, such as osteosarcoma, are also known to produce a variety of osteoblastic factors *(109–111)*. With such a large number of factors, it is difficult to determine which is the most appropriate one to evaluate, and, in fact, several of these osteogenic factors most likely work in concert to produce maximal bone production.

4. OSTEOCLASTIC BONE METASTASES

RANKL is a key osteoclastogenic factor. Several lines of evidence support the role of RANKL in CaP-mediated osteolysis. Although a bone metastatic CaP cell line has been shown to express OPG *(112)*, that same line overexpresses RANKL *(113)*. Additionally, in normal prostate, OPG protein was detected in luminal epithelial and stromal cells (5–65% and 15–70%, respectively) and RANKL immunoreactivity was observed in 15 to 50% of basal epithelial cells, 40 to 90% of luminal epithelial

Table 1
Osteogenic Factors Produced by Cancer Cells

Factor	Reference
Bone morphogenetic proteins (BMP)	*(100,206)*
Endothelin (ET)-1	*(101,207–209)*
Insulin-like growth factors (IGF)	*(210,211)*
Interleukin (IL)-1 and IL-6	*(212,213)*
Osteoprotegerin (OPG)	*(106,107)*
Parathyroid hormone-related peptide (PTHrP)	*(102,103)*
Transforming growth factor (TFG)-β	*(105)*
Urinary plasminogen activator (urokinase)	*(214)*
Vascular endothelial growth factor (VEGF)	*(215)*

cells, and 70 to 100% of stromal cells *(114)*. OPG was not detected in 8 of 10 primary CaP specimens, but RANKL was heterogeneously expressed in 10 of 11 CaP specimens *(114)*. Importantly, the percentage of tumor cells expressing OPG and RANKL was significantly increased in all CaP bone metastases compared with non-osseous metastases or primary CaP. Serum OPG levels are elevated in patients with advanced CaP compared with less-advanced CaP *(115)*. However, RANKL levels were not measured in that study, thus, one cannot determine whether the ratio of RANKL to OPG was altered in these patients. It is possible that RANKL is only expressed locally at the skeletal metastatic site and, therefore, not detectable in the serum. Regardless, taken together, these observations suggest that the RANKL–OPG axis may play an important role in CaP bone metastases. Further support for this possibility was demonstrated by the observation that administration of OPG prevented establishment of mixed osteoblastic/osteolytic CaP cells in the bones of SCID mice, although it had no effect on establishment of subcutaneous tumors in the same mice *(113)*. However, in another study, OPG administration did not prevent establishment of an osteoblastic CaP tumor, although it slowed the tumor growth *(116)*. OPG also diminished the progression of established osteoblastic CaP in human bone implants in mice *(117)*. Taken together, these studies suggest that OPG can inhibit prostate tumor growth in bone.

Matrix metalloproteinases (MMPs), a family of enzymes whose primary function is to degrade the extracellular matrix, play a role in bone remodeling. This activity occurs in the absence of osteoclasts *(118)*, suggesting that MMPs have a direct resorptive effect. Several have the ability to degrade the nonmineralized matrix of bone, including MMP-1, MMP-9, and MMP-13, which are collagenases. Other MMPs, such as stromelysin (MMP-3) activate MMP-1. Through their proteolytic activity, MMPs contribute to metastatic invasion, including destruction of bone *(119)*.

Prostate carcinomas and their cell lines express a large number of MMPs *(120–127)*. Levels of MMP-9 secretion in primary CaP cultures increased with Gleason histological grade *(122)*. Active MMP-9 species were detected in 15 cultures (31%) of primary CaP tissues. The presence of the mineralized matrix has been shown to induce MMP-9 expression from prostate carcinoma cells *(128)*.

The initial functional data that suggested prostate carcinoma bone metastasis modulates bone remodeling through MMPs was provided by in vitro studies. Specifically, blocking MMP activity with 1,10-phenanthroline, a MMP inhibitor, diminished bone matrix degradation induced by PC-3 cells in vitro *(129,130)*. Matrilysin (MMP-7) has been shown to be upregulated in DU-145 CaP cells and can enhance their invasive ability. Monoclonal antibody targeting the cytokine IL-6 has been shown to increase promatrilysin expression in DU-145 cultures *(131)*. This suggests that IL-6, which is increased in CaP (reviewed in ref. *132*), enhances CaP invasion through production of MMP-7.

The importance of MMPs in bone metastasis has been further confirmed in vivo. An MMP inhibitor, batimastat, has been shown to inhibit development bone resorption in vitro and in vivo in murine models of breast *(133)* and prostate carcinoma *(134)*. The mechanism through which prostate carci-

noma-produced MMPs induce bone resorption is not clear; however, it seems to involve induction of osteoclastogenesis, because inhibition of MMPs reduced the number of osteoclasts associated with prostate tumor growth in human bone implants in mice *(134)*. Additionally, the bisphosphonate alendronate blocked MMP production from PC-3 cells *(135)*. This was associated with diminished establishment of bone metastasis in mice injected with PC-3 tumors *(118)*.

PTHrP, a protein with limited homology to PTH, was originally identified as a tumor-derived factor responsible for humoral hypercalcemia of malignancy. PTH and PTHrP bind to the same receptor (the PTH-1 receptor) and evoke the same biological activity because of similarities in their steric configurations at the region of amino acids 25 to 34. Patients with solid tumors and hypercalcemia have increased serum PTHrP in 80% of the cases, emphasizing the impact of this peptide to increase bone resorption and renal tubular resorption of calcium *(136)*. Subsequent to its characterization in humoral hypercalcemia of malignancy, PTHrP was found to be produced by many normal tissues, including epithelium, lactating mammary gland, and cartilage (in which it has an autocrine, paracrine, or intracrine role) *(136)*.

PTHrP is an attractive candidate for influencing prostate carcinoma growth. PTHrP is produced by normal prostate epithelial cells, from which prostate carcinoma arises, and PTHrP is found in the seminal fluid *(96,137)*. PTHrP has been immunohistochemically identified in prostate carcinoma tissue in patients with clinically localized disease *(138)*, is found in higher levels in prostate intraepithelial neoplasia than in normal prostate epithelium, is found in higher levels in prostate carcinoma than in benign prostatic hyperplasia *(139,140)*, and is found in human metastatic lesions in bone *(141)*. However, in some studies, expression of PTHrP receptor in CaP seems to be more consistent than expression of PTHrP itself *(142)*. Overexpression of *ras* oncogene in immortalized prostate epithelial cells has been shown to promote PTHrP expression *(143)*. This may account for the increased expression of PTHrP as the cells progress to a malignant phenotype.

There is evidence that PTHrP can regulate malignant tumor growth in an autocrine manner in human renal cell carcinoma *(144)*, enhance breast cancer metastasis to bone *(145,146)*, and act as an autocrine growth factor for prostate carcinoma cells in vitro *(137)*, although it does not effect proliferation of normal prostate cells *(147)*. Recent evidence indicates that expression of nuclear-targeted PTHrP can protect prostate and other cells from apoptosis *(141,148)*, bind RNA *(149)*, and act as a mitogen *(150,151)*. PTHrP production by primary prostatic tumors is associated with increased tumor size and rate of growth in an animal model *(141)*, suggesting that PTHrP acts in an autocrine or intracrine mechanism to promote tumor growth. In contrast, in this same model and in an intracardiac injection model of prostate carcinoma, PTHrP was not associated with an increase in metastatic potential *(94,141)*. This suggests that PTHrP is not important in the process of metastasis to bone but, once in the bone microenvironment, where target cells with receptors are present (osteoblasts), it may play a critical role in the bone response to prostate carcinoma. Of particular interest to prostate carcinoma, PSA has been shown to cleave PTHrP, leading to an inactivation of the PTHrP-stimulation of cAMP, which is a key pathway for the actions of PTHrP in bone *(152)*. Overexpression of PTHrP in CaP cells has been shown to induce osteolytic lesions in the bone of rats *(153)*, although the level of expression may not directly correlate with the degree of osteolysis *(94)*. All of these data suggest that PTHrP has a critical role in the local bone microenvironment of metastatic prostate carcinoma; but what this precise role is has yet to be determined.

IL-6 belongs to the "IL-6 type cytokine" family, which also includes leukemia inhibitory factor, IL-11, ciliary neurotrophic factor, cardiotrophin-1, and oncostatin M *(154)*. Many physiological functions are attributed to IL-6, including promotion of antibody production from B lymphocytes, modulation of hepatic acute phase reactant synthesis, promotion of osteoclastic-mediated bone resorption, and induction of thrombopoiesis *(155)*. IL-6 mediates its activity through the IL-6 receptor complex, which is composed of two components; an 80-kDa transmembrane receptor (IL-6Rp80, IL-6R, α-subunit) that specifically binds IL-6, but has no signaling capability, and a 130-kDa membrane glycoprotein (gp130) that mediates signal transduction after IL-6R binding *(156)*. In addition to the

transmembrane IL-6R, a soluble form of IL-6R (sIL-6R) exists that is produced by either proteolytic cleavage of the 80-kDa subunit *(157,158)* or differential splicing of mRNA *(159)*. Although the sIL-6R does not posses a transmembrane component, it can still bind to IL-6, and the ligand-bound sIL-6R·IL-6 complex activates signal transduction and biological responses through membrane-bound gp130 *(160)*.

Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic CaP *(161–163)*. Adler et al. *(161)* demonstrated that serum levels of IL-6 and TGF-β1 are elevated in patients with metastatic CaP, and that these levels correlate with tumor burden as assessed by serum PSA or clinically evident metastases. In a similar fashion, Drachenberg et al. *(164)* reported elevated serum IL-6 levels in men with hormone-refractory CaP compared with normal controls, benign prostatic hyperplasia, prostatitis, and localized or recurrent disease. In an animal model, prostate tumor cells injected next to human bones implanted in the limb of mice demonstrated IL-6 expression *(165)*. In addition to IL-6, the IL-6R has been identified in human normal prostate and prostate carcinoma tissue *(166,167)*.

The secretion of IL-6 by CaP cells in the bone microenvironment may impact bone remodeling (reviewed in refs. *168* and *169*). IL-6 promotes osteoclastogenesis *(170–172)*, most likely by increasing osteoclastogenic precursors. IL-6-mediated osteoclastogenesis is directly related to the level of gp130 present on the precursor cells *(173)*. It seems that IL-6-mediated osteoclastogenesis is independent of promoting RANKL expression *(174)*. However, IL-6 has been shown to potentiate PTHrP-induced osteoclastogenesis *(175,176)*. Administration of an antibody to IL-6 has been shown to diminish growth of subcutaneously injected CaP cells in nude mice, thus, demonstrating the potential usefulness of this compound in clinical CaP *(177)*. These results strongly suggest that IL-6 may serve as a therapeutic target for the osteolytic component of CaP skeletal metastases.

5. THERAPY OF CANCER-ASSOCIATED OSTEOLYSIS

Bone metastases are associated with several clinical sequelae, including bone pain, neuralgia, pathologic bone fracture, and myelophthisis. Thus, targeting these lesions has received much research effort. Bisphosphonates are a group of chemicals that inhibit osteoclast activity, resulting in decreased bone resorption and, thus, have received much attention as inhibitors of clinical complications of bone metastases *(178–180)*. Bisphosphonates work directly on osteoclasts to induce their apoptosis *(181,182)*. Animal studies have demonstrated that bisphosphonates can diminish tumor-induced osteoclastogenesis and osteolysis *(183–187)*. In some instances, they seem to only reduce tumor-induced lysis, but not tumor burden *(188)*. Studies in breast cancer and myeloma patients have shown that these agents markedly inhibit the progression of bone disease, resulting in improved survival and decreased morbidity from bone pain and fracture *(189,190)*. These results have led to their incorporation into standard treatment regimens for skeletal metastases associated with these cancers.

In addition to inhibiting osteoclast survival, bisphosphonates may have direct effects on tumor cells *(191)*. For example, several bisphosphonates induce apoptosis in myeloma cells *(192–194)*. However, this is not the case for all bisphosphonates *(195)*. In addition to inducing apoptosis, bisphosphonates have been shown to inhibit breast carcinoma cell adhesion to bone *(196)*. Furthermore, alendronate blocked collagen degradation and MMP release from CaP cells *(135,197)*. Taken together, these findings suggest that bisphosphonate action is not limited to inhibition of osteoclasts.

Studies of bisphosphonate use in patients with CaP skeletal metastases have generally shown a decrease in bone pain, although some studies have shown no benefit *(198–200)*. A recent randomized study of the oral bisphosphonate, clodronate, showed an encouraging decrease in the rate of progression to symptomatic bone metastases in men with CaP *(201)*. Consistent with this observation, is the finding that zoledronic acid is a third-generation bisphosphonate that has demonstrated significantly increased activity in preclinical models when compared with early agents in this class. Exposure of CaP cell lines to zoledronic acid results in marked inhibition of cell proliferation, suggesting that this

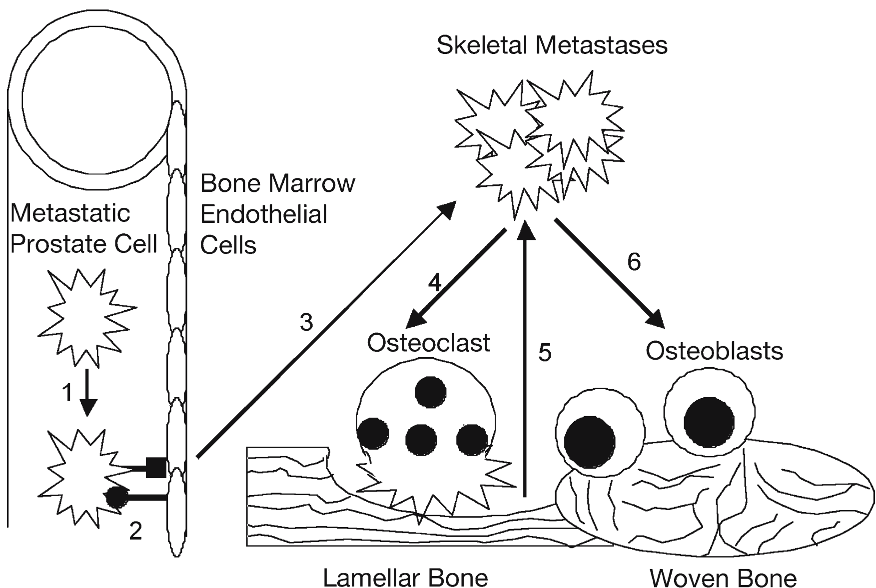


Fig. 3. Model of cross talk between prostate carcinoma cells and the bone microenvironment. The bone produces chemotactic factors that attract prostate carcinoma cells to migrate (1) through the vascular system toward the skeleton. The bone marrow endothelial displays adhesion molecules that are complement those expressed by the prostate carcinoma cell, resulting in attachment of the cell (2). The prostate carcinoma cell extravasates and invades into the skeletal extracellular tissue (3), at which point, it releases factors that stimulate osteoclastogenesis (4). The subsequent bone resorption is accompanied by release of growth factors that stimulate the prostate carcinoma proliferation (5). The progressing prostate carcinoma releases factors that promote osteoblast production and inhibit osteoblast apoptosis (6), resulting in production of woven bone and the characteristic osteosclerotic lesion. This process continues in a cyclical fashion, with continued induction of osteoclastic activity, carcinoma cell proliferation, and bone production.

agent may have a direct antitumor effect beyond its ability to inhibit osteoclast activity *(202,203)*. Zoledronic acid also has been shown to inhibit the invasion of prostate carcinoma cell lines in vitro *(204)*. Clinical studies have demonstrated efficacy in treating hypercalcemia of malignancy, leading to recent Food and Drug Administration (FDA) approval for use in this clinical setting *(205)*. Treatment with zoledronic acid results in a significant and sustained decrease in markers of bone metabolism.

6. SUMMARY

CaP metastases to bone are associated with a heterogenous mixture of grossly osteoblastic metastases that exist in parallel with osteoclastic activity. CaP induces exuberant mineralized weak bone production that results in pain and fracture. Through unknown mechanisms, CaP unbalances the normal bone remodeling cycle that is dependent on a balance between osteoclast and osteoblast activity. CaP cells seem to influence osteoclast activity through the RANKL–OPG pathway, as well as potentially through IL-6 and PTHrP. CaP cells also produce a variety of pro-osteoblastic activity, including BMPs, ET-1, and OPG. In turn, the bone produces factors that promote CaP growth (Fig. 3). Currently, targeting bone resorption with bisphosphonates has proven to reduce skeletal-related events in men with CaP bone metastases. However, there is much room for improving both our understanding of the biology of CaP bone metastases and therapies directed toward treating this debilitating aspect of CaP.

REFERENCES

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. (1999). Cancer statistics, 1999. *CA Cancer J. Clin.* **49**, 8–31.
2. Shah, R. B., Mehra, R., Chinnaiyan, A. M., et al. (2004). Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **64**, 9209–9216.
3. Abrams, H., Spiro, R., and Goldstein, N. (1950). Metastases in carcinoma. *Cancer* **3**, 74–85.
4. Bubendorf, L., Schopfer, A., Wagner, U., et al. (2000). Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. *Hum. Pathol.* **31**, 578–583.
5. Rana, A., Chisholm, G. D., Khan, M., Sekharjit, S. S., Merrick, M. V., and Elton, R. A. (1993). Patterns of bone metastasis and their prognostic significance in patients with carcinoma of the prostate. *Br. J. Urol.* **72**, 933–936.
6. Galasko, C. S. (1986). Skeletal metastases. *Clin. Orthop. Relat. Res.* **210**, 18–30.
7. Coleman, R. E. (1997). Skeletal complications of malignancy. *Cancer* **80**, 1588–1594.
8. Moul, J. W. and Lipo, D. R. (1999). Prostate cancer in the late 1990s: hormone refractory disease options. *Urol. Nurs.* **19**, 125–131; quiz 132–123.
9. Szostak, M. J. and Kyprianou, N. (2000). Radiation-induced apoptosis: predictive and therapeutic significance in radiotherapy of prostate cancer (review). *Oncol. Rep.* **7**, 699–706.
10. Papapoulos, S. E., Hamdy, N. A., and van der Pluijm, G. (2000). Bisphosphonates in the management of prostate carcinoma metastatic to the skeleton. *Cancer* **88**, 3047–3053.
11. Augat, P., Simon, U., Liedert, A., and Claes, L. (2005). Mechanics and mechano-biology of fracture healing in normal and osteoporotic bone. *Osteoporos. Int.* **16(Suppl 2)**, S36–S43.
12. Turner, C. H. and Pavalko, F. M. (1998). Mechanotransduction and functional response of the skeleton to physical stress: the mechanisms and mechanics of bone adaptation. *J. Orthop. Sci.* **3**, 346–355.
13. Hasegawa, K., Turner, C. H., and Burr, D. B. (1994). Contribution of collagen and mineral to the elastic anisotropy of bone. *Calcif. Tissue Int.* **55**, 381–386.
14. Frasca, P., Harper, R. A., and Katz, J. L. (1981). Scanning electron microscopy studies of collagen, mineral and ground substance in human cortical bone. *Scan. Electron. Microsc.* **Pt 3**, 339–346.
15. Burstein, A. H., Zika, J. M., Heiple, K. G., and Klein, L. (1975). Contribution of collagen and mineral to the elastic-plastic properties of bone. *J. Bone Joint Surg. Am.* **57**, 956–961.
16. Pinero, G. J., Farach-Carson, M. C., Devoll, R. E., Aubin, J. E., Brunn, J. C., and Butler, W. T. (1995). Bone matrix proteins in osteogenesis and remodelling in the neonatal rat mandible as studied by immunolocalization of osteopontin, bone sialoprotein, alpha 2HS-glycoprotein and alkaline phosphatase. *Arch. Oral. Biol.* **40**, 145–155.
17. Romanowski, R., Jundt, G., Termine, J. D., von der Mark, K., and Schulz, A. (1990). Immunoelectron microscopy of osteonectin and type I collagen in osteoblasts and bone matrix. *Calcif. Tissue Int.* **46**, 353–360.
18. Oguma, H., Murakami, G., Takahashi-Iwanaga, H., Aoki, M., and Ishii, S. (2001). Early anchoring collagen fibers at the bone-tendon interface are conducted by woven bone formation: light microscope and scanning electron microscope observation using a canine model. *J. Orthop. Res.* **19**, 873–880.
19. Pannarale, L., Braidotti, P., d'Alba, L., and Gaudio, E. (1994). Scanning electron microscopy of collagen fiber orientation in the bone lamellar system in non-decalcified human samples. *Acta Anat. (Basel)* **151**, 36–42.
20. Marotti, G., Muglia, M. A., and Palumbo, C. (1995). Collagen texture and osteocyte distribution in lamellar bone. *Ital. J. Anat. Embryol.* **100(Suppl. 1)**, 95–102.
21. Currey, J. D. (2003). The many adaptations of bone. *J. Biomech.* **36**, 1487–1495.
22. Currey, J. D. (2004). Tensile yield in compact bone is determined by strain, post-yield behaviour by mineral content. *J. Biomech.* **37**, 549–556.
23. Currey, J. (2004). Incompatible mechanical properties in compact bone. *J. Theor. Biol.* **231**, 569–580.
24. Currey, J. D. (1999). What determines the bending strength of compact bone? *J. Exp. Biol.* **202**, 2495–2503.
25. Zioupos, P., Currey, J. D., and Casinos, A. (2000). Exploring the effects of hypermineralisation in bone tissue by using an extreme biological example. *Connect. Tissue Res.* **41**, 229–248.
26. Lerner, U. H. (2002). Neuropeptidergic regulation of bone resorption and bone formation. *J. Musculoskelet. Neuronal Interact.* **2**, 440–447.
27. Chenu, C. (2004). Role of innervation in the control of bone remodeling. *J. Musculoskelet. Neuronal Interact.* **4**, 132–134.
28. Colnot, C. I. and Helms, J. A. (2001). A molecular analysis of matrix remodeling and angiogenesis during long bone development. *Mech. Dev.* **100**, 245–250.
29. Gerber, H. P., Vu, T. H., Ryan, A. M., Kowalski, J., Werb, Z., and Ferrara, N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med.* **5**, 623–628.
30. Kiliaridis, S., Engstrom, C., Lindskog-Stokland, B., and Katsaros, C. (1996). Craniofacial bone remodeling in growing rats fed a low-calcium and vitamin-D-deficient diet and the influence of masticatory muscle function. *Acta Odontol. Scand.* **54**, 320–326.
31. Bonewald, L. F. (2004). Osteocyte biology: its implications for osteoporosis. *J. Musculoskelet. Neuronal Interact.* **4**, 101–104.

32. Schinke, T. and Karsenty, G. (1999). Characterization of Osf1, an osteoblast-specific transcription factor binding to a critical cis-acting element in the mouse Osteocalcin promoters. *J. Biol. Chem.* **274**, 30,182–30,189.
33. Ducy, P. (2000). Cbfa1: a molecular switch in osteoblast biology. *Dev. Dyn.* **219**, 461–471.
34. Nakashima, K., Zhou, X., Kunkel, G., et al. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17–29.
35. Lee, M. H., Kwon, T. G., Park, H. S., Wozney, J. M., and Ryoo, H. M. (2003). BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem. Biophys. Res. Commun.* **309**, 689–694.
36. Vaananen, H. K., Zhao, H., Mulari, M., and Halleen, J. M. (2000). The cell biology of osteoclast function. *J. Cell. Sci.* **113(Pt 3)**, 377–381.
37. Uy, H. L., Mundy, G. R., Boyce, B. F., et al. A. (1997). Tumor necrosis factor enhances parathyroid hormone-related protein-induced hypercalcemia and bone resorption without inhibiting bone formation in vivo. *Cancer Res.* **57**, 3194–3199.
38. Lum, L., Wong, B. R., Josien, R., et al. (1999). Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. *J. Biol. Chem.* **274**, 13,613–13,618.
39. Lacey, D. L., Timms, E., Tan, H. L., et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**, 165–176.
40. Kong, Y. Y., Yoshida, H., Sarosi, I., et al. (1999). OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**, 315–323.
41. Yasuda, H., Shima, N., Nakagawa, N., et al. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA* **95**, 3597–3602.
42. Fuller, K., Wong, B., Fox, S., Choi, Y., and Chambers, T. J. (1998). TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J. Exp. Med.* **188**, 997–1001.
43. Shalhoub, V., Elliott, G., Chiu, L., et al. (2000). Characterization of osteoclast precursors in human blood. *Br. J. Haematol.* **111**, 501–512.
44. Matsuo, K., Owens, J. M., Tonko, M., Elliott, C., Chambers, T. J., and Wagner, E. F. (2000). Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation. *Nat. Genet.* **24**, 184–187.
45. Hofbauer, L. C. and Heufelder, A. E. (2001). The role of osteoprotegerin and receptor activator of nuclear factor kappaB ligand in the pathogenesis and treatment of rheumatoid arthritis. *Arthritis Rheum.* **44**, 253–259.
46. Menaa, C., Kurihara, N., and Roodman, G. D. (2000). CFU-GM-derived cells form osteoclasts at a very high efficiency. *Biochem. Biophys. Res. Commun.* **267**, 943–946.
47. Hofbauer, L. C., Neubauer, A., and Heufelder, A. E. (2001). Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases. *Cancer* **92**, 460–470.
48. Pettit, A. R., Ji, H., von Stechow, D., et al. (2001). TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. *Am. J. Pathol.* **159**, 1689–1699.
49. Li, J., Sarosi, I., Yan, X. Q., et al. (2000). RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl. Acad. Sci. USA* **97**, 1566–1571.
50. Simonet, W. S., Lacey, D. L., Dunstan, C. R., et al. (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**, 309–319.
51. Tsuda, E., Goto, M., Mochizuki, S., et al. (1997). Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem. Biophys. Res. Commun.* **234**, 137–142.
52. Tan, K. B., Harrop, J., Reddy, M., et al. (1997). Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. *Gene* **204**, 35–46.
53. Yasuda, H., Shima, N., Nakagawa, N., et al. (1998). Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* **139**, 1329–1337.
54. Yun, T. J., Chaudhary, P. M., Shu, G. L., et al. (1998). OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. *J. Immunol.* **161**, 6113–6121.
55. Hofbauer, L. C., Dunstan, C. R., Spelsberg, T. C., Riggs, B. L., and Khosla, S. (1998). Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines. *Biochem. Biophys. Res. Commun.* **250**, 776–781.
56. Hofbauer, L. C. and Heufelder, A. E. (1998). Osteoprotegerin and its cognate ligand: a new paradigm of osteoclastogenesis. *Eur. J. Endocrinol.* **139**, 152–154.
57. Vidal, N. O., Brandstrom, H., Jonsson, K. B., and Ohlsson, C. (1998). Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids. *J. Endocrinol.* **159**, 191–195.
58. Kartsogiannis, V., Zhou, H., Horwood, N. J., et al. (1999). Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. *Bone* **25**, 525–534.

59. Nagai, M. and Sato, N. (1999). Reciprocal gene expression of osteoclastogenesis inhibitory factor and osteoclast differentiation factor regulates osteoclast formation. *Biochem. Biophys. Res. Commun.* **257**, 719–723.
60. Bekker, P. J., Holloway, D., Nakanishi, A., Arrighi, M., Leese, P. T., and Dunstan, C. R. (2001). The effect of a single dose of osteoprotegerin in postmenopausal women. *J. Bone Miner. Res.* **16**, 348–360.
61. Thomas, G. P., Baker, S. U., Eisman, J. A., and Gardiner, E. M. (2001). Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts. *J. Endocrinol.* **170**, 451–460.
62. Fazzalari, N. L., Kuliwaba, J. S., Atkins, G. J., Forwood, M. R., and Findlay, D. M. (2001). The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. *J. Bone Miner. Res.* **16**, 1015–1027.
63. Roodman, G. D. (1999). Cell biology of the osteoclast. *Exp. Hematol.* **27**, 1229–1241.
64. Blair, H. C. and Athanasou, N. A. (2004). Recent advances in osteoclast biology and pathological bone resorption. *Histol. Histopathol.* **19**, 189–199.
65. Katunuma, N. (1997). Mechanism and regulation of bone resorption by osteoclasts. *Curr. Top. Cell. Regul.* **35**, 179–192.
66. Kiviranta, R., Morko, J., Uusitalo, H., Aro, H. T., Vuorio, E., and Rantakokko, J. (2001). Accelerated turnover of metaphyseal trabecular bone in mice overexpressing cathepsin K. *J. Bone Miner. Res.* **16**, 1444–1452.
67. Gowen, M., Lazner, F., Dodds, R., et al. (1999). Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. *J. Bone Miner. Res.* **14**, 1654–1663.
68. Yamashita, D. S. and Dodds, R. A. (2000). Cathepsin K and the design of inhibitors of cathepsin K. *Curr. Pharm. Des.* **6**, 1–24.
69. Stroup, G. B., Lark, M. W., Veber, D. F., et al. (2001). Potent and selective inhibition of human cathepsin K leads to inhibition of bone resorption in vivo in a nonhuman primate. *J. Bone. Miner. Res.* **16**, 1739–1746.
70. Lee, B. S., Holliday, L. S., Ojikutu, B., Krits, I., and Gluck, S. L. (1996). Osteoclasts express the B2 isoform of vacuolar H(+)-ATPase intracellularly and on their plasma membranes. *Am. J. Physiol.* **270**, C382–C388.
71. Lehenkari, P., Hentunen, T. A., Laitala-Leinonen, T., Tuukkanen, J., and Vaananen, H. K. (1998). Carbonic anhydrase II plays a major role in osteoclast differentiation and bone resorption by effecting the steady state intracellular pH and Ca2+. *Exp. Cell. Res.* **242**, 128–137.
72. Lalande, A., Roux, S., Denne, M. A., et al. (2001). Indapamide, a thiazide-like diuretic, decreases bone resorption in vitro. *J. Bone Miner. Res.* **16**, 361–370.
73. Rodan, G. A. (2003). The development and function of the skeleton and bone metastases. *Cancer* **97**, 726–732.
74. Parfitt, A. M. (2001). The bone remodeling compartment: a circulatory function for bone lining cells. *J. Bone Miner. Res.* **16**, 1583–1585.
75. Parfitt, A. M. (2004). What is the normal rate of bone remodeling? *Bone* **35**, 1–3.
76. Parfitt, A. M. (1995). Bone remodeling, normal and abnormal: a biological basis for the understanding of cancer-related bone disease and its treatment. *Can. J. Oncol.* **5(Suppl 1)**, 1–10.
77. Boyce, B. F., Xing, L., Shakespeare, W., et al. (2003). Regulation of bone remodeling and emerging breakthrough drugs for osteoporosis and osteolytic bone metastases. *Kidney Int.* **Suppl** S2–S5.
78. Migliaccio, S., Falcone, S., and Spera, G. (2004). Bone modeling and remodeling: from biology to clinical application. *Aging Clin. Exp. Res.* **16(Suppl.)**, 20–22.
79. Pogoda, P., Priemel, M., Rueger, J. M., and Amling, M. (2005). Bone remodeling: new aspects of a key process that controls skeletal maintenance and repair. *Osteoporos. Int.* **16(Suppl. 2)**, S18–S24.
80. Lange, P. H. and Vessella, R. L. (1998). Mechanisms, hypotheses and questions regarding prostate cancer micrometastases to bone. *Cancer Metastasis Rev.* **17**, 331–336.
81. Rosol, T. J. (2000). Pathogenesis of bone metastases: role of tumor-related proteins. *J. Bone Miner. Res.* **15**, 844–850.
82. Keller, E. T., Zhang, J., Cooper, C. R., et al. (2001). Prostate carcinoma skeletal metastases: cross-talk between tumor and bone. *Cancer Metastasis Rev.* **20**, 333–349.
83. Yamashita, K., Aoki, Y., and Hiroshima, K. (1996). Metastatic epidural bony tumor causing spinal cord compression: a case report. *Clin. Orthop. Relat. Res.* **34**, 231–235.
84. Munk, P. L., Poon, P. Y., O'Connell, J. X., et al. (1997). Osteoblastic metastases from breast carcinoma with false-negative bone scan. *Skeletal Radiol.* **26**, 434–437.
85. Berruti, A., Piovesan, A., Torta, M., et al. (1996). Biochemical evaluation of bone turnover in cancer patients with bone metastases: relationship with radiograph appearances and disease extension. *Br. J. Cancer* **73**, 1581–1587.
86. Vinholes, J., Coleman, R., and Eastell, R. (1996). Effects of bone metastases on bone metabolism: implications for diagnosis, imaging and assessment of response to cancer treatment. *Cancer Treat. Rev.* **22**, 289–331.
87. Urwin, G. H., Percival, R. C., Harris, S., Beneton, M. N., Williams, J. L., and Kanis, J. A. (1985). Generalised increase in bone resorption in carcinoma of the prostate. *Br. J. Urol.* **57**, 721–723.
88. Roudier, M., Sherrard, D., True, L., et al. (2000). Heterogenous bone histomorphometric patterns in metastatic prostate cancer. *J. Bone Miner. Res.* **15S1**, S567.
89. Carlin, B. I. and Andriole, G. L. (2000). The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. *Cancer* **88**, 2989–2994.

90. Zhang, J., Devalaraja, R., Dai, J., Baragi, V., and Keller, E. T. (2000). Osteoprotegerin (OPG) and anti-interleukin-6 (IL-6) antibody inhibit osteoclastogenesis induced by prostate cancer cells in vitro. *J. Bone Miner. Res.* **15S1**, S209.
91. Karsenty, G. (2000). Bone formation and factors affecting this process. *Matrix Biol.* **19**, 85–89.
92. Parfitt, A. M. (2000). The mechanism of coupling: a role for the vasculature. *Bone* **26**, 319–323.
93. Boyce, B. F., Hughes, D. E., Wright, K. R., Xing, L., and Dai, A. (1999). Recent advances in bone biology provide insight into the pathogenesis of bone diseases. *Lab. Invest.* **79**, 83–94.
94. Blomme, E. A., Dougherty, K. M., Pienta, K. J., Capen, C. C., Rosol, T. J., and McCauley, L. K. (1999). Skeletal metastasis of prostate adenocarcinoma in rats: morphometric analysis and role of parathyroid hormone-related protein. *Prostate* **39**, 187–197.
95. Boyce, B. F., Yoneda, T., and Guise, T. A. (1999). Factors regulating the growth of metastatic cancer in bone. *Endocr. Relat. Cancer* **6**, 333–347.
96. Deftos, L. J. (2000). Prostate carcinoma: production of bioactive factors. *Cancer* **88**, 3002–3008.
97. Yoneda, T. (1998). Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. *Eur. J. Cancer* **34**, 240–245.
98. Autzen, P., Robson, C. N., Bjartell, A., et al. (1998). Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. *Br. J. Cancer* **78**, 1219–1223.
99. Harris, S. E., Harris, M. A., Mahy, P., Wozney, J., Feng, J. Q., and Mundy, G. R. (1994). Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. *Prostate* **24**, 204–211.
100. Hullinger, T. G., Taichman, R. S., Linseman, D. A., and Somerman, M. J. (2000). Secretory products from PC-3 and MCF-7 tumor cell lines upregulate osteopontin in MC3T3-E1 cells. *J. Cell Biochem.* **78**, 607–616.
101. Nelson, J. B., Hedican, S. P., George, D. J., et al. (1995). Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat. Med.* **1**, 944–949.
102. Karaplis, A. C. and Vautour, L. (1997). Parathyroid hormone-related peptide and the parathyroid hormone/parathyroid hormone-related peptide receptor in skeletal development. *Curr. Opin. Nephrol. Hypertens.* **6**, 308–313.
103. Cornish, J., Callon, K. E., Lin, C., Xiao, C., Moseley, J. M., and Reid, I. R. (1999). Stimulation of osteoblast proliferation by C-terminal fragments of parathyroid hormone-related protein. *J. Bone Miner. Res.* **14**, 915–922.
104. Rabbani, S. A., Gladu, J., Mazar, A. P., Henkin, J., and Goltzman, D. (1997). Induction in human osteoblastic cells (SaOS2) of the early response genes fos, jun, and myc by the amino terminal fragment (ATF) of urokinase. *J. Cell Physiol.* **172**, 137–145.
105. Killian, C. S., Corral, D. A., Kawinski, E., and Constantine, R. I. (1993). Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF-beta and a proteolytic modulation of cell adhesion receptors. *Biochem. Biophys. Res. Commun.* **192**, 940–947.
106. Honore, P., Luger, N. M., Sabino, M. A., et al. (2000). Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. *Nat. Med.* **6**, 521–528.
107. Guise, T. A. (2000). Molecular mechanisms of osteolytic bone metastases. *Cancer* **88**, 2892–2898.
108. Chiao, J. W., Moonga, B. S., Yang, Y. M., et al. (2000). Endothelin-1 from prostate cancer cells is enhanced by bone contact which blocks osteoclastic bone resorption. *Br. J. Cancer* **83**, 360–365.
109. Laitinen, M., Marttinen, A., Aho, A. J., and Lindholm, T. S. (1998). Bone morphogenetic protein in bone neoplasms: comparison of different detection methods. *Eur. Surg. Res.* **30**, 168–174.
110. Raval, P., Hsu, H. H., Schneider, D. J., et al. (1996). Expression of bone morphogenetic proteins by osteoinductive and non- osteoinductive human osteosarcoma cells. *J. Dent. Res.* **75**, 1518–1523.
111. Wlosarski, K. and Reddi, A. H. (1987). Tumor cells stimulate in vivo periosteal bone formation. *Bone Miner.* **2**, 185–192.
112. Lin, D. L., Tarnowski, C. P., Zhang, J., et al. (2001). Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes in vitro. *Prostate* **47**, 212–221.
113. Zhang, J., Dai, J., Qi, Y., et al. (2001). Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. *J. Clin. Invest.* **107**, 1235–1244.
114. Brown, J. M., Corey, E., Lee, Z. D., et al. (2001). Osteoprotegerin and rank ligand expression in prostate cancer. *Urology* **57**, 611–616.
115. Brown, J. M., Vessella, R. L., Kostenuik, P. J., Dunstan, C. R., Lange, P. H., and Corey, E. (2001). Serum osteoprotegerin levels are increased in patients with advanced prostate cancer. *Clin. Cancer Res.* **7**, 2977–2983.
116. Kiefer, J. A., Vessella, R. L., Quinn, J. E., et al. (2004). The effect of osteoprotegerin administration on the intra-tibial growth of the osteoblastic LuCaP 23.1 prostate cancer xenograft. *Clin. Exp. Metastasis* **21**, 381–387.
117. Zhang, J., Dai, J., Yao, Z., Lu, Y., Dougall, W., and Keller, E. T. (2003). Soluble receptor activator of nuclear factor kappaB Fc diminishes prostate cancer progression in bone. *Cancer Res.* **63**, 7883–7890.
118. Stearns, M. E. and Wang, M. (1996). Effects of alendronate and taxol on PC-3 ML cell bone metastases in SCID mice. *Invasion Metastasis* **16**, 116–131.
119. John, A. and Tuszynski, G. (2001). The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. *Pathol. Oncol. Res.* **7**, 14–23.

120. Boag, A. H. and Young, I. D. (1993). Immunohistochemical analysis of type IV collagenase expression in prostatic hyperplasia and adenocarcinoma. *Mod. Pathol.* **6**, 65–68.
121. Bodey, B., Bodey, B., Jr., Siegel, S. E., and Kaiser, H. E. (2001). Immunocytochemical detection of matrix metalloproteinase expression in prostate cancer. *In Vivo* **15**, 65–70.
122. Festuccia, C., Bologna, M., Vicentini, C., et al. (1996). Increased matrix metalloproteinase-9 secretion in short-term tissue cultures of prostatic tumor cells. *Int. J. Cancer* **69**, 386–393.
123. Hamdy, F. C., Fadlon, E. J., Cottam, D., et al. (1994). Matrix metalloproteinase 9 expression in primary human prostatic adenocarcinoma and benign prostatic hyperplasia. *Br. J. Cancer* **69**, 177–182.
124. Hashimoto, K., Kihira, Y., Matuo, Y., and Usui, T. (1998). Expression of matrix metalloproteinase-7 and tissue inhibitor of metalloproteinase-1 in human prostate. *J. Urol.* **160**, 1872–1876.
125. Montironi, R., Fabris, G., Lucarini, G., and Biagini, G. (1995). Location of 72-kd metalloproteinase (type IV collagenase) in untreated prostatic adenocarcinoma. *Pathol. Res. Pract.* **191**, 1140–1146.
126. Montironi, R., Lucarini, G., Castaldini, C., Galluzzi, C. M., Biagini, G., and Fabris, G. (1996). Immunohistochemical evaluation of type IV collagenase (72-kd metalloproteinase) in prostatic intraepithelial neoplasia. *Anticancer Res.* **16**, 2057–2062.
127. Pajouh, M. S., Nagle, R. B., Breathnach, R., Finch, J. S., Brawer, M. K., and Bowden, G. T. (1991). Expression of metalloproteinase genes in human prostate cancer. *J. Cancer Res. Clin. Oncol.* **117**, 144–150.
128. Festuccia, C., Giunciuglio, D., Guerra, F., et al. (1999). Osteoblasts modulate secretion of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) in human prostate cancer cells promoting migration and matrigel invasion. *Oncol. Res.* **11**, 17–31.
129. Duivenvoorden, W. C., Hirte, H. W., and Singh, G. (1997). Use of tetracycline as an inhibitor of matrix metalloproteinase activity secreted by human bone-metastasizing cancer cells. *Invasion Metastasis* **17**, 312–322.
130. Sanchez-Sweatman, O. H., Orr, F. W., and Singh, G. (1998). Human metastatic prostate PC3 cell lines degrade bone using matrix metalloproteinases. *Invasion Metastasis* **18**, 297–305.
131. Stratton, M. S., Sirvent, H., Udayakumar, T. S., Nagle, R. B., and Bowden, G. T. (2001). Expression of the matrix metalloproteinase promatrilysin in coculture of prostate carcinoma cell lines. *Prostate* **48**, 206–209.
132. Smith, P. C., Hobish, A., Lin, D., Culig, Z., and Keller, E. T. (2001). Interleukin-6 and prostate cancer progression. *Cytokine Growth Factor Rev.* **12**, 33–40.
133. Lee, J., Weber, M., Mejia, S., Bone, E., Watson, P., and Orr, W. (2001). A matrix metalloproteinase inhibitor, batimastat, retards the development of osteolytic bone metastases by MDA-MB-231 human breast cancer cells in Balb C nu/nu mice. *Eur. J. Cancer* **37**, 106–113.
134. Nemeth, J. A., Yousif, R., Herzog, M., et al. (2002). Matrix metalloproteinase activity, bone matrix turnover, and tumor cell proliferation in prostate cancer bone metastasis. *J. Natl. Cancer Inst.* **94**, 17–25.
135. Stearns, M. E. and Wang, M. (1998). Alendronate blocks metalloproteinase secretion and bone collagen I release by PC-3 ML cells in SCID mice. *Clin. Exp. Metastasis* **16**, 693–702.
136. Strewler, G. J. (2000). The physiology of parathyroid hormone-related protein. *N. Engl. J. Med.* **342**, 177–185.
137. Iwamura, M., Abrahamsson, P. A., Foss, K. A., Wu, G., Cockett, A. T., and Deftos, L. J. (1994). Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. *Urology* **43**, 675–679.
138. Iwamura, M., di Sant'Agnese, P. A., Wu, G., et al. (1993). Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. *Cancer Res.* **53**, 1724–1726.
139. Asadi, F., Farraj, M., Sharifi, R., Malakouti, S., Antar, S., and Kukreja, S. (1996). Enhanced expression of parathyroid hormone-related protein in prostate cancer as compared with benign prostatic hyperplasia. *Hum. Pathol.* **27**, 1319–1323.
140. Iwamura, M., Gershagen, S., Lapets, O., et al. (1995). Immunohistochemical localization of parathyroid hormone-related protein in prostatic intraepithelial neoplasia. *Hum. Pathol.* **26**, 797–801.
141. Dougherty, K. M., Blomme, E. A., Koh, A. J., et al. (1999). Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. *Cancer Res.* **59**, 6015–6022.
142. Iddon, J., Bundred, N. J., Hoyland, J., et al. (2000). Expression of parathyroid hormone-related protein and its receptor in bone metastases from prostate cancer. *J. Pathol.* **191**, 170–174.
143. Kremer, R., Goltzman, D., Amizuka, N., Webber, M. M., and Rhim, J. S. (1997). ras Activation of human prostate epithelial cells induces overexpression of parathyroid hormone-related peptide. *Clin. Cancer Res.* **3**, 855–859.
144. Burton, P. B., Moniz, C., and Knight, D. E. (1990). Parathyroid hormone related peptide can function as an autocrine growth factor in human renal cell carcinoma. *Biochem. Biophys. Res. Commun.* **167**, 1134–1138.
145. Bouizar, Z., Spyratos, F., and De vernejoul, M. C. (1999). The parathyroid hormone-related protein (PTHrP) gene: use of downstream TATA promotor and PTHrP 1-139 coding pathways in primary breast cancers vary with the occurrence of bone metastasis. *J. Bone Miner. Res.* **14**, 406–414.
146. Yin, J. J., Selander, K., Chirgwin, J. M., et al. (1999). TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J. Clin. Invest.* **103**, 197–206.
147. Peehl, D. M., Edgar, M. G., Cramer, S. D., and Deftos, L. J. (1997). Parathyroid hormone-related protein is not an autocrine growth factor for normal prostatic epithelial cells. *Prostate* **31**, 47–52.

148. Henderson, J. E., Amizuka, N., Warshawsky, H., et al. (1995). Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Mol. Cell Biol.* **15**, 4064–4075.
149. Aarts, M. M., Rix, A., Guo, J., Bringhurst, R., and Henderson, J. E. (1999). The nucleolar targeting signal (NTS) of parathyroid hormone related protein mediates endocytosis and nucleolar translocation. *J. Bone Miner. Res.* **14**, 1493–1503.
150. Ye, Y., Wang, C., Du, P., Falzon, M., Seitz, P. K., and Cooper, C. W. (2001). Overexpression of parathyroid hormone-related protein enhances apoptosis in the rat intestinal cell line, IEC-6. *Endocrinology* **142**, 1906–1914.
151. Massfelder, T., Dann, P., Wu, T. L., Vasavada, R., Helwig, J. J., and Stewart, A. F. (1997). Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. *Proc. Natl. Acad. Sci. USA* **94**, 13,630–13,635.
152. Cramer, S. D., Chen, Z., and Peehl, D. M. (1996). Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. *J. Urol.* **156**, 526–531.
153. Rabbani, S. A., Gladu, J., Harakidas, P., Jamison, B., and Goltzman, D. (1999). Over-production of parathyroid hormone-related peptide results in increased osteolytic skeletal metastasis by prostate cancer cells in vivo. *Int. J. Cancer* **80**, 257–264.
154. Sehgal, P., Wang, L., Rayanade, R., Pan, H., and Margulies, L. (1995). Interleukin-6 type cytokines (Mackiewicz, A. Koji, A., and Sehgal, P., eds.). Ann. NY Acad. Sci. **762**, 1–14.
155. Hirano, T. (1992). The biology of interleukin-6. *Chem. Immunol.* **51**, 153–180.
156. Taga, T., Hibi, M., Murakami, M., et al. (1992). Interleukin-6 receptor and signals. In: Interleukins: Molecular Biology and Immunology. Chem Immunol., Vol. 51 (Kishimoto, T., ed.). Karger, Basel, Switzerland, pp. 181–204.
157. Mullberg, J., Oberthur, W., Lottspeich, F., et al. (1994). The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J. Immunol.* **152**, 4958–4968.
158. Rose-John, S. and Heinrich, P. C. (1994). Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem. J.* **300**, 281–290.
159. Lust, J. A., Donovan, K. A., Kline, M. P., Greipp, P. R., Kyle, R. A., and Maihle, N. J. (1992). Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine* **4**, 96–100.
160. Mackiewicz, A., Schooltink, H., Heinrich, P. C., and Rose-John, S. (1992). Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins. *J. Immunol.* **149**, 2021–2027.
161. Adler, H. L., McCurdy, M. A., Kattan, M. W., Timme, T. L., Scardino, P. T., and Thompson, T. C. (1999). Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma. *J. Urol.* **161**, 182–187.
162. Hoosien, N., Abdul, M., McCabe, R., et al. (1995). Clinical significance of elevation in neuroendocrine factors and interleukin-6 in metastatic prostate cancer. *Urol. Oncol.* **1**, 246–251.
163. Twillie, D. A., Eisenberger, M. A., Carducci, M. A., Hseih, W. S., Kim, W. Y., and Simons, J. W. (1995). Interleukin-6: a candidate mediator of human prostate cancer morbidity. *Urology* **45**, 542–549.
164. Drachenberg, D. E., Elgamal, A. A., Rowbotham, R., Peterson, M., and Murphy, G. P. (1999). Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer. *Prostate* **41**, 127–133.
165. Tsingotjidou, A. S., Zotalis, G., Jackson, K. R., et al. (2001). Development of an animal model for prostate cancer cell metastasis to adult human bone. *Anticancer Res.* **21**, 971–978.
166. Siegsmund, M. J., Yamazaki, H., and Pastan, I. (1994). Interleukin 6 receptor mRNA in prostate carcinomas and benign prostate hyperplasia. *J. Urol.* **151**, 1396–1399.
167. Hobisch, A., Rogatsch, H., Hittmair, A., et al. (2000). Immunohistochemical localization of interleukin-6 and its receptor in benign, premalignant and malignant prostate tissue. *J. Pathol.* **191**, 239–244.
168. Ershler, W. B. and Keller, E. T. (2000). Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. *Annu. Rev. Med.* **51**, 245–270.
169. Manolagas, S. C. (1998). The role of IL-6 type cytokines and their receptors in bone. *Ann. NY Acad. Sci.* **840**, 194–204.
170. Jilka, R. L., Passeri, G., Girasole, G., et al. (1995). Estrogen loss upregulates hematopoiesis in the mouse: a mediating role of IL-6. *Exp. Hematol.* **23**, 500–506.
171. Poli, V., Balena, R., Fattori, E., et al. (1994). Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *EMBO J.* **13**, 1189–1196.
172. de Grooth, R., Kawilarang-de Haas, E. W., van de Sande-Rijkers, C. M., and Nijweide, P. J. (1998). The role of osteoblast density and endogenous interleukin-6 production in osteoclast formation from the hemopoietic stem cell line FDCP-MIX C2GM in coculture with primary osteoblasts. *Calcif. Tissue Int.* **63**, 57–62.
173. O'Brien, C. A., Lin, S. C., Bellido, T., and Manolagas, S. C. (2000). Expression levels of gp130 in bone marrow stromal cells determine the magnitude of osteoclastogenic signals generated by IL-6-type cytokines. *J. Cell. Biochem.* **79**, 532–541.
174. Hofbauer, L. C., Lacey, D. L., Dunstan, C. R., Spelsberg, T. C., Riggs, B. L., and Khosla, S. (1999). Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin- 6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone* **25**, 255–259.

175. Greenfield, E. M., Shaw, S. M., Gornik, S. A., and Banks, M. A. (1995). Adenyl cyclase and interleukin 6 are downstream effectors of parathyroid hormone resulting in stimulation of bone resorption. *J. Clin. Invest.* **96**, 1238–1244.
176. de la Mata, J., Uy, H. L., Guise, T. A., et al. (1995). Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo. *J. Clin. Invest.* **95**, 2846–2852.
177. Smith, P. C. and Keller, E. T. (2001). Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. *Prostate* **48**, 47–53.
178. Major, P. P., Lipton, A., Berenson, J., and Hortobagyi, G. (2000). Oral bisphosphonates: A review of clinical use in patients with bone metastases. *Cancer* **88**, 6–14.
179. Mundy, G. R. (1999). Bisphosphonates as anticancer drugs. *Expert Opin. Investig. Drugs* **8**, 2009–2015.
180. Diel, I. J., Solomayer, E., and Bastert, G. (2000). Bisphosphonates and the prevention of metastasis. *Cancer* **88**, 3080–3088.
181. Rowe, D. J., Etre, L. A., Lovdahl, M. J., and Pietrzyk, D. J. (1999). Relationship between bisphosphonate concentration and osteoclast activity and viability. *In Vitro Cell Dev. Biol. Anim.* **35**, 383–388.
182. Fleisch, H. (1997). Mechanisms of action of the bisphosphonates. *Medicina (B Aires)* **57(Suppl. 1)**, 65–75.
183. Clohisy, D. R., O'Keefe, P. F., and Ramnaraine, M. L. (2001). Pamidronate decreases tumor-induced osteoclastogenesis in osteopetrotic mice. *J. Orthop. Res.* **19**, 554–558.
184. Kurth, A. H., Kim, S. Z., Sedlmeyer, I., Hovy, L., and Bauss, F. (2000). Treatment with ibandronate preserves bone in experimental tumour- induced bone loss. *J. Bone Joint Surg. Br.* **82**, 126–130.
185. Yoneda, T., Michigami, T., Yi, B., Williams, P. J., Niewolna, M., and Hiraga, T. (2000). Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. *Cancer* **88**, 2979–2988.
186. Hall, D. G. and Stoica, G. (1994). Effect of the bisphosphonate risedronate on bone metastases in a rat mammary adenocarcinoma model system. *J. Bone Miner. Res.* **9**, 221–230.
187. Yoneda, T., Sasaki, A., Dunstan, C., et al. (1997). Inhibition of osteolytic bone metastasis of breast cancer by combined treatment with the bisphosphonate ibandronate and tissue inhibitor of the matrix metalloproteinase-2. *J. Clin. Invest.* **99**, 2509–2517.
188. Dallas, S. L., Garrett, I. R., Oyajobi, B. O., et al. (1999). Ibandronate reduces osteolytic lesions but not tumor burden in a murine model of myeloma bone disease. *Blood* **93**, 1697–1706.
189. Lipton, A. (2000). Bisphosphonates and breast carcinoma: present and future. *Cancer* **88**, 3033–3037.
190. Apperley, J. F. and Croucher, P. I. (1999). Bisphosphonates in multiple myeloma. *Pathol. Biol. (Paris)* **47**, 178–181.
191. Shipman, C. M., Rogers, M. J., Apperley, J. F., Graham, R., Russell, G., and Croucher, P. I. (1998). Anti-tumour activity of bisphosphonates in human myeloma cells. *Leuk. Lymphoma* **32**, 129–138.
192. Shipman, C. M., Croucher, P. I., Russell, R. G., Helfrich, M. H., and Rogers, M. J. (1998). The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. *Cancer Res.* **58**, 5294–5297.
193. Takahashi, R., Shimazaki, C., Inaba, T., et al. (2001). A newly developed bisphosphonate, YM529, is a potent apoptosis inducer of human myeloma cells. *Leuk. Res.* **25**, 77–83.
194. Aparicio, A., Gardner, A., Tu, Y., Savage, A., Berenson, J., and Lichtenstein, A. (1998). In vitro cytoreductive effects on multiple myeloma cells induced by bisphosphonates. *Leukemia* **12**, 220–229.
195. Shipman, C. M., Vanderkerken, K., Rogers, M. J., et al. (2000). The potent bisphosphonate ibandronate does not induce myeloma cell apoptosis in a murine model of established multiple myeloma. *Br. J. Haematol.* **111**, 283–286.
196. Magnetto, S., Boissier, S., Delmas, P. D., and Clezardin, P. (1999). Additive antitumor activities of taxoids in combination with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. *Int. J. Cancer* **83**, 263–269.
197. Stearns, M. E. (1998). Alendronate blocks TGF-beta1 stimulated collagen 1 degradation by human prostate PC-3 ML cells. *Clin. Exp. Metastasis* **16**, 332–339.
198. Pelger, R. C., Hamdy, N. A., Zwinderman, A. H., Lycklama a Nijeholt, A. A., and Papapoulos, S. E. (1998). Effects of the bisphosphonate olpadronate in patients with carcinoma of the prostate metastatic to the skeleton. *Bone* **22**, 403–408.
199. Harvey, H. A. and Lipton, A. (1996). The role of bisphosphonates in the treatment of bone metastases—the U.S. experience. *Support Care Cancer* **4**, 213–217.
200. Heidenreich, A., Hofmann, R., and Engelmann, U. H. (2001). The use of bisphosphonate for the palliative treatment of painful bone metastasis due to hormone refractory prostate cancer. *J. Urol.* **165**, 136–140.
201. Fernandez-Conde, M., Alcover, J., Aaron, J. E., Ordi, J., and Carretero, P. (1997). Skeletal response to clodronate in prostate cancer with bone metastases. *Am. J. Clin. Oncol.* **20**, 471–476.
202. Dearnaley, D. and Sydes, M. (2001). Preliminary evidence that oral clodronate delays symptomatic progression of bone metastases from prostate cancer: first results of the MRC Pr05 Trial. *Am. Soc. Clin. Oncol.* Annual Meeting.
203. Coleman, R. E. (2000). Optimising treatment of bone metastases by Aredia(TM) and Zometa(TM). *Breast Cancer* **7**, 361–369.
204. Boissier, S., Ferreras, M., Peyruchaud, O., et al. (2000). Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. *Cancer Res.* **60**, 2949–2954.

205. Major, P., Lortholary, A., Hon, J., et al. (2001). Zoledronic acid is superior to pamidronate in the treatment of hypercalcemia of malignancy: a pooled analysis of two randomized, controlled clinical trials. *J. Clin. Oncol.* **19**, 558–567.
206. Bentley, H., Hamdy, F. C., Hart, K. A., et al. (1992). Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia. *Br. J. Cancer* **66**, 1159–1163.
207. Nelson, J. B. and Carducci, M. A. (2000). The role of endothelin-1 and endothelin receptor antagonists in prostate cancer. *BJU Int.* **85(Suppl 2)**, 45–48.
208. LeRoy, B. E., Sellers, R. S., and Rosol, T. J. (2004). Canine prostate stimulates osteoblast function using the endothelin receptors. *Prostate* **59**, 148–156.
209. Jimeno, A. and Carducci, M. (2004). Atrasentan: targeting the endothelin axis in prostate cancer. *Expert Opin. Investig. Drugs* **13**, 1631–1640.
210. Pirtskhalaishvili, G. and Nelson, J. B. (2000). Endothelium-derived factors as paracrine mediators of prostate cancer progression. *Prostate* **44**, 77–87.
211. Perkel, V. S., Mohan, S., Baylink, D. J., and Linkhart, T. A. (1990). An inhibitory insulin-like growth factor binding protein (In-IGFBP) from human prostatic cell conditioned medium reveals N-terminal sequence identity with bone derived In-IGFBP. *J. Clin. Endocrinol. Metab.* **71**, 533–535.
212. Taguchi, Y., Yamamoto, M., Yamate, T., et al. (1998). Interleukin-6-type cytokines stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage. *Proc. Assoc. Am. Physicians* **110**, 559–574.
213. Le Brun, G., Aubin, P., Soliman, H., et al. (1999). Upregulation of endothelin 1 and its precursor by IL-1beta, TNF-alpha, and TGF-beta in the PC3 human prostate cancer cell line. *Cytokine* **11**, 157–162.
214. Goltzman, D., Karaplis, A. C., Kremer, R., and Rabbani, S. A. (2000). Molecular basis of the spectrum of skeletal complications of neoplasia. *Cancer* **88**, 2903–2908.
215. Dai, J., Kitagawa, Y., Zhang, J., et al. (2004). Vascular endothelial growth factor contributes to the prostate cancer-induced osteoblast differentiation mediated by bone morphogenetic protein. *Cancer Res.* **64**, 994–999.

12

Molecular Imaging

Noninvasive Strategies to Visualize Gene Expression in Living Subjects

Meera Iyer and Sanjiv S. Gambhir

Summary

Recent advances in molecular imaging techniques have opened the door to a new universe that allows for early detection and characterization of disease and evaluation of therapy. Noninvasive imaging of reporter gene expression using different imaging modalities has proved to be valuable for the in vivo assessment of molecular events in several areas including reporter gene expression, immune cell trafficking, protein–protein interactions, and cancer gene therapy. Multimodality imaging offers the advantage of monitoring the expression of multiple genes using different imaging modalities and finds applications in many areas of biology and medicine. In the coming years, molecular imaging will play a key role in the screening of novel drugs and in the understanding of disease progression at the cellular and molecular level. This chapter provides a comprehensive review of different reporter gene imaging strategies, imaging modalities, and applications of molecular imaging for cancer gene therapy. The chapter also includes a discussion of transcriptional targeting using tissue-specific promoters, including prostate cancer imaging. The final section includes a brief review of the developments in viral and nonviral gene delivery.

Key Words: Bioluminescence imaging; firefly luciferase reporter gene; *HSV1-tk* reporter gene; molecular imaging; positron emission tomography; prostate cancer; two-step transcriptional amplification.

1. INTRODUCTION

Molecular imaging is a rapidly growing research field that encompasses several areas in biomedical research. It brings together scientific expertise from molecular biology, chemistry, physics, molecular pharmacology, bioengineering, biomathematics, and medicine. All of these research disciplines have helped channel molecular imaging into a novel archetype for studying biological processes at the cellular and molecular level in living subjects. Molecular imaging involves the noninvasive visualization, characterization, and quantitation of key biochemical/cellular events occurring within a living subject.

The field has undergone tremendous development in the last decade, mainly because of the emergence of new and advanced small-animal imaging technologies that permit rapid and sensitive visualization of cellular and molecular events in living subjects. The rapid growth of the field did not go unnoticed by the pharmaceutical industry, which has recently started to place emphasis on molecular imaging for accelerating drug development. In the years to come, molecular imaging will likely aid the pharmaceutical industry in the preclinical phases of drug development by validating better leads, and fast-tracking their optimization in preclinical models, thus, saving the industry valuable time and resources.

Imaging technologies provide a better understanding of tumor biology and play a vital role in the drug development process by allowing researchers to evaluate the effectiveness of therapies. It has

From: *Contemporary Cancer Research: Prostate Cancer:*
Biology, Genetics, and the New Therapeutics, Second Edition
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

long been known that changes at the cellular level are responsible for the origin of many oncological abnormalities. These changes can occur long before any morphological changes are observed and provide an ideal target for molecular imaging strategies. Malignant lesions express many targets suitable for imaging; molecular imaging can distinguish them from the adjacent healthy tissues.

Noninvasive imaging in small-animal models is an essential platform for preclinical drug development, because it allows serial measurements without having to sacrifice animals at each time point and permits longitudinal monitoring of response to drug therapy in individual animals. Noninvasive imaging offers the ability to perform preclinical studies in small-animal models of disease and subsequently validate a potential drug in a relatively short time. The findings from preclinical studies can then be translated to human subjects rapidly, accelerating the drug development process. This chapter discusses the significance of molecular imaging, strategies for imaging gene expression, and different imaging modalities. Approaches for molecular imaging in cancer gene therapy are discussed, with special emphasis on prostate cancer imaging.

2. WHY MOLECULAR IMAGING?

Molecular imaging offers several advantages over the conventional *ex vivo* molecular assays. Conventional microscopic methods required the use of postmortem tissue for histological analysis to obtain conclusive information at any given time. The two main limitations of conventional microscopic methods include the need for invasive procedures to obtain tissue samples and the inability to survey multiple tissues simultaneously *(1)*. With molecular imaging, however, it is now possible to noninvasively follow the same animal during an extended period, revealing a more meaningful picture of the progressive changes taking place within the entire animal, usually at the expense of some spatial resolution. Noninvasive imaging also reduces the number of animals required for a particular study by allowing repetitive imaging of the same animal, thereby circumventing the need to procure many animals for a given study. Imaging also permits monitoring the expression of multiple genes in the same animal using different imaging modalities. Molecular imaging methods are also less labor intensive compared with conventional methods and yield quantitative data in relatively less time. The ability to perform tomographic imaging and 3D reconstruction and simultaneous quantification of these biological measures yields 3D information regarding the spatial distribution of a probe within the body of the animal.

3. IMAGING STRATEGIES

The strategies for imaging gene expression can be broadly categorized as direct and indirect. Direct imaging strategies are based on imaging the molecular target (e.g., a protein) directly using a target-specific probe. The resulting image of the localized probe represents its interaction with the target protein. Each new target requires the synthesis of a new chemical compound or a structural modification of an existing compound to render it specific for that target. In addition to the need for a new probe for each new target, various other characteristics must be optimized. These include specificity of interaction, pharmacokinetics, and sensitivity of detection. To conform to these requirements, researchers must invest considerable effort and time, and this often slows down the validation process. Although most imaging assays are based on the direct strategy, there is no one generalizable probe for use with different molecular targets, which limits this approach. There are several examples of the direct imaging strategy, a few are discussed later in Section 4.2 of this chapter. Other examples of specific applications of molecular imaging in living subjects are described in a recent review by Massoud and Gambhir *(2)*.

Indirect imaging strategies are based on the use of a reporter gene and a reporter probe, which together report back on the status of gene expression within a cell. The product of a reporter gene can be an enzyme that converts a reporter probe into a metabolite, which gets selectively trapped within cells that carry the reporter gene. Alternatively, the reporter gene product can be a receptor that

sequesters the probe in an irreversible manner. The main advantage of the indirect strategy is that it allows the validation of a generalizable system that can be used to image several different biological processes (for example, gene therapy monitoring, protein–protein interactions, and cell trafficking in living subjects) using the same reporter probe. Indirect imaging strategies have been used in radionuclide-based imaging *(3–6)* as well as for optical *(7–9)* and MRI *(10,11)*. Though generalizable, indirect imaging requires the incorporation of foreign genes into cells. Gene delivery into living subjects can pose a challenge for the widespread use of this strategy. Nevertheless, the issue of gene delivery in living subjects is not as difficult to resolve as synthesizing a new probe for every new gene under investigation (as in the direct strategy). The last decade has witnessed remarkable progress by several research groups developing and validating indirect imaging assays using reporter gene/reporter probe systems.

4. IMAGING MODALITIES

Sensitive and quantitative analysis of protein function in living subjects has now been made possible by recent developments in molecular imaging techniques. Several imaging assays use optical reporters composed of either fluorescent proteins or bioluminescent proteins (bioluminescence imaging [BLI]) to monitor gene expression. Other technologies, such as positron emission tomography (PET), and single-photon emission computed tomography (SPECT), rely on radiopharmaceuticals (tracers) and reporter genes. MRI requires magnetic reporters or spectroscopic signatures (magnetic resonance spectroscopy) to study biological function. The choice of a molecular imaging strategy should seek to address a few important questions.

1. Is there a molecular target relevant to the disease being studied?
2. After identification of a target, is there an affinity ligand that can bind to the target?
3. What are the imaging requirements (single or repeated), the intended uses (animal or human), and spatial requisites?

Significant progress has been made in developing different imaging modalities, such as PET, BLI, MRI, SPECT, fluorescence, CT, ultrasound, intravital microscopy, and near-infrared fluorescence. Space limitations prohibit the discussion of every imaging modality in detail. Therefore, in this chapter, we have highlighted the advances in imaging with PET, SPECT, BLI, and MRI. Detailed information regarding other imaging techniques, such as fluorescence, ultrasound, CT, intravital microscopy, and near-infrared fluorescence, can be found in several review articles *(2,12,13)*.

4.1. Radionuclide Imaging—PET

PET uses molecules labeled with positron-emitting radionuclides, injected intravenously in trace quantities, to measure biochemical organ and tissue functions in living subjects *(14)*. Typically, either positron-labeled substrates for enzymes or positron-labeled ligands for receptors are used as imaging probes. Retention of the positron-emitting chemical probe in tissue is detected with PET, as a result of either conversion of positron-emitter-labeled enzyme substrate to a trapped product or positron-emitting ligand binding to a receptor. A general paradigm for reporter gene imaging using the enzyme-based and receptor-based methods is illustrated in Fig. 1.

4.2. Direct Imaging

There are several examples of molecular imaging probes for direct imaging using radionuclide techniques. The most commonly used PET radiopharmaceutical is [^{18}F]-2-fluoro-2-deoxyglucose (FDG). The probe is incorporated into cells by facilitated glucose transport and undergoes phosphorylation by hexokinase. The elevated rate of glycolysis in many tumor cells increases the uptake of FDG in these cells relative to normal cells. FDG-PET is used for the metabolic evaluation of many forms of cancer, such as lung, breast, colon, melanoma, and ovarian cancer *(15,16)*. However, FDG was not found to be very useful in the detection of slow-growing tumors, such as prostate carcinoma,

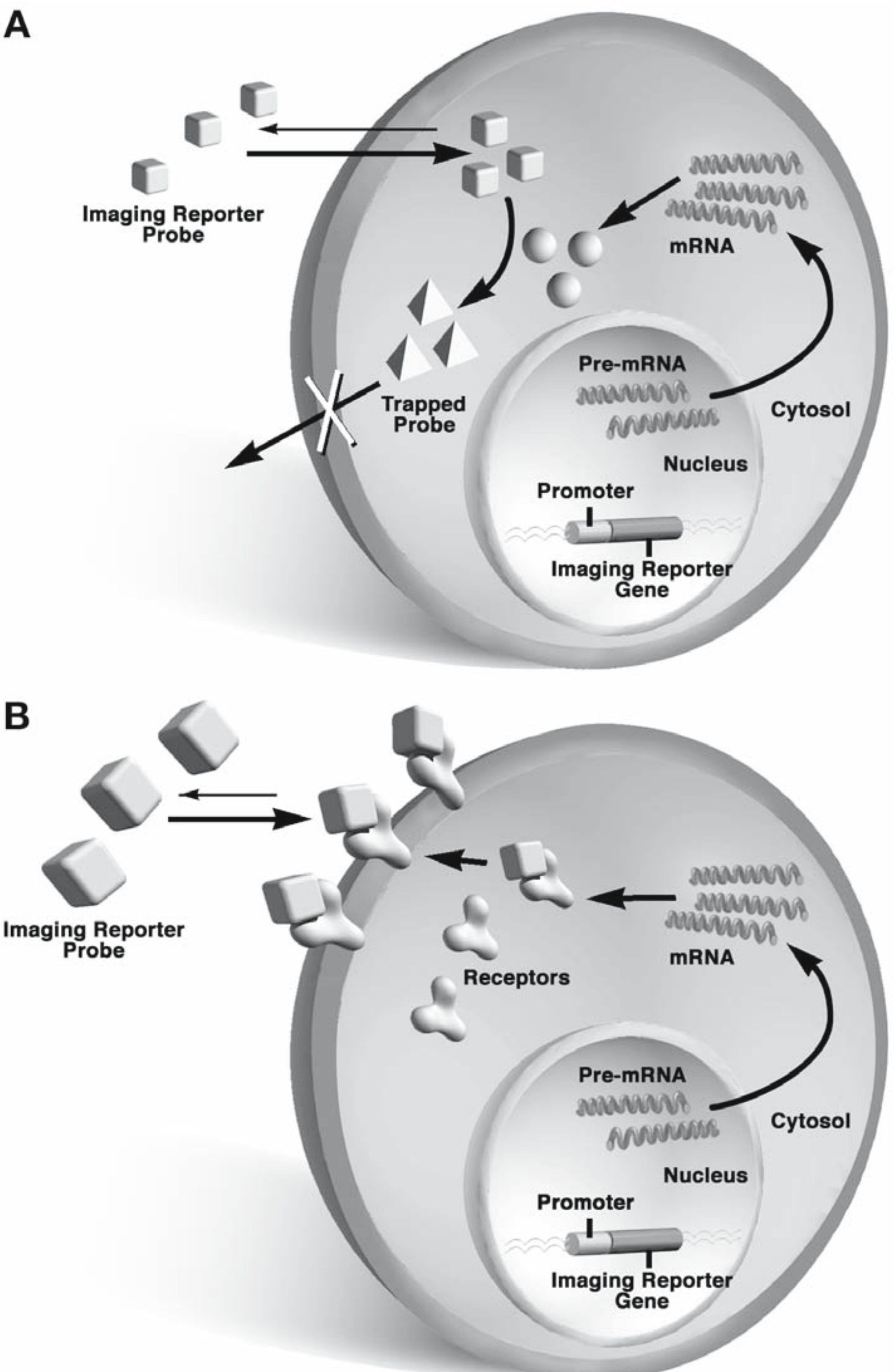


Fig. 1. A schematic diagram illustrating two different approaches for imaging reporter gene expression using PET. A reporter gene introduced into the cell can encode for **(A)** an enzyme (e.g., HSV1-*tk*) that leads to trapping of a radiolabeled probe ([^{18}F]-FPCV/[[^{18}F]-FHBG) or **(B)** an intracellular and/or extracellular receptor (e.g., D2R), which leads to trapping of a radiolabeled ligand ([^{18}F]-FESP). In both cases, significant trapping of the reporter probe will occur only if the reporter gene is expressed. Reproduced with permission from ref. *132*).

because of the slow rate of glycolysis *(17,18)*. Added to this, intense tracer activity in the bladder limited the detection of primary tumor and metastases in pelvic lymph nodes. These issues warranted the continued development of new oncological tracers for PET.

Choline, a precursor for phospholipids synthesis, has been evaluated as an alternate probe for several tumor types. A study by Hara et al. *(19)* showed increased accumulation of [^{11}C]-choline in prostate cancer and improved detection of metastases as compared with FDG. However, the short half-life of this tracer (20 minutes) made it useful only for those PET centers that had inhouse cyclotrons. To overcome this problem, two new analogs of choline labeled with ^{18}F were synthesized. [^{18}F]-fluoroethylcholine and [^{18}F]-fluoromethylcholine demonstrated increased accumulation in primary and metastatic prostate cancer as compared with FDG *(20)*. However, both tracers showed rapid excretion in urine. The short residence time of the tracers in the blood may limit their distribution into areas that are not well-perfused.

Another tracer used for PET imaging of prostate cancer is [^{11}C]-acetate. This tracer has been used to study cardiac oxidative metabolism and blood flow. A study by Oyama et al. *(21)* compared [^{11}C]-acetate with FDG and found that 100% of primary lesions were identified with [^{11}C]-acetate compared with 83% with FDG-PET. Seven out of 22 patients had bone metastases identified on [^{99m}Tc]-hydroxy-methylene diphosphate scintigraphy, [^{11}C]-acetate PET showed corresponding bone lesions in 6 of the patients as compared to only 4 out of 7 patients with FDG-PET. However, the short half-life of the isotope precluded its widespread use.

PET imaging of carcinoembryonic antigen (CEA) expression in living mice is another example of a direct imaging strategy. Using microPET, Sundaresan et al. *(22)* evaluated the efficiency of [^{124}I]-labeled diabody and minibody for tumor imaging in living subjects. In vivo distribution of the antibodies was monitored in athymic nude mice bearing LS174T human colon carcinoma (CEA^{+}) and C6 rat glioma (CEA^{-}) xenografts. The mice were imaged 4 hours and 18 hours after injection of the [^{124}I]-labeled diabody and minibody. PET images showed specific localization to the CEA-positive xenografts and relatively low activity elsewhere in the mice, particularly at 18 hours. Target-to-background ratios for the LS174T tumors vs soft tissues using [^{124}I]-minibody were 3.05 at 4 hours and 11.03 at 18 hours. Similar values were obtained for the [^{124}I]-diabody (3.95 at 4 hours and 10.93 at 18 hours). The [^{124}I]-diabody yielded high-contrast images at 18 hours because of a significant reduction in normal tissue activity in the abdominal area. These results suggest that [^{124}I]-labeled engineered antibody fragments can be used as tumor-specific probes for PET imaging.

Monoclonal antibodies have proven valuable in the treatment of several diseases, including cancer. They act by focusing an immune response on or by targeting delivery of highly cytotoxic agents to the cancer cells without targeting normal cells. Prostate-specific membrane antigen (PSMA) is a type II integral membrane cell surface glycoprotein expressed by virtually all prostate cancers, and is an ideal target for targeted therapy of prostate cancer *(23)*. PSMA is the most well-established, highly restricted prostate cancer cell surface antigen. It is expressed at high density on the cell membrane of all prostate cancers, and, after antibody binding, the PSMA–antibody complex is rapidly internalized along with any payload carried by the antibody. The first antibody to PSMA (7E11) was evaluated in vivo and commercialized as an imaging agent (capromab pendetide). In vivo studies showed that capromab could target known sites of soft tissue metastases in two-thirds of patients *(24)*. However, it did not target the most common site of metastases, the bone. The inability of capromab to target bone metastases was caused by its recognition of an intracytoplasmic site of the PSMA molecule. In viable cells, the capromab binding site is masked by the intact cell membrane and is invisible to the circulating antibody. It was subsequently hypothesized that an antibody to the extracellular domain of PSMA would result in improved targeting because of the large amount of PSMA present on the exterior of prostate cancer cells.

J591 is a monoclonal antibody to PSMA that binds with high affinity to the extracellular domain of PSMA. Murine J591 antibody was deimmunized using a novel method involving specific deletion of human B- and T-cell-recognized epitopes. Radiolabeled J591 (^{111}In/^{90}Y) demonstrated excellent

tumor targeting in prostate cancer patients without previous selection for PSMA expression *(25)*. [^{90}Y]-J591 was well-tolerated, with measurable tumor responses. A second radiometal, ^{177}Lu, was also evaluated for imaging. ^{177}Lu has the advantage of a longer half-life, lower energy, and a shorter range of β-emission, which are useful for detection of metastases that generally are small-volume sites. [^{177}Lu]-J591 has been shown to be well-tolerated, nonimmunogenic, can be administered in multiple doses, and targets metastases with sensitivity and specificity *(26)*. These results have now led to the initiation of phase II trials to assess antitumor activity.

Prostate stem cell antigen (PSCA) is a 123-amino acid glycoprotein first identified in the LAPC-4 prostate xenograft mode of human prostate cancer. PSCA expression in normal tissues is largely prostate specific, but is also expressed in many bladder and pancreatic cancers *(27)*. PSCA is an attractive target for high-risk and metastatic prostate cancer. Monoclonal antibodies against PSCA inhibit tumor growth and metastasis formation, leading to prolonged survival in mice bearing human prostate cancer xenografts *(28)*. In another preclinical study, Ross et al. demonstrated the regression of established tumors treated with a PSCA–maytansinoid immunotoxin conjugate *(29)*. These conjugates showed in vitro cytotoxicity and marked in vivo efficacy, with complete tumor eradication in a large number of treated animals. These encouraging preclinical studies demonstrate the therapeutic potential for PSCA monoclonal antibody immunotherapy for advanced and metastatic prostate cancer.

4.3. Indirect Imaging Strategies

4.3.1. Enzyme-Based Approach

One of the most commonly used reporter genes is the herpes simplex virus type 1 thymidine kinase (HSV1-*tk*). It was originally used as a marker for detection of viral encephalitis *(30)*. The HSV1-TK enzyme has broad substrate specificity and can phosphorylate nucleoside analogs that differ in structure from deoxythymidine. *Note that tk denotes the gene and* TK *denotes the protein.* The two main categories of substrates for HSV1-TK include halogenated derivatives of acycloguanosine and thymidine. When HSV1-*tk* is expressed in cells, the substrates are phosphorylated by the viral thymidine kinase enzyme and subsequently become trapped as a result of phosphorylation by HSV1-TK (Fig. 1A). We and others have validated the use of HSV1-*tk* as a reporter gene with fluorinated ganciclovir (FGCV), penciclovir (PCV), and iodinated 5-iodo-2'-fluoro-2'-deoxy-1-b-D-arabinofuranosyl-5-iodouracil (FIAU) *(4,5,31–34)*. In our first study, we used an adenoviral-mediated delivery of HSV1-*tk* gene to murine liver. After injection of the reporter probe, [^{14}C]-ganciclovir, whole-body autoradiography was used to evaluate the specificity and sensitivity of the imaging assay *(31)*. MicroPET imaging using the same murine model, but a different reporter probe, [^{18}F]-FGCV showed similar results, demonstrating the ability to image reporter gene expression in living mice *(5)*.

Our subsequent efforts to optimize the imaging assay resulted in the validation of two additional reporter probes, [^{18}F]-PCV ([^{18}F]-FPCV) and [^{18}F]-9-(4-fluoro-3-hydroxymethylbutyl)guanine ([^{18}F]-FHBG), and a mutant version of HSV1-*tk* gene, HSV1-sr39tk, (sr39tk). [^{18}F]-FPCV showed a twofold to threefold greater accumulation in cells expressing HSV1-*tk* as compared with [^{18}F]-FGCV *(35)*. The mutant HSV1-sr39TK protein showed an increased affinity for acycloguanosine analogs, and a decreased affinity for the native substrate, thymidine. [^{18}F]-FHBG was found to be superior to [^{18}F]-FPCV as a reporter probe for monitoring HSV1-*tk* gene expression (Iyer and Gambhir, unpublished results). The potential to image two reporter genes simultaneously was also demonstrated in this study. Further, the mutant *sr39tk* reporter gene and [^{18}F]-FHBG reporter probe offered the maximum sensitivity in vivo *(36)*. The mutant sr39TK protein had an increased affinity for acycloguanosine analogs, but not for uracil derivatives. Therefore, [^{124}I]-FIAU is currently the probe of choice for imaging wild-type HSV1-*tk* reporter gene expression in living subjects.

Several studies have attempted to compare acycloguanosine and uracil derivatives as reporter probes for imaging HSV1-*tk* and HSV1-*sr39tk* gene expression *(36–39)*. It is difficult to compare the results from the different studies because they differ in the method of gene delivery (adenoviral mediated vs stably transfected cells) and the cell type. To address this issue, we carried out an in-depth study to compare the two reporter probes, [^{18}F]-FHBG and [^{14}C]-FIAU. Results from our studies indicated that the mechanism of reporter gene introduction is an important variable for determining the optimal reporter gene/reporter probe combination *(36)*. We observed greater accumulation of [^{18}F]-FHBG in adenoviral-mediated HSV1-*tk* gene delivery and [^{14}C]-FIAU in HSV1-*tk* stably transfected cells (Fig. 2). In addition, [^{18}F]-FHBG/HSV1-*sr39tk* was found to be a more sensitive combination than [^{14}C]-FIAU/HSV1-*tk* for adenoviral-mediated gene delivery. In a recent study, the uptake characteristics of [^{14}C]-FMAU, [^{3}H]-FEAU, [^{14}C]-FIAU, and [^{3}H]-PCV were directly compared in HSV1-*tk*- and HSV1-*sr39tk*-expressing cells. C6 rat glioma cells stably transfected with HSV1-*tk* (C6-stb-tk+) showed slightly increased accumulation of the four tracers as compared with C6 cells. In C6-stb-sr39tk+ cells, accumulations of [^{3}H]-FEAU and [^{3}H]-PCV were higher than accumulations of [^{14}C]-FIAU *(40)*. The uptake ratio between transduced and nontransduced cells (selectivity index) was also compared. In C6-stb-tk+ cells, selectivity indices among the four substrates were not significantly different. However, in Morris hepatoma cells stably transfected with the HSV1-*tk*+ gene (MH3924A-stb-tk+), the selectivity index of [^{3}H]-FEAU was significantly greater than that of [^{14}C]-FMAU, [^{14}C]-FIAU, or [^{3}H]-PCV. Moreover, in the selectivity index in C6 cells infected with different titers of viral vectors, [^{3}H]-FEAU showed the highest selectivity index in cells infected with HSV1-*tk*. At high titers of the mutant *sr39tk* adenovirus, [^{3}H]-FEAU again showed greater selectivity compared with [^{3}H]-PCV. [^{14}C]-FMAU and [^{14}C]-FIAU showed poor selectivity indices in cells expressing either HSV1-*tk* or HSV1-*sr39tk*. The results from this study suggest that [^{3}H]-FEAU may be a useful substrate for both HSV1-*tk* and HSV1-*sr39tk*. Continued efforts in the development of newer reporter probes/reporter proteins should lead to further improvements in sensitivity of the PET imaging assays.

In these studies, the expression of the HSV1-*tk* gene was driven by the CMV promoter. Although the CMV promoter yields robust gene expression, it is often prone to transcriptional inactivation. The inactivation occurs via different mechanisms, including DNA methylation and histone deacetylation *(41,42)*. In a recent study, we demonstrated the role of DNA methylation in the silencing of firefly luciferase (*fluc*) gene expression in cardiomyoblast cells. Rat H9c2 embryonic cardiomyoblasts stably transfected with *fluc* gradually lost transgene expression during a period of 8 months *(43)*. We further showed the reversal of this event using 5-azacytidine, an inhibitor of DNA methyltransferase, and tricostatin A, an inhibitor of histone deacetylase. The recovery of gene expression was demonstrated in living rats using BLI. H9c2-*fluc* cells treated with 5-azacytidine showed a significantly higher bioluminescence signal compared with untreated cells during 2 weeks. The results from this study suggest that transcriptional inactivation of the CMV promoter can be reversed in cell culture and imaged in living subjects.

The usefulness of the reporter gene–reporter probe was subsequently evaluated in a clinical trial for gene therapy. PET imaging using [^{124}I]-FIAU was used to demonstrate HSV1-*tk* suicide gene treatment of glioblastomas *(44)*. Vector-mediated HSV1-*tk* gene expression was monitored by PET and [^{124}I]-FIAU in five patients with recurrent glioblastoma. The gene was delivered using a cationic liposome vector, 3-β-(*N-N*, *N*'-dimethyl-aminoethane-carbamoxyl-cholesterol (DAC-30)/1,2-dioleoylphosphatidylethanolamine (DOPE). After intratumoral infusion of cationic liposome–gene complex, FIAU-PET scans were performed 68 to 72 hours after [^{124}I]-FIAU administration. Ganciclovir treatment was initiated 4 days after vector infusion. Treatment responses were recorded using MRI, FDG-PET, and [^{11}C]-methionine (MET)-PET. In one of five patients, specific [^{124}I]-FIAU-associated radioactivity was detected within the infused tumor and the radioactivity was localized at the site of infusion within the center of the tumor. After ganciclovir treatment, signs of necrosis

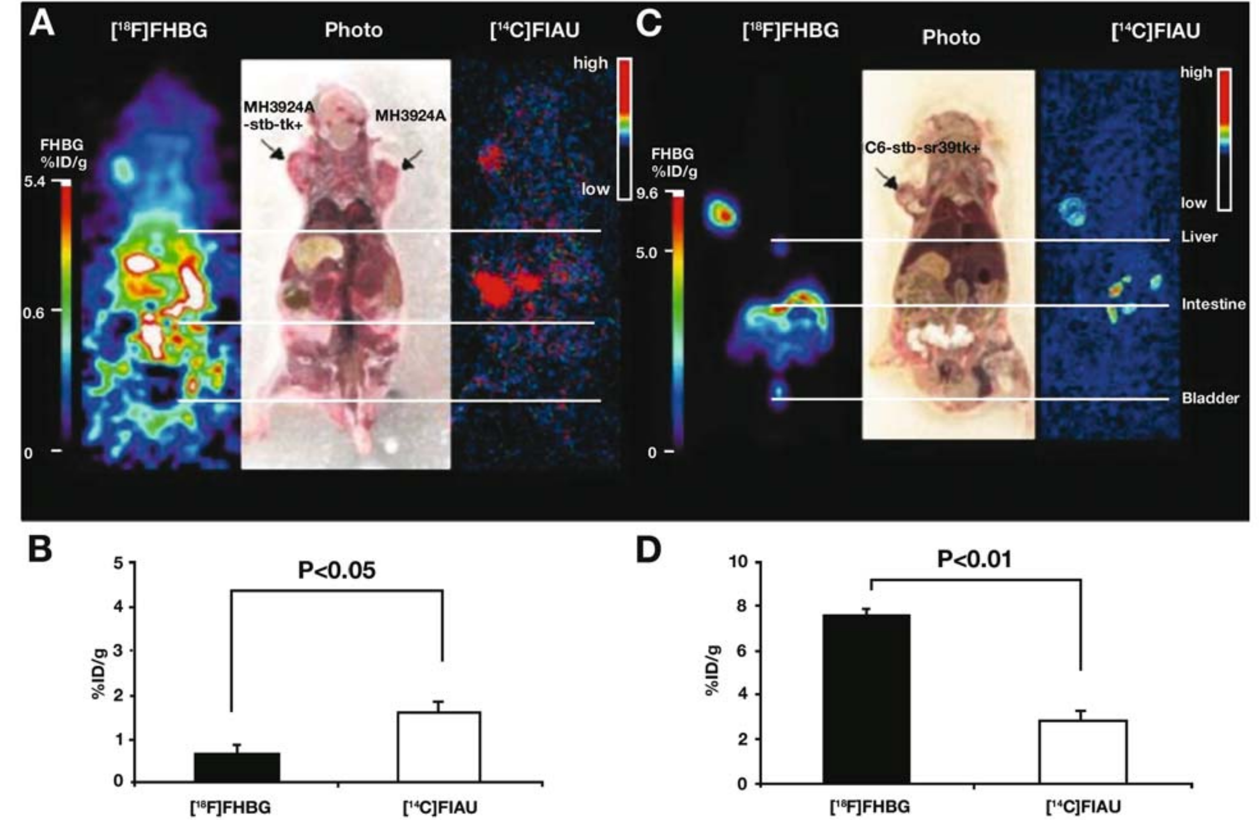


Fig. 2. (**A**) [18F]-FHBG microPET and [14C]-FIAU digital whole-body autoradiography (DWBA) imaging in MH3924A and MH3924A-stb-tk$^+$ tumors. A whole-body coronal projection image from the [18F]-FHBG scan is displayed on the left. The MH3924A-stb-tk$^+$ tumor showed increased signal with [18F]-FHBG. The right panel shows [14C]-FIAU accumulation in an MH3924A-stb-tk+ tumor. The middle panel is a photograph of a tissue section (45-mm thick) corresponding to a DWBA image. (**B**) Comparison of percent injected dose per gram (%ID/g) of tissue between [18F]-FHBG microPET and [14C]-FIAU biodistribution study in MH3924A-stb-tk$^+$ tumor. [14C]-FIAU accumulation was significantly higher than [18F]-FHBG (%ID/g: 1.6 + 0.2% vs 0.7 + 0.2%). (**C**) [18F]-FHBG microPET and [14C]-FIAU DWBA imaging of a mouse carrying C6-stb-sr39tk$^+$ tumors. A whole-body coronal projection image from the [18F]-FHBG scan is displayed on the left. A C6-stb-sr39tk$^+$ tumor shows intense signal with [18F]-FHBG. The right panel shows [14C]-FIAU accumulation in a C6-stb-sr39tk$^+$ tumor. The middle panel is a photograph of a tissue section (45-μm thick) corresponding to a DWBA image. (**D**) Comaprison of %ID/g of tissue between [18F]-FHBG microPET and [14C]-FIAU biodistribution study in a C6-stb-sr39tk$^+$ tumor. [18F]-FHBG accumulation is significantly higher than that of [14C]-FIAU (%ID/g: 7.6% vs 2.8%). (Reproduced with permission from ref. *36*).

were observed by FDG-PET and MET-PET, indicating the HSV1-*tk*-mediated treatment response. However, in four patients, no specific [^{124}I]-FIAU accumulation was observed. Histology of these four patients revealed a low number of proliferating tumor cells per voxel, suggesting that a certain critical number of HSV1-*tk*-transduced tumor cells per voxel are needed to detect and measure [^{124}I]-FIAU accumulation. This preliminary study demonstrated the use of FIAU-PET imaging for monitoring HSV1-*tk* gene expression in patients.

Parallel studies by our group investigated the biodistribution and route of clearance of [^{18}F]-FHBG reporter probe in healthy human volunteers. The phase I study showed that [^{18}F]-FHBG was safe, nontoxic, and had low background activity and rapid clearance *(45)*. The biodistribution studied showed that the tracer cleared through the renal and hepatobiliary pathways. The amount of [^{18}F]-FHBG in the liver and kidneys was initially high, but declined rapidly thereafter. The amount of tracer in the gut increased over time because of the biliary excretion. The tracer was not observed to cross the blood–brain barrier. These results supported the use of [^{18}F]-FHBG for PET imaging of HSV1-*tk* gene expression in most regions except the lower abdomen and the central nervous system.

The preliminary study in human volunteers paved the way for the evaluation of HSV1-*tk* reporter gene expression in patients. Recently, we demonstrated that [^{18}F]-FHBG-PET imaging can be successfully used to image HSV1-*tk* gene expression in liver cancer patients *(46)*. Gene expression was monitored in patients with hepatocellular carcinoma after intratumoral injection of a recombinant replication-incompetent adenovirus encoding the HSV1-*tk* gene. The patients received a dose of 900 mg of valganciclovir immediately after the [^{18}F]-FHBG-PET scan. Gene expression was in the treated tumor was detected in all patients who received a viral dose of 10^{12} pfu or more. The signal to background ratio between the lesion and the nontreated liver increased with time after injection of the tracer. However, when the patients were imaged 9 days after vector injection, no HSV1-*tk* gene expression was detected. The magnitude of [^{18}F]-FHBG accumulation in the treated lesion showed variability from patient to patient. The lack of good correlation between the adenoviral dose and tumor transduction may be attributed to different transduction efficiencies in the tumors or differences in tracer incorporation in the tumor and are exactly the reason why imaging of gene expression will be important in the eventual success of gene therapy. The lack of signal 9 days after vector administration indicated the elimination of cells transduced by the virus after valganciclovir therapy and the immune response against the viral antigens expressed by the HSV1-*tk*-infected cells. This is the first study that supports the use of PET to assess tissue distribution and transduction efficiency of adenoviral vectors in cancer patients. Further studies with more patients are needed to confirm these results and address issues regarding correlation and repeated virus administration.

Gene therapy trials for prostate cancer can also greatly benefit from the use of adenoviral vectors carrying PET and other reporter genes. In a recent study, we demonstrated the ability to monitor gene expression patterns in hormone-refractory prostate cancer (HRPC) using an adenoviral vector carrying the HSV1-*tk* reporter gene *(47)*. PET imaging in living mice showed robust activity in HRPC and androgen-dependent (AD) tumors. The development of viral vectors for prostate cancer imaging is discussed in further detail later in Section 6 of this chapter.

4.3.2. Receptor-Based Approach

In the receptor-based approach to indirect imaging, the reporter gene encodes for a receptor that binds a radiolabeled or paramagnetic probe. A schematic illustration of this approach is shown in Fig. 1B. The dopamine-2 receptor (D2R) is an example of such a reporter gene. D2R is expressed primarily in the brain striatum and in the pituitary glands. A reporter gene–reporter probe imaging assay based on D2R/[^{18}F]-fluoroethyl spiperone (FESP) has been developed in our laboratories. This system was studied both in an adenoviral-directed hepatic gene delivery system and in stable tumor xenografts *(48)*. The D2R reporter gene, when expressed, leads to accumulation of [^{18}F]-FESP by direct binding to D2R (extracellular and intracellular). The D2R system has the advantage of using a reporter probe with a high specific activity (5–10 Ci/μmole), but is limited by a ligand-binding mecha-

nism as opposed to enzymatic conversion of substrate, as in the case with HSV1-*TK*. Although D2R has been validated as a PET reporter gene using [^{18}F]-FESP, dopamine binding to the D2R can modulate cAMP levels. For optimal use of D2R as a reporter gene, it is important to uncouple ligand binding from Gi-protein-mediated inhibition of cAMP production. Mutation of Asp80 or Ser194 produced D2Rs that retained the ability to bind [^{3}H]-spiperone in transfected cells. The D2R80A mutation completely eliminated the ability of the D2R to suppress forskolin-stimulated cAMP accumulation in response to dopamine in cells transfected with a D2R80A expression plasmid and in cells infected with replication-defective adenovirus expressing D2R80A. The D2R194A mutation was substantially reduced, but did not completely eliminate dopamine modulation of cAMP levels. Cultured cells infected with adenoviruses expressing D2R and D2R80A demonstrated equivalent [^{3}H]-spiperone binding activity. Moreover, hepatic [^{18}F]-FESP sequestration was found to be equivalent after intravenous injection of adenoviruses expressing D2R and D2R80A *(49)*.

The sodium iodide symporter (NIS) is another reporter protein that has the characteristics of both a reporter and a therapeutic gene. NIS is expressed primarily on the basolateral membrane of thyroid epithelial cells *(50)*. It is responsible for active uptake of iodine in thyrocytes. It is also expressed in many other organs, including the salivary and lacrimal glands, stomach, kidney epithelial cells, and placenta *(51,52)*. NIS plays a key role in thyroid hormone biosynthesis by concentrating the iodide ion into thyroid cells. Electrophysiological studies of cells have shown that NIS transports two sodium ions with one iodide ion across the cell membrane *(53)*. The ubiquitously expressed sodium–potassium ATPase maintains the sodium concentration within the cells at a low level. The cloning of its cDNA has resulted in the use of NIS as a tool for gene therapy imaging *(54,55)*. NIS gene expression has also been used recently for serial imaging of replication competent vectors. Recombinant, replication-competent measles virus (MV) from the Edmonston vaccine strain (Edm) is selectively oncolytic. To enhance the oncolytic potency of MV-Edm against radiosensitive malignancies and to monitor imaging of infected tissues, a recombinant MV-Edm encoding the human NIS was generated. Intratumoral distribution of MV-NIS was demonstrated by serial γ-camera imaging of ^{123}I uptake in MV-sensitive KAS-6/1 myeloma xenografts *(56)*. The tumors regressed completely after a single intravenous dose of MV-NIS. Iodide uptake in the tumors decreased considerably after 17 days, indicating the elimination of tumors. Although further studies are warranted to evaluate the potential hematopoietic toxicity, this image-guided radiovirotherapy approach seems promising for the treatment of multiple myeloma.

Targeted NIS gene transfer using tissue-specific promoters (TSPs) offers the ability to target the gene to malignant cells while minimizing toxic side effects in normal cells. One example makes use of the prostate-specific antigen (PSA) promoter. The therapeutic effects of ^{131}I were demonstrated in prostate cancer in cell culture and in vivo using PSA promoter-directed NIS expression *(57)*. The prostate cancer cell line, NP-1, was selectively killed by ^{131}I after inducing prostate-specific uptake by PSA promoter-driven NIS expression in vitro. In athymic nude mice, a significant reduction in tumor size was observed in the therapy group compared with the normal group after a single intraperitoneal injection of a therapeutic dose of ^{131}I. Because of the weak transcriptional activity of the PSA promoter, radionuclide accumulation can be considerably low in tumor cells. The ability to accumulate sufficiently high levels of the radionuclide in the prostate tumors can be significantly enhanced using the two-step transcriptional amplification (TSTA) strategy, which is discussed in Section 6 of this chapter. As an imaging reporter gene, NIS has several advantages. Because it originates from a human source, problems associated with immunogenicity of the transgene product can be circumvented. Secondly, in addition to ^{124}I, other γ-isotopes, such as ^{123}I, ^{131}I, and ^{99m}Tc-pertechnetate can be used as reporter probes. These are readily available at clinical imaging centers with conventional γ-systems. A third advantage is that the NIS gene is not likely to interact with the underlying cell biochemistry. All of these characteristics make NIS a favorable choice for the molecular imaging of NIS function in the detection and treatment of cancer.

Somatostatin receptor (SSTR) type 2 is yet another reporter gene that is used in molecular imaging assays. Somatostatin is a cyclic 14-amino acid peptide that is responsible for the regulation of both endocrine and exocrine secretion through inhibition of the release of many hormones, such as growth hormone, insulin, and vasoactive intestinal peptide. The biological activity of the peptide is mediated by SSTRs, members of the super family of G protein-coupled receptors with seven transmembrane spanning domains. Five SSTR subtypes have been cloned (SSTR1–5) *(58)*. In addition to their wide distribution throughout the body, SSTRs are also expressed in many tumors of neuroendocrine origin, such as pancreatic islet-cell tumors. Tumors that are of nonendocrine origin, such as lymphomas and breast cancer, also express SSTRs *(59,60)*. SSTR-targeted radiolabeled peptides have been explored as radiopharmaceuticals for molecular imaging of cancer.

Somatostatin undergoes rapid proteolytic degradation resulting in a short half-life of approx 3 minutes. Hence, for imaging applications, a continuous infusion of the native labeled peptide would be required. The most well-known and well-characterized SSTR targeted radiopharmaceutical is an [^{111}In]-labeled analog of somatostatin containing eight amino acid residues (octreotide). It is stable under physiological conditions and has an efficient renal clearance, making it a versatile agent for imaging somatostatin-expressing tumors *(61)*. SSTR2 has been used for imaging gene transfer to solid subcutaneous tumors. SSTR2 expression was imaged using radiolabeled peptide ligands, such as [^{99m}Tc]-P289 and [^{111}In]-octreotide *(62,63)*. Results from these studies demonstrated rapid clearance of the tracer from normal tissues, leading to specific accumulation relative to background radioactivity. A replication-incompetent adenovirus encoding the SSTR2 gene was used to demonstrate noninvasive imaging of gene transfer in a xenograft model of ovarian cancer *(64)*. Five hours after injection of [^{99m}Tc]-P2045, significant uptake was observed in the region of the peritoneal tumor. The radiotracer uptake was also confirmed by biodistribution studies. The vector-mediated expression of SSTR2 in the subcutaneous tumors was significantly greater than that observed in normal tissues. This study showed the feasibility of imaging gene transfer to ovarian cancer. In a recent report by McCart et al. *(65)*, an oncolytic vaccinia virus (VV) expressing the SSTR2 gene was generated. Nude mice bearing colon cancer xenografts were injected intraperitoneally with the SSTR2 expressing VV. Six days later, the mice received an injection of [^{111}In]-pentetreotide and were imaged. Tumors infected with the SSTR2 expressing VV showed significantly greater accumulation of radioactivity compared with the control tumors. Repeated injection of the radiotracer enabled the visualization of tumor-specific signal for up to 3 weeks after virus injection. The results from this study demonstrated tumor-specific imaging with [^{111}In]-pentetreotide after systemic delivery of VV, and the ability to repetitively image the tumors during a 3-week period. Further studies will be needed to optimize these results and address issues such as the renal uptake of the radiotracer, which may impair the ability to visualize tumors proximal to the peritoneal cavity.

4.4. SPECT Imaging

Noninvasive imaging in living subjects can also be performed using SPECT. This technique requires the use of γ-cameras, which are rotated around the subject to track the location and concentration of the radionuclide from multiple angles. γ-Emitting radionuclides, such as ^{99m}Tc, ^{111}In, ^{123}I, and ^{131}I, are frequently used in SPECT imaging. Cell surface receptors and transporters have been imaged using SPECT. The gene encoding the human SSTR2 was used as a marker gene in combination with the specific receptor-binding compound, [^{99m}Tc]-P2045, which was detected by SPECT imaging *(66,67)*. Adenoviral-mediated delivery of the CEA into experimental human D54MG gliomas was imaged using a [^{131}I]-labeled monoclonal antibody to CEA, COL-1 *(68)*. SPECT has also been used in the noninvasive imaging of resistance to chemotherapeutic drugs. For example, the expression of the multiple drug resistance P-glycoprotein with hepatocellular carcinoma was monitored in patients using [^{99m}Tc]-MIBI SPECT *(69)*. SPECT is a less complex and more widely available imaging modality than PET. However, it has two major limitations: loss in sensitivity (because of the

need for collimators, which reduces the radioactivity counts available for quantitation), and accuracy (correction of radioactivity measurements for attenuation of emitted radiation by tissue presents a challenge).

PET imaging offers a high degree of sensitivity in the range of 10^{-11} to 10^{-12} *M*, unlimited depth penetration, and a broad range of clinically tested molecular imaging agents. Development of molecular imaging assays using PET is of particular significance because the results obtained from cell culture and small-animal models can be directly translated into the clinical setting. It is this ability to conduct translational research that sets PET apart from its other counterparts in the imaging arena. PET also has the advantage of being fully quantitative and provides a higher spatial resolution than SPECT. The short half-life of ^{18}F requires PET centers to either have an inhouse cyclotron or be located within a few hours of a PET center housing a cyclotron. The lower resolution that PET offers compared with other molecular imaging modalities is a limiting factor. The recent introduction of fusion systems, such as PET-CT and SPECT-CT should overcome this limitation by assimilating the information obtained from a lower-resolution PET scanner with the high-resolution anatomic features from CT.

4.5. Bioluminescence Imaging

BLI is a class of optical imaging methods that enable rapid analysis of a variety of cellular and molecular events in living subjects. The technique is based on the emission of visible light at specific wavelengths after energy-dependent reactions catalyzed by luciferases. The light is blue to yellow-green in color and has a wavelength range of 490 to 620 nm *(70)*. The emitted photons can be detected using a cooled charge-coupled device (CCD) camera. Luciferase genes have been cloned from different organisms, such as bacteria, firefly, and coral. Firefly luciferase (*fluc*) is the most commonly used luciferase in biomedical research *(71,72)* and has proved to be a useful marker in monitoring the persistence of gene expression. It was used to image gene expression in the liver after delivery of human clotting factor IX using HIV-1-derived lentiviral vectors in mice *(73)*. A recombinant adeno-associated virus (AAV) vector carrying the *fluc* reporter gene was used to determine the efficacy, safety, and long-term expression of foreign genes delivered to fetuses of mice *in utero (74)*. The detection of bioluminescent signal *in utero* was indicative of the substrate, D-luciferin crossing the placental barrier. BLI was also used to demonstrate long-term *fluc* gene expression in murine liver after intravenous injection of plasmid DNA *(75)*. In this study, the authors showed that the form of DNA delivered via tail-vein injection did not affect long-term expression levels. BLI has contributed to a better understanding of cell-trafficking patterns of immune cells, which led to optimization of local delivery of immunoregulatory proteins to sites of autoimmune reactivity.

BLI has been used to study gene delivery, immune cell therapies, protein–protein interactions, and tumor metastasis *(9,76–78)*. We first evaluated the potential of BLI to image *fluc* reporter gene expression in the skeletal muscles of living mice *(79)*. We studied the ability of the CCD camera to monitor *fluc* gene expression in mice after injection of an E1-deleted adenovirus. We also evaluated the kinetics of *fluc* gene expression in immunocompetent and nude mice. In the Swiss-Webster mice, *fluc* gene expression was found to peak in the first 48 hours and declined rapidly thereafter. In nude mice, however, *fluc* gene expression remained robust for several weeks and gradually declined thereafter. We have used BLI to study a variety of small-animal models of biology and disease. We reported the noninvasive imaging of both bioluminescent (*fluc*) and a PET (HSV1-*sr39tk*) reporter genes in living mice *(80)*. The aim of this study was to evaluate the gene transfer efficiency of cationic liposomes in vivo. We further extended the usefulness of BLI to image the temporal and spatial characteristics of cardiac gene expression in living rats *(81)*. An adenovirus carrying the *fluc* gene was injected directly into the rat myocardium. Reporter gene expression was followed in the same animal for 2 weeks. Cardiac FLUC activity was found to peak within the first 3 to 5 days and declined rapidly thereafter. *Note that fluc refers to the gene and* FLUC *refers to the protein.* Gene

expression was localized to the area surrounding the injection site. In continuation with our work in cardiac imaging, we demonstrated the feasibility of monitoring transplanted cell survival in living animals *(82)*. Rats were injected in the myocardium with embryonic rat H9c2 cardiomyoblasts expressing the *fluc* gene. BLI revealed *fluc* gene expression in the heart, which persisted for longer than 2 weeks. These noninvasive approaches to cardiac imaging will undoubtedly enable researchers to rapidly evaluate the efficiency of repeated transplantations, and screen for novel drugs designed to reduce acute donor cell death. With further development, molecular imaging studies should add critical insights into cardiac cell transplantation biology.

BLI has also been used to demonstrate the ability to identify metastases in a human prostate cancer model, using a prostate-specific adenovirus vector. The adenovirus vector, which expresses *fluc* from an enhanced PSA promoter, restricted expression in the liver but produced robust signals in prostate tumors (Fig. 3). Gene expression was higher in advanced, androgen-independent tumors than in AD lesions. Repetitive imaging during a 3-week period after virus injection into tumor-bearing mice revealed that the virus could locate and illuminate metastases in the lung and spine. These results demonstrate the potential use of a noninvasive imaging modality in therapeutic and diagnostic strategies to manage prostate cancer *(78)*. BLI has also made a significant impact on the understanding of immune responses by revealing lymphocyte trafficking patterns in vivo. It has the potential to elucidate the temporal patterns of immune responses and the spatial distribution of lymphocytes within the body *(83)*.

Luciferases other than firefly are also being evaluated as reporter genes with potential in vivo usefulness. Luciferase from the sea pansy (*Renilla reniformis*) is a blue light-emitting luciferase that uses the substrate coelenterazine. This enzyme derives its energy from the substrate and does not require ATP *(84)*. We have demonstrated the use of *Renilla luciferase* (*rluc*) reporter gene in conjunction with *fluc* for dual reporter gene imaging in vivo *(85)*. Transiently transfected C6 rat glioma cells were evaluated for RLUC enzyme activity. The kinetics of light production from cell lysates was rapid, with a quick peak at 10 seconds followed by a rapid decline during 10 minutes (Fig. 4). Because RLUC and FLUC use two different substrates and their light emission follows different time kinetics, they form an ideal pair for use in imaging dual reporters. *Note that rluc denotes the gene and RLUC denotes the protein.* Renilla luciferase has also been used as a fusion protein with glial-derived neurotrophic factor, and has been studied in a glial cell line transplanted into the central nervous system of rats *(86)*. This study demonstrated that *rluc* could be functionally fused to other open-reading frames and used to assay expression levels. We have used *fluc* and *rluc* in the development of bi-fusion and triple fusion vectors for imaging in living mice *(87,88)*. Recently, we have characterized mutants of RLUC for serum stability and light output. One of the mutants, an eight-mutation form of RLUC (RLUC8) exhibited a 150-fold improvement in stability in murine serum and a 4-fold improvement in light output from the native RLUC *(89)*. The enhancement in light output resulted from increases in quantum yield and improved kinetics. A double mutant yielded a fivefold improvement in light output and was more labile than the native RLUC. The development of such mutated versions of RLUC will yield luciferase proteins optimized for different purposes. Overall, the development and validation of multigene fusions will allow researchers to monitor gene expression at a single cell level (using fluorescence), in preclinical mouse models (using bioluminescence), and in a clinical setting (using PET).

BLI has several advantages compared with the radionuclide imaging modalities. It is rapid, can increase the number of quantitative data points for a given subject, and can improve the statistical significance. It allows whole-body imaging of an animal, allowing all organs to be visualized simultaneously. BLI has gained immense popularity because of its usefulness, cost, flexibility, and sensitivity. However, this technique also has certain limitations. First, it is limited to detection at the surface level. The efficiency of light transmission through the body of an animal is rather limited and depends on the type of tissue. Organs with the highest transmission include skin and muscle; those

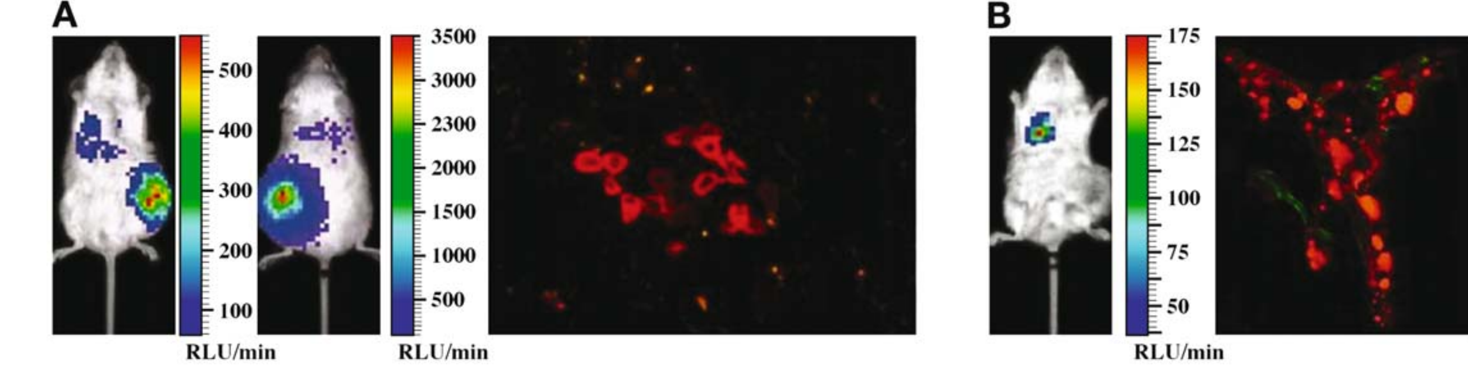


Fig. 3. Bioluminescence imaging and histological analysis of lung metastases. **(A)** Detection of lung metastases by CCD camera imaging and confocal microscopy. In mice bearing LAPC-4 androgen-independent (AI) tumors, the signal in the upper chest area was greater in the image on the right after intratumoral injection of 1.8×10^9 IU of an adenoviral vector carrying the *fluc* reporter gene whose expression was driven by an enhanced prostate-specific promoter (Ad-PSE-BC-*fl*). The right panel shows a confocal microscopic image of metastatic cells in the lung. On the far right, the cluster of cells stained positive for cytokeratin. Original magnification ×400. **(B)** Detection of lung metastases after systemic injection of Ad-PSE-BC-*fl*. The virus was injected (3.6×10^7 IU) via the tail-vein in a LAPC-4 androgen-dependent (AD) tumor-bearing mouse. The left panel shows the CCD camera image 12 days after injection. The right panel shows a section of the lung stained positive using an antibody to cytokeratin. Original magnification ×400. (Reproduced with permission from ref. *78*).

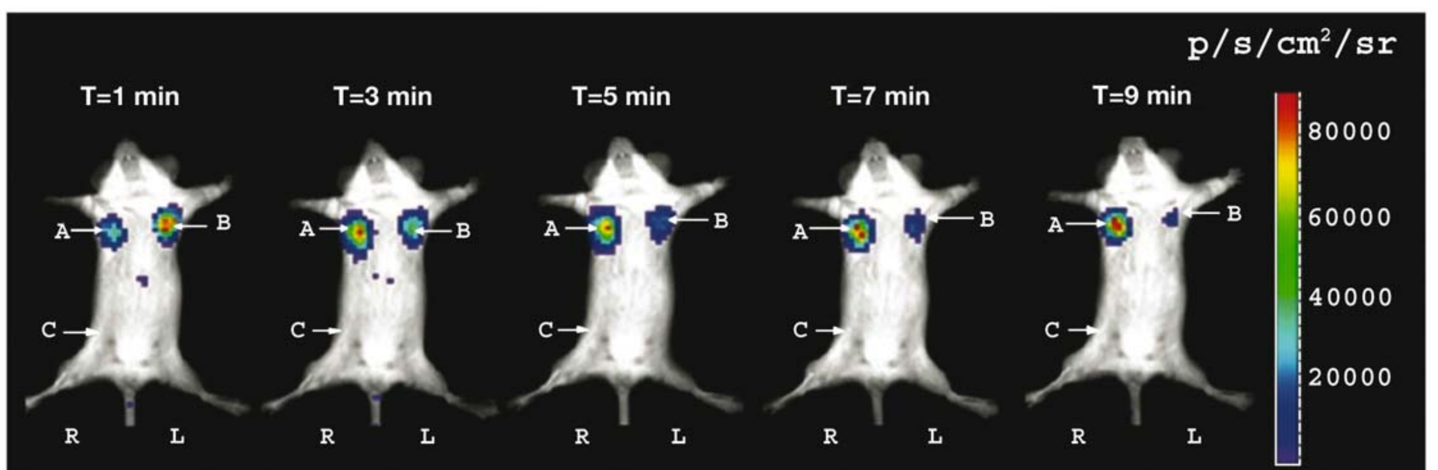


Fig. 4. Kinetics of light production from mice carrying subcutaneous C6-*fluc* and C6-rluc cells after simultaneous tail-vein injection of both D-luciferin and coelenterazine. The mouse was injected with C6-*fluc* (**A**), C6-*rluc* (**B**), and C6 control cells (**C**) in the right forearm, left forearm, and right thigh regions, respectively. Simultaneous injection of the two substrates shows bioluminescence signal from both the sites simultaneously but with different light kinetics. A series of images were acquired every 2 minutes, each image represents a scan time of 1 minute. The *rluc* signal peaks early and is almost zero within 10 minutes. The *fluc* signal persists beyond 10 minutes. R and L represent the right and left side of the mouse resting in supine position. (Reproduced with permission from ref. *85*).

with the lowest transmission include liver and spleen. The low transmission through these tissues is caused by the absorption of light by oxyhemoglobin and deoxyhemoglobin. The net reduction in bioluminescence signal has been estimated to be approx 10-fold for every centimeter of tissue depth *(90)*. A second limitation of BLI is that the images obtained from the CCD camera are 2D and lack depth information. This issue can be overcome by the development of CCD cameras that allow multiple views of the same animal. The third limitation may be the most significant in terms of translation from a preclinical mouse model to a clinical platform. The extensive loss in signal intensity from deep tissue caused by scattering and absorption precludes the use of BLI in clinical applications. Despite its limitations, BLI will have wide-ranging applications in cancer research and the information obtained from preclinical studies will accelerate the discovery of novel therapies for disease treatment.

4.6. Magnetic Resonance Imaging

In MRI, the imaging signal is generated as a result of water proton spin relaxation effects that are augmented by certain atoms with high magnetic moments (e.g., gadolinium and iron). The basic principle in MRI involves the alignment of unpaired nuclear spins when they are placed in a magnetic field. A temporary radiofrequency pulse is then applied to cause a change in the alignment of the spins, and their return to baseline is detected as a change in electromagnetic flux. Every imaging technique has certain advantages and disadvantages. In the case of MRI, the two main advantages include high spatial resolution (10–100 μm) and the ability to measure physiological and anatomic information simultaneously. However, the sensitivity of MRI is considerably lower than the radionuclide and bioluminescent techniques. This necessitates the use of large amounts of molecular probes, which can pose a physical limitation in terms of the injectable volume. Because of the low sensitivity of MRI, robust cellular amplification strategies are required to confer ample sensitivity of label detection. This is achieved by using targeted or smart contrast agents together with biological amplification strategies. Tumor cells exhibit a higher rate of endocytosis when compared with normal cells, and this feature has been exploited for their selective labeling. The human transferrin receptor (TfR) is expressed at high levels in a variety of tumor cell types and can be imaged using transferrin-bound superparamagnetic particles *(91)*. A genetically engineered form of the human TfR (ETR) lacking the negative feedback mechanism that leads to downregulation of endogenous TfR expression was expressed in glioma tumors. Twenty-four hours after perfusion of superparamagnetic TfR particles into tumor-bearing mice, ETR^+ tumors showed increased signal intensity when compared with ETR^- tumors. In another report, HSV-based amplicon vectors were used to deliver ETR, and its expression was imaged with dextran-crosslinked iron oxide superparamagnetic nanoparticles. The ETR expression was found to correlate well with the expression of therapeutic genes placed on the same amplicon vector *(92)*.

Another way to image gene expression using MRI is using paramagnetic chelates that change magnetic properties on enzymatic hydrolysis *(11)*. For example, a paramagnetic galactopyranoside/galactosidase system was injected into cells and used to image β-galactosidase activity in *Xenopus* embryos *(11)*. A paramagnetic galactopyranose chelator substrate for β-galactosidase was developed based on the observation that the relaxivity of gadolinium can be modulated by conjugating into β-galactose via a spacer. The removal of β-galactose from the paramagnetic conjugate by β-galactosidase-mediated hydrolysis results in an increase in relaxivity by approx 40%, which was detected by MRI.

A third strategy to visualize gene expression by MRI is based on the ability of a single enzyme to catalyze the production of multiple molecules in cells. This was demonstrated using the tyrosinase–melanin system, wherein melanin becomes detectable by MRI *(93)*. Tyrosinase catalyzes two main reactions during melanogenesis: hydroxylation of tyrosine to yield dihydroxyphenylalanine and its subsequent oxidation to dopaquinone. Dopaquinone undergoes spontaneous cyclization and polymerization to yield melanin. Melanins have a very high metal-binding capacity, which results in high

signal intensity of melanotic tumors on T1-weighted MRI. Despite its ability to provide soft tissue and functional information, the use of MRI has been rather restricted. This is mainly because of its intrinsically lower sensitivity compared with other imaging modalities. Efforts are underway to overcome the sensitivity issue using signal amplification strategies that will offer a higher contrast between the target and background *(94)*.

In a recent report, Genove et al. *(95)* described a novel method to visualize gene expression in living subjects using MRI. In this study, a replication-deficient adenoviral vector carrying the MRI reporter, ferritin, was introduced into host tissues. The concept was based on the production of superparamagnetic crystals by many organisms. Ferritin exhibits high superparamagnetism because of its crystalline ferrihydrite core. The novelty of this approach is that the vector-encoded reporter is made superparamagnetic when cells sequester endogenous iron from the organism. When the adenovirus–ferritin vector was delivered into murine brain, the virus-transduced neurons and glia showed strong image contrast, which was monitored for up to 5 weeks.

In a second study by Cohen et al. *(96)*, ferritin expression was regulated by tetracycline (TET) in a multimodality reporter cassette. This enabled the detection of TET-regulated gene expression by MRI as well as independent confirmation with fluorescence microscopy and histology. The authors demonstrated that overexpression of ferritin heavy chain in C6 rat glioma cells under TET regulation increased cellular iron content in vitro. For animal studies, the cells were implanted subcutaneously in nude mice. Overexpression of EGFP and ferritin was suppressed by supplying TET in the drinking water, and withdrawal of TET led to overexpression (Fig. 5). In three different experiments, both R1 and R2 relaxation times were found to be high in the ferritin-overexpressing tumors (without TET). The sensitivity of detection was high as shown by the number of cells that were detected. The cell number was 40-fold lower than that reported for TET-regulated expression of tyrosinase. Moreover, tyrosinase contrast was detectable after external supplementation with iron, unlike with ferritin, in which the contrast was achieved with endogenous iron. These new approaches present exciting opportunities for studying gene activation in disease models and are valuable in cases in which external administration of a contrast agent presents a challenge.

5. MOLECULAR IMAGING FOR CANCER GENE THERAPY

With a better understanding of the molecular mechanisms underlying cancer development and the rapid advances in imaging capabilities, it is now possible to detect tumors, monitor their growth, measure their invasive ability, and visualize the effect of anticancer therapy in living subjects. Previously, the two ways to detect tumors and monitor their growth were biopsy or surgery, both invasive procedures. In the last decade, several noninvasive imaging technologies have been developed, which have provided researchers with a new way of looking at cancer development.

For preclinical imaging studies, mice are the preferred choice, mainly because of their short breeding times and well-characterized genetics. In addition, many human genes have a related mouse gene, thereby allowing mice to be used as a platform for mimicking many human diseases. Advances in molecular biology have enabled scientists to “knock-in” and “knockout” genes to generate different kinds of transgenic mouse models. Many of the imaging modalities have been scaled down to allow sensitive and high-resolution imaging of mouse models of disease. Some of the miniaturized imaging instruments are shown in Fig. 6. Imaging technologies can be used to detect the onset and progression of tumors as well as to visualize physiological changes associated with cancer, such as changes in blood volume and perfusion. The different imaging modalities rely on energy–tissue interactions or reporter probe administration. The reporter probes can be classified into two main categories; constitutive (e.g., radiolabeled) and activatable. The former produce signal in a continuous manner, before and after interaction with their target, for example, through the decay of a radioisotope. Activatable probes produce signal only when they interact with their target. Molecular imaging probes can be small molecules, such as receptor ligands or enzyme substrates, or high-molecular weight affinity ligands, such as monoclonal antibodies.

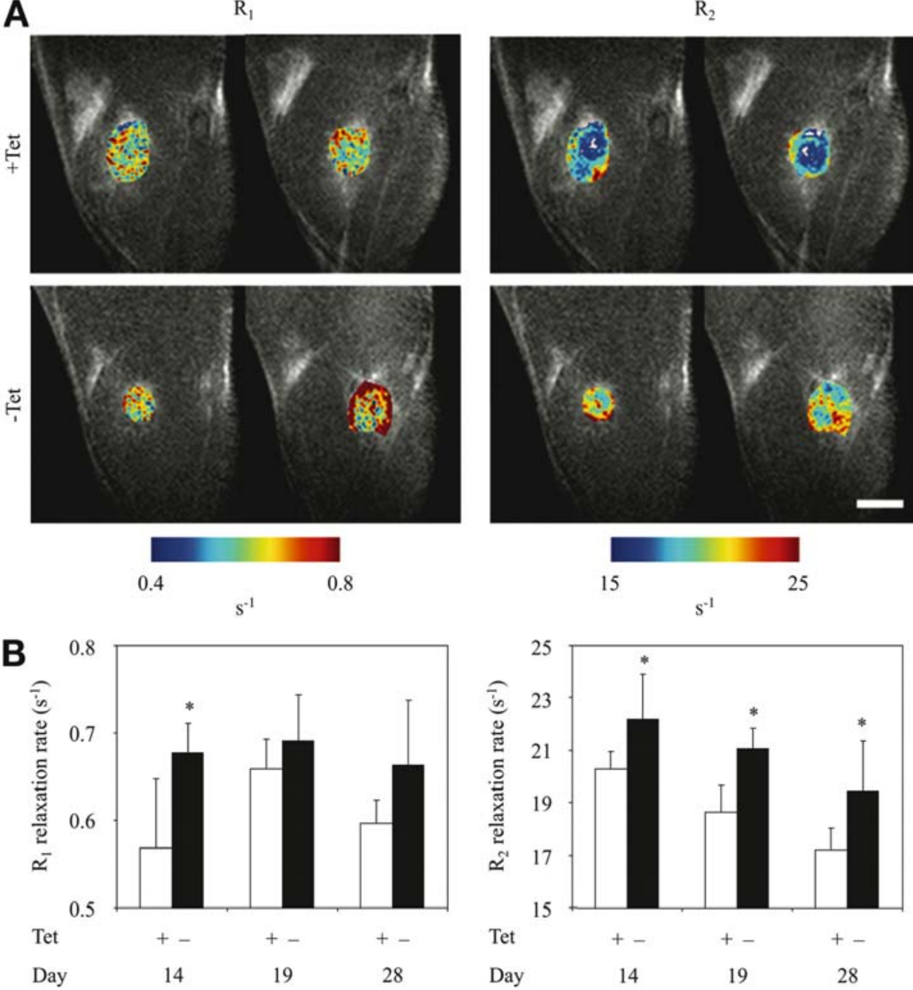


Fig. 5. In vivo MRI detection of switchable ferritin expression in C6 rat glioma tumors. MRI of ferritin expressing tumors at different times after inoculation of C6-TET-EGFP-HA-ferritin tumor cells in the hind limb of nude mice. Tetracycline (TET) and sucrose were supplied in drinking water, starting 2 days before inoculation. **(A)** R_1 and R_2 maps of tumor regions overlaid on the MRI scans are shown for two representative mice from each group. **(B)** R_1 and R_2 values at the tumor site in the presence (ferritin off, $n = 7$) or absence (ferritin on, $n = 4$) of TET in drinking water. All four TET^- mice were imaged three times each (on days 14, 19, and 28 after tumor inoculation). All seven TET^+ mice were imaged in the first (day 14) and second (day 19) MRI sessions, and five of these mice were scanned in the third (day 28) session. (Reproduced with permission from ref. *96*).

Gene therapy offers tremendous promise for the treatment of cancer. It can provide highly selective cancer treatment without adverse systemic toxicity. Advances in this field have led to a better understanding of cancer at the molecular level and new strategies for selectively eradicating cancer cells. There are three main persistent premises in cancer gene therapy. The first premise entails the development of approaches to specifically target cancer cells to destroy the cancer cells or curtail their growth. The second factor is the development of vectors/delivery vehicles to achieve gene transfer in a safe, efficient, and targeted manner, and, lastly, the results obtained from preclinical studies should be readily translated into clinical applications. As advances continue to be made in under-

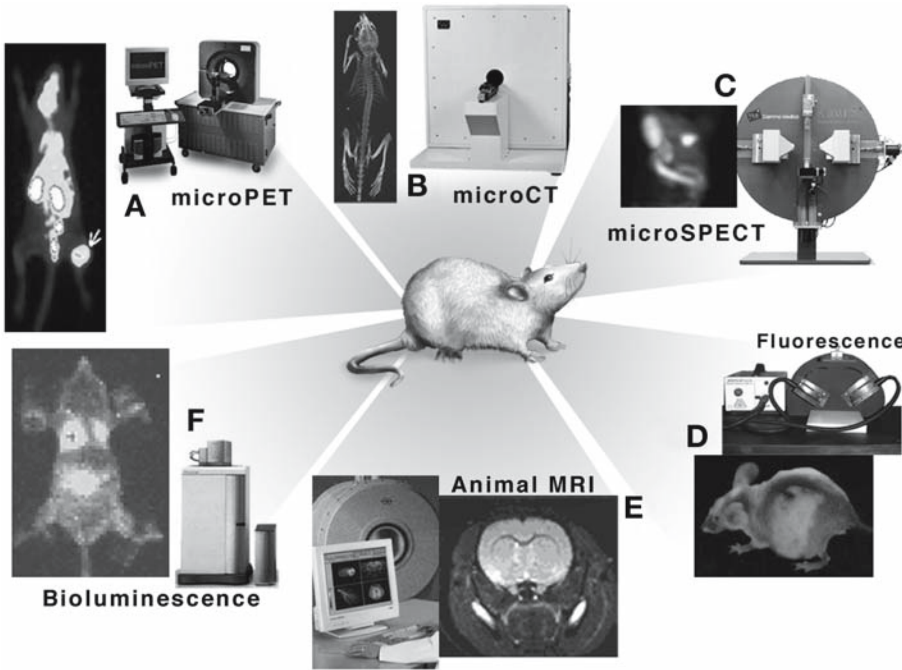


Fig. 6. Multiple imaging modalities available for small-animal molecular imaging. Shown are views of typical instruments available, and their illustrative examples. **(A)** microPET whole-body coronal image of a rat injected with [^{18}F]-FDG, showing uptake of tracer in muscles, heart, brain, and bladder. **(B)** MicroCT coronal image of a mouse abdomen after intravenous injection of iodinated contrast medium. **(C)** MicroSPECT coronal image of a mouse abdomen and pelvis regions after injection of [^{99m}Tc]-methylene diphosphonate, showing spine, pelvis, tai vertebrae, femurs, and knee joints because of tracer accumulation in bone. **(D)** Optical reflectance fluorescence image of a mouse showing green fluorescence protein fluorescence from the liver, abdomen, spine, and brain. **(E)** MicroMRI coronal T2-weighted image of a mouse brain. **(F)** Bioluminescence image of a mouse carrying a subcutaneous tumor xenograft expressing *rluc* in the left shoulder. Images were obtained using a cooled CCD camera. Reproduced with permission from ref. *2*).

standing the molecular pathways of disease and developing new molecular imaging strategies, the knowledge gained can be applied to earlier detection of cancer and more efficient therapy, ultimately resulting in improved patient care.

The primary goal in cancer gene therapy is to selectively destroy cancer cells. Selective targeting of cancer cells will lead to improved safety and increased efficacy in patients undergoing treatment. The basic notion is to reduce toxicity through precise targeting of therapy, leading to an overall increase in the therapeutic index. Three different strategies are currently being investigated to achieve selective targeting in vivo. These include transcriptional targeting, transductional targeting, and activation of therapy based on the activation of cancer-associated cellular pathways. Transcriptional targeting approaches make use of cancer-specific promoters to express the gene of interest, based on the fact that certain cancers express a subset of exclusive genes *(97)*. In transductional targeting, the surface of the gene delivery vehicle is modified to increase the interactions with the cancer cell membrane antigen. This, in turn, results in improved gene transfer to the cancer cells. The third approach is a newer and exciting kind of cancer gene therapy and makes use of oncolytic viruses, which replicate only in cancer cells. The E1B-deleted adenovirus, ONYX-015, has been shown to selectively replicate and kill p53-deficient tumor cells only *(98,99)*. Yet another strategy to destroy cancer cells makes use of apoptosis induction. The three common pathways for induction of apopto-

sis include the tumor necrosis factor, TNF-related apoptosis-inducing ligand, and Fas pathway; the cytotoxic T-cell pathway through perforin; and the mitochondrial pathway through BAX and BCL-2 *(100,101)*. These pathways work through a common pathway involving caspase 3 and all potential targets for anticancer gene therapy. Among the different strategies in use for cancer gene therapy, this chapter focuses on transcriptional targeting approaches using TSPs and the use of molecular imaging to monitor gene expression in living animals.

Transcriptional targeting is based on the use of TSPs or tumor-specific promoters to limit the expression of transgenes specifically to tumor cells *(102)*. Some of the promoters that have been studied include the α-fetoprotein promoter for hepatic carcinoma, the tyrosinase promoter for melanoma *(103)*, the PSA promoter for prostate cancer *(104)*, and the cyclooxygenase-2 promoter for gastrointestinal cancer *(105)* and melanoma *(106)*. The use of tissue-targeted gene therapy has been reported for breast *(107)*, melanoma *(108)*, and prostate cancer *(109)*. The key enzyme in melanin synthesis, tyrosinase, is highly expressed in melanoma cells and its promoter was used to target human and murine melanoma cells using recombinant adenoviruses *(110)*. The enzyme human α-lactalbumin is a key player in lactose production and is expressed in a high percentage of breast cancer cases. Selective expression in breast cancer cells was demonstrated using a human α-lactalbumin promoter-driven HSV-*tk* gene *(107)*.

The PSA gene is expressed in the epithelial cells of normal and cancerous prostate. PSA is a known tumor marker and is used in the diagnosis of prostate cancer. Because of its highly tissue-specific characteristics, the PSA promoter has been extensively used in developing new approaches to prostate cancer gene therapy *(111,112)*. There is a large body of evidence that suggests that transcriptional targeting can enhance tumor specificity and therapeutic index. However, the transcriptional activities of TSPs are often considerably weaker when compared with the traditional CMV promoter. Therefore, attempts to enhance the potency of such weak promoters are actively underway with the aim of also maintaining the specificity of the promoter to reap therapeutic benefits.

6. ENHANCING THE ACTIVITY OF TISSUE-SPECIFIC PROMOTERS

As stated in the preceding discussion, promoters that are active only in certain tissues or inducible promoters play an essential role in the development of site-selective vectors for cancer gene therapy. Some promoters can be used directly because they combine strong transcriptional activity with high specificity. A good example of this category is the melanocyte-specific tyrosinase promoter *(113)*. Commonly, however, TSPs suffer from weak transcriptional activity, which greatly limits their applicability. Two typical examples are the von Willebrand factor promoter and the PSA promoter. The von Willebrand factor promoter demonstrates a high degree of endothelial cell-type specificity, but is a poor activator of transcription *(114)*. The same is true in the case of the PSA promoter, which demonstrates high specificity for prostate cells, but is a relatively weak promoter.

The use of TSPs in cancer gene therapy offers a means to restrict transgene expression to specific cells/tissues. To use TSPs effectively in vivo, strategies to augment their transcriptional activity are highly warranted. There are several strategies to enhance the transcriptional activity of weak promoters, some of which have resulted in the development of promoters that are considerably smaller than the corresponding wild-type sequences. This offers a tremendous advantage in view of the size limitations of gene transfer vectors used in gene therapy. The different strategies have been well-described in a review article by Nettelbeck et al. *(97)*. One of the most effective methods to augment the transcriptional activity of weak promoters involves either removal of inert regulatory sequences or multimerization of positive sequences to promote synergistic interactions, with subsequent enhancement of transcription *(115)*. This was demonstrated in a study of the PSA promoter, in which the activity of the native PSA promoter and prostate-specific enhancer (PSE) was augmented by modifying the androgen receptor (AR) elements *(112,116)*. By adopting either of two ways, duplication of a 400-base pair enhancer core element or insertion of four tandem copies of the synthetic androgen-

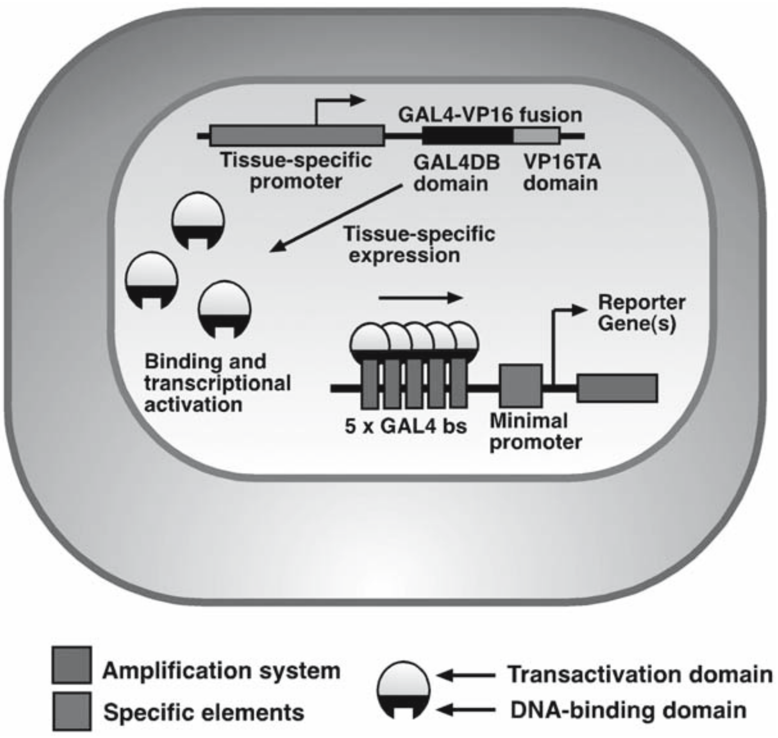


Fig. 7. Schematic diagram of the two-step transcriptional amplification (TSTA) system. The first step consists of activation of the GAL4-VP16 transactivator by a tissue-specific promoter. This is followed by binding of the transactivator complex to five Gal4 binding sites placed upstream of the target gene and a minimal promoter. Transcription of the reporter gene leads to reporter protein, which, in turn, leads to a detectable signal in the presence of a reporter probe. (Reproduced with permission from ref. *122*).

responsive element, Wu et al. showed that the activity of the PSE promoter can be enhanced nearly 20-fold over the parental PSE promoter *(117)*.

In further studies, the PSE was used in an expression cassette to amplify *fluc* reporter gene expression in a two-tiered approach. The PSE was inserted upstream of the GAL4-VP16 transactivator to generate the effector plasmid and the *fluc* gene was cloned downstream of five Gal4 binding sites in the reporter template. The PSE promoter directed the fusion protein, GAL4-VP16, and the chimeric transcription factor, in turn, activated the gene of interest through Gal4-binding sites positioned upstream of a TATA region from the minimal adenovirus E1b promoter. This approach is referred to as the TSTA approach (Fig. 7) and offers a significant advantage for gene expression monitoring using relatively weak TSPs *(118–121)*. Using the TSTA system, we have demonstrated that the activity of the prostate-specific promoter can be amplified over an ~800-fold range *(122,123)*. In the first study, we used the TSTA system to amplify the expression of *fluc* and HSV1-*sr39tk* reporter genes in prostate cancer cells *(122)*. The system was also shown to maintain a high degree of tissue specificity.

We further sought to augment promoter activity and generate a titratable system for eventual transition to clinically relevant methodologies. The TSTA system was optimized by modifying the potency of the prostate-specific promoter and activator. The synergistic behavior observed by multimerizing the activator domain and Gal4-binding sites was exploited to yield an approx 800-fold range in activity *(123)*. The optimized system was used to demonstrate in vivo efficacy and cell-specificity using BLI. To further evaluate the potential of the TSTA system in gene therapy applica-

tions, the TSTA cassette was incorporated into an adenoviral vector. Using BLI, the in vivo specificity of the system was studied in different adenoviral configurations *(124)*. The activity of the TSTA-based adenoviral vector (Ad-TSTA-*fluc*) was shown to exceed that of a CMV promoter-driven vector (Ad-CMV-*fluc*), while maintaining tissue specificity. Using Ad-TSTA-*fluc*, we further interrogated AR signaling in AD and recurrent prostate cancer xenografts using BLI. The results from the study supported the concept that AR is fully functional in recurrent cancer *(125)*.

In a recent study, Sato et al. *(47)* demonstrated that the PSA-based TSTA system is active in hormone refractory prostate cancer (HRPC) models. Using both BLI and PET, the authors demonstrated that the prostate-specific TSTA vectors display robust activity in HRPC and AD tumors. Adenoviruses carrying *fluc* and HSV1-*sr39tk* reporter genes were administered to AD and hormone refractory LAPC-4 tumors in SCID mice and the animals were imaged using BLI and combined microPET/microCT. The combined imaging technique allowed precise localization of the PET signal with the anatomic information acquired from CT. Results from the study revealed robust signals in both AD and hormone-refractory tumors, with the activity being greater in the latter. The results suggested that the presence of functional AR is essential to activate PSA-based promoter vectors and AR function is activated in HRPC regardless of androgen withdrawal. Because a large number of prostate cancers express AR and PSA, the TSTA-based strategy should be a useful tool for the diagnosis and treatment of HRPC.

A potentially useful approach to monitor adenoviral biodistribution after delivery in vivo involves the use of radiolabeled adenoviral particles. Gene therapy protocols will benefit from simultaneous monitoring of virus biodistribution and reporter gene expression. We have investigated the potential of ^{124}I labeling of adenovirus to study its biodistribution as a function of time in living mice using microPET and BLI *(126)*. A recombinant adenovirus carrying the *fluc* reporter gene (Ad-CMV-*fluc*) was labeled with ^{124}I by the iodogen method. [^{124}I]-Adenovirus purified by size exclusion chromatography was injected by tail vein in nude mice and whole-body dynamic microPET images were obtained. Previous administration of potassium iodide (drinking water) and potassium perchlorate (gastric lavage) blocked thyroid and stomach uptake of free iodine. After injection of the [^{124}I]-adenovirus, activity was observed exclusively in the heart during the first 15 seconds and subsequently in the lungs and liver. BLI showed *fluc* expression in the liver 3 weeks after [^{124}I]-adenovirus injection, which was comparable to mice injected with nonlabeled adenovirus indicating the viability of labeled virus. This preliminary study supports the use of radiolabeled adenoviruses for imaging virus trafficking in gene therapy.

Among the different vectors currently in use for in vivo gene transfer, lentiviruses possess the unique ability to transduce quiescent cells and this feature makes them particularly attractive as gene delivery vectors. The use of a TSP together with a lentivirus vector would lead to targeted, long-term gene expression that can be monitored in a noninvasive manner with molecular imaging techniques. The TSTA cassette was incorporated into a third-generation self-inactivating lentivirus vector carrying a central polypurine tract and a posttranscriptional regulatory element. The TSTA-based lentivirus vector was evaluated for its in vivo delivery and efficacy *(127)*. Long-term, sustained *fluc* gene expression was demonstrated in subcutaneous prostate tumor xenografts after intratumoral injection of the vector (Fig. 8A) and in the prostate of living mice after direct injection into the prostate tissue (Fig. 8B). Furthermore, the androgen dependence of the prostate-specific promoter was demonstrated by monitoring *fluc* gene expression after androgen withdrawal (by castration).

In a parallel study, we recently reported the development and imaging of a transgenic mouse model carrying the *fluc* reporter gene in a TSTA system *(128)*. The primary goal in this study was to evaluate the negative effects, if any, of the strong transcriptional activator, GAL4-VP16 in the offspring of transgenic mice. In dealing with a strong transcriptional activator, it is important to attain different levels of expression as a safeguard in gene therapy. Although not common, GAL4-VP16 has been known to exert toxic effects when expressed at high levels *(129,130)*. We demonstrated that the enhanced prostate-specific promoter mediated strong AD *fluc* gene expression, primarily in the dor-

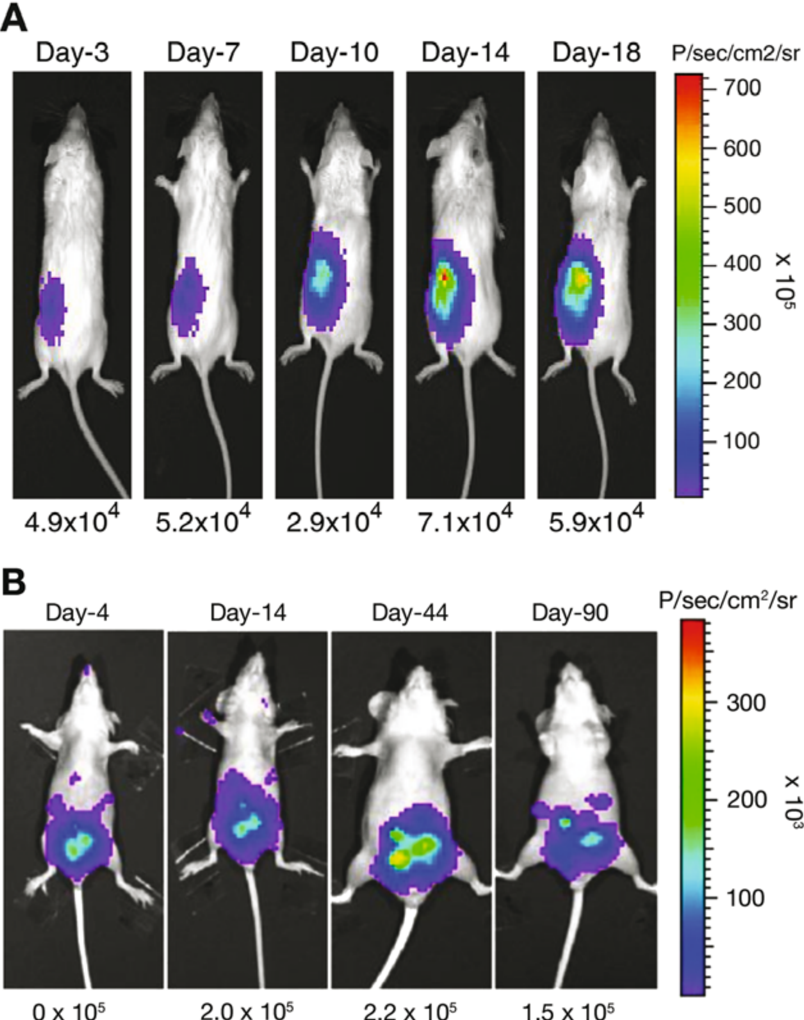


Fig. 8. Tumor specific expression of *fluc* reporter gene in male SCID mice after intratumoral injection of a lentivirus carrying the two-step transcriptional amplification (TSTA) system. **(A)** Six-week-old male SCID mice were implanted with prostate tumor LAPC-9 xenografts in the left, bottom flank. When the tumor size reached 0.5 to 0.6 cm in diameter, lentivirus carrying the TSTA system (LV-TSTA) was injected directly into the tumor. The mice were imaged using a CCD camera on days 3, 7, 10, 14, and 18 using 150 mg/kg d-luciferin as the substrate (intraperitoneal). Color images of visible light are superimposed on photographic images of mice, with a scale in photons per second per square centimeter per steradian (sr). A significantly high level of *fluc* gene expression is observed in the tumor, which continues to persist to day 18. **(B)** In vivo optical imaging of mice after direct intraprostatic delivery of LV-TSTA. Six-week-old male nude mice were injected with the lentivirus in the dorsal lobe of the prostate. The mice were imaged using the CCD camera 2 to 4 days after virus injection. Subsequent imaging was performed every 3 to 4 days till day 21, then once a week until day 90. By day 4, a high level of bioluminescence signal is observed in the prostate (2.2×10^5 photons/s/cm^2/sr). The *fluc* expression shows strong persistence with time. (Reproduced with permission from ref. *127*).

solateral and ventral lobes of the prostate (Fig. 9). No deleterious effects were observed from the potent transactivator in the transgenic offspring. The founder animals and their progeny were healthy, bred well, and demonstrated no sign of spontaneous tumor development. A comparative analysis of relative areas of tissue and cell density revealed histological similarities between the transgenic and

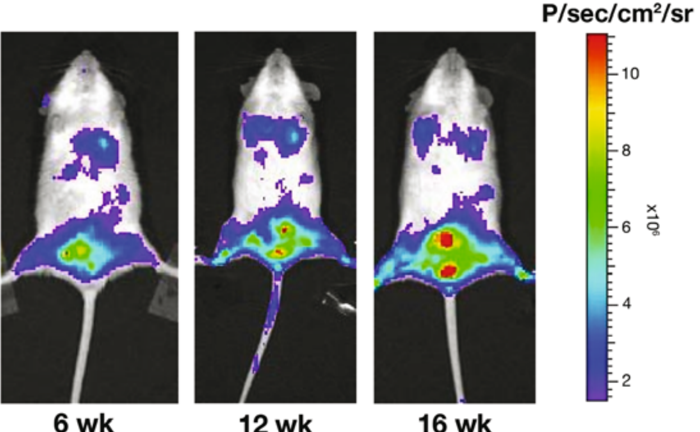


Fig. 9. Repetitive optical imaging of two-step transcriptional amplification (TSTA)-*fluc* transgenic mice. A transgenic male mouse was imaged in the CCD camera at 8 weeks of age, then subsequently re-imaged at 12 and 16 weeks. Color images of visible light are superimposed on photographic images of mice, with a scale in photons per second per square centimeter per steradian (sr). Gene expression is primarily observed in the prostate tissue and showed persistence with time (2–3 × 10^6 p/sec/cm^2/sr). Reproduced with permission from ref. *128*).

wild-type mice. These observations may not necessarily exclude the likelihood of toxic effects in all other tissues; the data suggest that VP16 expression may be well-endured in the prostate tissue.

The TSTA transgenic mouse model is useful for monitoring the onset of cancer and further progression to metastasis by crossing the mice with other transgenic mice that have the ability to develop spontaneous tumors. Our work with the prostate-specific TSTA system has spanned 5 years; the end objective of the study being the development of enhanced prostate-specific vectors for prostate cancer imaging. Our studies involving molecular imaging of enhanced gene expression has not been limited to the prostate-specific promoter. We have recently shown that the TSTA system can be used to amplify the activity of the hypoxia-inducible vascular endothelial growth factor (VEGF) promoter *(131)*. To enable sensitive monitoring of VEGF induction using in living subjects, a transgenic mouse model carrying the TSTA system in a VEGF promoter was developed. The induction of VEGF gene expression was demonstrated in the transgenic mice using a wound-healing model and a subcutaneous mammary tumor model. The VEGF-TSTA mouse model will allow monitoring of early events during angiogenesis in living subjects in a noninvasive and quantitative manner.

7. LINKING A REPORTER GENE TO A THERAPEUTIC GENE

In clinical trials for gene therapy, a quantitative assessment of the location, magnitude, and persistence of gene expression is highly desirable. In many cases, it would be ideal if the expression of a therapeutic gene of interest can be monitored noninvasively using a specific reporter probe. However, this is not practical to achieve, because a new probe would have to be synthesized and validated for every new target protein. Therefore, alternative methods would be highly desirable that can be generalized to image a gene product arising from expression of any gene of interest. Therefore, indirect imaging strategies are being increasingly used to monitor the level of therapeutic gene expression based on levels of reporter gene expression *(132)*. Indirect imaging can be accomplished in one of the following several ways.

7.1. *Bi-cistronic Approach*

The bi-cistronic approach involves the insertion of an internal ribosomal entry site (IRES) sequence between the reporter gene and the therapeutic gene. This leads to transcription of both genes into a

single mRNA from the same promoter, followed by translation into two different proteins. Using this approach, we previously demonstrated microPET imaging of D2R and HSV1-*sr39tk* genes in living mice using stably transfected tumors *(133)*. Although the IRES-based approach is one of the more popular approaches to link two genes, it suffers from a major drawback. The expression of the gene placed downstream of the IRES sequence is often attenuated, resulting in lower imaging sensitivity *(134)*. Attempts to improve the sensitivity of this approach include the use of 10 linked copies of the Gtx (homeodomain protein) IRES (termed SIRES) in place of the encephalomyocarditis IRES in mediating downstream reporter gene expression *(135)*. We have recently demonstrated the use of this approach to augment the expression of the downstream genes (*fluc* and HSV1-*sr39tk*) in cell culture and in living mice using BLI and microPET imaging *(136)*. With the new and improved SIRES vectors, the IRES-based approach is ideally suited for imaging therapeutic gene expression by inferring the levels of reporter gene expression.

7.2. Fusion Approach

A second strategy to link two genes involves a fusion gene construct, wherein the coding sequences of the two genes are in the same reading frame, thereby, producing a single protein with properties of both the original proteins. Using this approach, we have demonstrated the ability to image the expression of an HSV1-*sr39tk-rluc* fusion protein in living mice using microPET imaging and BLI *(87)*. This approach has an advantage in that the expression of the two genes is stoichiometrically coupled. However, this approach is not a generalizable one because many fusion proteins do not yield functional activity for the two individual proteins or may not localize in an appropriate subcellular compartment.

7.3. Bi-directional Transcriptional Approach

In many gene therapy applications, it would be desirable to not only deliver the gene of interest to the desired tissue, but also to be able to regulate the level of expression. This will be especially useful with systems that are known to be potent transcriptional activators, such as the GAL4-VP16 fusion protein. The ability to turn on gene expression and to turn it off when not required provides a safety switch to avoid potential toxicity issues. A few years ago, we demonstrated the use of such a system to monitor the induction of *D2R* and HSV1-*sr39tk* genes in the presence of doxycycline *(137)*. The expression of the two genes was highly correlated. The bidirectional strategy has an advantage over the IRES-based approach because it avoids attenuation of the therapeutic and reporter genes. More recently, we have reported the development of a new bidirectional vector system based on the TSTA strategy, which can simultaneously amplify the expression of a reporter gene and a therapeutic gene using a relatively weak promoter *(138)*.

7.4. Co-Vector Administration Approach

In this approach, the two genes are cloned into two different vectors, but their expression is driven by the same promoter (CMV). We validated this approach using *D2R* and HSV1-*tk* reporter genes driven by the CMV promoter *(139)*. The expression of the two genes showed a very good correlation both in cell culture and in living mice. Despite the limitation associated with the delivery of multiple vectors in vivo, this approach for indirectly imaging ectopic gene expression is an effective alternative to other delivery approaches.

8. IMAGING OTHER INTERACTIONS

Gene expression is no longer the only cellular activity that can be imaged in living subjects. Molecular imaging has now been applied to monitor the interactions between different proteins inside a cell; these proteins regulate a multitude of cellular activities, which include, among others, gene transcription and response to hormones, growth factors, and other signaling molecules. Because these interactions are known to be responsible for the onset of cancer, molecular imaging of such interac-

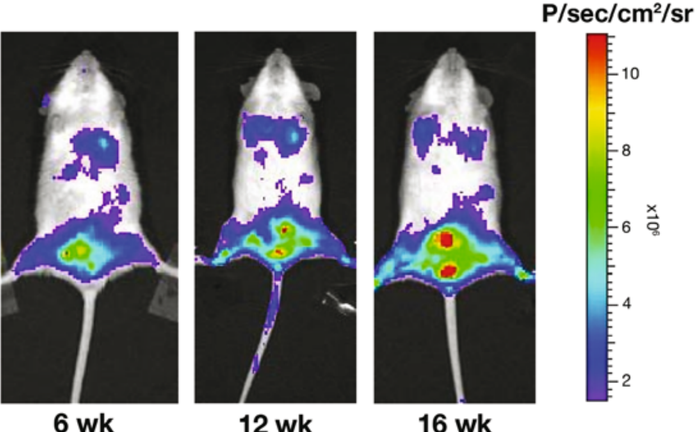


Fig. 9. Repetitive optical imaging of two-step transcriptional amplification (TSTA)-*fluc* transgenic mice. A transgenic male mouse was imaged in the CCD camera at 8 weeks of age, then subsequently re-imaged at 12 and 16 weeks. Color images of visible light are superimposed on photographic images of mice, with a scale in photons per second per square centimeter per steradian (sr). Gene expression is primarily observed in the prostate tissue and showed persistence with time (2–3 × 10^6 p/sec/cm^2/sr). Reproduced with permission from ref. *128*).

wild-type mice. These observations may not necessarily exclude the likelihood of toxic effects in all other tissues; the data suggest that VP16 expression may be well-endured in the prostate tissue.

The TSTA transgenic mouse model is useful for monitoring the onset of cancer and further progression to metastasis by crossing the mice with other transgenic mice that have the ability to develop spontaneous tumors. Our work with the prostate-specific TSTA system has spanned 5 years; the end objective of the study being the development of enhanced prostate-specific vectors for prostate cancer imaging. Our studies involving molecular imaging of enhanced gene expression has not been limited to the prostate-specific promoter. We have recently shown that the TSTA system can be used to amplify the activity of the hypoxia-inducible vascular endothelial growth factor (VEGF) promoter *(131)*. To enable sensitive monitoring of VEGF induction using in living subjects, a transgenic mouse model carrying the TSTA system in a VEGF promoter was developed. The induction of VEGF gene expression was demonstrated in the transgenic mice using a wound-healing model and a subcutaneous mammary tumor model. The VEGF-TSTA mouse model will allow monitoring of early events during angiogenesis in living subjects in a noninvasive and quantitative manner.

7. LINKING A REPORTER GENE TO A THERAPEUTIC GENE

In clinical trials for gene therapy, a quantitative assessment of the location, magnitude, and persistence of gene expression is highly desirable. In many cases, it would be ideal if the expression of a therapeutic gene of interest can be monitored noninvasively using a specific reporter probe. However, this is not practical to achieve, because a new probe would have to be synthesized and validated for every new target protein. Therefore, alternative methods would be highly desirable that can be generalized to image a gene product arising from expression of any gene of interest. Therefore, indirect imaging strategies are being increasingly used to monitor the level of therapeutic gene expression based on levels of reporter gene expression *(132)*. Indirect imaging can be accomplished in one of the following several ways.

7.1. *Bi-cistronic Approach*

The bi-cistronic approach involves the insertion of an internal ribosomal entry site (IRES) sequence between the reporter gene and the therapeutic gene. This leads to transcription of both genes into a

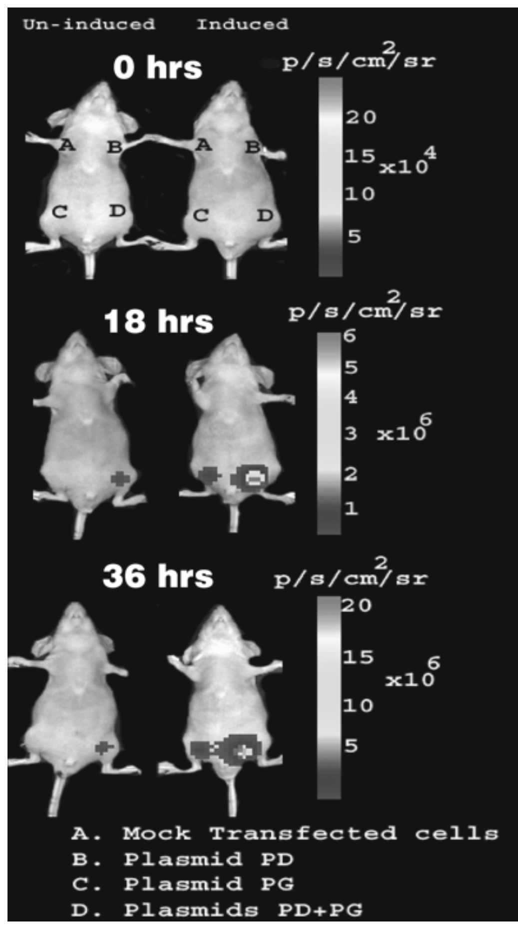


Fig. 10. (*continued from opposite page*) MyoD-C*fluc*, site B), PG (NfκB-N*fluc*-ID, site C), PD + PG (site D), and mock-transfected cells (site A). Only one group of mice was induced with tumor necrosis factor (TNF)-α. Images are from one representative mouse from each group immediately after cell implantation (0 hours), 18 hours, and 36 hours after TNF-α induction. The bioluminescence signal in the induced mouse was higher at site D when compared with the mouse that did not get TNF-α. (Reproduced with permission from ref. *146*).

the p53 tumor suppressor gene and the large-T antigen of simian virus 40 *(143)*. Although widely used, the yeast two-hybrid system is limited to interactions occurring in the nucleus. To circumvent this requirement, we used the split reporter technology (complementation and intein-mediated reconstitution) to visualize protein–protein interactions in living mice (Fig. 10) *(77,144–147)*. In this study, split FLUC reporter proteins consisting of N-terminal and C-terminal ends are inactive until closely approximated (complementation) or spliced together (reconstitution) through the interactions of the MyoD and Id proteins. We have also recently demonstrated the ability to monitor protein–protein interactions in living subjects using bioluminescence resonance energy transfer (BRET) *(148)*. Using a well-characterized BRET system *(149)*, we studied the small molecule rapamycin-mediated heterodimerizations of two human proteins, FRB and FKBP12, in cell culture and in living mice. The measured BRET ratio from rapamycin-mediated interaction of FRB and FKBP12 indicated a strong interaction between the two proteins. The ability to modulate the BRET signal was demonstrated by repeated imaging after addition or withdrawal of rapamycin. The BRET approach can be combined with the split reporter complementation strategy to study interactions in multiprotein complexes.

9. MULTIMODALITY IMAGING

Recent advances in the field of small-animal imaging instrumentation have now made it possible to merge one or more imaging modalities to facilitate imaging of multiple genes. In the preceding discussion, we mentioned fusion gene constructs carrying either two PET reporter genes, two bioluminescent reporter genes, or a PET and a bioluminescent reporter gene, and the use of different imaging techniques to monitor the expression of these genes. Multimodality systems can provide the ability to nearly perfectly register images to improve interpretation and quantification of data across multiple experimental systems *(150)*. Combinations of radionuclide, bioluminescent, MRI, and CT will enable the generation of multimodality images, which will permit better quantitation of biological processes and characterization of new imaging probes. In the future, multimodality imaging will play a key role in assessing therapeutic gene delivery and monitoring the effects of pharmacological agents in disease progression.

10. VECTOR-MEDIATED GENE DELIVERY

The main objective in gene therapy is to develop efficient and safe gene transfer vehicles that can deliver genetic material into specific cell types. The last two decades have seen enormous growth in the field of gene delivery, in particular for cancer gene therapy. Viral vectors are derived from naturally evolved viruses capable of transferring their genetic material into the host cells. Although several different viruses have been studied for their gene transfer efficiency, their use has been rather restricted. This is mainly because of their immunogenicity and toxicity and their limited capacity. These factors have led researchers to increasingly focus on nonviral vectors, which include cationic liposomes and cationic polymers. The efficiency of nonviral vectors is lower than the viral vectors; but their three main advantages of safety, ease of preparation, and high gene encapsulation ability have made them increasingly popular for use in gene therapy protocols. Gene transfer in vivo is accompanied by certain risks, such as induction of malignant transformations or evolution of new viral diseases in humans. Keeping the best interests of the patient foremost in mind, the development of vectors with the highest possible safety profile has become mandatory. Significant improvements in transduction efficiency and selectivity will be seen.

10.1. Adenoviruses

Adenoviruses are linear double-stranded DNA viruses. The uptake of viral particles occurs via binding of viral fiber coat proteins with cell receptors and integrins followed by internalization via receptor-mediated endocytosis *(151,152)*. The viral genome does not integrate into the host genome, instead replicates as episomal elements in the nucleus of the host cell with no risk for insertional mutagenesis. Adenoviral-mediated transfections are efficient, but transient in nature. A major limitation of adenoviral vectors is the highly immunogenic nature of adenoviral vector particles. In addition to eliciting inflammatory and toxic responses in the host, immunogenicity also causes the depletion of adenoviral-transduced cells. These properties limit the use of adenoviral vectors for pathological conditions that require long-term transgene expression. The issue of overcoming immune response has been dealt with from different angles. Newer generation of adenoviruses have been developed either by deletion of E1, E2, and E4 genes to avoid the expression of immunogenic viral proteins within transduced cells *(153)*, or by overexpression via a constitutive promoter of the E3-encoded 19-kDa glycoprotein in adenoviral vectors lacking the E1 gene *(154,155)*. Another strategy to minimize the adverse effects of immune responses consists of reducing the administration load of the vector into patients by developing high-efficiency expression vectors with short-term immune suppression of the subject *(156,157)*. In yet another study, adenoviral particles were PEGylated to protect them from neutralizing antibodies in vivo *(158)*. The PEG-modified adenoviral particles were efficiently protected from humoral immune responses after readministration of the virus into mouse lung.

10.2. Retroviruses

Retroviruses have attracted a great deal of interest as gene transfer vectors. Such interest has been fostered by the biological characteristics of retroviruses. Lentiviruses and foamiviruses also belong to the category of retroviruses. The retroviral genome can be easily rearranged to generate recombinant viral vector particles, which are noncompetent for replication. They are mainly based on the amphotropic Moloney murine leukemia virus *(159)* and have been used in many clinical trials for cancer *(160)*, inherited or acquired monogenic disorders *(161)* and AIDS *(162)*. Retroviruses can infect only dividing cells and, therefore, cannot be used for a variety of therapeutic applications, such as neurological diseases and genetic diseases that require transduction of hepatocytes, because neurons and hepatocytes are nondividing cells. However, their selective transduction of dividing cells makes them ideal for use in cancer therapy.

All retroviruses can be used to transduce a wide variety of cell types. This is based on the ability to pseudotype HIV-1, FIV, and foamivirus cores with the Moloney murine leukemia virus envelope or vesicular stomatitis virus glycoprotein *(163)*. Pseudotyping with the vesicular stomatitis virus glycoprotein enables easy purification by simple ultracentrifugation *(164)*. The transduction efficiency for *ex vivo* and in vivo applications can be improved by concentrating the viral vector particles. A major safety concern surrounding retroviruses is the formation of viral-competent viruses in patients, which occurs by homologous recombination events within the packaging cell lines. Techniques used to monitor the absence of replication-competent retroviruses are based on sensitive PCR and serological ELISA *(165)*. Although the formation of replication-competent retrovirus is highly unlikely because of the design of the retroviral vector, the current trend is to generate high-titer vector stocks in a transient manner to minimize the risk of recombination events among the retroviral components in the packaging cell line. These transient systems are based on three plasmid co-transfections of 293T cell line *(166)*. Studies are currently underway to generate clinical grade retroviral vector by transient transfection systems *(167)*. Overall, in vivo administration of retroviral vectors still has technical limitations and safety concerns. Generation of tissue-specific retroviral vectors is warranted to achieve efficient and safe transduction in vivo.

10.3. Adeno-Associated Viruses

Adeno-Associated Viruses (AAVs) are human single-stranded DNA viruses without an envelope. AAV-based vectors are typically based on serotype 1 or 2. They have the ability to transduce a variety of cell types, including nondividing cells. In AAV, the viral genome gets stably integrated into the chromosomal DNA, thus, allowing for stable transgene expression. The capacity for DNA insertion is limited to approx 4 kb. Recombinant AAV vectors are produced by infecting the AAV packaging cell line with an adenovirus or a herpes virus *(168)*. They can be produced at high titers. However, the stocks are almost always contaminated with helper virus. This limits the use of AAV vector-mediated gene transfer in clinical trials. This disadvantage was overcome by inducing viral replication through genotoxic stimuli such as heat shock, chemicals, or irradiation *(169)*. AAV vector-mediated gene transduction and antitumor effects in vitro and in vivo were significantly enhanced when combined with UV and γ-radiation treatments *(170,171)*.

10.4. Nonviral Vectors

Gene transfer systems based on nonviral vectors mainly consist of cationic liposomes, polyethyleneimines, and naked DNA. They are cationic in nature and interact with negatively charged DNA through electrostatic interactions. The total charge remains a positive net value. This allows the vector to interact efficiently with the negatively charged cell membrane and internalize into the cell through endocytosis *(172)*. Although they are reported to be nontoxic and nonimmunogenic, their efficiency of gene transfer is considerably lower than the viral vectors *(173)*.

Cationic liposomes are positively charged lipid bilayers that form vesicles with negatively charged DNA on the surface. They have the ability to encapsulate large transgenes, up to even 50 kb in size. Their efficiency of gene transfer can be improved by incorporating a small amount of an anionic lipid, which allows the DNA to be integrated into the internal surface of the liposome, where it is protected from enzymatic degradation *(174)*. A distinct feature of cationic liposomes is their preferential uptake by cells in the reticuloendothelial system. This uptake was decreased by incorporating sialic acid residues in the liposomes *(175)*.

DNA–protein complexes have been developed using naturally occurring or synthetic peptides as gene delivery vehicles. Targeting proteins include asiaglycoprotein, transferrin, and polymeric IgA *(176–178)*. DNA binding peptides coupled to cell-specific ligands allow receptor-mediated targeting of the peptide–DNA complexes to specific cell types.

Naked DNA vectors are yet another class of nonviral gene delivery system. They are usually administered systemically; rapid injection of DNA in large volume resulted in efficient gene transfer and high level of gene expression in vivo *(179)*. Among all organs, liver showed the highest level of gene expression. In another study, matrix attachment regions were added as *cis*-acting DNA elements flanking the human coagulation factor IX expression cassette *(180)*. A fivefold higher expression was detected in vivo for up to 1 year posttransfection from a vector containing the chicken matrix attachment region from the lysozyme locus. This method of injection is not considered beneficial because serum nucleases degrade the DNA. Therefore, direct injection of DNA vectors into specific tissues, such as muscle, is generally used and has been shown to produce transient low levels of gene expression *(181)*.

Overall, for cancer gene therapy, the ideal gene delivery formulation should seek to encapsulate and protect DNA, escape the endosomal degradation, and specifically target the tumor site. With continued modifications to the current delivery systems (viral and nonviral), the availability of an optimized vector in the next few years seems highly likely.

11. CLOSING REMARKS

Molecular imaging has already had a significant impact on many areas of biology. The next few years will see further advances in this field, including gene therapy monitoring, where it will provide a realistic hope for better assessment of efficacy and safety aspects of preclinical and clinical development of gene therapy. The recent advances made in gene transfer imaging using enhanced PSA promoters and TSTA-based vector systems will substantially contribute to the development of gene therapy strategies for monitoring prostate cancer. The use of the TSTA system (to significantly enhance the activity of the prostate-specific promoter) and PET imaging (a clinically applicable modality) will likely foster the development of diagnostic and therapeutic strategies to manage prostate cancer. BLI, at the present time, is limited to detection of light output at the surface; translation of findings from preclinical studies to humans poses a considerable challenge because of factors such as greater depth penetration and the requirement of large amounts of substrate. However, with continued progress in instrumentation methodology (fluorescence and bioluminescence tomography), translation of the current optical imaging approaches into clinically applicable modalities in the near future should be feasible. The advantages and benefits that molecular imaging has to offer in several areas of biomedical research are clear; the ability to visualize transcriptional and posttranscriptional regulation of gene expression, specific cellular interactions, and cell-trafficking will provide an opportunity for new experimental venues in patients.

ACKNOWLEDGMENTS

We acknowledge the many graduate students, post-doctoral fellows, and research staff who helped to develop the molecular imaging assays discussed in this chapter. We also thank the National Cancer Institute and the Department of Energy for supporting this work. This work was supported in part by the National Institutes of Health grant ICMIC P50CA114747 (SSG).

REFERENCES

1. Phair, R. D. and Misteli, T. (2001). Kinetic modelling approaches to in vivo imaging. *Nat. Rev. Mol. Cell Biol.* **2**, 898–907.
2. Massoud, T. F. and Gambhir, S. S. (2003). Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* **17**, 545–580.
3. Tjuvajev, J. G., Avril, N., Oku, T., et al. (1998). Imaging herpes virus thymidine kinase gene transfer and expression by positron emission tomography. *Cancer Res.* **58**, 4333–4341.
4. Tjuvajev, J. G., Finn, R., Watanabe, K., Joshi, R., et al. (1996). Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a potential method for monitoring clinical gene therapy. *Cancer Res.* **56**, 4087–4095.
5. Gambhir, S. S., Barrio, J. R., Phelps, M. E., et al. (1999). Imaging adenoviral-directed reporter gene expression in living animals with positron emission tomography. *Proc. Natl. Acad. Sci. USA* **96**, 2333–2338.
6. Gambhir, S. S., Herschman, H. R., Cherry, S. R., et al. (2000). Imaging transgene expression with radionuclide imaging technologies. *Neoplasia* **2**, 118–138.
7. Rehemtulla, A., Stegman, L. D., Cardozo, S. J., et al. (2000). Rapid and quantitative assessment of cancer treatment response using in vivo bioluminescence imaging. *Neoplasia* **2**, 491–495.
8. Contag, C. H., Spilman, S. D., Contag, P. R., et al. (1997). Visualizing gene expression in living mammals using a bioluminescent reporter. *Photochem. Photobiol.* **66**, 523–531.
9. Contag, C. H., and Bachmann, M. H. (2002). Advances in in vivo bioluminescence imaging of gene expression. *Annu. Rev. Biomed. Eng.* **4**, 235–260.
10. Weissleder, R., Simonova, M., Bogdanova, A., Bredow, S., Enochs, W., and Bogdanov, A. J. (1997). MR imaging and scintigraphy of gene expression through melanin induction. *Radiology* **204**, 425–429.
11. Louie, A. Y., Huber, M. M., Ahrens, E. T., et al. (2000). In vivo visualization of gene expression using magnetic resonance imaging. *Nat. Biotechnol.* **18**, 321–325.
12. Shah, K., Jacobs, A., Breakefield, X. O., and Weissleder, R. (2004). Molecular imaging of gene therapy for cancer. *Gene Ther.* **11**, 1175–1187.
13. Jaffer, F. A. and Weissleder, R. (2005). Molecular imaging in the clinical arena. *JAMA* **293**, 855–862.
14. Gambhir, S. S. (2002). Molecular imaging of cancer with positron emission tomography. *Nat. Rev. Cancer* **2**, 683–693.
15. Conti, P. S., Lilien, D. L., Hawley, K., Keppler, J., Grafton, S. T., and Bading, J. R. (1996). PET and [18F]-FDG in oncology: a clinical update. *Nucl. Med. Biol.* **23**, 717–735.
16. Delbeke, D. (1999). Oncological applications of FDG-PET imaging: brain tumors, colorectal cancer, lymphoma, and melanoma. *J. Nucl. Med.* **40**, 591–603.
17. Effert, P. J., Bares, R., Handt, S., Wolff, J. M., Bull, U., and Jakse, G. (1996). Metabolic imaging of untreated prostate cancer by positron emission tomography with 18-fluorine-labeled deoxyglucose. *J. Urol.* **155**, 994–998.
18. Shreve, P. D., Grossman, H. B., Gross, M. D., and Wahl, R. L. (1996). Metastatic prostate cancer: initial findings of PET with 2-deoxy-2-[F-18]-fluoro-D-glucose. *Radiology* **199**, 751–756.
19. Hara, T., Kosaka, N., and Kishi, H. (1998). PET imaging of prostate cancer using carbon-11-choline. *J. Nucl. Med.* **39**, 990–995.
20. DeGrado, T. R., Baldwin, S. W., Wang, S., et al. (2001). Synthesis and evaluation of (18)F-labeled choline analogs as oncologic PET tracers. *J. Nucl. Med.* **42**, 1805–1814.
21. Oyama, N., Akino, H., Kanamaru, H., et al. (2002). 11C-acetate PET imaging of prostate cancer. *J. Nucl. Med.* **43**, 181–186.
22. Sundaresan, G., Yazaki, P. J., Shively, J. E., et al. (2003). 124I-labeled engineered anti-CEA minibodies and diabodies allow high-contrast, antigen-specific small-animal PET imaging of xenografts in athymic nude mice. *J. Nucl. Med.* **44**, 1962–1969.
23. Israeli, R. S., Powell, C. T., Corr, J. G., Fair, W. R., and Heston, W. D. (1994). Expression of the prostate-specific membrane antigen. *Cancer Res.* **54**, 1807–1811.
24. Wynant, G. E., Murphy, G. P., Horoszewicz, J. S., et al. (1991). Immunoscintigraphy of prostatic cancer: preliminary results with 111In-labeled monoclonal antibody 7E11-C5.3 (CYT-356). *Prostate* **18**, 229–241.
25. Milowsky, M. I., Nanus, D. M., Kostakoglu, L., Vallabhajosula, S., Goldsmith, S. J., and Bander, N. H. (2004). Phase I trial of Yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer. *J. Clin. Oncol.* **22**, 2522–2531.
26. Bander, N. H., Milowsky, M. I., Nanus, D. M., Kostakoglu, L., Vallabhajosula, S., and Goldsmith, S. J. (2005). Phase I trial of 177Lu-labeled J591, a monoclonal antibody to prostate-specific membrane antigen in patients with androgen-independent prostate cancer. *J. Clin. Oncol.* **23**, 4591–4601.
27. Amara, N., Palapattu, G. S., Schrage, M., et al. (2001). Prostate stem cell antigen is overexpressed in human transitional cell carcinoma. *Cancer Res.* **61**, 4660–4665.
28. Saffran, D. C., Raitano, A. B., Hubert, R. S., Witte, O. N., Reiter, R. E., and Jakobovits, A. (2001). Anti-PSCA mAbs inhibit tumor growth and metastasis formation and prolong the survival of mice bearing human prostate cancer xenografts. *Proc. Natl. Acad. Sci. USA* **98**, 2658–2663.
29. Ross, S., Spencer, S. D., Holcomb, I., et al. (2002). Prostate stem cell antigen as therapy target: tissue expression and in vivo efficacy of an immunoconjugate. *Cancer Res.* **62**, 2546–2553.

30. Iwashina, T., Tovell, D. R., Xu, L., Tyrrell, D. L., Knaus, E. E., and Wiebe, L. I. (1988). Synthesis and antiviral activity of IVFRU, a potential probe for the non-invasive diagnosis of herpes simplex encephalitis. *Drug Des. Deliv.* **3**, 309–321.
31. Gambhir, S. S., Barrio, J. R., Wu, L., et al. (1998). Imaging of adenoviral-directed herpes simplex virus type 1 thymidine kinase reporter gene expression in mice with radiolabeled ganciclovir. *J. Nucl. Med.* **39**, 2003–2011.
32. Gambhir, S. S., Bauer, E., Black, M. E., et al. (2000). A mutant herpes simplex virus type 1 thymidine kinase reporter gene shows improved sensitivity for imaging reporter gene expression with positron emission tomography. *Proc. Natl. Acad. Sci. USA* **97**, 2785–2790.
33. Tjuvajev, J. G., Chen, S. H., Joshi, A., et al. (1999). Imaging adenoviral-mediated herpes virus thymidine kinase gene transfer expression in vivo. *Cancer Res.* **59**, 5186–5193.
34. Tjuvajev, J. G., Joshi, R., Lindsley, L., et al. (1998). Noninvasive imaging of HSV1-tk marker gene with FIAU for monitoring transfer and expression of other therapeutic genes by multi-gene delivery vectors. *J. Nucl. Med.* **39**, 130P.
35. Iyer, M., Barrio, J. R., Namavari, M., et al. (2001). 8-[18F]Fluoropenciclovir: an improved reporter probe for imaging HSV1-tk reporter gene expression in vivo using PET. *J. Nucl. Med.* **42**, 96–105.
36. Min, J. J., Iyer, M., and Gambhir, S. S. (2003). Comparison of [18F]FHBG and [14C]FIAU for imaging of HSV1-tk reporter gene expression: adenoviral infection vs stable transfection. *Eur. J. Nucl. Med.* **30**, 1547–1560.
37. Iyer, M., Bauer, E., Barrio, J. R., et al. (2000). Comparison of FPCV, FHBG, and FIAU as reporter probes for imaging herpes simplex virus type 1 thymidine kinase reporter gene expression [abstract]. *J. Nuc. Med.* **41**, 80P–81P.
38. Wang, H. E., Deng, W. P., Chang, P. F., et al. (2002). Evaluation and comparison of [18F]-FHBG and [131I]-FIAU as gene probes in gene therapy [abstract]. *J. Nucl. Med.* **43(Suppl.)**, 274.
39. Tjuvajev, J. G., Doubrovin, M., Akhurst, T., et al. (2002). Comparison of radiolabeled nucleoside probes (FIAU, FHBG, and FHPG) for PET imaging of HSV1-tk gene expression. *J. Nucl. Med.* **43**, 1072–1083.
40. Kang, K. W., Min, J. J., Chen, X., and Gambhir, S. S. (2005). Comparison of [14C]FMAU, [3H]FEAU, [14C]FIAU and [3H]PCV for monitoring reporter gene expression of wild type and mutant herpes simplex virus type 1 thymidine kinase in cell culture. *Mol. Imag. Biol.* **7,** 296–303.
41. Loser, P., Jennings, G. S., Strauss, M., and Sandig, V. (1998). Reactivation of the previously silenced cytomegalovirus major-immediate early promoter in the mouse liver: involvement of NFkappaB. *J. Virol.* **72**, 180–190.
42. Meier, J. L. (2001). Reactivation of the human cytomegalovirus major immediate-early regulatory region and viral replication in embryonal NTera2 cells: the role of trichostatin A, retinoic acid and deletion of the 21-base pair repeats and modulator. *J. Virol.* **75**, 1581–1593.
43. Krishnan, M., Park, J. M., Cao, F., et al. (2006). Effects of epigenetic modulation of reporter gene expression: implications for stem cell imaging. *FASEB J.* **20**, 106–108.
44. Jacobs, A., Voges, J., Reszka, R., et al. (2001). Positron-emission tomography of vector-mediated gene expression in gene therapy for gliomas. *Lancet* **358**, 727–729.
45. Yaghoubi, S., Barrio, J. R., Dahlbom, M., et al. (2001). Human pharmacokinetic and dosimetry studies of [F-18]FHBG: A reporter probe for imaging herpes simplex virus type-1 thymidine kinase reporter gene expression. *J. Nucl. Med.* **42**, 1225–1234.
46. Penuelas, I., Mazzolini, G., Boan, J. F., et al. (2005). Positron emission tomography imaging of adenoviral-mediated transgene expression in liver cancer patients. *Gastroenterology* **128**, 1787–1795.
47. Sato, M., Johnson, M., Zhang, L., Gambhir, S. S., Carey, M., and Wu, L. (2005). Functionality of androgen receptor-based gene expression imaging in hormone refractory prostate cancer. *Clin. Cancer Res.* **11**, 3743–3749.
48. MacLaren, D. C., Gambhir, S. S., Satyamurthy, N., et al. (1999). Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. *Gene Ther.* **6**, 785–791.
49. Liang, Q., Satyamurthy, N., Barrio, J. R., et al. (2001). Noninvasive, quantitative imaging in living animals of a mutant dopamine D2 receptor reporter gene in which ligand binding is uncoupled from signal transduction. *Gene Ther.* **8**, 1490–1498.
50. Jhiang, S. M., Cho, J. Y., Ryu, K. Y., et al. (1998). An immunohistochemical study of Na+/I- symporter in human thyroid tissues and salivary gland tissues. *Endocrinology* **139**, 4416–4419.
51. Spitzweg, C., Joba, W., Schriever, K., Goellner, J. R., Morris, J. C., and Heufelder, A. E. (1999). Analysis of human sodium iodide symporter immunoreactivity in human exocrine glands. *J. Clin. Endocrinol. Metab.* **84**, 4178–4184.
52. Spitzweg, C., Joba, W., Eisenmenger, W., and Heufelder, A. E. (1998). Analysis of human sodium iodide symporter gene expression in extrathyroidal tissues and cloning of its complementary deoxyribonucleic acids from salivary gland, mammary gland and gastric mucosa. *J. Clin. Endocrinol. Metab.* **83**, 1746–1751.
53. Eskandari, S., Loo, D. D., Dai, G., Levy, O., Wright, E. M., and Carrasco, N. (1997). Thyroid Na+/I- symporter. Mechanism, stoichiometry and specificity. *J. Biol. Chem.* **272**, 2,7230–27,238.
54. Mandell, R. B., Mandell, L. Z., and Link, C. J. (1999). Radioisotope concentrator gene therapy using the sodium/iodide symporter gene. *Cancer Res.* **59**, 661–668.
55. Groot-Wassink, T., Aboagye, E. O., Glaser, M., Lemoine, N. R., and Vassaux, G. (2002). Adenovirus biodistribution and noninvasive imaging of gene expression in vivo by positron emission tomography using human sodium iodide symporter as reporter gene. *Hum. Gen. Ther.* **13**, 1723–1735.

56. Dingli, D., Peng, K. W., Harvey, M. E., et al. (2004). Image-guided radiovirotherapy for multiple myeloma using a recombinant measles virus expressing the thyroidal sodium iodide symporter. *Blood* **103**, 1641–1646.
57. Spitzweg, C., O'Connor, M. K., Bergert, E. R., Tindall, D. J., Young, C. Y., and Morris, J. C. (2000). Treatment of prostate cancer by radioiodine therapy after tissue-specific expression of the sodium iodide symporter. *Cancer Res.* **60**, 6526–6530.
58. Bell, G. and Reisine, T. (1993). Molecular biology of somatostatin receptors. *Trends Neurosci.* **16**, 34–38.
59. Reubi, J., Waser, B., and Vanhagen, M. (1992). In vitro and in vivo detection of somatostatin receptors in human malignant lymphomas. *Int. J. Cancer* **50**, 895–900.
60. Reubi, J., Waser, B., and Foekens, J. (1990). Somatostatin receptor incidence and distribution in breast cancer using receptor autoradiography: relationship to EGF receptors. *Int. J. Cancer* **46**, 416–420.
61. Krenning, E. P., Bakker, W. H., Kooij, P. P., et al. (1992). Somatostatin receptor scintigraphy with indium-111-DTPA-D-Phe-1-octreotide in man: metabolism, dosimetry and comparison with iodine-123-Tyr-3-octreotide. *J. Nucl. Med.* **33**, 652–658.
62. Zinn, K. R., Buchsbaum, D. J., Chaudhuri, T. R., et al. (2000). Noninvasive monitoring of gene transfer using a receptor reporter imaged with a high affinitypeptide radiolabeled with 99mTc or 188Re. *J. Nucl. Med.* **41**, 887–895.
63. Rogers, B. E., Zinn, K. R., and Buchsbaum, D. J. (2000). Gene transfer strategies for improving radiolabeled peptide imaging and therapy. *Q. J. Nucl. Med.* **44**, 208–223.
64. Chaudhuri, T. R., Rogers, B. E., Buchsbaum, D. J., Mountz, J. M., and Zinn, K. R. (2001). A noninvasive reporter system to image adenoviral-mediated gene transfer to ovarian cancer xenografts. *Gynecol. Oncol.* **83**, 432–438.
65. McCart, J. A., Mehta, N., Scollard, D., et al. (2004). Oncolytic vaccinia virus expressing the human somatostatin receptor SSTR2: molecular imaging after systemic delivery using 111In-pentetreotide. *Mol. Ther.* **10**, 553–561.
66. Zinn, K. R., Chaudhuri, T. R., Buchsbaum, D. J., Mountz, J. M., and Rogers, B. E. (2001). Detection and measurement of in vitro gene transfer by gamma camera imaging. *Gene Ther.* **8**, 291–299.
67. Zinn, K. R. and Chaudhuri, T. R. (2002). The type 2 human somatostatin receptor as a platform for reporter gene imaging. *Eur. J. Nucl. Med.* **29**, 388–399.
68. Raben, D., Buchsbaum, D. J., Khazaeli, M. B., et al. (1996). Enhancement of radiolabeled antibody binding and tumor localization through adenoviral transduction of the human carcinoembryonic antigen gene. *Gene Ther.* **3**, 567–580.
69. Kim, Y. S., Cho, S. W., Lee, K. J., et al. (1999). Tc-99m MIBI SPECT is useful for noninvasively predicting the presence of NDR1 gene-encoded P-glycoprotein in patients with hepatocellular carcinoma. *Clin. Nucl. Med.* **24**, 874–879.
70. Wilson, T. and Hastings, J. W. (1998). Bioluminescence. *Annu. Rev. Cell. Dev. Biol.* **14**, 197–230.
71. Contag, C. and Bachmann, M. H. (2002). Advances in in vivo bioluminescence imaging of gene expression. *Annu. Rev. Biomed. Eng.* **4**, 235–260.
72. Contag, C. and Ross, B. D. (2002). It's not just about anatomy: in vivo bioluminescence imaging as an eyepiece into biology. *J. Magn. Reson. Imaging* **16**, 373–387.
73. Tsui, L. V., Kelly, M., Zayek, N., et al. (2002). Production of human clotting factor IX without toxicity in mice after vascular delivery of a lentiviral vector. *Nat. Biotechnol.* **20**, 53–57.
74. Lipshutz, G. S., Gruber, C. A., Cao, Y., Hardy, J., Contag, C. H., and Gaensler, K. M. (2001). In utero delivery of adeno-associated viral vectors: intraperitoneal gene transfer produces long-term expression. *Mol. Ther.* **3**, 284–292.
75. Chen, Z. Y., Yant, S. R., He, C. Y., Meuse, L., Shen, S., and Kay, M. A. (2001). Linear DNAs concatemerize in vivo and result in sustained transgene expression in mouse liver. *Mol. Ther.* **3**, 403–410.
76. Costa, G. L., Sandora, M. R., Nakajima, A., et al. (2001). Adoptive immunotherapy of experimental autoimmune encephalomyelitis via T cell delivery of the IL-12 p40 subunit. *J. Immunol.* **167**, 2379–2387.
77. Paulmurugan, R., Umezawa, Y., and Gambhir, S. S. (2002). Noninvasive imaging of protein–protein interactions in living subjects by using reporter protein complementation and reconstitution strategies. *Proc. Natl. Acad. Sci. USA* 15,608–15,613.
78. Adams, J. Y., Johnson, M., Sato, M., et al. (2002). Visualization of advanced human prostate cancer lesions in living mice by a targeted gene transfer vector and optical imaging. *Nat. Med.* **8**, 891–897.
79. Wu, J. C., Sundaresan, G., Iyer, M., and Gambhir, S. S. (2001). Noninvasive optical imaging of firefly luciferase reporter gene expression in skeletal muscles of living mice. *Mol. Ther.* **4**, 297–306.
80. Iyer, M., Berenji, M., Templeton, N. S., and Gambhir, S. S. (2002). Noninvasive imaging of cationic lipid-mediated delivery of optical and PET reporter genes in living mice. *Mol. Ther.* **6**, 555–562.
81. Wu, J. C., Inubushi, M., Sundaresan, G., Schelbert, H. R., and Gambhir, S. S. (2002). Optical imaging of cardiac reporter gene expression in living rats. *Circulation* **105**, 1631–1634.
82. Wu, J. C., Chen, I. Y., Sundaresan, G., et al. (2003). Molecular imaging of cardiac cell transplantation in living animals using optical bioluminescence and positron emission tomography. *Circulation* **108**, 1302–1305.
83. Hardy, J., Edinger, M., Bachmann, M. H., Negrin, R. S., Fathman, C. G., and Contag, C. H. (2001). Bioluminescence imaging of lymphocyte trafficking in vivo. *Exp. Hematol.* **29**, 1353–1360.
84. Lorenz, W. W., McCann, R. O., Longiaru, M., and Cormier, M. J. (1991). Isolation and expression of a cDNA encoding Renilla reniformis luciferase. *Proc. Natl. Acad. Sci. USA* **88**, 4438–4442.

85. Bhaumik, S. and Gambhir, S. S. (2002). Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc. Natl. Acad. Sci. USA* **99**, 377–382.
86. Liu, H., Iacono, R. P., and Szalay, A. A. (2001). Detection of GDNF secretion in glial cell culture and from transformed cell implants in the brains of live animals. *Mol. Genet. Genomics* **266**, 614–623.
87. Ray, P., Wu, A. M., and Gambhir, S. S. (2003). Optical bioluminescence and positron emission tomography imaging of a novel fusion reporter gene in tumor xenografts of living mice. *Cancer Res.* **63**, 1160–1165.
88. Ray, P., De, A., Min, J. J., Tsien, R. Y., and Gambhir, S. S. (2004). Imaging tri-fusion multimodality reporter gene expression in living subjects. *Cancer Res.* **64**, 1323–1330.
89. Loening, A. M., Fenn, T. D., Wu, A. M., and Gambhir, S. S. (2006). Consensus guided mutagenesis of renilla luciferase yields enhanced stability and light output. *Protein Engineering and Design* **19**, 453–460..
90. Contag, C. H., Contag, P. R., Mullins, J. I., Spilman, S. D., Stevenson, D. K., and Benaron, D. A. (1995). Photonic detection of bacterial pathogens in living hosts. *Mol. Microbiol.* **18**, 593–603.
91. Moore, A., Josephson, L., Bhorade, R. M., Basilion, J. P., and Weissleder, R. (2001). Human transferrin receptor gene as a marker gene for MR imaging. *Radiology* **221**, 244–250.
92. Ichikawa, T., Hogemann, D., Saeki, Y., et al. (2002). MRI of transgene expression: correlation to therapeutic gene expression. *Neoplasia* **4**, 523–530.
93. Enochs, W. S., Petherick, P., Bogdanova, A., Mohr, U., and Weissleder, R. (1997). Paramagnetic metal scavenging by melanin: MR imaging. *Radiology* **204**, 417–423.
94. Jaffer, F. A. and Weissleder, R. (2004). Seeing within molecular imaging of the cardiovascular system. *Circ. Res.* **94**, 433–445.
95. Genove, G., DeMarco, U., Xu, H., Goins, W. F., and Ahrens, E. T. (2005). A new transgene reporter for in vivo magnetic resonance imaging. *Nat. Med.* **11**, 450–454.
96. Cohen, B., Dafni, H., Meir, G., Harmelin, A., and Neeman, M. (2005). Ferritin as an endogenous MRI reporter for noninvasive imaging of gene expression in C6 glioma tumors. *Neoplasia* **7**, 109–117.
97. Nettelbeck, D. M., Jerome, V., and Muller, R. (2000). Gene therapy: designer promoters for tumour targeting. *Trends Genet.* **16**, 174–181.
98. Yoon, T. K., Shichinohe, T., Laquerre, S., and Kasahara, N. (2001). Selectively replicating adenoviruses for oncolytic therapy. *Curr. Cancer Drug Targets* **1**, 85–107.
99. Biederer, C., Ries, S., Brandts, C. H., and McCormick, F. (2002). Replication-selective viruses for cancer therapy. *J. Mol. Med.* **80**, 163–175.
100. Roth, W., and Reed, J. C. (2002). Apoptosis and cancer: When BAX is TRAILing away. *Nat. Med.* **8**, 216–218.
101. Reed, J. C. (2002). Apoptosis-based therapies. *Nat. Rev. Drug Discov.* **1**, 111–121.
102. Zhu, Z. B., Makhija, S. K., Lu, B., et al. (2004). Transcriptional targeting of tumors with a novel tumor-specific survivin promoter. *Cancer Gene Ther.* **11**, 256–262.
103. Easty, D. J. and Bennett, D. C. (2000). Protein tyrosine kinases in malignant melanoma. *Melanoma Res.* **10**, 401–411.
104. Lee, S. E., Jin, R. J., Lee, S. G., et al. (2000). Development of a new plasmid vector with PSA-promoter and enhancer expressing tissue-specificity in prostate carcinoma cell lines. *Anticancer Res.* **20**, 417–422.
105. Yamamoto, M., Alemany, R., Adachi, Y., Grizzle, W. E., and Curiel, D. T. (2001). Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers. *Mol. Ther.* **3**, 385–394.
106. Nettelbeck, D. M., Rivera, A. A., Davydova, J., Dieckmann, D., Yamamoto, M., and Curiel, D. T. (2003). Cyclooxygenase-2 promoter for tumour-specific targeting of adenoviral vectors to melanoma. *Melanoma Res.* **13**, 287–292.
107. Anderson, L. M., Swaminathan, S., Zackon, I., Tajuddin, A. K., Thimmapaya, B., and Weitzman, S. A. (1999). Adenovirus-mediated tissue-targeted expression of the HSV-tk gene for the treatment of breast cancer. *Gene Ther.* **6**, 854–864.
108. Nettelbeck, D. M., Rivera, A. A., Balague, C., Alemany, R., and Curiel, D. T. (2002). Novel oncolytic adenoviruses targeted to melanoma: specific viral replication and cytolysis by expression of E1A mutants from the tyrosinase enhancer/promoter. *Cancer Res.* **62**, 4663–4670.
109. Wu, L. and Sato, M. (2003). Integrated, molecular engineering approaches to develop prostate cancer gene therapy. *Curr. Gene Ther.* **3**, 452–467.
110. Siders, W. M., Halloran, P. J., and Fenton, R. G. (1996). Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res.* **56**, 5638–5646.
111. Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., and Henderson, D. R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* **57**, 2559–2563.
112. Pang, S., Dannull, J., Kaboo, R., et al. (1997). Identification of a positive regulatory element responsible for tissue-specific expression of prostate-specific antigen. *Cancer Res* **57**, 495–499.
113. Vile, R. G., Nelson, J. A., Castleden, S., Chong, H., and Hart, I. R. (1994). Systemic gene therapy of murine melanoma using tissue-specific expression of the HSVtk gene involves an immune component. *Cancer Res.* **54**, 6228–6234.

114. Nettelbeck, D. M., Jerome, V., and Muller, R. (1998). A strategy for enhancing the transcriptional activity of weak cell type-specific promoters. *Gene Ther.* **5**, 1656–1664.
115. Wu, L., Johnson, M., and Sato, M. (2003). Transcriptionally targeted gene therapy to detect and treat cancer. *Trends Mol. Med.* **9**, 421–429.
116. Schuur, E. R., Henderson, G. A., Kmetec, L. A., Miller, J. D., Lamparski, H. G., and Henderson, D. R. (1996). Prostate-specific antigen expression is regulated by an upstream enhancer. *J. Biol. Chem.* **271**, 7043–7051.
117. Wu, L., Matherly, J., Smallwood, A., Belldegrun, A. S., and Carey, M. (2001). Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vector. *Gene Ther.* **8**, 1416–1426.
118. Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**, 563–564.
119. Triezenberg, S. J., LaMarco, K. L., and McKnight, S. L. (1988). Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. *Genes Dev.* **2**, 730–742.
120. Carey, M., Lin, Y. S., Green, M. R., and Ptashne, M. (1990). A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. *Nature* **345**, 361–364.
121. Carey, M. and Smale, S. T. (2000). Transcriptional Regulation in Eukaryotes. Cold Spring Harbor Laboratory Press, New York, NY.
122. Iyer, M., Wu, L., Carey, M., Wang, Y., Smallwood, A., and Gambhir, S. S. (2001). Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proc. Natl. Acad. Sci USA* **98**, 14,595–14,600.
123. Zhang, L., Adams, J. Y., Billick, E., et al. (2002). Molecular engineering of a two-step transcription amplification (TSTA) system for transgene delivery in prostate cancer. *Mol. Ther.* **5**, 223–232.
124. Sato, M., Johnson, M., Zhang, L., et al. (2003). Optimization of adenoviral vectors to direct highly amplified prostate-specific gene expression for imaging and gene therapy. *Mol. Ther.* **8**, 726–737.
125. Zhang, J., Johnson, M., Le, K. H., et al. (2003). Interrogating androgen receptor function in recurrent prostate cancer. *Cancer Res.* **63**, 4552–4560.
126. Sundaresan, G., Murugesan, S., and Gambhir, S. S. (2003). The Society for Molecular Imaging. 2nd Annual Meeting, San Fransisco, CA.
127. Iyer, M., Salazar, F., Lewis, X., et al. (2004). Noninvasive imaging of enhanced prostate-specific gene expression using a two-step transcriptional amplification-based lentivirus vector. *Mol. Ther.* **10**, 545–552.
128. Iyer, M., Salazar, F., Lewis, X., et al. (2005). Noninvasive imaging of a transgenic mouse model using a prostate-specific two-step transcriptional amplification strategy. *Transgenic Res.* **14**, 47–55.
129. Gill, G. and Ptashne, M. (1988). Negative effect of the transcriptional activator GAL4. *Nature* **334**, 721–724.
130. Yueh, Y. G., Yaworsky, P. J., and Kappen, C. (2000). Herpes simplex virus transcriptional activator VP16 is detrimental to preimplantation development in mice. *Mol. Reprod. Dev.* **55**, 37–46.
131. Wang, Y., Iyer, M., Annala, A., Wu, L., Carey, M., and Gambhir, S. S. (2006). Non-invasive indirect imaging of vascular endothelial growth factor gene expression using bioluminescence imaging in living transgenic mice. *Phys. Genom.* **24**, 173–180.
132. Sundaresan, G. and Gambhir, S. S. (2002). Radionuclide imaging of reporter gene expression. In Brain Mapping: The Methods (Toga, A. W., Mazziotta, J. C., ed.). Academic Press, San Diego, CA, pp. 799–818.
133. Yu, Y., Annala, A. J., Barrio, J. R., et al. (2000). Quantification of target gene expression by imaging reporter gene expression in living animals. *Nat. Med.* **6**, 933–937.
134. Kamoshita, N., Tsukiyama-Kohara, K., Kohara, M., and Nomoto, A. (1997). Genetic analysis of internal ribosomal entry site on hepatitis C virus RNA: implication for involvement of the highly ordered structure and cell type-specific transacting factors. *Virology* **233**, 9–18.
135. Chappell, S., Edelman, G., and Mauro, V. (2000). A 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity. *Proc. Natl. Acad. Sci. USA* **97**, 1536–1541.
136. Wang, Y., Iyer, M., Annala, A. J., Chappell, S., Mauro, V., and Gambhir, S. S. (2005). Noninvasive imaging of target gene expression by imaging reporter gene expression in living animals using improved bicistronic vectors. *J. Nucl. Med.* **46**, 667–674.
137. Sun, X., Annala, A. J., Yaghoubi, S. S., et al. (2001). Quantitative imaging of gene induction in living animals. *Gene Ther.* **8**, 1572–1579.
138. Ray, S., Paulmurugan, R., Hildebrandt, I., et al. (2004). Novel bidirectional vector strategy for amplification of therapeutic and reporter gene expression. *Hum. Gen. Ther.* **15**, 681–690.
139. Yaghoubi, S. S., Nguyen, K., Bauer, E., et al. (2000). Imaging adenoviral mediated therapeutic gene delivery by co-administration of a second adenovirus carrying a PET reporter gene. *J. Nucl. Med.* **41**, 37P–38P.
140. Chien, C. T., Bartel, P. L., Sternlglanz, R., and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* **88**, 9578–9582.
141. Bai, C. and Elledge, S. J. (1996). Gene identification using the yeast two-hybrid system. *Methods Enzymol.* **273**, 331–347.

142. Ray, P., Pimenta, H., Paulmurugan, R., et al. (2002). Noninvasive quantitative imaging of protein–protein interactions in living subjects. *Proc. Natl. Acad. Sci. USA* **99**, 3105–3110.
143. Luker, G. D., Sharma, V., Pica, C. M., et al. (2002). Noninvasive imaging of protein–protein interactions in living animals. *Proc. Natl. Acad. Sci. USA* **99**, 6961–6966.
144. Paulmurugan, R. and Gambhir, S. S. (2003). Monitoring protein–protein interactions using split synthetic renilla luciferase protein-fragment-assisted complementation. *Anal. Chem.* **75**, 1584–1589.
145. Massoud, T. F., Paulmurugan, R., and Gambhir, S. S. (2004). Molecular imaging of homodimeric protein–protein interactions in living subjects. *FASEB J.* **18**, 1105–1107.
146. Paulmurugan, R. and Gambhir, S. S. (2005). Firefly luciferase enzyme fragment complementation for imaging in cells and living animals. *Anal. Chem.* **77**, 1295–1302.
147. Paulmurugan, R. and Gambhir, S. S. (2005). Novel fusion protein approach for efficient high-throughput screening of small molecule-mediating protein–protein interactions in cells and living animals. *Cancer Res.* **65**, 7413–7420.
148. De, A. and Gambhir, S. S. (2005). Non-invasive imaging of protein–protein interactions from live cells and living subjects using bioluminescence resonance energy transfer. *FASEB J.* **19**, 2017–2019.
149. Jensen, A. A., Hansen, J. L., Sheikh, S. P., and Brauner-Osborne, H. (2002). Probing intermolecular protein–protein interactions in the calcium-sensing receptor homodimer using bioluminescence resonance energy transfer (BRET). *Eur. J. Biochem.* **269**, 5076–5087.
150. Jacobs, R. E. and Cherry, S. R. (2001). Complementary emerging techniques: high-resolution PET and MRI. *Curr. Opin. Neurobiol.* **11**, 621–629.
151. Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v and beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**, 309–319.
152. Bergelson, J. M., Cunningham, J. A., Droguett, G., et al. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320–1323.
153. Ferry, N. and Heard, J. M. (1998). Liver-directed gene transfer vectors. *Hum. Gene Ther.* **9**, 1975–1981.
154. Bruder, J. T., Jie, T., McVey, D. L., and Kovesdi, I. (1997). Expression of gp 19K increases the persistence of transgene expression from an adenovirus vector in the mouse lung and liver. *J. Virol.* **71**, 7623–7628.
155. Ilan, Y., Droguett, G., Chowdhury, N. R., et al. (1997). Insertion of the adenoviral E3 region into a recombinant viral vector prevents antiviral humoral and cellular immune responses and permits long-term gene expression. *Proc. Natl. Acad. Sci. USA* **94**, 2587–2592.
156. Vilquin, J. T., Guereatte, B., Kinoshita, I., et al. (1995). FK 506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. *Hum. Gen. Ther.* **6**, 1391–1401.
157. Kass-Eisler, A., Leinwand, L., Gall, J., Bloom, B., and Falck-Pedersen, E. (1996). Circumventing the immune response to adenovirus-mediated gene therapy. *Gene Ther.* **3**, 154–162.
158. O'Riordan, C. R., Lachapelle, A., Delgado, C., et al. (1999). PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum. Gen. Ther.* **10**, 1349–1358.
159. Shinnick, T. M., Lerner, R. A., and Sutcliffe, J. G. (1981). Nucleotide sequence of Moloney murine leukemia virus. *Nature* **293**, 543–548.
160. Roth, J. A. and Cristiano, R. (1997). Gene therapy for cancer: what have we done and where are we going? *J. Natl. Cancer Inst.* **89**, 21–39.
161. Romano, G., Pacilio, C., and Giordano, A. (1999). Gene transfer technology in therapy: current applications and future goals. *Stem Cells* **17**, 191–202.
162. Amado, R. G., Mitsuyasu, R. T., Symonds, G., et al. (1999). A phase I trial of autologous CD34+ hematopoietic prgenitor cells transduced with an anti-HIV ribozyme. *Hum. Gen. Ther.* **10**, 2255–2270.
163. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee J. K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90**, 8033–8037.
164. Takeuchi, Y., Cosset, F. L., Lachmann, P. J., Okada, H., Weiss, R. A., and Collins, M. K. (1994). Type C retrovirus inactivation by human complement is determined by both the viral genome and the producer cell. *J. Virol.* **68**, 8001–8007.
165. Martineau, D., Klump, W. M., McCormack, J. E., et al. (1997). Evaluation of PCR and ELISA assays for screening clinical trial subjects for replication-competent retrovirus. *Hum. Gen. Ther.* **8**, 1231–1241.
166. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* **90**, 8392–8396.
167. Yang, S., Delgado, R., King, S. R., et al. (1999). Generation of retroviral vector for clinical studies using transient transfection. *Hum. Gen. Ther.* **10**, 123–132.
168. Weindler, F. W. and Heilbronn, R. (1991). A subset of heres simplex virus replication genes provides helper functions for productive adeno-associated virus replication. *J. Virol.* **65**, 2476–2483.
169. Yakinoglu, A. O., Heilbronn, R., Burkle, A., Schlehofer, J. R., and zur Hausen, H. (1988). DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. *Cancer Res.* **48**, 3123–3129.
170. Alexander, I. E., Russell, D. W., and Miller, A. D. (1994). DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *J. Virol.* **68**, 8282–8287.

171. Kanazawa, T., Mizukami, H., Okada, T., et al. (2003). Suicide gene therapy using AAV-HSVtk/ganciclovir in combination with irradiation results in regression of human head and neck cancer xenografts in nude mice. *Gene Ther.* **10**, 51–58.
172. Behr, J. P. (1994). Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. *Bioconjug. Chem.* **5**, 382–389.
173. Nishikawa, M. and Huang, L. (2001). Nonviral vectors in the new millennium: Delivery barriers in gene transfer. *Hum. Gen. Ther.* **12**, 861–870.
174. Alio, S. F. (1997). Long term expression of the human alpha I antitrypson gene in mice employing anionic and cationic liposome vector. *Biochem. Pharmacol.* **54**, 9–13.
175. Lasic, D. D., Martin, F. J., Gabizon, A., Huang, S. K., and Papahadjopoulos, D. (1991). Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. *Biochim. Biophys. Acta.* **1070**, 187–192.
176. Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991). Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery into cells. *Proc. Natl. Acad. Sci. USA* **88**, 4255–4259.
177. Wu, C. H., Wilson, J. M., and Wu, G. Y. (1989). Targeting genes: Delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo. *J. Biol. Chem.* **264**, 16985–16987.
178. Ferkol, T., Kaetzel, C. S., and Davis, P. B. (1993). Gene transfer into respiratory epithelial cells by targeting the polymeric immunoglobin receptor. *J. Clin. Invest.* **92**, 2394–2400.
179. Liu, F., Song, Y. K., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**, 1258–1266.
180. Ehrhardt, A., Peng, P. D., Xu, H., Meuse, L., and Kay, M. A. (2003). Optimization of cis-acting elements for gene expression from nonviral vectors in vivo. *Hum. Gen. Ther.* **14**, 215–225.
181. Wolff, J. A., Malone, R. W., Williams, P., et al. (1990). Direct gene transfer into mouse muscle in vivo. *Science* **247**, 1465–1468.

13

Quantum Dot Nanotechnology for Prostate Cancer Research

Xiaohu Gao, Yun Xing, Leland W. K. Chung, and Shuming Nie

Summary

Quantum dots (QDs), tiny light-emitting particles on the nanometer scale, are emerging as a new class of fluorescent probes for cancer cell imaging and molecular profiling. In comparison with organic dyes and fluorescent proteins, QDs have unique optical and electronic properties, such as size-tunable light emission, improved signal brightness, resistance against photobleaching, and simultaneous excitation of multiple fluorescence colors. These properties are most promising for improving the sensitivity of molecular imaging and quantitative cellular analysis by one to two orders of magnitude. Recent advances have led to multifunctional nanoparticle probes that are highly bright and stable under complex biological conditions. A new structural design involves encapsulating luminescent QDs with amphiphilic block copolymers, and linking the polymer coating to tumor-targeting ligands and drug-delivery functionalities. Polymer-encapsulated QDs are essentially nontoxic to cells and animals, but their long-term in vivo toxicity and degradation need more studies that are careful. Nonetheless, bioconjugated QDs have raised new possibilities for ultrasensitive and multiplexed imaging of molecular targets in living cells, animal models, and, possibly, in human patients.

Key Words: Fluorescence; molecular imaging; nanotechnology; quantum dots; tumor targeting.

1. INTRODUCTION

Semiconductor quantum dots (QDs) have captivated scientists and engineers during the past two decades because of their fascinating optical and electronic properties, which are not available from either isolated molecules or bulk solids. Recent research has stimulated considerable interest in developing these quantum-confined nanocrystals as fluorescent probes for biomedical applications. In comparison with organic dyes and fluorescent proteins, semiconductor QDs offer several unique advantages, such as size- and composition-tunable emission from visible to infrared wavelengths, large absorption coefficients across a wide spectral range, and very high levels of brightness and photostability *(1)*. Because of their broad excitation profiles and narrow/symmetric emission spectra, high-quality QDs are also well-suited for combinatorial optical encoding, in which multiple colors and intensities are combined to encode thousands of genes, proteins, or small-molecule compounds *(2–4)*.

Despite their relatively large sizes (2–8 nm diameter), recent research has shown that bioconjugated QD probes behave like fluorescent proteins (4–6 nm), and do not suffer from serious binding kinetic or steric-hindrance problems *(5–12)*. In this "mesoscopic" size range, QDs also have more surface areas and functionalities that can be used for linking to multiple diagnostic (e.g., radioisotopic or magnetic) and therapeutic (e.g., anticancer) agents. Furthermore, polymer-encapsulated QDs have been found to be essentially nontoxic to cells and animals, an essential requirement for future clinical applications. In the chapter, we briefly discuss new developments and recent applications of QD probes for cancer research. Particularly, using prostate cancer as a model system, we

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

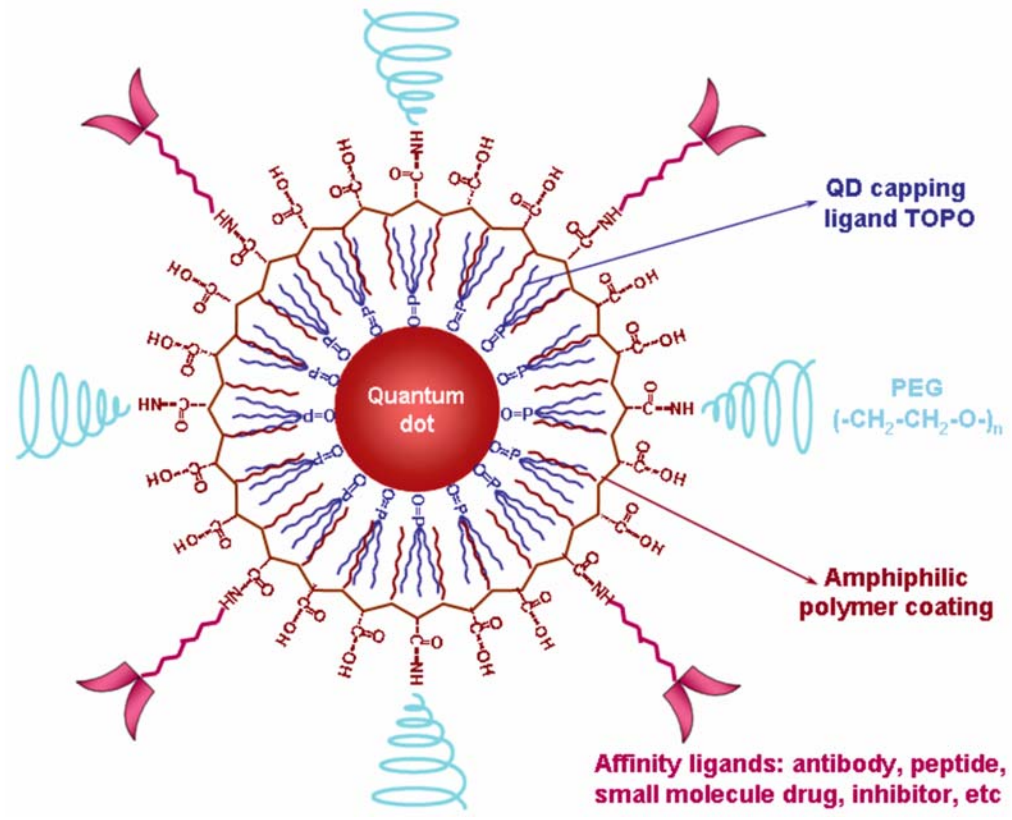


Fig. 1. Structure of a multifunctional quantum dot (QD) probe, showing the capping ligand trioctylphosphineoxide (TOPO), an encapsulating copolymer layer, tumor-targeting ligands (such as peptides, antibodies, or small-molecule inhibitors), and polyethylene glycol (PEG).

discuss the use of multicolor QDs for molecular profiling of clinical tissue specimens and in vivo tumor detection and imaging. For detailed information on QD fundamentals and applications, excellent review articles are available in the literature *(1,13,14)*.

2. PROBE DEVELOPMENT

Research in probe development has focused on synthesis, solubilization, and bioconjugation of highly luminescent and stable QDs. The particles are generally made from hundred to thousands of atoms of groups II and VI (e.g., CdSe and CdTe) or groups III and V (e.g., InP and InAs) elements. Recent advances have allowed the precise control of particle sizes, shapes (dots, rods, or tetrapods) *(15–18)*, as well as internal structures (core-shell, gradient alloy, or homogeneous alloy) *(19–22)*. In particular, QDs have been synthesized using both two-element systems (binary dots) as well as three-element systems (ternary alloy dots). Their fluorescence emission wavelength can be continuously tuned from 400 to 2000 nm by changing both the particle size and chemical composition, with fluorescence quantum yields as high as 85% at room temperature *(23)*.

High-quality QDs are typically prepared at elevated temperatures in organic solvents, such as trioctylphosphineoxide (TOPO); trioctylphosphine (TOP), and hexadecylamine (all of which are high boiling-point solvents containing long alkyl chains). These hydrophobic organic molecules not only serve as the reaction medium, but also coordinate with unsaturated metal atoms on the QD surface to

prevent formation of bulk semiconductors. As a result, the nanoparticles are capped with a monolayer of the organic ligands and are soluble only in nonpolar hydrophobic solvents, such as chloroform. For biological imaging applications, these hydrophobic dots can be solubilized by using amphiphilic polymers that contain both a hydrophobic segment or side-chain (mostly hydrocarbons) and a hydrophilic segment or group (such as polyethylene glycol [PEG] or multiple carboxylate groups). A number of polymers have been reported, including octylamine-modified low molecular weight polyacrylic acid, PEG-derivatized phospholipids, block copolymers, and polyanhydrides *(5,24–26)*. As schematically illustrated in Fig. 1, the hydrophobic domains strongly interact with TOPO on the QD surface, whereas the hydrophilic groups face outward and render QDs water-soluble. Note that the coordinating organic ligands (TOP or TOPO) are retained on the inner surface of QDs, a feature that is important for maintaining the optical properties of QDs and for shielding the core from the outside environment. To achieve binding specificity or targeting abilities, polymer-coated QDs are linked to bioaffinity ligands, such as monoclonal antibodies, peptides, oligonucleotides, or small-molecule inhibitors, and also to PEGs or similar ligands (leading to improved biocompatibility and reduced nonspecific binding).

QD bioconjugation can be achieved by several approaches, including passive adsorption, multivalent chelation, or covalent bond formation (Fig. 2). Two popular cross-linking reactions are carbodiimide-mediated amide formation, and active ester maleimide-mediated amine and sulfhydryl coupling. An advantage for the carboxylate-amine condensation method is that most proteins contain primary amine and carboxylic acid groups, and do not need any chemical modifications before QD conjugation. In contrast, free and accessible sulfhydryl groups are rare in native biomolecules and are often unstable in the presence of oxygen. Depending on the available chemical groups, other conjugation reactions can also be used. For example, Pellegrino et al. reported the use of a pre-activated amphiphilic polymer for nanoparticle solubilization *(26)*. This polymer contains multiple anhydride units, and is highly reactive toward primary amines without addition of coupling reagents. This procedure deserves further attention because polyanhydrides represent a class of biodegradable polymers that are under intense development for use in sustained drug delivery and tissue engineering *(27,28)*.

Several strategies can be used to manipulate the molecular orientations of the attached ligands as well as their molar ratios with respect to QDs. However, "perfect" QD probes with precisely controlled ligand orientations and molar ratios are still not available. Mattoussi et al. first explored the use of a fusion protein as an adaptor for IgG antibody coupling *(29)*. The adaptor protein has a positively charged leucine zipper domain for electrostatic interaction with QDs, and a protein G domain that binds to the antibody Fc region. As a result, the Fc end of the antibody is connected to the QD surface, with the target-specific $F(ab')_2$ domain facing outward (Fig. 2C). In a dramatically different approach, we have linked QDs to a chelating compound (nickel-nitrilotriacetic acid) that quantitatively binds to hexahistidine-tagged biomolecules with controlled molar ratio and molecular orientation (Fig. 2D). Early studies using genetically engineered peptides showed excellent tumor targeting abilities (X. Gao, L. Yang, and S. Nie, unpublished data). This indirect "his-tag" coupling method has several advantages, such as a controlled or known orientation of the binding ligand (a histidine tag can be conveniently fused to proteins and peptides at a particular site), compact overall probe sizes (which should improve binding efficiencies), and low production costs (direct coupling and rapid purification).

3. NOVEL OPTICAL PROPERTIES

As briefly noted above in Section 2, Probe Development, QDs are made from inorganic semiconductors, and have novel optical properties that can be used to optimize signal-to-background ratios. First, QDs have very large molar extinction coefficients, on the order of 0.5 to 5×10^6 /*M*/cm *(30)*, which makes them brighter probes under photon-limited in vivo conditions (in which light intensities

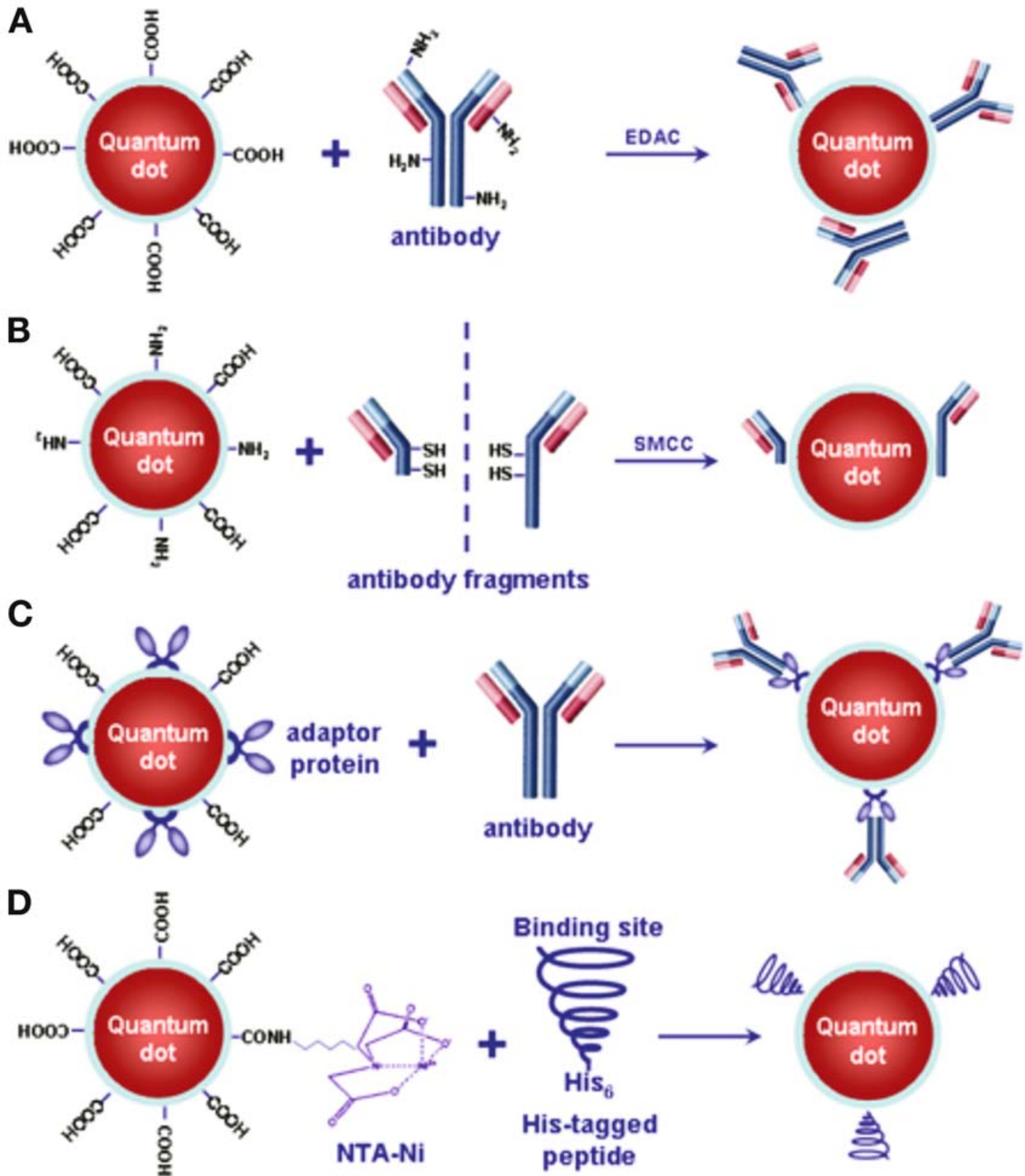


Fig. 2. Methods for conjugating quantum dots (QDs) to biomolecules. **(A)** Traditional covalent cross-linking chemistry using (1-Ethyl-3-[3-dimethyl-aminopropyl]carbodiimide hydrochloride) (EDAC) as a catalyst. **(B)** Conjugation of antibody fragments to QDs via reduced sulfhydryl-amine coupling. succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). **(C)** Conjugation of antibodies to QDs via an adaptor protein. **(D)** Conjugation of histidine-tagged peptides and proteins to nitrilotriacetic acid (NTA)-Ni-modified QDs, with potential control of the attachment site and QD:ligand molar ratios.

are severely attenuated by scattering and absorption). In theory, the lifetime-limited emission rates for single QDs are 5 to 10 times lower than those of single organic dyes because of their longer excited state lifetimes (20–50 ns). In practice, however, fluorescence imaging usually operates under

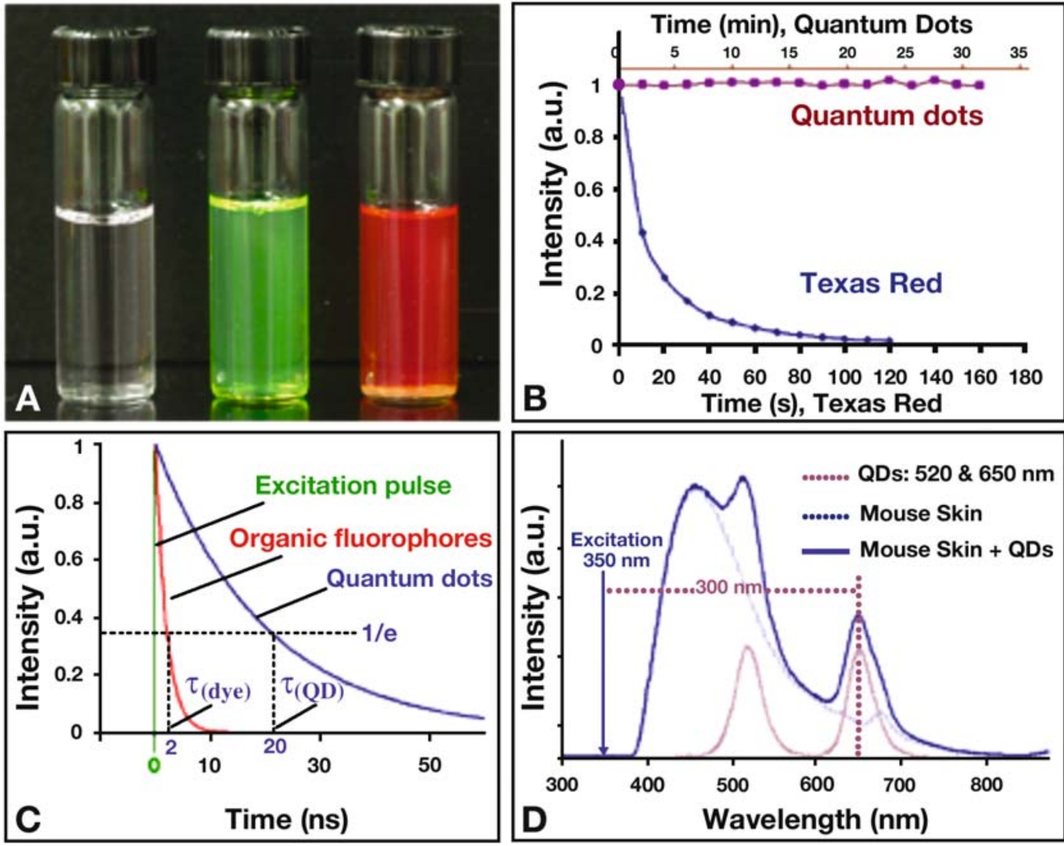


Fig. 3. Novel optical properties of quantum dot (QDs) for improving the sensitivity of in vivo bioimaging. **(A)** Comparison of fluorescence light emission from organic dyes (TRITC, left vial), green QDs (middle vial), and red QDs (right vial) under normal room light illumination and at the same molar concentration (1.0 μ*M* for dyes and QDs). Bright fluorescence emission is observed from QDs but not from the dye, because of the large absorption cross sections of QDs. **(B)** Photobleaching curves showing that QDs are several thousand times more photostable than organic dyes under the same excitation conditions. **(C)** A comparison of the excited-state decay curves (monoexponential model) between QDs and common organic dyes. The longer excited-state lifetimes of QD probes allow the use of time-domain imaging to discriminate against the background fluorescence (short lifetimes). **(D)** Comparison of mouse skin and QD emission spectra obtained under the same excitation conditions, demonstrating that the QD signals can be shifted to a spectral region where the autofluorescence is reduced.

absorption-limited conditions, in which the rate of absorption is the main limiting factor of fluorescence emission. Because the molar extinction coefficients of QDs are approx 10 to 50 times larger than those of organic dyes ($5–10 \times 10^4$ /*M*/cm), the QD absorption rates will be 10 to 50 times faster than that of organic dyes at the same excitation photon flux (number of incident photons per unit area). Because of this increased rate of light emission, individual QDs have been found to be 10 to 20 times brighter than organic dyes (Fig. 3A) *(31,32)*. In addition, QDs are several thousand times more stable against photobleaching than dye molecules (Fig. 3B), and are, thus, well-suited for continuous tracking studies during a long period of time.

Second, the longer excited state lifetimes of QDs provide a means for separating the QD fluorescence from background fluorescence, in a technique known as time-domain imaging *(33,34)*. Figure

3C shows a comparison of the excited state decay curves of QDs and organic dyes. Assuming that the initial fluorescence intensities of QDs and dyes after a pulse excitation are the same, and that the fluorescence lifetime of QDs is one order of magnitude longer, one can estimate that the QD and dye intensity ratio (I_{QD}/I_{dye}) will increase rapidly from 1 at time t = 0 to approx 100 in only 10 ns (t = 10 ns). Thus, the image contrast (measured by signal-to-noise or signal-to-background ratios) can be dramatically improved by time-relayed data acquisition.

Third, the large Stokes shifts of QDs (measured by the distance between the excitation and emission peaks) can be used to further improve detection sensitivity. This factor becomes especially important for in vivo molecular imaging because of the high autofluorescence background often seen in complex biomedical specimens. As shown in Fig. 3D, the Stokes shifts of semiconductor QDs can be as large as 300 to 400 nm, depending on the wavelength of the excitation light. Organic dye signals with a small Stokes shift are often buried by strong tissue autofluorescence, whereas QD signals with a large Stokes shift are clearly recognizable above the background. This "color contrast" is only available to QD probes because the signals and background can be separated by wavelength-resolved or spectral imaging *(25)*.

A further advantage is that multicolor QD probes can be used to image and track multiple molecular targets simultaneously. This is a very important feature because most complex human diseases, such as cancer and atherosclerosis, involve a large number of genes and proteins. Tracking a panel of molecular markers at the same time will allow scientists to understand, classify, and differentiate complex human diseases *(35)*. Multiple parameter imaging, however, represents a significant challenge for MRI, positron emission tomography (PET), CT, and related imaging modalities. On the other hand, fluorescence optical imaging provides both signal intensity and wavelength information, and multiple wavelengths or colors can be resolved and imaged simultaneously (color imaging). Therefore, different molecular or cellular targets can be tagged with different colors. In this regard, QD probes are particularly attractive because their broad absorption profiles allow simultaneous excitation of multiple colors, and their emission wavelengths can be continuously tuned by varying particle size and chemical composition. For organ and vascular imaging in which micrometer-sized particles could be used, optically encoded beads (polymer beads embedded with multicolor QDs at controlled ratios) could allow multiplexed molecular profiling in vivo at high sensitivities *(35–40)*.

4. CELLULAR IMAGING AND TRACKING

The use of QDs for sensitive and multicolor cellular imaging has seen major recent advances, because of significant improvements in QD synthesis, surface chemistry, and conjugation (Fig. 4). Wu et al. linked polymer-protected QDs to streptavidin and showed detailed cell skeleton structures using confocal microscopy *(5)*. The improved photostability of QDs allowed acquisition of many consecutive focal-plane images and their reconstruction into a high-resolution 3D projection. The high electron density of QDs also allowed correlated optical and electron microscopy studies of cellular structures *(41)*. One step further, Dahan, Jovin, and their coworkers achieved real-time visualization of single molecule movement in single living cells *(6,7)*, a task that would be extremely difficult or impossible to do with organic dyes. The achieved single-molecule sensitivity should open a new avenue for studying receptor diffusion dynamics, ligand-receptor interaction, biomolecular transport, enzyme activity, and molecular motors.

For long-term cell imaging and tracking, Dubertret et al. *(24)* encapsulated QDs with PEG-derivatized phospholipid micelles, and injected them into frog embryos. The resulting PEG-coated dots were highly stable and biocompatible, with normal embryo development, for up to 4 days. Other recent studies also took advantage of the extraordinary photostability of QD probes, and achieved real-time tracking of molecules and cells during extended periods of time *(6,7,9)*. For example, QDs were observed to be taken up by lymph nodes and were observable for more than 4 months in mice *(42)*.

Semiconductor QDs have also been used as cell "taggants" for in vivo imaging of prelabeled cells (Fig. 4C) *(24,25,43–46)*. The results indicate that large amounts of QDs can be delivered into live

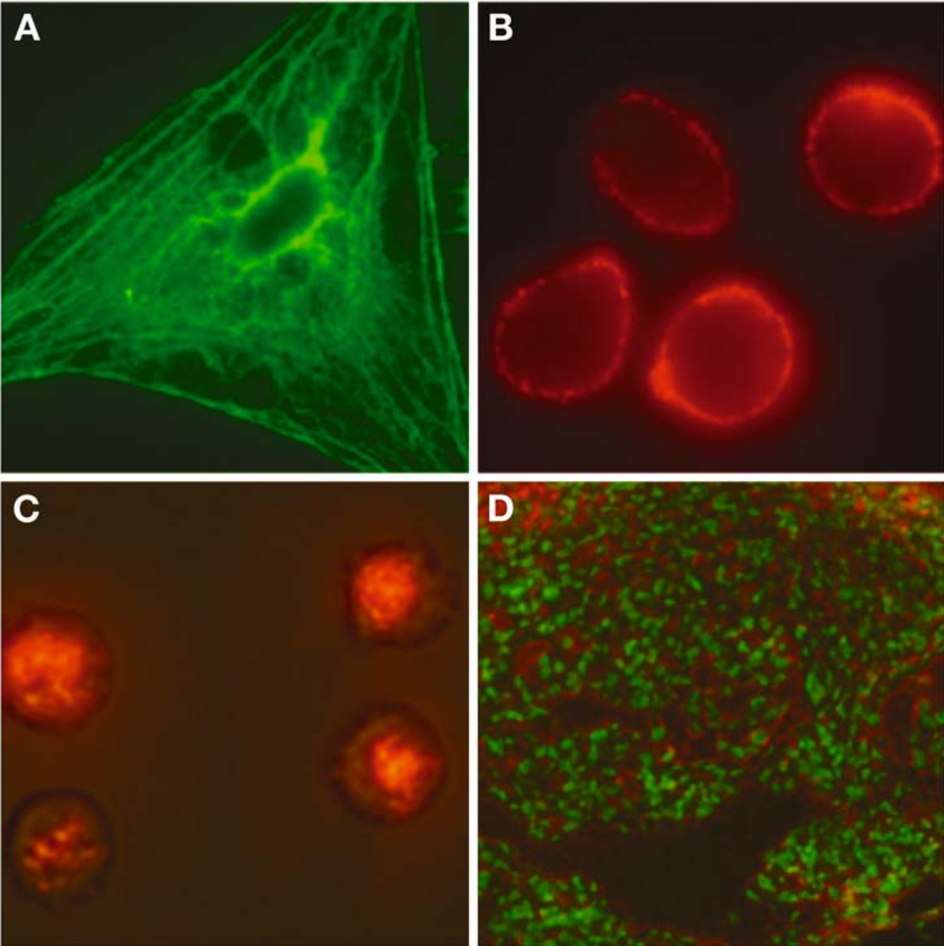


Fig. 4. Fluorescence micrographs of quantum dot (QD)-stained cells and tissues. **(A)** Actin staining (green QDs) on fixed 3T3 fibroblast cells. **(B)** Live MDA-MB-231 breast tumor cells labeled with red QD-antibody targeting the urokinase plasminogen receptor. **(C)** Intracellular labeling of live mammalian cells using QD-tat peptide conjugates *(25)*. **(D)** Frozen tissue specimens stained with QDs (targeting the CXCR4 receptor, red) and a nuclear dye (green).

mammalian cells via three different mechanisms: nonspecific pinocytosis, microinjection, and peptide-induced transport (e.g., protein transduction domain of HIV-1 tat peptide) *(25)*. A surprising finding is that 2 billion QDs could be delivered into the nucleus of a single cell, without compromising its viability, proliferation, or migration *(24,44,47)*. The ability to image single-cell migration and differentiation in real time is expected to be important to a number of research areas, such as embryogenesis, cancer metastasis, stem cell therapeutics, and lymphocyte immunology.

5. MOLECULAR PROFILING OF HUMAN PROSTATE TUMOR SPECIMENS

For many anticancer agents, prediction of therapeutic response or resistance in a clinical trial can be made with a biopsy. This requires the simultaneous detection and quantification of multiple protein biomarkers on tissue specimens. Current technologies allow neither simultaneous detection nor exact quantification of multiple therapeutic target proteins in cancer specimens. QDs conjugated to high avidity directed antibodies, permits a rapid, simultaneous assessment and quantification of can-

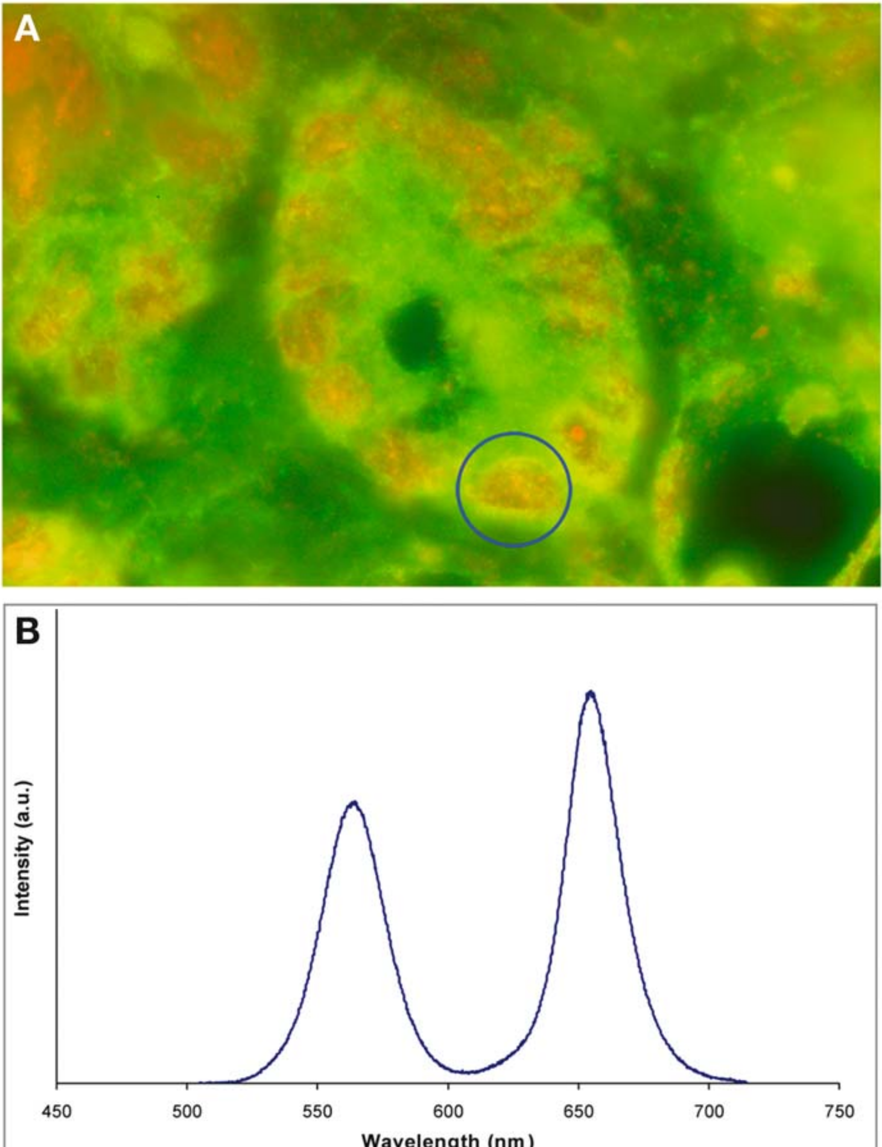


Fig. 5. Quantum dot (QD)-immunostaining of formalin-fixed, paraffin-embedded human prostate tumor specimens. **(A)** Fluorescence micrograph of dual-color QD-stained formalin-fixed, paraffin-embedded tissue, *see* text for details. **(B)** Quantitative measurement of QD fluorescence intensity. The region of interest can be selected manually (e.g., the blue circle in panel A) and quantified using a wavelength-resolved spectrometer.

cer biomarkers. The simultaneous detection of multiple proteins allows interrogation of entire signal transduction pathways in cancer tissues in response to novel agents. Ultimately, the use of QD antibody nanotyping may allow the exact tailoring of specific therapies to individual patients. Figure 5A shows the immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections of human prostate cancer using QDs. Mutated p53 phosphoprotein overexpressed in the nuclei of androgen-independent prostate cancer cells is labeled with red-color QDs (antibody DO-7; DAKO); whereas early growth response protein, egr-1, is tagged with green-color QDs. Quantitative spectro-

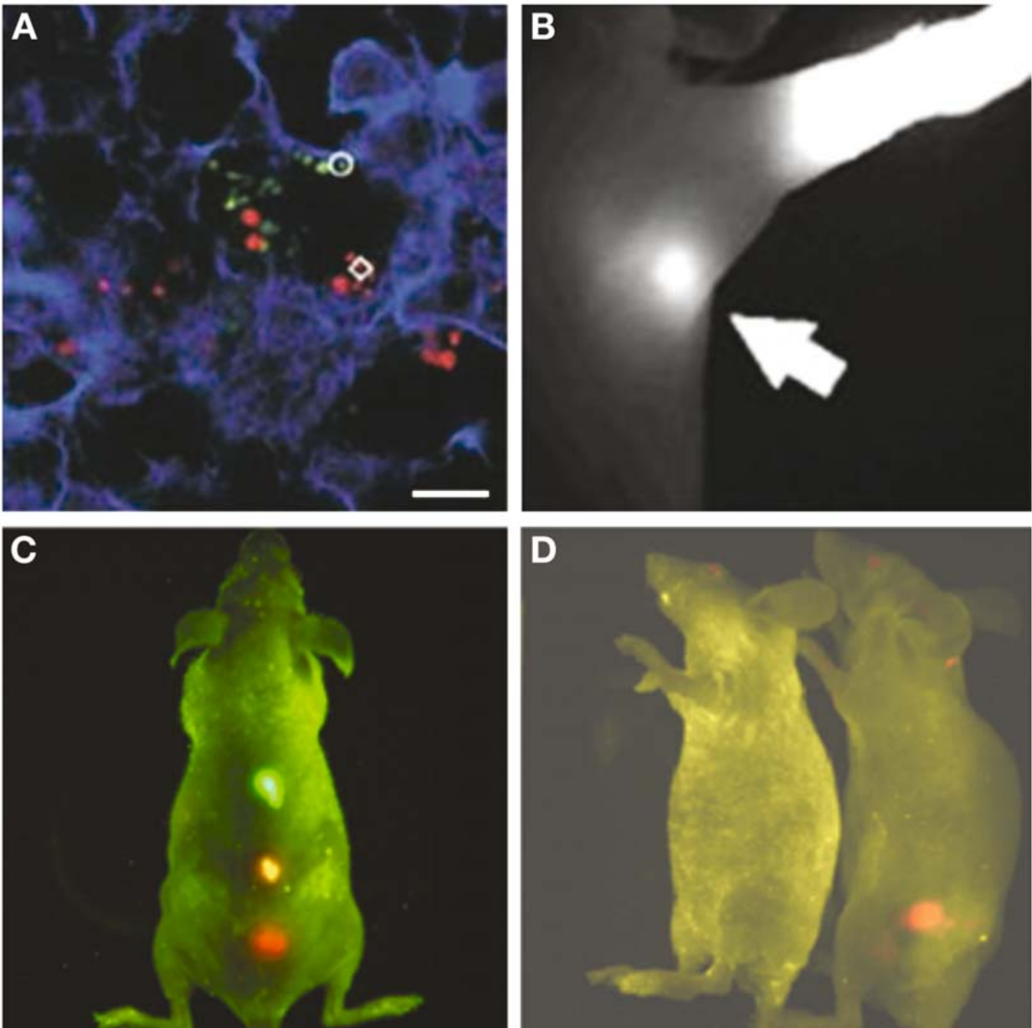


Fig. 6. In vivo targeting and imaging with quantum dots (QDs). **(A)** *Ex vivo* tissue examination of QD-labeled cancer cells trapped in a mouse lung *(44)*. **(B)** Near infrared fluorescence of water-soluble type II QDs taken up by sentinel lymph nodes *(49)*. **(C)** In vivo simultaneous imaging of multicolor QD-encoded microbeads injected into a live mouse *(25)*. **(D)** Molecular targeting and in vivo imaging of prostate tumor using a QD–antibody conjugate *(25)*.

scopic analysis of each targets at cellular level can also be performed by using a spectrometer (Fig. 5B) or an automated laser-scanning microscope.

6. MAPPING SENTINEL LYMPH NODES AND TUMOR ANGIOGENESIS

In vivo imaging with QDs has been reported for lymph node mapping, blood pool imaging, angiogenic vessels, and cell subtype isolation (Fig. 6A–C). Ballou and coworkers injected PEG-coated QDs into the mouse blood stream, and studied how the surface coating would affect their circulation lifetime *(42)*. In contrast to small organic dyes (which are eliminated from circulation within minutes after injection), PEG-coated QDs were found to stay in blood circulation for an extended period of time (half-life longer than 3 hours). This long-circulating feature can be explained by the unique

structural properties of QD nanoparticles. PEG-coated QDs are in an intermediate size range—they are small and hydrophilic enough to slow down opsonization and reticuloendothelial uptake, and they are large enough to avoid renal filtration. Webb and coworkers took advantage of this property, and reported the use of QDs and two-photon excitation to image small blood vessels *(48)*. They found that the two-photon absorption cross sections of QDs are two to three orders of magnitude larger than that of traditional organic fluorophores. Most recently, Jain and coworkers have used of QDs and QD-doped silica beads for differentiating tumor vessels from perivascular cells and matrix. The results demonstrated a much clearer boundary between blood vessels and cells than using traditional high-molecular weight dextran *(49)*.

For improved tissue penetration, Frangioni and Bawendi prepared a novel core-shell nanostructure called type II QDs *(50)*, with fairly broad emission at 850-nm emission and a moderate quantum yield of approx 13%. In contrast to the conventional QDs (type I), the shell materials in type II QDs have valence and conduction band energies both lower than those of the core materials. As a result, the electrons and holes are physically separated and the nanoparticles emit light at reduced energies (longer wavelengths). Their results showed rapid uptake of bare QDs into lymph nodes, and clear imaging and delineation of involved sentinel nodes (which could then be removed). This work points to the possibility that QD probes could be used for real-time intraoperative optical imaging, providing an *in situ* visual guide so that a surgeon could locate and remove small lesions (e.g., metastatic tumors) quickly and accurately. At the present, however, high-quality QDs with near-infrared-emitting properties are not yet available. Most materials (e.g., PdS, PdSe, InGaP, CdHgTe, and CdSeTe) are either not bright enough or not stable enough for cancer-imaging applications. As such, there is an urgent need to develop bright and stable near-infrared-emitting QDs that are broadly tunable in the far-red and infrared spectral regions. Theoretical modeling studies by Kim et al. indicate that two spectral windows are excellent for in vivo QD imaging, one at 700 to 900 nm and another at 1200 to 1600 nm *(51)*.

7. IN VIVO PROSTATE TUMOR TARGETING AND IMAGING

Akerman et al. first reported the use of QD–peptide conjugates to target tumor vasculatures, but the QD probes were not detected in living animals *(52)*. Nonetheless, their in vitro histological results revealed that QDs homed to tumor vessels guided by the peptides and were able to escape clearance by the reticuloendothelial system. Most recently, Gao et al. reported a new class of multifunctional QD probes for simultaneous targeting and imaging of tumors in live animals *(25)*. This class of QD conjugates contains an amphiphilic triblock copolymer for in vivo protection, targeting ligands for tumor antigen recognition, and multiple PEG molecules for improved biocompatibility and circulation. The use of an ABC triblock copolymer has solved the problems of particle aggregation and fluorescence loss previously encountered for QDs stored in physiological buffer or injected into live animals *(52–54)*. Detailed studies were reported on the in vivo behaviors of QDs probes, including biodistribution, nonspecific uptake, cellular toxicity, and pharmacokinetics.

Under in vivo conditions, QD probes can be delivered to tumors by both a passive targeting mechanism and an active targeting mechanism. In the passive mode, macromolecules and nanometer-sized particles are accumulated preferentially at tumor sites through an enhanced permeability and retention effect *(55,56)*. This effect is thought to arise from two factors:

1. Angiogenic tumors, which produce vascular endothelial growth factors that hyperpermeabilize the tumor-associated neovasculatures and cause the leakage of circulating macromolecules and small particles.
2. Tumors lack an effective lymphatic drainage system, which leads to subsequent macromolecule or nanoparticle accumulation.

For active tumor targeting, Gao et al. have used antibody-conjugated QDs to target a prostate-specific cell surface antigen, prostate-specific membrane antigen (PSMA) (Fig. 6D). Previous research has identified PSMA as a cell surface marker for both prostate epithelial cells and neovascular endothelial cells *(57)*. PSMA has been selected as an attractive target for both imaging

and therapeutic intervention of prostate cancer *(58)*. Accumulation and retention of PSMA antibody at the site of tumor growth is the basis of radioimmunoscintigraphic scanning (e.g., ProstaScint scan) and targeted therapy for human prostate cancer metastasis *(59)*.

8. TOXICITY AND POTENTIAL CLINICAL USE

The potential toxic effects of semiconductor QDs have recently become a topic of considerable importance and discussion. Indeed, in vivo toxicity is likely a key factor in determining whether QD imaging probes would be approved by regulatory agencies for human clinical use. Recent work by Derfus et al. indicates that CdSe QDs are highly toxic to cultured cells under UV illumination for extended periods of time *(60)*. This is not surprising, because the energy of UV-irradiation is close to that of covalent chemical bond and dissolves the semiconductor particles in a process known as photolysis, which releases toxic cadmium ions into the culture medium. In the absence of UV irradiation, QDs with a stable polymer coating have been found to be essentially nontoxic to cells and animals, for example no effect on cell division or ATP production (D. Stuart, X. Gao, and S. Nie, unpublished data). It has also been reported that the polymer-coated QDs could be toxic if significant aggregates are formed on the cell surface *(61)*. In vivo studies by Ballou and coworkers also confirmed the nontoxic nature of stably protected QDs *(42)*. Still, there is an urgent need to study the cellular toxicity and in vivo degradation mechanisms of QD probes. For polymer-encapsulated QDs, chemical or enzymatic degradations of the semiconductor cores are unlikely to occur. However, the polymer-protected QDs might be cleared from the body by slow filtration and excretion out of the body. This and other possible mechanisms must be carefully examined before any human applications in tumor or vascular imaging.

9. CONCLUDING REMARKS

QDs have already fulfilled some of their promises as a new class of molecular probes for cancer research. Through their versatile polymer coatings, QDs have also provided a "building block" to assemble multifunctional nanostructures and nanodevices. Multi-modality imaging probes could be created by integrating QDs with paramagnetic or superparamagnetic agents. Indeed, researchers have recently attached QDs to Fe_2O_3 and FePt nanoparticles *(62,63)* and even to paramagnetic gadolinium chelates (X. Gao and S. Nie, unpublished data). By correlating the deep imaging capabilities of MRI with ultrasensitive optical imaging, a surgeon could visually identify tiny tumors or other small lesions during an operation and remove the diseased cells and tissue completely. Medical imaging modalities such as MRI and PET can identify diseases noninvasively, however, they do not provide a visual guide during surgery. The development of magnetic or radioactive QD probes could solve this problem.

Another desired multifunctional device would be the combination of a QD imaging agent with a therapeutic agent. Not only would this allow tracking of pharmacokinetics, but diseased tissue could be treated and monitored simultaneously and in real time. Surprisingly, QDs may be innately multimodal in this fashion, because they have been shown to have potential activity as photodynamic therapy agents *(64)*. These combinations are only a few possible achievements for the future. Practical applications of these multifunctional nanodevices will not come without careful research, but the multidisciplinary nature of nanotechnology may expedite these goals by combining the great minds of many different fields. The success seen so far with QDs points toward the success of QDs in biological systems, and predicts the success of other nanotechnologies for biomedical applications.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (grants R01 CA108468, R01 GM 60562, and P20 GM 072069 to S.N.), the Georgia Cancer Coalition Distinguished Cancer Scholar Awards (to S.N. and LCWC), and the Coulter Translational Research Program at Georgia Tech Emory University and University of Washington.

REFERENCES

1. Chan, W. C. W., Maxwell, D. J., Gao, X. H., Bailey, R. E., Han, M. Y., and Nie, S. M. (2002). Luminescent quantum dots for multiplexed biological detection and imaging. *Curr. Opin. Biotechnol.* **13**, 40–46.
2. Han, M. Y., Gao, X. H., Su, J. Z., and Nie, S. M. (2001). Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat. Biotechnol.* **19**, 631–635.
3. Gao, X. H. and Nie, S. M. (2003). Doping mesoporous materials with multicolor quantum dots. *J. Phys. Chem. B* **107**, 11,575–11,578.
4. Gao, X. H. and Nie, S. M. (2004). Quantum dot-encoded mesoporous beads with high brightness and uniformity: rapid readout using flow cytometry. *Anal. Chem.* **76**, 2406–2410.
5. Wu, X. Y., Liu, H. J., Liu, J. Q., et al. (2003). Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. *Nat. Biotechnol.* **21**, 41–46.
6. Dahan, M., Levi, S., Luccardini, C., Rostaing, P., Riveau, B, and Triller, A. (2003). Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. *Science* **302**, 442–445.
7. Lidke, D. S., Nagy, P., Heintzmann, R., et al. (2004). Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nat. Biotechnol.* **22**, 198–203.
8. Xiao, Y. and Barker, P. E. (2004). Semiconductor nanocrystal probes for human metaphase chromosomes. *Nucleic Acids Res.* **32**, e28.
9. Jaiswal, J. K., Mattoussi, H., Mauro, J. M., Simon, S. M. (2003). Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat. Biotechnol.* **21**, 47–51.
10. Medintz, L., Clapp, A. R., Mattoussi, H., Goldman, E. R., Fisher, B., Mauro, J. M. (2003). Self-assembled nanoscale biosensors based on quantum dot FRET donors Igor. *Nat. Mat.* **2**, 630–638.
11. Medintz, I. L., Konnert, J. H., Clapp, A. R., et al. (2004). A fluorescence resonance energy transfer-derived structure of a quantum dot-protein bioconjugate nanoassembly. *Proc. Natl. Acad. Sci. USA* **29**, 9612–9617.
12. Rosenthal, S. J., Tomlinson, I., Adkins, E. M., et al. (2002). Targeting cell surface receptors with ligand-conjugated nanocrystals. *J. Am. Chem. Soc.* **124**, 4586–4594.
13. Alivisatos, A. P. (1996). Semiconductor clusters, nanocrystals, and quantum dots, *Science* **271**, 933–937.
14. Alivisatos, A. P. (2004). The use of nanocrystals in biological detection. *Nat. Biotechnol.* **22**, 47–52.
15. Manna, L., Milliron, D. J., Meisel, A., Scher, E. C., and Alivisatos, A. P. (2003). Controlled growth of tetrapod branched inorganic nanocrystals. *Nat. Mat.* **2**, 382–385.
16. Milliron, D. J., Hughes, S. M., Cui, Y., et al. (2004). Colloidal nanocrystal heterostructures with linear and branched topology. *Nature* **430**, 190–195.
17. Dick, K. A., Deppert, K., Larsson, M. W., et al. (2004). Synthesis of branched 'nanotrees' by controlled seeding of multiple branching events. *Nat. Mat.* **3**, 380–384.
18. Yu, W. W., Wang, Y. A., and Peng, X. G. (2003). Formation and stability of size-, shape-, and structure-controlled CdTe nanocrystals: Ligand effects on monomers and nanocrystals. *Chem. Mat.* **15**, 4300–4308.
19. Hines, M. A. and Guyot-Sionnest, P. (1996). Synthesis of strongly luminescing ZnS-capped CdSe nanocrystals. *J. Phys. Chem. B* **100**, 468–471.
20. Peng, X. G., Schlamp, M. C., Kadavanich, A. V., and Alivisatos, A. P. (1997). Epitaxial growth of highly luminescent CdSe/CdS core/shell nanocrystals with photostability and electronic accessibility. *J. Am. Chem. Soc.* **119,** 7019–7029.
21. Dabbousi B. O., Rodriguez-Viejo J., Mikulec F. V., et al. (1997). (CdSe)ZnS core-shell quantum dots: synthesis and characterization of a size series of highly luminescent nanocrystallites. *J. Phys. Chem. B* **101**, 9463–9475.
22. Bailey, R. E. and Nie, S. (2003). Alloyed semiconductor quantum dots: tuning the optical properties without changing the particle size. *J. Am. Chem. Soc.* **125**, 7100–7106.
23. Qu, L. H. and Peng, X. G. (2002). Control of photoluminescence properties of CdSe nanocrystals in growth. *J. Am. Chem. Soc.* **124**, 2049–2055.
24. Dubertret, B., Skourides, P, Norris, D. J., Noireaux, V., Brivanlou, A. H., Libchaber, A. (2002). In vivo imaging of quantum dots encapsulated in phospholipid micelles. *Science* **298**, 1759–1762.
25. Gao, X. H., Cui, Y. Y., Levenson, R. M., Chung, L. W. K., and Nie, S. M. (2004). In vivo cancer targeting and imaging with semiconductor quantum dots. *Nat. Biotechnol.* **22**, 969–976.
26. Pellegrino, T., Manna, L., and Kudera, S. (2004). Hydrophobic nanocrystals coated with an amphiphilic polymer shell: a general route to water soluble nanocrystals. *Nano. Lett.* **4**, 703–707.
27. Ron, E., Turek, T., and Mathiowitz, E. (1993). Controlled release of polypeptides from polyanhydrides. *Proc. Natl. Acad. Sci. USA* **90**, 4176–4180.
28. Anseth, K. S., Shastri, V. R., and Langer, R. (1999). Photopolymerizable degradable polyanhydrides with osteocompatibility. *Nat. Biotechnol.* **17**, 156–159.
29. Goldman, E. R., Anderson, G. P., Tran, P. T., Mattoussi, H., Charles, P. T., and Mauro, J. M. (2002). Conjugation of luminescent quantum dots with antibodies using an engineered adaptor protein to provide new reagents for fluoroimmunoassays. *Anal. Chem.* **74**, 841–847.

30. Leatherdale, C. A., Woo, W. K., Mikulec, F. V., and Bawendi, M. G. (2002). On the absorption cross section of CdSe nanocrystal quantum dots. *J. Phys. Chem. B* **106**, 7619–7622.
31. Bruchez, J. M., Moronne, M., Gin, P., Weiss, S., and Alivisatos, A. P. (1998). Semiconductor nanocrystals as fluorescent biological labels. *Science* **281**, 2013–2015.
32. Chan, W. C. W. and Nie, S. M. (1998). Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science* **281**, 2016–2018.
33. Jakobs, S., Subramaniam, V., Schonle, A., Jovin, T. M., and Hell, S. W. (2000). EGFP and DsRed expressing cultures of Escherichia coli imaged by confocal, two-photon and fluorescence lifetime microscopy. *FEBS Lett.* **479**, 131–135.
34. Pepperkok, R., Squire, A., Geley, S., and Bastiaens, P. I. H. (1999). Simultaneous detection of multiple green fluorescent proteins in live cells by fluorescence lifetime imaging microscopy. *Curr. Bio.* **9**, 269–272.
35. Gao, X. H. and Nie, S. M. (2003). Molecular profiling of single cells and tissue specimens with quantum dots. *Trends Biotechnol.* **21**, 371–373.
36. Ogawara, K., Yoshida, M., Higaki, K., et al. (1999). Hepatic uptake of polystyrene microspheres in rats: Effect of particle size on intrahepatic distribution. *J. Control Release* **59**, 15–22.
37. Flacke, S., Fischer, S., Scott M. J., et al. (2001). Novel MRI contrast agent for molecular imaging of fibrin implications for detecting vulnerable plaques. *Circulation* **104**, 1280–1285.
38. Katz, L. C., Burkhalter, A., and Dreyer, W. J. (1984). Fluorescent latex microspheres as a retrograde neuronal marker for in vivo and in vitro studies of visual-cortex. *Nature* **310**, 498–500.
39. Chien, G. L., Anselone, C. G., Davis, R. F., and Van Winkle, D. M. (1995). Fluorescent vs. radioactive microsphere measurement of regional myocardial blood flow. *Cardiovasc Res.* **30**, 405–412.
40. Pasqualini, R. and Ruoslahti, E. (1996). Organ targeting in vivo using phage display peptide libraries. *Nature* **380**, 364–366.
41. Nisman, R., Dellaire, G., Ren, Y., Li, R., and Bazett-Jones, D. P. (2004). Application of quantum dots as probes for correlative fluorescence, conventional, and energy-filtered transmission electron microscopy. *J. Histochem. Cytochem.* **52**, 13–18.
42. Ballou B., Lagerholm B. C., Ernst L. A., Bruchez M. P., and Waggoner A. S. (2004). Noninvasive imaging of quantum dots in mice. *Bioconj. Chem.* **15**, 79–86.
43. Hoshino A., Hanaki K., Suzuki K., and Yamamoto K. (2004). Applications of T-lymphoma labeled with fluorescent quantum dots to cell tracing markers in mouse body. *Biochem. Biophy. Res. Com.* **314**, 46–53.
44. Voura E. B., Jaiswal J. K., Mattoussi H., and Simon S. M. (2004). Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. *Nat. Med.* **9**, 993–998.
45. Mattheakis L. C., Dias J. M., Choi Y. .J, et al. (2004). Optical coding of mammalian cells using semiconductor quantum dots. *Anal. Chem.* **327**, 200–208.
46. Lagerholm, B. C., Wang, M., Ernst, L. A., et al. (2004). Multicolor coding of cells with cationic peptide coated quantum dots. *Nano Lett.* **4(10)**, 2019–2022.
47. Lewin, M., Carlesso, N., Tung, C. H., et al. (2000). Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat. Biotechnol.* **18**, 410–414.
48. Larson, D. R., Zipfel, W. R., Williams, R. M., et al. (2003). Water-soluble quantum dots for multiphoton fluorescence imaging in vivo. *Science* **300**, 1434–1436.
49. Stroh, M., Zimmer, J. P., Duda, D. G., et al. (2005). Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo. *Nat. Med.* **11**, 678–682.
50. Kim, S., Lim, Y. T., Soltesz, E. G., et al. (2004). Near-infrared fluorescent type II quantum dots for sentinel lymph node mapping. *Nat. Biotechnol.* **22**, 93–97.
51. Lim, Y. T., Kim S., Nakayama, A., Stott, N. E., Bawendi, M. G., and Frangioni, J. V. (2003). Selection of quantum dot wavelengths for biomedical assays and imaging. *Mol. Imaging* **2**, 50–64.
52. Akerman, M. E., Chan, W. C. W., Laakkonen, P., Bhatia S. N., and Ruoslahti, E. (2002). Nanocrystal targeting in vivo. *Proc. Natl. Acad. Sci. USA* **99**, 12,617–12,621.
53. Mattoussi, H., Mauro, J. M., Goldman, E. R., et al. (2000). Self-assembly of CdSe-ZnS quantum dot bioconjugates using an engineered recombinant protein. *J. Am. Chem. Soc.* **122**, 12,142–12,150.
54. Gao, X. H., Chan, W. C. W., and Nie, S. M. (2002). Quantum-dot nanocrystals for ultrasensitive biological labeling and multicolor optical encoding. *J. Biomed. Opt.* **7**, 532–537.
55. Jain, R. K. (1999). Transport of molecules, particles, and cells in solid tumors. *Ann. Rev. Biomed. Eng.* **1**, 241–263.
56. Jainm R. K. (2001). Delivery of molecular medicine to solid tumors: lessons from in vivo imaging of gene expression and function. *J. Control Release* **74**, 7–25.
57. Chang, S. S., Reuter, V. E., Heston, W. D. W., and Gaudin, P. B. (2001). Metastatic renal cell carcinoma neovasculature expresses prostate-specific membrane antigen. *Urology* **57**, 801–805.
58. Schulke, N., Varlamova, O. A., Donovan, G. P., et al. (2003). The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy. *Proc. Natl. Acad. Sci. USA* **100**, 12,590–12,595.
59. Bander, N. H., Trabulsi, E. J., Kostakoglu, L., et al. (2003). Targeting metastatic prostate cancer with radiolabeled monoclonal antibody J591 to the extracellular domain of prostate specific membrane antigen. *J. Urol.* **170**, 1717–1721.

60. Derfus, A. M., Chan, W. C. W., and Bhatia, S. N. (2004). Probing the cytotoxicity of semiconductor quantum dots. *Nano Lett*. **4**, 11–18.
61. Kirchner, C., Liedl, T., Kudera, S., et al. (2005). Cytotoxicity of colloidal CdSe and CdSe/ZnS nanoparticles. *Nano Lett*. **5**, 331–338.
62. Wang, D. S., He, J. B., Rosenzweig, N., and Rosenzweig, Z. (2004). Superparamagnetic Fe_2O_3 Beads-CdSe/ZnS quantum dots core-shell nanocomposite particles for cell separation. *Nano Lett*. **4**, 409–413.
63. Gu, H. W., Zheng, R. K., Zhang, X. X., and Xu, B. (2004). Facile one-pot synthesis of bifunctional heterodimers of nanoparticles: a conjugate of quantum dot and magnetic nanoparticles. *J. Am. Chem. Soc*. **126**, 5664–5665.
64. Samia, A. C. S., Chen, X., and Burda, C. (2003). Semiconductor quantum dots for photodynamic therapy. *J. Am. Chem. Soc*. **125**, 15,736–15,737.

14

Expression Profiling of Prostate Cancer Progression

Scott A. Tomlins and Arul M. Chinnaiyan

Summary

DNA microarray technology has revolutionized cancer research through the ability to obtain a genome-wide perspective of gene expression. More than 25 published studies have profiled human prostate tissues, and the number of dysregulated genes identified through microarray studies is still expanding. However, although we have gained tremendous insight into expression differences between benign and cancerous prostate tissue, much remains to be understood regarding gene expression in the context of progression from benign to clinically localized prostate cancer to metastatic disease. In this review, after briefly introducing DNA microarrays and the current state of expression profiling in prostate cancer (CaP), we focus on how profiling studies have contributed to our knowledge of gene expression in CaP progression, particularly in advanced disease. Studies attempting to identify expression signatures correlating with Gleason grade are discussed. In addition, studies characterizing expression in aggressive tumors, either by profiling metastatic samples directly or identifying gene signatures characteristic of localized tumors likely to recur after surgical resection, are analyzed. Results from these studies will be synthesized to characterize CaP progression and identify focused areas of future analysis. The limitations of current approaches are also addressed, particularly in translating conclusions from these studies to clinical practice. We conclude with a discussion of important advances and future areas for expression profiling in the context of understanding CaP progression.

Key Words: Example: DNA microarrays; expression profiling; prostate cancer; Gleason grade; PSA recurrence.

1. INTRODUCTION

Prostate cancer (CaP) is remarkable for its heterogeneity at the clinical, histological, and biological level. In a single prostate, cancerous epithelial cells in different architectural patterns and degrees of differentiation (reflected in the Gleason score; ref. *1*), fibromuscular stromal cells, multiple foci of prostatic intraepithelial neoplasia (PIN), atrophic lesions, nodules of benign prostatic hypertrophy (BPH), and benign-appearing glands may all coexist. Further, although nomograms and other models based on clinical and pathological parameters are effective at identifying the likelihood of an individual tumor recurring after surgical resection *(2)*, they do not explain why two tumors with identical clinical and pathological parameters may show remarkably different clinical courses. Classically, many human cancers are thought to develop through a series of sequentially accumulated genetic events, culminating in carcinoma and metastatic disease, and these results are reflected at the histological, genetic, and clinical level. Many aspects of CaP biology do not fit with this model, and it is unclear whether this framework should be applied to CaP progression. For example, classic genetic techniques have provided only limited information regarding the process of CaP progression. There are no obvious syndromes predisposing to CaP, and, whereas CaP clearly has a familial component, little functional information has been gained from characterizing genes at the implicated loci. How-

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

ever, several candidate genes and pathways have been implicated in CaP progression, such as *GSTP1 (3)*, *PTEN (4)*, *NKX3.1 (5)*, *AR (6,7)*, *c-MYC (8,9)*, and the hedgehog pathway *(10)*. Although this chapter focuses on the contributions of expression profiling studies to understanding CaP progression, several excellent reviews discuss these genes and others that have been implicated through other techniques *(11,12)*. Although many lines of evidence support the role of these genes and pathways in CaP progression, few tumors share many or all of the same genetic lesions and there is little evidence for a clearly defined series of genetic events leading to CaP progression.

The advent of the prostate-specific antigen (PSA) screening test has led to an increasing number of cancers being detected at an early stage. Coupled with the genetic and clinical heterogeneity of CaP, treatment decisions have become complicated, and a more thorough understanding of CaP progression is urgently needed. The unique biological and clinical aspects of CaP, as described in the preceding paragraph, have led to speculation that characterizing CaP at the molecular level may be the best way to identify clinically relevant tumor subtypes and provide insight into disease pathogenesis and progression. Several techniques exist for characterizing gene expression at a global level, including DNA microarray analysis, which has become the standard research tool for high-throughput examination of genome-wide expression changes in cancer.

2. DNA MICROARRAYS

DNA microarrays are based on the arrangement of thousands or tens of thousands of "probes" (cDNA clones or oligonucleotides [25–80mers]) on a solid support, with each feature identified by its location. Total RNA is isolated from samples of interest and converted to antisense RNA (aRNA) or complementary DNA (cDNA), which is labeled with a fluorescent tag. The labeled target is allowed to hybridize to the microarray, and the array is scanned by a laser, with the resulting fluorescence of each spot proportional to the amount of target hybridized. By profiling a large number of samples (i.e., groups of cancerous and benign samples) expression patterns across groups of samples are identified. Because of the massive amounts of data obtained during microarray experiments, numerous bioinformatic tools have been developed for data storage, normalization, and analysis, which can profoundly influence the final conclusions drawn from a microarray study *(13–15)*.

Because of the popularity of microarrays, a variety of technologies have emerged, which can have important effects on experimentation and design of profiling studies. The first microarrays were developed by Patrick Brown's group at Stanford University, and are commonly referred to as cDNA microarrays *(16,17)*. On these arrays, each probe consists of a cDNA clone of an individual transcript produced by PCR. These clones are much longer than oligonucleotide arrays and, importantly, two samples are hybridized competitively to the array. Thus, for each experiment, cDNA from an experimental sample is fluorescently labeled with one tag and cDNA from a control sample is labeled with a different tag. The array is scanned to excite both tags, and image analysis programs quantify the ratio of experimental to control fluorescence for each feature as a measure of transcript abundance in the experimental sample compared with the control. The requirement for a control sample influences experimental design. For example, profiling studies of CaP often use pooled cell line RNA or pooled normal prostate tissue as the control sample.

The first commercial microarray system was developed by Affymetrix, whose GeneChip system uses millions of 25-mer oligonucleotide probes, each designed to hybridize to a particular part of a transcript *(18,19)*. The probes are synthesized *in situ* onto silicone wafers using photolithography. Each transcript on the array is represented by a number of different probe pairs. Each probe pair consists of a 25-base pair oligonucleotide perfectly matched to the consensus sequence of the transcript and a 25-base pair "mismatch" probe, in which the 13th position is designed not to match the transcript sequence. The information across the different matched and mismatched probes is integrated by Affymetrix software to calculate the output signal for each feature. Importantly, RNA from a single sample is hybridized to each oligonucleotide array. Recently, other commercial suppliers

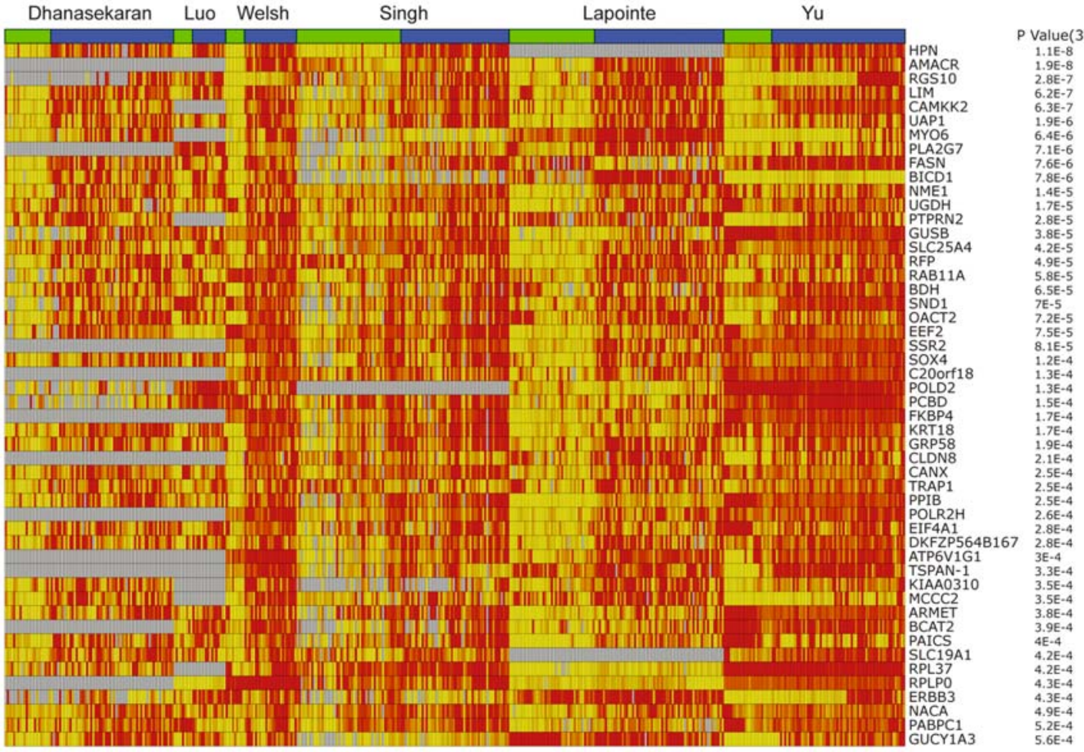


Fig. 1. Meta-analysis of genes overexpressed in prostate cancer (blue) compared with benign prostate tissue (green) from six independent profiling studies *(26,28,29,40,47,51)*, indicated by the last name of the first author, using Oncomine. Columns represent individual arrays and rows represent individual genes. Yellow and red saturation of cells represent relative under and overexpression respectively. Gray cells represent features not monitored or technically inadequate in the individual study. Genes present in at least four of the six studies were ranked by the third lowest *p* value from the individual studies, and the top 50 genes are listed.

have begun offering microarrays in a variety of formats with distinct advantages and limitations *(20)*. For example, Agilent offers 60-mer oligonucleotide arrays printed using ink-jet technology, which can be used for hybridizations with or without a control sample, although competitive hybridizations give more precise data *(21)*.

Because of the differences in technology, experimental design, and output, profiles obtained from different arrays are often difficult to compare directly *(22)*. Our group has developed a meta-analysis method to compare microarray studies from different platforms, which we applied to four of the earliest prostate-profiling studies from cDNA and oligonucleotide microarrays to identify genes consistently dysregulated across the data sets *(23)*. Several of the genes identified in the meta-analysis as being consistently dysregulated in CaP, such as *AMACR* and *Hepsin*, have been identified in almost every subsequent study and likely represent potential biomarkers as well as genes involved in CaP biology. We have also developed a web-based resource, Oncomine (www.oncomine.org), to catalog expression-profiling studies and allow data mining across multiple studies *(24)*. As an update, we

Table 1
DNA Microarray Studies Profiling Human CaP Tissues [a]

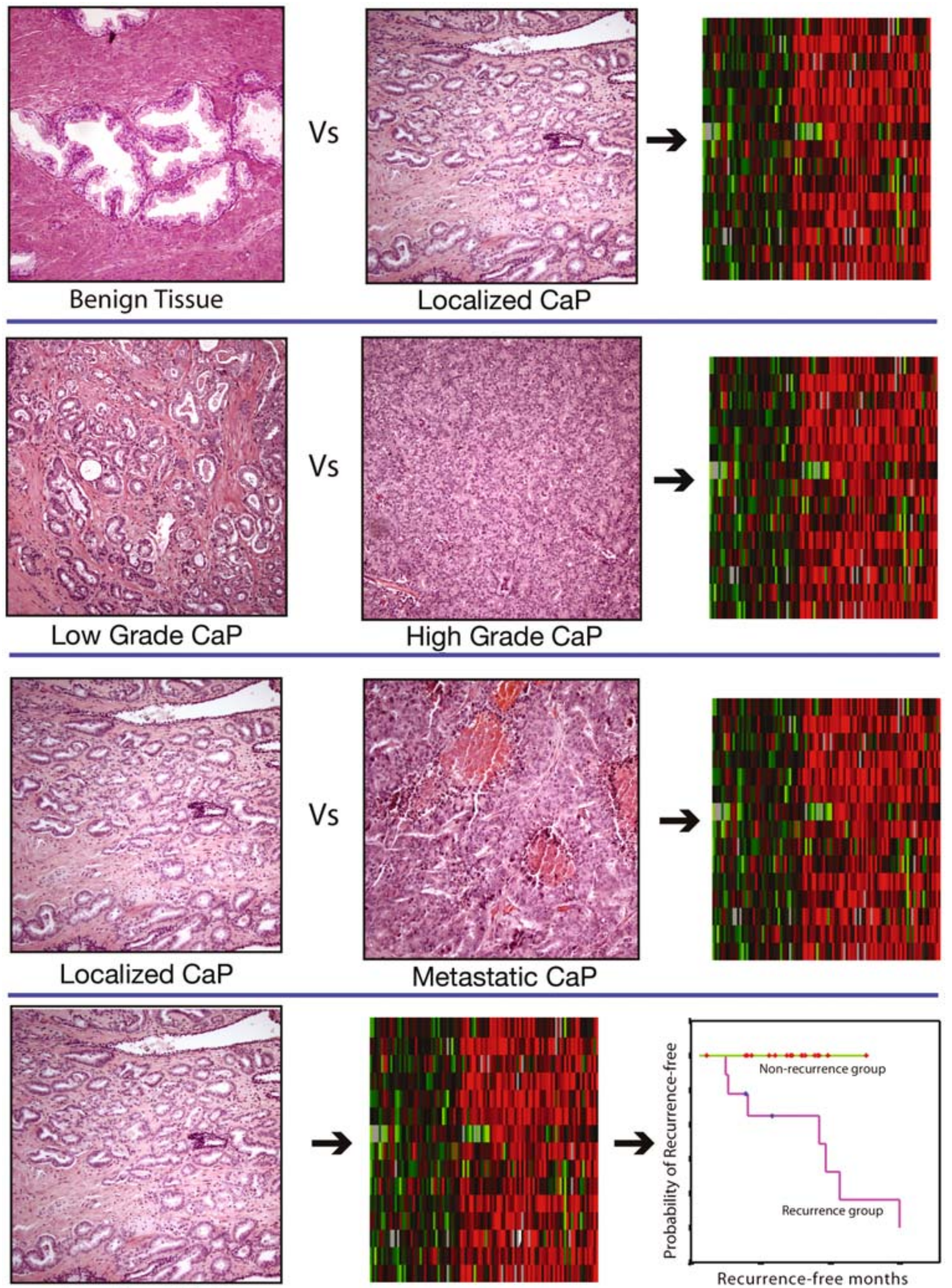
			Samples				
Author	Array type	Probes	NOR	PIN	CaP	MET	References
Ashida	cDNA	23,040		10	20		*(31)*
Best	cDNA	6400			13		*(32)*
Bull	cDNA	1877	11	2	16		*(25)*
Chen	Affymetrix	6800	3	1	4		*(33)*
Dhanasekaran	cDNA	9984	22		59	20	*(26,34)*
Ernst	Affymetrix	12,625	9		17		*(35)*
Glinsky	Affymetrix	12,625			79		*(36)*
Henshall	Affymetrix	59,619			72		*(37,38)*
Kristiansen	Affymetrix	3950	42		42		*(39)*
Lapointe	cDNA	26,000	41		62	9	*(40)*
LaTulippe	Affymetrix	63,175	3		23	9	*(41)*
Luo	cDNA	6500	9		16		*(29)*
Luo	Affymetrix	>47,000	15		15		*(42)*
Luo	cDNA	12,000	25		25		*(43)*
Magee	Affymetrix	6800	4		9	2	*(27)*
Moore	cDNA	6200	12		12		*(44)*
Ramachandran	Affymetrix	39,000	12		28		*(45)*
Rossi	Affymetrix	12,625				7	*(46)*
Singh	Affymetrix	12,625	50		52		*(47)*
Stamey	Affymetrix	6800	8		9		*(30)*
Stamey	Affymetrix	6800	26		12		*(48)*
Stuart	Affymetrix	12,625	50		38		*(49)*
Vanaja	Affymetrix	12,625	8		23	5	*(50)*
Welsh	Affymetrix	12,625	9		23	1	*(28)*
Yu	Affymetrix	37,777	83		66		*(51)*

[a] Studies are identified by the last name of the first author from the appropriate reference. For each study, the type of array (Affymetrix or cDNA), the number of probes or genes monitored on the array (as reported by the authors), and the number of benign samples (normal [NOR], including benign prostatic hypertrophy [BPH]), prostatic intraepithelial neoplasia (PIN), localized prostate cancer (CaP), and metastatic samples (MET) are indicated.

provide a meta-analysis of genes identified as being overexpressed in CaP compared with benign prostate tissue across six large-scale profiling studies in Fig. 1.

3. PROFILING CAP PROGRESSION

The first studies to profile CaP using large-scale DNA microarrays were published in 2001 *(25–30)*. These studies identified genes that were dysregulated between "normal" samples (benign adjacent tissue and BPH) and cancerous samples (in some cases including PIN or metastatic tissues). Several subsequent studies have also profiled normal and cancerous tissue, and almost all studies are able to differentiate cancerous samples from benign prostate tissues. Recently, studies have begun to focus on profiling aspects of CaP progression, as shown in Fig. 2. These studies include identifying expression profiles characteristic of tumors of different Gleason scores, and characterizing aggressive and metastatic CaP. Studies have also been conducted with the goal of identifying expression signatures that predict which localized CaPs will recur after surgical resection. Studies examining



PE, submit to ART: Please change labeling to CaP to match book style.

Fig. 2. Different aspects of prostate cancer (CaP) progression examined by expression-profiling studies. The earliest profiling studies were designed to identify differences in gene expression between benign prostate tissue and localized CaP. Studies that are more recent have focused on identifying expression patterns correlating with different Gleason scores. Other studies have focused on identifying genes differentially expressed between localized and metastatic CaP. Several studies have also profiled localized CaPs in an attempt to identify expression signatures that can be used to identify which localized CaPs will be most likely to experience PSA recurrence after surgical resection.

these aspects of CaP progression will be the focus of this review, although a more thorough list of profiling studies is presented in Table 1.

4. IDENTIFYING GENE EXPRESSION CORRELATES OF GLEASON SCORE

Several studies, including those that also profiled metastatic samples or attempted to predict recurrence, have attempted to identify expression patterns that correlate with Gleason grade or score. Theoretically, these studies should be able to shed biological insight into molecular changes that characterize the observed histological patterns. Welsh et al. profiled 9 normal prostate samples, 23 localized tumors, and 1 metastatic CaP sample on Affymetrix U95a GeneChips containing probes for 12,625 transcripts *(28)*. The authors performed an analysis of variance (ANOVA) on the data set, with the tumors divided into three groups (Gleason score 5 or 6, Gleason score 7, and Gleason score 8 or 9). Although the number of genes and level of significance was not stated, the authors noted that 2 of the top 20 most significant genes, *IGFBP-2* and *IGFBP-5* (up in Gleason score 8 or 9), had been identified previously as being elevated in high-grade cancer *(52)*. A study by Best et al. profiled six high Gleason grade tumors (score 9 or 10) and seven low-grade tumors (score 5, 6, or 7) on 6400-element cDNA microarrays *(32)*. They identified 21 genes that showed differential expression between high- and low-grade tumors ($p < 0.03$) that could accurately cluster their samples, although the high- and low-grade samples did not form separate clusters when the full data set was used. Singh et al. profiled 52 tumors and 50 normal prostate specimens on Affymetrix U95Av2 arrays *(47)* and attempted to identify genes that correlated with clinical and pathological features. The authors reported that 29 genes significantly correlated with Gleason score, with 15 genes positively correlated with increasing Gleason score, and 14 genes negatively correlating with Gleason score ($p < 0.001$). Importantly, Singh et al. validated their signatures on Welsh and colleagues' data set *(28)*, and demonstrated that the positively and negatively correlated gene sets remained separate, although the genes were not able to separate high- and low-grade tumors robustly. Lapointe et al. profiled 62 primary prostate tumors, 41 normal adjacent tissues, and 9 lymph node metastases using cDNA microarrays containing 26,260 genes *(40)*. The authors identified 41 genes associated with high-grade disease, of which four, *COL1A2*, *BGN*, *SPARC*, and *ABCA5*, were reported by Singh et al. to correlate with tumor grade.

If gene signatures distinguishing Gleason patterns are reflective of underlying biological differences, we would expect that the same genes should appear in multiple studies. However, a single set of candidates has not emerged from these studies and there seems to be a limited overlap of genes between studies. There are several reasons for this discrepancy, which are also problematic and often more apparent in studies attempting to predict recurrence. In addition to profiling different samples on multiple platforms that do not contain the same sets of genes, these studies also treated Gleason scores differently when attempting to identify expression signatures. Some studies looked for expression differences between two defined groups of high and low Gleason scores, whereas other studies treated each Gleason score as an individual group for a correlation analysis. Another important factor is that because Gleason scoring is based on subjective criteria, interobserver variation could influence these studies. Further, most of these studies are limited by the number of samples, both in total and at the upper and lower extremes of Gleason scores, which is vitally important to developing a robust classifier.

Nevertheless, several important conclusions can be taken from these studies. Although only limited, the observed overlap does suggest that genetic differences exist between high and low Gleason patterns, which is expected because of the general biological and clinical differences shown by these classes of tumor. However, not discounting the factors described in the preceding paragraph, heterogeneity should also be expected, because of the different clinical courses that can be observed from tumors of the same grade. In terms of specific genes and features discriminating different Gleason scores, additional validation is needed for all of the identified candidates. In particular, validation of

transcript expression using *in situ* hybridization or protein expression using immunohistochemistry (IHC) on tissue microarrays (TMAs) will help determine whether expression changes in the identified genes truly represent different Gleason patterns. However, it is interesting to note that many of the differential genes encode proteins that contribute to the basement membrane and extracellular matrix, such as *COL1A2*, *SPARC*, and *BGN*, which correlated with high grade in the studies of both Singh et al. and Lapointe et al. For example, expression of SPARC has been assessed using IHC on TMAs in an independent study on a limited number samples, and, although expression did not seem to correlate with Gleason score, expression was increased in metastatic carcinoma compared with localized CaP, suggesting a possible role in CaP progression *(53)*.

5. IDENTIFYING AGGRESSIVE TUMORS THROUGH EXPRESSION PROFILING

Gleason score has proven to be a significant predictor of patient outcome *(1)*, and determining expression patterns that distinguish different grade cancers should help to identify fundamental molecular differences that distinguish these architectural patterns. However, a more powerful use of microarray technology is to identify expression signatures that reflect the molecular differences that drive the clinical progression of CaP, which may or may not be shared by cancers of the same grade. In this manner, aggressive tumors can be characterized regardless of clinical or pathological characteristics. In addition, these expression profiles should help in understanding the biology of CaP progression. Following this idea, several groups have tried to identify expression changes characteristic of aggressive tumors using different experimental strategies. Some groups have chosen to profile advanced and metastatic tumors directly and identify expression patterns that distinguish these samples from localized cancer, whereas others have focused on identifying expression patterns that predict biochemical or clinical recurrence after treatment.

6. PROFILING METASTATIC CAP

Several groups have attempted to characterize CaP progression using DNA microarrays to profile advanced cancers, including metastatic samples. Some studies have attempted to identify expression patterns that differentiate between organ-confined CaP and locally advanced or metastatic CaP, with the thought that differentially expressed genes may serve as biomarkers and help explain CaP progression. For example, Luo et al. profiled 15 cancerous and 15 adjacent normal tissues on Affymetrix Hu35k and U95a GeneChips containing more than 47,000 probes *(42)*. Seven cancerous samples represented invasive CaP characterized by capsular penetration, with or without invasion of other organs or distant metastasis. Clustering revealed that three highly aggressive tumors had profiles distinct from organ-confined tumors and were themselves very heterogeneous, although the authors were able to identify 12 genes with at least a twofold difference between invasive CaPs and normal samples or organ-confined tumors. Magee et al. profiled four normal prostate samples, eight organ-confined tumors, and three advanced samples (two lymph node metastases and one tumor with seminal vesicle invasion) on Affymetrix GeneChips representing 6800 transcripts *(27)*. Using a filter to identify genes showing a threefold differential between the three advanced samples and seven of the eight organ-confined tumors, the authors identified two upregulated (advanced vs organ-confined) genes and nine downregulated genes. Vajana et al. profiled 23 organ-confined CaP samples (12 Gleason score 9, and 11 Gleason score 6), 5 metastatic samples, and 8 benign adjacent prostate tissues on Affymetrix U95Av2 GeneChips *(50)*. The authors did not report on expression patterns that could differentiate between the metastatic and localized cancers. However, the authors validated several genes by quantitative real-time PCR (QPCR) that showed progressive dysregulation through localized and metastatic samples. The authors focused on *ZNF185*, a gene they identified as being downregulated in cancer and metastatic samples, and demonstrated that *ZNF185* is epigenetically silenced in localized and metastatic samples.

Other studies have chosen to focus on genes that are differentially expressed between localized and metastatic CaP, with the idea that these alterations may more directly address the process of metastasis. Rossi et al. extended the study of Singh et al. *(47)* by profiling an additional seven metastatic lesions in bone *(46)*. They identified fatty acid synthase (*FASN*) as being significantly overexpressed in the metastatic samples compared with the 52 localized CaP samples and validated this finding by examining protein expression using IHC on TMAs.

LaTulippe et al. profiled 3 noncancerous prostate samples, 23 localized CaP samples, and 9 metastatic CaPs on the Affymetrix U95 GeneChip set containing 63,175 probe sets *(41)*. Unsupervised hierarchical clustering of the 32 CaPs separated the localized and metastatic tumors and the authors chose to focus on differential expression between the subset of primary cancers without recurrence and the metastatic samples. They identified 3436 probe sets that showed at least a threefold mean differential gene expression between these two groups. In an attempt to identify biological differences between early stage and metastatic tumors, the authors functionally annotated 100 of the most differentially expressed genes. Twenty-six of the top 100 genes play a role in cell cycle regulation (including mitosis, DNA replication, and DNA repair), which is consistent with the authors' observation that the metastatic tumors had a higher proliferation index than the localized samples, based on Ki67 IHC analysis of the samples used for profiling. Other functional categories with a large proportion of dysregulated genes include genes regulating transcription and protein expression, as well as cell signaling. Although localized and metastatic tumors clustered separately using the entire gene set, it would be interesting to determine the extent of shared gene expression between the localized CaP samples with PSA recurrence and the metastatic samples.

In two studies, our group *(26,34)* has profiled 22 benign prostate tissues (BPH and normal adjacent tissue), 59 localized tumors, and 20 metastatic CaPs. We analyzed the data set in an attempt to identify genes specifically overexpressed in metastatic samples compared with localized CaP, and found *EZH2* to be the most overexpressed gene by significance of microarray analysis *(54)*. Using IHC on TMAs, we demonstrated that EZH2 protein expression increased from benign to localized CaP to metastatic cancer samples. In a multivariate Cox model including surgical margin status, tumor size, Gleason score, and preoperative PSA level, increased EZH2 protein expression was the best predictor of recurrence ($p = 0.02$).

7. PREDICTING RECURRENCE FROM EXPRESSION SIGNATURES

As an alternative to studies profiling aggressive and metastatic samples in an attempt to understand CaP progression, several groups have attempted to use expression patterns to predict which patients will demonstrate biochemical or clinical recurrence after treatment. These studies have been encouraged by demonstrations that microarray profiling can stratify patients into "good prognosis" vs "bad prognosis" groups in other cancers, including diffuse large B-cell lymphoma *(55,56)*, acute lymphoblastic leukemia *(57)*, acute myeloid leukemia *(58,59)*, and breast cancer *(60)*. In addition to providing valuable diagnostic markers, functional examination of implicated genes should aid in understanding the molecular alterations that drive CaP progression.

In the study by Singh et al. *(47)* described in Section 4, the authors also attempted to identify genes whose expression correlated with outcome. Unfortunately, only 21 of the 52 patients with localized CaP were evaluable at the time of publication with respect to recurrence during at least 4 years of follow-up, and there were only eight recurrence events. The authors reported that they were not able to identify a single gene that was statistically associated with recurrence. Instead, they applied k-nearest neighbor classification and developed a five-gene model reaching 90% accuracy in predicting recurrence during leave-one-out cross validation. Five genes were identified as being used in more than half of the prediction models, including *chromogranin A*, *PDGFRB*, *HOXC6*, *ITPR3*, and *SIAT1*.

Similarly, Lapointe et al. also analyzed their study in an attempt to identify genes associated with recurrence *(40)*. Twenty-three genes associated with early recurrence were identified, although the false discovery rate *(61)* was 16%, which the authors attributed to the short period of clinical follow-

up. It should also be noted that the cohort only contained seven tumors with recurrence events, and the authors reported that recurrence information was missing for 31 of the 62 tumors. Although no genes overlapped with the five outcome predictor genes of Singh et al., the authors demonstrated that their 23-gene signature was able to accurately predicted recurrence in 15 of the 21 samples in the study by Singh et al. (although only 9 of the 23 genes in their signature were available on the Affymetrix GeneChips used in the study by Singh et al.).

Three recent studies have been designed with a more-focused objective of identifying genes associated with recurrence or aggressive cancers. Henshall et al. profiled 72 localized CaPs on custom Affymetrix GeneChips containing 59,619 probe sets *(37)*. Seventeen of the 72 patients in their cohort had PSA recurrence and their analysis was designed to identify genes that were able to predict recurrence better than preoperative PSA level. They identified 266 probes that were significant predictors at $p < 0.01$, although the false discovery rate *(61)* was 23%. Clustering samples by these 266 probes separated the recurrence samples from the nonrecurrence samples, as would be expected by the selection criteria, and also grouped 22 patients who were disease free at the time of censoring with the recurrence samples. The authors focused on one gene, *trp-p8* (also known as *TRPM8)*, encoding a putative Ca^{2+} channel, which showed downregulation in samples with recurrence compared with nonrecurrence samples. The loss of *TRPM8* remained a significant predictor of PSA relapse in a multivariate Cox proportional hazards model ($p = 0.0008$). *TRPM8* was further characterized as being androgen-regulated using xenograft models. Unfortunately, *TRPM8* had not been monitored in any other published microarray studies, so it could not be validated in independent data sets; however, the authors noted that none of the 11 genes commonly appearing in Singh's cross-validation model appeared in their list of 266 probe sets.

Glinsky et al. used a unique approach to predict prognosis from expression patterns *(36)*. The authors began by analyzing the data set from Singh et al. *(47)*, identifying 218 genes differentially regulated in the 8 recurrence samples and 13 nonrecurrence samples ($p < 0.05$). These genes were compared with expression profiles in three xenograft models of highly aggressive human CaP cells and the authors developed an algorithm that combined small gene clusters exhibiting highly concordant expression patterns across the clinical and model system samples. This algorithm, using 14 genes, was able to classify 90% of the samples correctly with respect to recurrence. Importantly, this algorithm was then validated on Glinsky et al.'s own data set, containing 79 tumor specimens, of which, 37 had recurrence events. This algorithm was found to be an independent predictor of disease recurrence in the Cox multivariate analysis ($p = 0.0001$) and was able to correctly classify 75% (59 of 79) of the samples correctly.

Yu et al. profiled 152 prostate samples, including 23 prostate samples from patients showing no prostate pathology, 60 normal tissues adjacent to CaP, and 66 CaP samples on the Affymetrix U95 GeneChip series consisting of probe sets for 37,777 genes *(51)*. The authors divided the CaP samples into two groups for predicting tumor aggressiveness. Tumors showing invasion into seminal vesicles or adjacent organs, PSA recurrence, or distant metastasis were classified as aggressive, and the remainder were considered nonaggressive. Seventy-two genes were identified as differentially expressed between the two groups ($p < 0.002$). To classify the samples, the five most differentially expressed genes were identified and groups of five genes were added sequentially and the classification rate was evaluated by leave-one-out cross validation. A 70-gene model was found to perform the best, correctly classifying 89% of the samples. This 70-gene model was validated on an independent data set of 23 cases of aggressive and nonaggressive cases CaP (78% overall accuracy), although no information was provided regarding the samples. The authors noted that 60% of the 70-gene model were expressed sequence tags or genes with unknown functions, although it was unclear whether any genes in the model overlapped with other predictors.

Comparing these studies that attempted to predict outcome, a similar picture to the comparison of studies that attempted to distinguish Gleason patterns emerges. A single set of candidates does not readily emerge and, again, the overlap between gene sets from different studies is limited. Many

reasons again account for the observed discrepancies, some of which were shared by studies attempting to profile Gleason patterns. Again, these studies were conducted on different platforms that do not contain probes for the same genes, as pointed out by many of the authors. Inclusion criteria were also different between the studies. Perhaps the biggest limitation of many of these studies is the limited number of samples used in the analysis, particularly with regard to the number of recurrence events. As described, two of the studies were only able to use approximately half of their localized CaP samples in the analyses. Several important reasons for the observed discrepancies between the outcome studies are also unique to this type of analysis. Many of the studies rely on arbitrary cut-off points for inclusion into recurrence or recurrence-free classification. Whether tumors recurring at 1 or 5 years after surgery, which would be classified as two distinct categories using most of the analysis methods, are molecularly and biologically different is unknown. Each group also used a unique set of data-processing steps, algorithms, and significance cut-offs to define their classifiers, and these can profoundly influence the classification signatures and success rates *(62,63)*. For example, using different methods, Singh et al. found no genes in their data set that significantly associated with outcome ($p < 0.05$), whereas Glinsky et al., analyzing the same data set, identified 218 genes (91 upregulated and 127 downregulated transcripts) that were differentially regulated ($p < 0.05$).

8. SYNTHESIZING PROFILING STUDIES OF AGGRESSIVE AND METASTATIC SAMPLES TO UNDERSTAND CAP PROGRESSION

Although these studies do not seem to have uncovered the defining expression signature associated with CaP recurrence, they provide evidence that genetic differences can distinguish between high- and low-risk tumors. Further, these genetic differences may be poorly accounted for by, or independent of, the clinical and pathological parameters currently used to predict recurrence. Perhaps more importantly, these current studies provide important insight into CaP progression, particularly in the context of studies profiling metastatic samples, and suggest areas of focus in future studies. When synthesizing the results of these studies and subsequent studies to validate identified genes, one of the most consistent observations across these studies is that regardless of their clinical and pathological characteristics, primary tumors with expression signatures similar to metastatic samples, including cell cycle genes, are likely to have a poor outcome. This observation is not surprising, but results from several studies argue strongly for the conclusion and it can help focus future studies into CaP progression.

One of the first candidate genes to be associated with metastatic tumors and localized CaP samples with poor outcome was *EZH2*. We identified *EZH2* as being overexpressed in metastatic samples compared with localized CaP, and increased protein expression in localized CaP was associated with recurrence *(34)*. *EZH2* has also been found to be significantly overexpressed in metastatic samples compared with localized CaP in other studies *(40,41)*. Besides acting as a histone methyltransferase responsible for gene silencing, recent evidence has demonstrated that *EZH2* is regulated by the pRB/E2F pathway and is essential for proliferation in several cell types *(34,64–67)*. These results, along with observations that *EZH2* transcripts are often increased in tumors with increased proliferation as assessed by Ki67 staining, have prompted speculation that *EZH2* expression may merely represent a marker of proliferation *(40,41)*.

However, several lines of evidence suggest that *EZH2* has additional roles in CaP progression besides serving as a marker of proliferation. In addition to being overexpressed in CaP, *EZH2* has been found to be overexpressed or amplified in other cancers, suggesting a role as a general oncogene *(67)*. In breast cancer, in which increased EZH2 protein also predicts disease recurrence *(68)*, immunofluorescence demonstrated that expression of EZH2 precedes high frequencies of proliferation *(69)*. Recently, EZH2 expression in the content of a polycomb complex was also examined during CaP progression in the *NKX3.1*$^{+/-}$; *PTEN* $^{+/-}$ mouse model, which develops PIN and subsequent carcinoma *(70,71)*. PIN lesions displayed moderate elevation in the number of cells expressing EZH2,

and cancerous tissues displayed further elevations, as assayed at the transcript and protein level. Importantly, the authors noted that increased EZH2 expression did not correlate with increased Ki67 levels *(66)*. However, even if EZH2 is merely a marker of proliferation, localized tumors that express high levels of EZH2 are more likely to recur than tumors expressing low levels of EZH2.

As another example, *JAG1*, a notch signaling ligand, was identified by LaTullippe et al. in their study *(41)* as one of the most overexpressed genes in metastatic samples compared with localized nonrecurrence samples (mean 32.8-fold difference). This gene was also upregulated in our study in metastatic samples compared with localized CaP *(34)*. Subsequently, JAG1 protein expression was evaluated using IHC on TMAs. As expected, significantly higher expression in metastatic cancer compared with localized cancer and benign tissues was observed. Most importantly, increased JAG1 expression was found to be significantly associated with recurrence in localized CaP samples, independent of other clinical parameters *(72)*.

Further support for this concept was provided by results from the studies of Glinsky et al. and Lapointe et al. Although they did not profile metastatic tumors directly, Glinsky et al. built their predictive algorithm around expression differences between highly aggressive and less aggressive xenograft models. Using unsupervised hierarchal clustering, Lapointe et al. identified three clusters of tumor samples. Interestingly, high Gleason grade samples, advanced stage samples, and tumors with PSA recurrence or clinical metastasis were disproportionately represented in two of the three clusters. These clusters also contained all of the lymph node metastases. To investigate the clinical relevance of the tumor subtypes observed, the authors focused on two genes, *AZGP1* and *MUC1*, which showed differential expression between the observed tumor clusters. IHC on TMAs composed of 225 primary tumors demonstrated that increased MUC1 expression ($p = 0.0005$) and decreased AZGP1 expression ($p = 0.002$) were predictors of recurrence independent of grade, stage, or preoperative PSA in a multivariate proportional hazards analysis.

Taken together, these studies suggest an important role for genes that show dysregulation in localized tumors and metastatic cancer in CaP progression. In addition to serving as potential biomarkers, genes consistently identified across studies should also provide focused targets for functional studies. As an example, *HOXC6* has been found to be overexpressed in recurrent localized or metastatic samples by multiple cDNA microarray studies *(34,41,47)*, and a recent functional study demonstrated that HOXC6 silencing resulted in decreased proliferation through increased apoptosis in LnCaP cells *(45)*. These results argue strongly for increased profiling of metastatic CaP, as Table 1 reveals that just greater than 50 metastatic samples have been profiled. Further, because metastatic cancer shows heterogeneity between individual cases as well as heterogeneity at different foci within a single patient *(53,73)*, much remains to be understood regarding gene expression in metastatic CaP. To identify possible targets for functional studies, we performed a meta-analysis of genes overexpressed during CaP progression from three large-scale studies profiling benign prostate, localized CaP, and metastatic CaP tissues, as shown in Fig. 3.

9. LIMITATIONS OF CURRENT APPROACHES TO EXPRESSION PROFILING

Although the outcome studies described in Sections 6 and 7 suggest that expression signatures can be used to identify patients at high risk for recurrence, these models do not seem to be ready for clinical application. Many of the outcome studies have demonstrated that their classifiers can predict outcome independent of other known risk factors using multivariate models. However, established models, such as the GPSM *(74)* and Kattan nomogram *(75)*, have already been developed that optimally incorporate these factors. Because it seems unlikely that expression profiling will replace monitoring of Gleason scoring, preoperative PSA levels, seminal vesicle, and margin status, these variables will be available for any model, including one incorporating expression signatures. Thus, it has been suggested that the question that should be asked is: How accurate is the best prediction model without

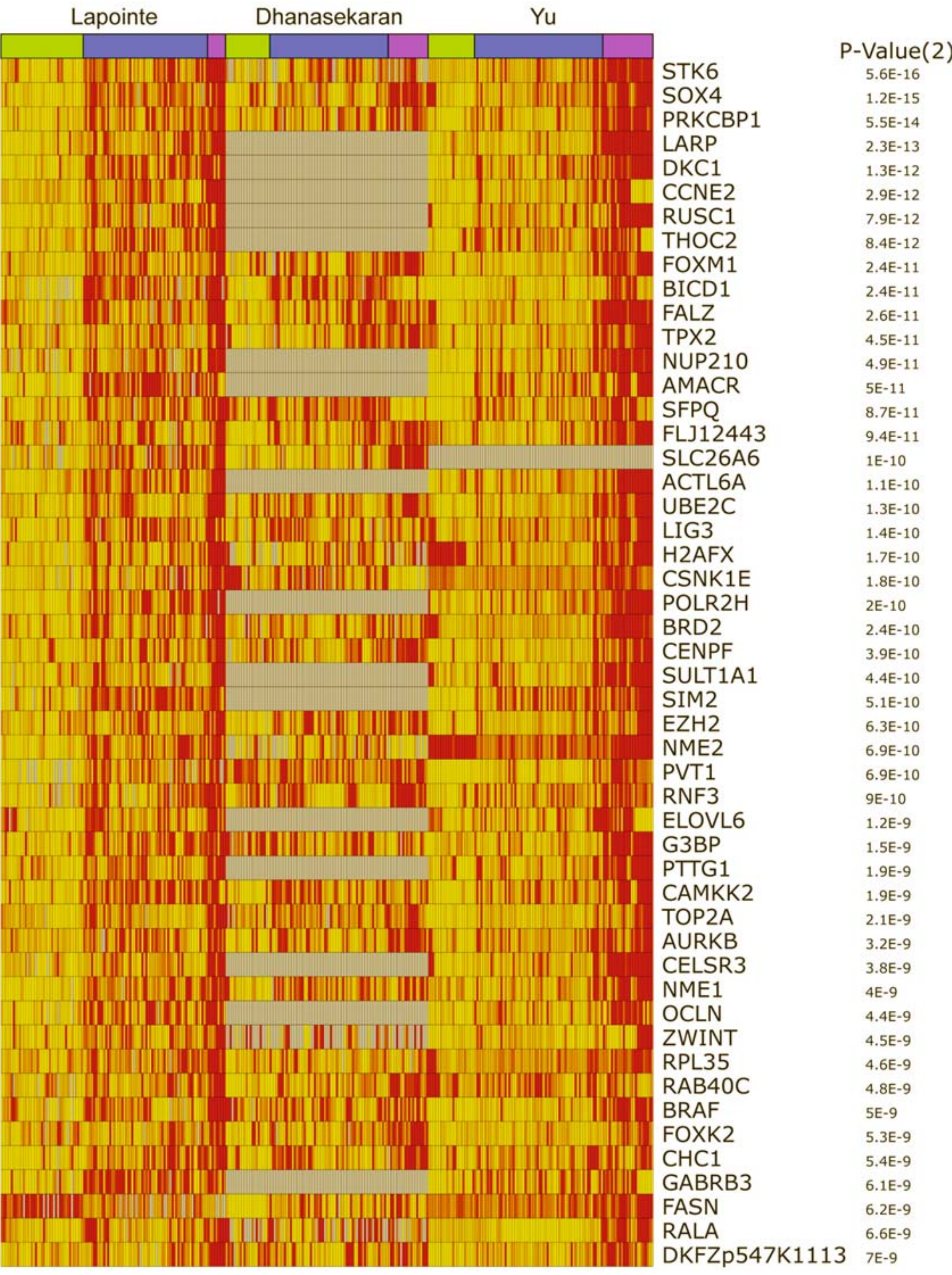


Fig. 3. Meta-analysis of genes overexpressed during prostate cancer (CaP) progression from three studies *(26,40,51)*, indicated by the last name of the first author, profiling benign prostate tissue (green), localized CaP (blue) and metastatic CaP (purple) using Oncomine. See Fig. 1 for the matrix color scheme. Genes present in two of the three studies were ranked by the second lowest p value from the individual studies, and the top 50 genes are listed.

Table 2
Meta-Analysis of Three Independent Data Sets [a]

Gene symbol	Gene name	Gene symbol	Gene name
BPAG1	Bullous pemphigoid antigen 1	*ENSA*	Endosulfine-α
EDNRB	Endotehlin receptor type B	*NICE-1*	NICE-1 protein
CDC27	Cell division cycle 27	*SVIL*	Supervillin
HLF	Hepatic leukemia factor	*GPS2*	G protein pathway suppressor 2
FLJ12443	Hypothetical protein FLJ12443	*HDAC7A*	Histone deacetylase 7A
RBBP6	Retinoblastoma binding protein 6	*CNTN1*	Contactin 1
GSTM3	Gluathione-*S*-transferase M3 (brain)	*SNX5*	Sorting nexin 5
C7	Complement component 7	*FBXO6*	F-box protein 6
SF1	Splicing factor 1	*LIFR*	Leukemia inhibitory factor receptor
PRKWNK1	Protein kinase, lysine deficient 1	*SYNPO2*	Synaptopodin 2
GPATC1	G patch domain containing 1	*FBXW2*	F-box and WD-40 domain protein 2
LOC55971	Insulin receptor tyrosine kinase substrate	*USP53*	Ubiquitin specific protease 53
NDP	Norrie disease (pseudoglioma)	*NT5C3*	5'-nucleotidase, cytosolic III
INPP4B	Inositol polyphosphate-4phosphatase	*TPR*	Translocated promoter region
SLC39A13	Solute carrier family 39, member 13	*RGS5*	Regulator of G protein signaling 5
EST	Transcribed sequences	*ASAHL*	*N*-acylsphingosine aminohydrolase-like
PLXNC1	Plexin C1	*CBX3*	Chromobox homolog 3

[a] Meta-analysis *(23)* of three independent data sets *(26,36,51)* identified 34 dysregulated genes at a Q value (estimated false discovery rate) of 0.3 between localized CaP samples with or without PSA recurrence. Gene symbols and names are listed. EST, expressed sequence tag.

the new marker relative to the model with the new marker *(2,76)*? Only the study by Glinsky et al. directly addressed this question. They found that their classifier alone or incorporated with the nomogram *(75)* was able to classify 59 (75%) of 79 samples, whereas the nomogram alone was able to classify 56 (71%) of 79 samples.

Examining the limitations of these current studies can help identify how gene expression might eventually be used clinically to influence treatment decisions. Perhaps the biggest limitation of most of the published studies is the limited number of samples profiled, particularly the number of recurrence events. Recent analysis of several published studies attempting to predict outcome in other cancers suggests that the number of samples profoundly influences the accuracy and stability of the classifier *(77,78)*. One way to accumulate a larger sample size would be to combine multiple independent data sets. At present, a large data set consisting of 300 localized CaP samples could be created by combining the data sets just using the Affymetrix U95A GeneChip. Such comparisons and validations can only be accomplished through the deposition of data in publicly available databases, such as GEO (http://www.ncbi.nlm.nih.gov/geo/) *(79)*, Array Express (http://www.ebi.ac.uk/arrayexpress/) *(80)*, or Oncomine. In an attempt to synthesize current studies attempting to predict outcome, we used our meta-analysis method *(23)* to identify a meta-signature of 34 genes that are dysregulated between tumors with or without PSA recurrence across three independent data sets *(26,36,51)* at a Q value (estimated false discovery rate) of 0.3, as shown in Table 2. We then validated this meta-signature on a fourth independent data set *(40)*, and demonstrated that our signature was greater than 75% sensitive and specific in predicting the prognosis of clinically localized disease (J. Yu et al., manuscript submitted). Although this study is still limited by the same factors as the individual studies, it provides support for the existence of genes consistently dys-regulated across studies and provides candidates for functional studies.

However, single studies and meta-analyses alike face serious challenges that reveal fundamental limitations of using microarrays to develop classifiers. At present, samples that can be used for

microarray analysis are limited to frozen tissues, because formalin-fixed, paraffin-embedded (FFPE) tissues yield degraded RNA, which cannot be used with current microarray techniques. Although this is an area of intense investigation, at present it represents a fundamental problem, because samples with considerable clinical follow up, which are essential for developing robust and clinically usable classifiers, are poorly represented in frozen tissue banks.

At present, microarray analysis is still prohibitively expensive and labor intensive. It also seems very unlikely that the measurement of tens of thousands of genes will be required to monitor the biological effects of the cancer transcriptome. Alternative technologies, such as QPCR, allow for much higher throughput measurement of gene expression for tens or hundreds of genes at reduced expense *(81,82)*. QPCR also offers increased sensitivity and precision along with a much broader dynamic range. A recent study by Paik et al. *(83)* demonstrates the enormous power of combining bioinformatics analysis of DNA microarray data with QCPR. In this study, the authors mined breast cancer microarray data to select a panel of genes to predict recurrence of tamoxifen-treated breast cancer. After testing 250 candidate genes on three independent data sets consisting of a total of 447 tumors, the authors used 21 genes to create an algorithm for use in a prospective study using 675 tissues. The sensitivity of QPCR allowed the algorithm, which calculates a “recurrence score,” to stratify patients into low-, intermediate-, or high-risk groups. Importantly, using a multivariate Cox model, the recurrence score provided significant predictive power that was independent of age and tumor size ($p < 0.001$). Further, the recurrence score was also predictive of overall survival ($p < 0.001$), and the authors demonstrated that it could be used as a continuous function to predict distant recurrence in individual patients. An important feature of this study was the prospective selection of the 21-gene panel. In addition to five reference genes, the included genes represented a proliferation cluster, an estrogen cluster, a *HER2* cluster, an invasion cluster, and three individual genes. These genes and clusters may be representative of hundreds or thousands that show similar expression patterns across the transcriptome-wide analysis that microarrays reveal. Importantly, this study demonstrates that accurate monitoring of representative genes from important biological processes in breast cancer and metastasis in general, along with only a few “outcome” genes, can effectively predict clinical behavior based on global gene expression.

A similar study for identifying aggressive CaP can be easily imagined. Eventually, with enough profiling studies, or by meta-analysis of current studies, a small group of genes (hundreds) should emerge as candidates for predicting PSA recurrence. These genes could then be monitored using QPCR or other high-throughput techniques. Perhaps the most important part of such an analysis would be the opening of the large collection of fixed tissues with long-term follow-up that are unavailable in studies limited to frozen tissues. The study by Paik et al. was performed exclusively using FFPE tissues, and their study used the extended follow-up that is generally unavailable in frozen tissue cohorts. Primer sets for QPCR are designed to amplify regions less than 100 base pairs, and the technology is sensitive enough to accurately quantify transcripts even in severely degraded material. Being able to use FFPE tissues would also allow for focused studies with controlled clinical and pathological parameters, such as identifying a poor prognosis signature for low Gleason score tumors, which are very poorly represented in most frozen tissue banks. Taken together, these results suggest that current and future expression profiling studies in combination with other techniques, such as QPCR, may be able to guide treatment options for CaP.

10. FUTURE AREAS OF FOCUS FOR PROFILING IN THE CONTEXT OF CAP PROGRESSION

In this final section, we provide a brief discussion of important advancements and future areas of focus for expression profiling that are essential for understanding CaP progression. An important aspect of CaP progression involves characterizing precursor lesions, such as PIN, which has recently become possible because of technological advances. These technological advances, such as laser

capture microdissection (LCM), as well as *in silico* techniques are also being applied to minimize the effect of contaminating stromal cells on expression signatures. Finally, we briefly discuss how alternative ways of analyzing microarray data sets may help to identify genetic lesions that drive CaP progression in individual tumors.

11. PROFILING PRECURSOR LESIONS USING LCM

Gene expression profiling generally requires micrograms of total RNA. Because of these requirements, little is known regarding genome wide expression in precursor lesions of CaP, such as PIN. One of the first expression-profiling studies by Bull et al. in 2001 profiled two grossly dissected specimens containing PIN on 1877-element nylon arrays and identified nine genes as being overexpressed in PIN *(25)*. Unfortunately, since this study, there has been little progress in characterizing global gene expression in PIN. Because of the multifocal and heterogeneous nature of CaP, profiling PIN has important implications for understanding CaP development and progression. Many prostate tumors contain foci of PIN and it is unknown whether expression in these foci reflect changes observed in CaP. For example, if a set of genes is identified that predict a poor outcome in primary CaP, it will be important to see whether this signature is present at the stage of PIN, or if these expression changes only occur after the development of carcinoma. To enable the profiling of smaller lesions, groups are combining two technologies, LCM and RNA amplification. LCM allows the specific capturing of cells of interest from a tissue section using a laser beam *(84)*. Typically, nanograms of total RNA can be isolated from hundreds to thousands of cells. To profile the captured cells, the RNA must be amplified before hybridization. Variations of the Eberwine in vitro transcription-based linear amplification method are the most thoroughly validated for expression profiling *(85,86)*. Recently, several exponential PCR-based amplification techniques have also been developed and used for expression profiling *(87,88)*.

A recent study by Ashida et al. represents the first study combining LCM and RNA amplification to characterize gene expression in PIN. The authors captured, amplified, and generated expression profiles on 23,040-element cDNA microarrays from cancerous epithelia from 20 CaP samples, epithelium from 10 PIN samples, and normal adjacent epithelial cells from an unstated number of cases *(31)*. The authors reported 21 and 63 genes as being overexpressed or underexpressed respectively in both PIN and CaP compared with normal tissue. Forty-one and 98 genes were identified as being upregulated or downregulated, respectively, during the transition from PIN to CaP. Hierarchical clustering using the 63 genes showing the highest standard deviation across the PIN and CaP samples successfully separated the CaP and PIN samples.

Unfortunately, although PIN and CaP samples were profiled from the same patient in four cases, the authors did not compare the profiles from these samples directly. Eight of the 20 CaP samples were Gleason score 5 and only 1 of the 20 samples had a Gleason score greater than 7, therefore, these samples are not entirely representative of samples observed in the Western world. However, in addition to providing a list of genes that may be differentially expressed between PIN and CaP, this study represents an important proof of concept for profiling PIN lesions in the context of understanding CaP progression.

12. ACCOUNTING FOR STROMAL CONTAMINATION IN EXPRESSION PROFILING

When selecting tissues for profiling, investigators have noticed that cancerous tissues consistently contain more epithelium than benign samples. Groups have used different techniques to control for this difference, most commonly trying to minimize the difference between the percentage of epithelial cells in each case. However, an elegant study combining expression profiling with *in silico* analysis revealed that stromal contamination has an important effect on profiling studies. By accurately determining the percentages of various cell types, including cancerous epithelium, benign hyperplas-

tic epithelium and stroma, Stuart et al. were able to determine each cell type's relative contribution to the observed expression profile *(49)*. They showed that although most genes identified as upregulated from benign tissue to CaP in the meta-analysis by Rhodes et al. *(23)* accurately reflect genes upregulated specifically in cancer, the majority of genes identified as downregulated represent stromal transcripts. In particular, they noticed a large number of archetypal smooth muscle transcripts, including smooth muscle myosin and actin, dominating the stromal profile. LaTuillipe et al. removed genes that showed high levels of expression in benign tissues, effectively removing smooth muscle transcripts, although they were comparing localized CaP to metastatic CaP *(41)*. In their study, Lapointe et al. attempted to determine the influence of epithelial content by identifying genes whose expression correlated with epithelial content in tumor samples or normal samples *(40)*. However, although they only identified nine genes as being negatively correlated with epithelial content, their study shows significant downregulation from normal to localized to metastatic cancer of smooth muscle actin (*ACTG2* and *ACTGA2*), smooth muscle myosin (*MYH11*, *MYL9*, and *MYLK*), and tropomyosin (*TMP1* and *TMP2*), among other transcripts identified by Stuart et al. as being stromal transcripts. These results suggest that this method may not be ideal for minimizing the effects of stromal contamination.

As an alternative to *in silico* techniques, some investigators have begun to use LCM to obtain pure populations of cancerous and benign epithelial cells as well as stroma *(33,35)*. As an example, Moore et al. profiled cancerous and benign epithelium from 12 samples, identifying stearoyl-CoA desaturase as being specifically downregulated from normal to cancerous epithelium, and the authors confirmed this result using IHC on TMAs *(44)*. Kristiansen et al. profiled cancerous and benign epithelial cells from 42 samples *(39)*. In agreement with Stuart et al.'s observations, Kristiansen et al. identified many of the genes identified by Rhodes' meta-analysis as being upregulated in cancer. Kristiansen et al. also validated two proteins encoded by differentially expressed genes, ALCAM and CD24 (upregulated and downregulated, respectively), using IHC on TMAs and demonstrated significant prognostic information with regard to PSA relapse in a COX multivariate analysis. It should be noted that they and others had previously identified these targets as being dysregulated, although their data set and this technique should provide new candidates for future studies.

It is important to realize that the epithelium to stroma ratio most likely represents an important feature of CaP (partially captured in Gleason patterns) and that there are very important changes in the stroma during the progression from benign to advanced CaP. However, typical profiling studies are most likely amplifying the apparent effects. More importantly, the attempt to find tumor suppressor genes or other genes that show specific downregulation during the progression to advanced CaP is being hindered, and bioinformatics and/or experimental approaches should be used to address this during expression profiling. As an example, we used Oncomine to visualize expression of the archetypal smooth muscle transcript myosin light chain kinase (*MYLK*) across eight profiling studies. Each study showed downregulation from benign tissue to CaP, however, when we examined MYLK expression using IHC on TMAs, we found that MYLK was expressed exclusively in stroma and the decreased staining observed from benign to localized to metastatic tissue was dependent on decreased stromal content, as shown in Fig. 4. In addition, by performing the same meta-analysis as described in Fig. 3, except looking for transcripts downregulated during the progression of CaP, *MYLK* was the most downregulated transcript. Other smooth muscle transcripts, such as *ACTG2*, *ACTA2*, *MYL9*, *TMP1*, and *TMP2* were also in the top 50 downregulated transcripts in the meta-analysis. These results demonstrate the impact that stromal transcripts have on CaP profiling studies.

13. CHARACTERIZING BIOLOGICALLY IMPORTANT EXPRESSION CHANGES IN CAP PROGRESSION

Expression profiling may also be very important in identifying the driving genetic changes in individual prostate tumors. For example, despite its importance in terms of therapeutics and biologi-

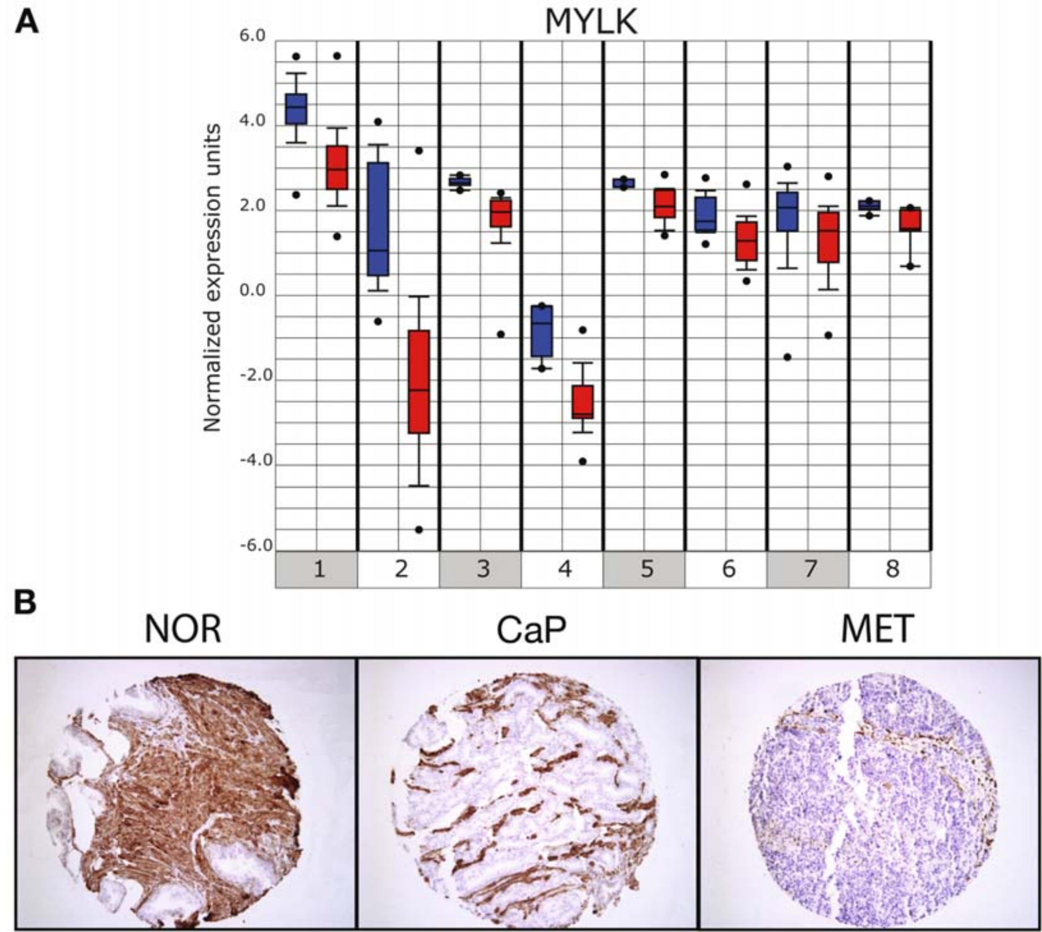


Fig. 4. Transcript and protein expression of myosin light chain kinase (MYLK). **(A)** Oncomine (www.oncomine.org) was used to visualize the normalized expression of *MYLK* across eight data sets (Lapointe et al. *[40]* = 1; Dhanasekaran et al. *[26]* = 2; Welsh et al. *[28]* = 3; Luo et al. *[29]* = 4; LaTulippe et al. *[41]* = 5; Luo et al. *[42]* = 6; Singh et al. *[47]* = 7; and Magee et al. *[27]* = 8), comparing grossly dissected benign tissue (blue) vs localized prostate cancer (CaP) (red). **(B)** Tissue microarray analysis of MYLK protein expression. Representative cores of benign (NOR), localized CaP, and metastatic CaP (MET) tissues from a tissue microarray stained for MYLK expression demonstrating stromal expression.

cal behavior, in breast cancer profiling studies, *ERBB2* does not appear on the list of the most upregulated genes between normal breast tissue and breast cancer. Most commonly, studies attempt to identify the genes that are most differentially expressed (often using a *t*-test) between two classes of samples. In these types of analyses, *ERBB2* is not highly significant, because it is not upregulated in all cases of cancer, even though it is very highly expressed when it is upregulated. A similar situation has recently been discovered during trials evaluating the epidermal growth factor receptor (EGFR) inhibitor, gefitinib, in the treatment of non-small cell lung cancer. During the trial, only 20% of patients had major objective responses and several groups have now determined that most patients experiencing a response have tumors that demonstrate mutations associated with the ATP-binding

site of *EGFR*, the target of gefitinib *(89,90)*. Again, if researchers were mining lung cancer microarray data for genes differentiating between lung cancer and benign, the downstream targets of activated *EGFR* would be missed, because *EGFR* is mutated in only 10 to 20% of cases. Recent work has confirmed that signatures in individual tumors can reveal oncogene activation or amplification when compared with mouse models *(91,92)*. For example, a study by Ellwood-Yen et al. used expression signatures from mouse tumors driven by prostate specific expression of Myc to identify a subset of human CaPs that have a "Myc-like" signature *(9)*. Taken together, these results indicate that directed analysis of microarray data may be able to identify genetic alterations that drive CaP progression as well as identify potential therapeutic targets. Recently, we developed a bioinformatics algorithm termed COPA to identify genes with marked-over-expression in a fraction of cancer cases from DNA microarray data. This led to the discovery of recurrent gene fusions in the majority of prostate cancers, where the 5' region of the androgen regulated gene TMPR552 is fused to the ETS transcription factors ERG or ETV1 in cases with outlier expression of the respective gene *(92)*.

14. CONCLUSION

Our understanding of gene expression dysregulation in CaP has increased dramatically since the advent of DNA microarrays. Unfortunately, molecular changes controlling the progression of CaP, from benign glands, through precursor lesions, localized CaP and finally metastatic disease, are poorly understood. Recently, aspects of CaP progression have been profiled by groups using DNA microarrays. In this review, we have attempted to analyze these studies critically and identify what they reveal regarding CaP progression, particularly in the context of advanced disease. Importantly, these studies also demonstrate some of the limitations of DNA microarrays. Nevertheless, the power of DNA microarrays to characterize expression changes at the genome-wide level ensures they will remain a valuable tool in the attempt to determine the molecular events controlling CaP progression.

ACKNOWLEDGMENTS

We thank Rohit Mehra for selecting histopathology images as well as Jianjun Yu for carrying out the meta-analysis in Table 2. This work was supported by grants from the American Cancer Society (RSG-02-179-01-MGO), the National Institutes of Health (EDRN UO1 CA111275, R01 CA07063, RO1 AG0214104, and the U of M Prostate SPORE P50 CA69568), and the Department of Defense (PC020322). S.A.T. is a Fellow of the Medical Scientist Training Program. A.M.C. is a Pew Biomedical Scholar.

REFERENCES

1. Gleason, D. F. and Mellinger, G. T. (1974). Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J. Urol.* **111**, 58–64.
2. Kattan, M. W. (2003). Judging new markers by their ability to improve predictive accuracy. *J. Natl. Cancer Inst.* **95**, 634–635.
3. Nakayama, M., Gonzalgo, M. L., Yegnasubramanian, S., Lin, X., De Marzo, A. M., and Nelson, W. G. (2004). GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. *J. Cell. Biochem.* **91**, 540–552.
4. Sansal, I. and Sellers, W.R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. *J. Clin. Oncol.* **22**, 2954–2963.
5. Bhatia-Gaur, R., Donjacour, A.A., Sciavolino, P.J., et al. (1999). Roles for Nkx3.1 in prostate development and cancer. *Genes Dev.* **13**, 966–977.
6. Linja, M. J. and Visakorpi, T. (2004). Alterations of androgen receptor in prostate cancer. *J. Steroid Biochem. Mol. Biol.* **92**, 255–264.
7. Feldman, B.J. and Feldman, D. (2001). The development of androgen-independent prostate cancer. *Nat. Rev. Cancer* **1**, 34–45.
8. Nupponen, N. N., Kakkola, L., Koivisto, P., and Visakorpi, T. (1998). Genetic alterations in hormone-refractory recurrent prostate carcinomas. *Am. J. Pathol.* **153**, 141–148.
9. Ellwood-Yen, K., Graeber, T. G., Wongvipat, J., et al. (2003). Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer Cell* **4**, 223–238.

10. Karhadkar, S. S., Bova, G. S., Abdallah, N., et al. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* **431**, 707–712.
11. Nelson, W. G., De Marzo, A. M., and Isaacs, W. B. (2003). Prostate cancer. *N. Engl. J. Med.* **349**, 366–381.
12. Rubin, M. A. and De Marzo, A. M. (2004) Molecular genetics of human prostate cancer. *Mod. Pathol.* **17**, 380–388.
13. Slonim, D. K. (2002). From patterns to pathways: gene expression data analysis comes of age. *Nat. Genet.* **32(Suppl.)**, 502–508.
14. Quackenbush, J. (2002). Microarray data normalization and transformation. *Nat. Genet.* **32(Suppl.)**, 496–501.
15. Churchill, G. A. (2002). Fundamentals of experimental design for cDNA microarrays. *Nat. Genet.* **32(Suppl.)**, 490–495.
16. DeRisi, J., Penland, L., Brown, P. O., et al. (1996). Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat. Genet.* **14**, 457–460.
17. Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**, 467–470.
18. Wodicka, L., Dong, H., Mittmann, M., Ho, M. H., and Lockhart, D. J. (1997). Genome-wide expression monitoring in Saccharomyces cerevisiae. *Nat. Biotechnol.* **15**, 1359–1367.
19. Chee, M., Yang, R., Hubbell, E., et al. (1996). Accessing genetic information with high-density DNA arrays. *Science* **274**, 610–614.
20. Gershon, D. (2004). Microarrays go mainstream. *Nat. Meth.* **1**, 263–270.
21. Hughes, T. R., Mao, M., Jones, A. R., et al. (2001). Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nat. Biotechnol.* **19**, 342–347.
22. Rhodes, D. R. and Chinnaiyan, A. M. (2004). Bioinformatics strategies for translating genome-wide expression analyses into clinically useful cancer markers. *Ann. NY Acad. Sci.* **1020**, 32–40.
23. Rhodes, D. R., Barrette, T. R., Rubin, M. A., Ghosh, D., and Chinnaiyan, A. M. (2002). Meta-analysis of microarrays: interstudy validation of gene expression profiles reveals pathway dysregulation in prostate cancer *Cancer. Res.* **62**, 4427–4433.
24. Rhodes, D. R., Yu, J., Shanker, K., et al. (2004). ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* **6**, 1–6.
25. Bull, J. H., Ellison, G., Patel, A., et al. (2001). Identification of potential diagnostic markers of prostate cancer and prostatic intraepithelial neoplasia using cDNA microarray. *Br. J. Cancer* **84**, 1512–1519.
26. Dhanasekaran, S. M., Barrette, T. R., Ghosh, D., et al. (2001). Delineation of prognostic biomarkers in prostate cancer. *Nature* **412**, 822–826.
27. Magee, J. A., Araki, T., Patil, S., et al. (2001). Expression profiling reveals hepsin overexpression in prostate cancer. *Cancer Res.* **61**, 5692–5696.
28. Welsh, J. B., Sapinoso, L. M., Su, A. I., et al. (2001). Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. *Cancer Res.* **61**, 5974–5978.
29. Luo, J., Duggan, D. J., Chen, Y., et al. (2001). Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. *Cancer Res.* **61**, 4683–4688.
30. Stamey, T. A., Warrington, J. A., Caldwell, M. C., et al. (2001). Molecular genetic profiling of Gleason grade 4/5 prostate cancers compared to benign prostatic hyperplasia. *J. Urol.* **166**, 2171–2177.
31. Ashida, S., Nakagawa, H., Katagiri, T., et al. (2004). Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs. *Cancer Res.* **64**, 5963–5972.
32. Best, C. J., Leiva, I. M., Chuaqui, R. F., et al. (2003). Molecular differentiation of high- and moderate-grade human prostate cancer by cDNA microarray analysis. *Diagn. Mol. Pathol.* **12**, 63–70.
33. Chen, Z., Fan, Z., McNeal, J. E., et al. (2003). Hepsin and maspin are inversely expressed in laser capture microdissectioned prostate cancer. *J. Urol.* **169**, 1316–1319.
34. Varambally, S., Dhanasekaran, S. M., Zhou, M., et al. (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**, 624–629.
35. Ernst, T., Hergenhahn, M., Kenzelmann, M., et al. (2002). Decrease and gain of gene expression are equally discriminatory markers for prostate carcinoma: a gene expression analysis on total and microdissected prostate tissue. *Am. J. Pathol.* **160**, 2169–2180.
36. Glinsky, G. V., Glinskii, A. B., Stephenson, A. J., Hoffman, R. M., and Gerald, W. L. (2004). Gene expression profiling predicts clinical outcome of prostate cancer. *J. Clin. Invest.* **113**, 913–923.
37. Henshall, S. M., Afar, D. E., Hiller, J., et al. (2003). Survival analysis of genome-wide gene expression profiles of prostate cancers identifies new prognostic targets of disease relapse. *Cancer Res.* **63**, 4196–4203.
38. Henshall, S. M., Afar, D. E., Rasiah, K. K., et al. (2003). Expression of the zinc transporter ZnT4 is decreased in the progression from early prostate disease to invasive prostate cancer. *Oncogene* **22**, 6005–6012.
39. Kristiansen, G., Pilarsky, C., Wissmann, C., et al. (2005). Expression profiling of microdissected matched prostate cancer samples reveals CD166/MEMD and CD24 as new prognostic markers for patient survival. *J. Pathol.* **205**, 359–376.

40. Lapointe, J., Li, C., Higgins, J. P., et al. (2004). Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc. Natl. Acad. Sci. USA* **101**, 811–816.
41. LaTulippe, E., Satagopan, J., Smith, A., et al. (2002). Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. *Cancer Res.* **62**, 4499–4506.
42. Luo, J. H., Yu, Y. P., Cieply, K., et al. (2002). Gene expression analysis of prostate cancers. *Mol. Carcinog.* **33**, 25–35.
43. Luo, J., Dunn, T. A., Ewing, C. M., Walsh, P. C., and Isaacs, W.B. (2003). Decreased gene expression of steroid 5 alpha-reductase 2 in human prostate cancer: implications for finasteride therapy of prostate carcinoma. *Prostate* **57**, 134–139.
44. Moore, S., Knudsen, B., True, L. D., et al. (2005). Loss of stearoyl-CoA desaturase expression is a frequent event in prostate carcinoma. *Int. J. Cancer* **114**, 563–571.
45. Ramachandran, S., Liu, P., Young, A. N., et al. (2005). Loss of HOXC6 expression induces apoptosis in prostate cancer cells. *Oncogene* **24**, 188–198.
46. Rossi, S., Graner, E., Febbo, P., et al. (2003). Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. *Mol. Cancer Res.* **1**, 707–715.
47. Singh, D., Febbo, P. G., Ross, K., et al. (2002). Gene expression correlates of clinical prostate cancer behavior. *Cancer Cell* **1**, 203–209.
48. Stamey, T. A., Caldwell, M. C., Fan, Z., et al. (2003). Genetic profiling of Gleason grade 4/5 prostate cancer: which is the best prostatic control tissue? *J. Urol.* **170**, 2263–2268.
49. Stuart, R. O., Wachsman, W., Berry, C. C., et al. (2004). In silico dissection of cell-type-associated patterns of gene expression in prostate cancer. *Proc. Natl. Acad. Sci. USA* **101**, 615–620.
50. Vanaja, D. K., Cheville, J. C., Iturria, S. J., and Young, C. Y. (2003). Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer Res.* **63**, 3877–3882.
51. Yu, Y. P., Landsittel, D., Jing, L., et al. (2004). Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. *J. Clin. Oncol.* **22**, 2790–2799.
52. Figueroa, J. A., De Raad, S., Tadlock, L., Speights, V. O., and Rinehart, J. J. (1998). Differential expression of insulin-like growth factor binding proteins in high versus low Gleason score prostate cancer. *J. Urol.* **159**, 1379–1383.
53. Thomas, R., True, L. D., Bassuk, J. A., Lange, P. H., and Vessella, R. L. (2000). Differential expression of osteonectin/SPARC during human prostate cancer progression. *Clin. Cancer Res.* **6**, 1140–1149.
54. Tusher, V. G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* **98**, 5116–5121.
55. Rosenwald, A., Wright, G., Chan, W. C., et al. (2002). The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N. Engl. J. Med.* **346**, 1937–1947.
56. Shipp, M. A., Ross, K. N., Tamayo, P., et al. (2002). Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. *Nat. Med.* **8**, 68–74.
57. Yeoh, E. J., Ross, M. E., Shurtleff, S. A., et al. (2002). Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell.* **1**, 133–143.
58. Bullinger, L., Dohner, K., Bair, E., et al. (2004). Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N. Engl. J. Med.* **350**, 1605–1616.
59. Valk, P. J., Verhaak, R. G., Beijen, M. A., et al. (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. *N. Engl. J. Med.* **350**, 1617–1628.
60. van 't Veer, L. J., Dai, H., van de Vijver, M. J., et al. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530–536.
61. Storey, J. D. and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA* **100**, 9440–9445.
62. Bair, E. and Tibshirani, R. (2004). Semi-supervised methods to predict patient survival from gene expression data. *PLoS Biol.* **2**, E108.
63. Ebert, B. L. and Golub, T. R. (2004). Genomic approaches to hematologic malignancies. *Blood* **104**, 923–932.
64. Tonini, T., Bagella, L., D'Andrilli, G., Claudio, P. P., and Giordano, A. (2004). Ezh2 reduces the ability of HDAC1-dependent pRb2/p130 transcriptional repression of cyclin A. *Oncogene* **23**, 4930–4937.
65. Tang, X., Milyavsky, M., Shats, I., Erez, N., Goldfinger, N., and Rotter, V. (2004). Activated p53 suppresses the histone methyltransferase EZH2 gene. *Oncogene* **23**, 5759–5769.
66. Attwooll, C., Oddi, S., Cartwright, P., et al. (2005). A novel repressive E2F6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. *J. Biol. Chem.* **280**, 1199–1208.
67. Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003). EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *Embo. J.* **22**, 5323–5335.
68. Kleer, C. G., Cao, Q., Varambally, S., et al. (2003). EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc. Natl. Acad. Sci. USA* **100**, 11,606–11,611.
69. Raaphorst, F. M., Meijer, C. J., Fieret, E., et al. (2003). Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. *Neoplasia* **5**, 481–488.

70. Abate-Shen, C., Banach-Petrosky, W. A., Sun, X., et al. (2003). Nkx3.1; Pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases. *Cancer Res.* **63**, 3886–3890.
71. Kim, M. J., Cardiff, R. D., Desai, N., et al. (2002). Cooperativity of Nkx3.1 and Pten loss of function in a mouse model of prostate carcinogenesis. *Proc. Natl. Acad. Sci. USA* **99**, 2884–2889.
72. Santagata, S., Demichelis, F., Riva, A., et al. (2004). JAGGED1 expression is associated with prostate cancer metastasis and recurrence. *Cancer Res.* **64**, 6854–6857.
73. Shah, R. B., Mehra, R., Chinnaiyan, A. M., et al. (2004). Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **64**, 9209–9216.
74. Blute, M. L., Bergstralh, E. J., Iocca, A., Scherer, B., and Zincke, H. (2001). Use of Gleason score, prostate specific antigen, seminal vesicle and margin status to predict biochemical failure after radical prostatectomy. *J. Urol.* **165**, 119–125.
75. Kattan, M. W., Wheeler, T. M., and Scardino, P.T. (1999). Postoperative nomogram for disease recurrence after radical prostatectomy for prostate cancer. *J. Clin. Oncol.* **17**, 1499–1507.
76. Kattan, M. W. (2004). Evaluating a new marker's predictive contribution. *Clin. Cancer Res.* **10**, 822–824.
77. Ntzani, E. E. and Ioannidis, J. P. (2003). Predictive ability of DNA microarrays for cancer outcomes and correlates: an empirical assessment. *Lancet* **362**, 1439–1444.
78. Michiels, S., Koscielny, S. and Hill, C. (2005). Prediction of cancer outcome with microarrays: a multiple random validation strategy. *Lancet* **365**, 488–492.
79. Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucl. Acids Res.* **30**, 207–210.
80. Brazma, A., Parkinson, H., Sarkans, U., et al. (2003). ArrayExpress—a public repository for microarray gene expression data at the EBI. *Nucl. Acids Res.* **31**, 68–71.
81. Mocellin, S., Rossi, C. R., Pilati, P., Nitti, D., and Marincola, F. M. (2003) Quantitative real-time PCR: a powerful ally in cancer research. *Trends Mol. Med.* **9**, 189–195.
82. Brazeau, D. A. (2004) Combining genome-wide and targeted gene expression profiling in drug discovery: microarrays and real-time PCR. *Drug Discov. Today* **9**, 838–845.
83. Paik, S., Shak, S., Tang, G., et al. (2004). A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N. Engl. J. Med.* **351**, 2817–2826.
84. Emmert-Buck, M. R., Bonner, R. F., Smith, P.D., et al. (1996). Laser capture microdissection. *Science* **274**, 998–1001.
85. Wang, E., Miller, L. D., Ohnmacht, G. A., Liu, E. T., and Marincola, F. M. (2000). High-fidelity mRNA amplification for gene profiling. *Nat. Biotechnol.* **18**, 457–459.
86. Van Gelder, R. N., von Zastrow, M. E., Yool, A., Dement, W. C., Barchas, J. D., and Eberwine, J. H. (1990). Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl. Acad. Sci. USA* **87**, 1663–1667.
87. Makrigiorgos, G. M., Chakrabarti, S., Zhang, Y., Kaur, M., and Price, B. D. (2002). A PCR-based amplification method retaining the quantitative difference between two complex genomes. *Nat. Biotechnol.* **20**, 936–939.
88. Iscove, N. N., Barbara, M., Gu, M., Gibson, M., Modi, C., and Winegarden, N. (2002). Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. *Nat. Biotechnol.* **20**, 940–943.
89. Paez, J. G., Janne, P. A., Lee, J. C., et al. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497–1500.
90. Lynch, T. J., Bell, D. W., Sordella, R., et al. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* **350**, 2129–2139.
91. Sweet-Cordero, A., Mukherjee, S., Subramanian, A., et al. (2005). An oncogenic KRAS2 expression signature identified by cross-species gene-expression analysis. *Nat. Genet.* **37**, 48–55.
92. Lamb, J., Ramaswamy, S., Ford, H. L., et al. (2003) A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. *Cell* **114**, 323–334.
93. Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., et al. (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* **310**, 644–648.

15

Somatic Alterations in Prostate Cancer Progression

Kati P. Porkka and Tapio Visakorpi

Summary

The chromosomal aberrations typical for prostate cancer have been quite well characterized by using molecular genetic tools, such as the analysis of loss of heterozygosity (LOH), comparative genomic hybridization (CGH), and the array-based CGH (aCGH). Consequently, the major challenge during recent years has been the identification of the individual genes targeted by these chromosomal alterations. Although some target genes, including the androgen receptor gene (*AR*) at Xq and the *PTEN* tumor suppressor gene at the 10q, are already known, most target genes are yet to be discovered. Overall, only a few genes have been found to be mutated in prostate cancer. The most common alterations of individual genes, detected so far, are hypermethylation of the *GSTP1* gene, amplification of the *AR* gene, and mutations in the *TP53* and *PTEN* genes. Because genetic alterations seem to point out the weak spots of cancer and have, thus, been used as markers for druggable targets in many other cancer types, it would be extremely important to carry out systematic mutation screening programs to find more genes that are frequently mutated also in prostate cancer.

Key Words: CGH; comparative genomic hybridization; genetics; LOH; loss of heterozygosity; oncogene; prostatic carcinoma; tumor suppressor gene.

1. INTRODUCTION

Prostate cancer arises from glandular epithelium, most often in the peripheral zone of the prostate. High-grade prostatic intraepithelial neoplasia (PIN) is thought to be a premalignant stage of prostate carcinoma. Other premalignant lesions, such as proliferative inflammatory atrophy, have been suggested *(1)*. Microscopic lesions of cancer have been found in autopsies from more than 50% of 70- to 80-year-old men. A vast majority of these histological cancers would most probably never develop into a clinical cancer. Whether these incidentally found small carcinomas represent the same disease entity as the clinically relevant, life-threatening tumors, is not really known. Organ-confined prostate cancer invades through the capsule of the prostate into its surroundings and metastasizes to local lymph nodes and to distant organs, mainly bones. The growth and progression of prostate cancer is dependent on androgens. Thus, for more than half a century, the standard treatment for advanced prostate cancer has been hormonal therapy, such as castration or anti-androgens. However, during the treatment, an androgen-independent cancer cell population will eventually arise *(2)*. For such hormone-refractory tumors, no effective treatments are available.

The described clinical development and progression of prostate cancer is underlain by genetic alterations. The novel genomic tools have helped to identify some critical genetic aberrations in prostate cancer, but many are to be discovered. In this chapter, we describe chromosomal and gene aberrations that have been found in prostate cancer.

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

	Chromosomal changes		Gene alterations
	Losses	*Gains*	*Hypermethylation, amplification and mutation*
Normal epithelium			
Prostatic intraepithelial neoplasia	8p ~ 30% 13q ~ 30%		GSTP1 ~ 90%
Localized clinical cancer	6q ~ 20% 7q ~ 30% 10q ~ 10% 16q ~ 20% 18q ~ 20%	8q ~ 5% 7p/q ~ 10%	KLF6 ~ ? ATBF1 ~ ?
Metastatic cancer Hormone-refractory cancer	8p ~ 80% 13q ~ 60% 6q ~ 40% 7q ~ 30% 10q ~ 50% 16q ~ 55% 18q ~ 20%	8q ~ 80% 7p/q ~ 50% Xq ~ 40%	PTEN ~ 60% TP53 ~ 30% EPHB2 ~ ? AR (mutation) ~ 10% AR (amplification) ~ 30%

Fig 1. Summary of genetic aberrations in different stages of prostate cancer. The frequencies of gains and losses of chromosomal regions are based on loss of heterozygosity and comparative genomic hybridization studies. Gene mutations, amplifications, and hypermethylations have been detected by various methods. The references for the loci and genes are given in the main text.

2. CHROMOSOMAL ABERRATIONS

Prostate cancer cells do not grow well in vitro, which is why traditional cytogenetics (G-banding) has been very uninformative in elucidating genetic alterations of prostate cancer. Typically, karyotyping has indicated normal 46XY chromosomes, obviously caused by overgrowth of normal fibroblasts *(3)*. The development of modern molecular genetic methods, such as the analysis of loss of heterozygosity (LOH) and comparative genomic hybridization (CGH), has, however, enabled characterization of the typical genomic aberrations in prostate cancer (Fig. 1). LOH studies using restriction fragment length polymorphism, microsatellite, or single-nucleotide polymorphism markers have been used to detect regions of deletion and to narrow the putative tumor suppressor gene loci. Although LOH studies are applicable mainly in search of deletions, usually concerning rather restricted chromosomal areas, CGH enables the detection of both losses and gains of genetic material simultaneously, and has been the most informative tool in genome-wide studies. On the other hand, the resolution of LOH analysis can be significantly higher than resolution of CGH if only a sufficient number of genetic markers are used. The recently developed array-based CGH (aCGH) methods have, however, significantly increased the resolution of CGH.

2.1. LOH Studies

Only a few LOH studies with markers in every chromosome arm (so-called allelotyping) have been published thus far *(4–7)*. LOH studies have detected allelic imbalance in prostate cancer most

often at chromosomal arms 6q, 7q, 8p, 10q, 13q, 16q, and 18q (reviewed in ref. *8* and Fig. 1). Fine mapping of these chromosomal areas has successfully narrowed the minimal regions of deletions, however, in most cases, the individual tumor suppressor genes have still not been identified.

The highest rate of LOH has been detected at the chromosomal regions of 8p, 13q, and 16q (reviewed in ref. *8*). Within 8p, at least two minimal regions of deletion, 8p21 and 8p22, have been identified *(9–11)*, suggesting that several tumor suppressor genes may be located at 8p. LOH at 8p also seems to be an early event, because it has been found in high-grade PIN *(12)*. The most promising candidate gene is a homeobox gene, *NKX3-1 (13)*, located at 8p21, although no mutations in the remaining allele of the *NKX3-1* have been found *(14)*. However, the *NKX3-1* knock-out mouse models have demonstrated that even a loss of one allele can lead to hyperplasias and PIN-like lesions in the mouse prostate *(15,16)*, suggesting haploinsufficiency of the *NKX3-1* gene. Another promising candidate gene has been the macrophage scavenger receptor 1 (*MSR1*) at 8p22, whose germline mutations and sequence variants have been shown to be associated with prostate cancer risk in some studies *(17–19)*. In sporadic prostate cancer, however, mutations in the *MSR1* gene have not been found *(20)*.

LOH at 16q is often observed in advanced prostate cancer and is associated with poor prognosis *(21–23)*. The commonly deleted regions detected in various studies are at 16q22–q24 *(21,24,25)*. Perhaps the most intensively studied putative target gene has been E-cadherin (*CDH1*) at 16q22.1. E-cadherin is a Ca^{2+}-dependent adhesion molecule, loss of which is associated with metastatic capability of cancer cells *(26)*. Early immunohistochemical studies suggested that loss of CDH1 expression is relatively common, and that it is associated with poor prognosis *(27–29)*. However, later studies have shown that CDH1 is expressed strongly, even in metastases *(30)*. The discrepancy is probably caused by new immunostaining technologies, including better antibodies and antigen retrievals. Because single-nucleotide polymorphism markers within the *CDH1* gene have revealed no LOH *(31)*, and no somatic mutations have been detected *(21,24)*, *CDH1* might not be the target of this deletion.

Very recently, another putative target for 16q loss was reported *(32)*. The *ATBF1* gene, encoding for an AT-binding transcription factor 1, was found to be mutated in almost 40% of prostate cancers. Transfection of *ATBF1* into an *ATBF1*-negative cell line suppressed growth of the cells, indicating a tumor suppressor function of the gene. The finding is obviously very intriguing, and should be rapidly confirmed.

More than half of prostate carcinomas show LOH at the long arm of chromosome 13. At least three distinct regions of allelic loss, 13q14, 13q21–22, and 13q33, have been detected *(33)*. The target genes suggested for these regions include, for example, *RB1*, *BRCA2*, and *EDNRB*, but no strong evidence in support of any of these genes has been produced.

In 10q, the highest rate of LOH has been reported at the region 10q23–q24 *(34,35)*, where the well-known tumor suppressor gene, *PTEN*, is located *(36–38)*. *PTEN* is most likely one of the target genes of this region, and will be discussed in more detail in the following sections. However, because LOH at 10q23–q24 has been detected much more frequently than mutations in the *PTEN* gene, it has been suggested that it is not the only tumor suppressor gene in this region. Another candidate gene has been *MXI1*, which has an interesting function as an antagonist of MYC. Mutations in the *MXI1* gene are, however, rare according to most studies *(34,39,40)*, and the gene is also located outside the minimal regions of deletion.

Perhaps the most controversial finding in LOH studies on prostate cancer has been the allelic loss at the chromosomal region 7q31 *(41–44)*. This is because both CGH and fluorescence *in situ* hybridization studies have shown that extra copies of the entire chromosome 7 are very common in prostate cancer *(45–51)*, and 7q31 has been shown to be one of the minimal regions of gain *(52)*. A candidate target gene for both the deletion and the gain of the 7q31 locus has been the caveolin gene (*CAV1*). According to some studies, CAV1 displays tumor suppressive activities, and its expression is decreased in human malignancies *(53–57)*. Paradoxically, positive immunostaining of the CAV1

protein has been shown to be associated with poor prognosis of prostate cancer, and functional studies have demonstrated the oncogenic potential of the *CAV1* gene in prostate cancer cells *(58–61)*. Therefore, it is unclear whether *CAV1* is acting like an oncogene or like a tumor suppressor gene. Possibly, the specific function of CAV1 is highly cell and context dependent, which is why further studies with prostate cancer cells and samples are required to elucidate its role in the progression of prostate cancer.

LOH at 6q has been detected in approx 30 to 50% of primary prostate carcinomas *(62–64)*. Several minimal regions of LOH have been found at 6q14–q22 *(62–65)*, but no promising candidate tumor suppressor have thus far been suggested for this region of deletion. Deletion of 18q, on the other hand, has been detected mainly in advanced stages of prostate cancer *(66,67)*. The common minimally deleted region is at 18q21, in which the *MADR2/SMAD2*, *SMAD4*, and *DCC* genes at located. However, no mutations in these genes have been found *(68,69)*.

2.2. CGH Studies

CGH studies have revealed two main features characteristic to prostate cancer progression. First, in the early stages of prostate cancer, losses of the genetic material are more common than gains or amplifications *(48)*, indicating that tumor suppressor genes probably play an important role in the early tumorigenesis of the prostate. Second, gains and amplifications are typical for metastatic and hormone-refractory tumors, suggesting that the late stage of the disease is characterized by oncogenic activation *(48)*. The chromosomal regions in which losses have most commonly been detected by CGH are mainly the same that often have shown LOH in prostate cancer, thus, confirming the significance of these regions. In early prostate cancer, losses of 6q (~20%), 8p (~30%), 13q (~30%), 16q (~20%), and 18q (~20%) are the most common. In advanced disease, the frequency of alterations in these chromosomal arms is higher, for example, losses of 8p and 13q are found in up to 80% of cases. In addition, losses of 10q (~40%) and 17p (~40%), as well as gains of 7p/q (40%), 8q (~80%), and Xq (~40%) are common in the advanced stage of the disease (reviewed in ref. *70*, and Fig. 1).

Because detection of gains and amplifications is difficult and unreliable with LOH analysis, the main contribution of CGH studies has been the identification of gains of chromosomal regions. In the late stage of prostate cancer, gains of 7p/q, 8q, and Xq are often detected. Of those, the target gene for only Xq gain, the androgen receptor (*AR*) gene, is known (discussed in detail in Subheading 3.2.). The most common chromosomal alteration detected in hormone-refractory and metastatic prostate carcinomas by CGH is gain of 8q, with the frequency of approx 70 to 90% *(48,52,71)*. Gain of 8q has been shown to be associated with an aggressive phenotype and poor prognosis of prostate cancer *(72,73)*. Although the gain usually covers the entire long arm of chromosome 8, two independently amplified subregions, 8q21 and 8q23–q24, have been identified *(52,71)*. Probably the most intensively studied putative target gene for the 8q gain is the well-characterized oncogene *MYC*, located at 8q24. Recently, it was shown that transgenic mice expressing human *MYC* in prostate develop PIN lesions as well as invasive adenocarcinomas *(74)*. In humans, *MYC* has been shown to be amplified in prostate carcinomas *(75,76)*, and its increased copy number seems to be associated with poor prognosis *(77)*. Even though overexpression of MYC, determined by immunohistochemistry, has been reported in prostate cancer *(75)*, we have not found any difference in MYC expression, detected by quantitative RT-PCR (Q-RT-PCR), in benign prostatic hyperplasia (BPH) and prostate carcinoma *(78)*. In addition, MYC expression in prostate and breast cancer cell lines was not associated with the copy number of the *MYC* gene, suggesting that *MYC* is probably not a common target gene of the 8q gain.

By using suppression subtraction and complementary DNA (cDNA) microarray hybridization (and a combination of these), we have recently identified three putative target genes for the 8q23–24 gain: *EIF3S3*, *KIAA0196*, and *RAD21 (76,79)*. These genes seem to be amplified in approx 30% of hormone-refractory prostate carcinomas. We have also already shown that the amplification of *EIF3S3*

is associated with high Gleason score and advanced clinical stage of the disease *(80)*. According to the Q-RT-PCR analyses, *EIF3S3*, *KIAA0196*, and *RAD21* are also overexpressed in prostate carcinomas compared with BPH tumors *(78,79)*.

The *EIF3S3* gene encodes for a p40 subunit of eukaryotic translation initiation factor 3 (*eIF3*), which plays a key role in the translation initiation pathway by binding to 40S ribosomal subunits in the absence of other translational components and helps to maintain the 40S and 60S subunits in a dissociated state *(81)*. However, the function of the p40 subunit itself is unknown. *RAD21* (also known as *hr21/Scc1/Mcd1/NXP1/KIAA0078*), on the other hand, is a human ortholog of the *S. pombe RAD21*. It is a component of the cohesin complex that holds sister chromatids together during mitosis *(82,83)*. It has also been reported that a caspase-mediated cleavage of the RAD21 protein occurs during apoptosis, causing amplification of the proapoptotic death signal *(84,85)*. The function of *KIAA0196* is unknown, and it shows no homologies to known genes.

Other target genes suggested for the 8q23–24 amplification are, for example, prostate stem cell antigen (*PSCA*) and *TRPS1/GC79 (86,87)*. *PSCA* was identified by using the representational difference analysis method, in search for genes that are upregulated during prostate cancer progression *(86)*. PSCA protein expression has been reported to correlate with prostate tumor stage, grade, and androgen independence *(88)*, but studies on the messenger RNA (mRNA) level have not confirmed this correlation *(89)*. *TRPS1* was first discovered by differential display as a transcript showing higher expression in androgen-dependent than in androgen-independent prostate cell line *(90)*. We have found amplifications of the *TRPS1* gene in approximately one-third of hormone-refractory prostate carcinomas, but have not detected any differences in *TRPS1* expression between BPH and prostate carcinoma by Q-RT-PCR *(78)*.

For the minimal amplification region at 8q21, only a few candidate target genes have been suggested. Using the cDNA microarray technique, we and others have detected two overexpressed genes from this region: Elongin C (*TCEB1*) and *PrLZ/TPD52 (91,92)*. Both genes have been found to be amplified in prostate carcinomas, especially in metastatic and hormone-refractory tumors *(91–93)*. PrLZ protein has been shown to be overexpressed in clinical prostate carcinomas, but also in PIN lesions *(92,93)*, suggesting that increased expression of PrLZ is an early event in the development of prostate cancer and may, thus, not be related to 8q gain *(92)*. Elongin C, on the other hand, is amplified in approx 20% of hormone-refractory prostate carcinomas, but not in early cancer *(91)*. In addition, the expression of Elongin C is significantly higher in hormone-refractory than untreated carcinoma. Elongin C seems to have at least two functions. First, it is a subunit of the elongin complex, containing also subunits A and B, that activates transcription performed by RNA polymerase II. Binding of Elongin A to the subunits B and C reactivates the RNA polymerase, preventing pausing of the transcription *(94)*. For tumorigenesis, a potentially more important second function of Elongin C relates to the degradation of hypoxia-inducible factor (HIF)-1α. A complex formed by Elongin B, Elongin C, Cul2, Rbx1, and von Hippel-Lindau (VHL) proteins targets other proteins, such as HIF-1α, for ubiquitinylation-mediated degradation. Mutations in *VHL*, which are often found in renal cancer, abrogate the binding of Elongin C to VHL *(95–98)*, preventing the ubiquitination and degradation of HIF-1α. This, in turn, results in increased expression of their target genes, such as vascular endothelial growth factor, platelet-derived growth factor B chain, and transforming growth factor-α, thus, promoting tumor growth (reviewed in ref. *98*). Increased expression of HIF-1α has been found in prostate cancer *(99)*, whereas mutations in *VHL* have not been found. The role of Elongin C in the interaction with VHL seems to be protective against tumorigenesis. Thus, the functional significance of the overexpression and amplification of the gene in prostate cancer remains unclear. Because it has been shown that the Elongin C protein is capable of forming complexes with several proteins *(100)*, it is possible that it has other, unidentified functions that could promote tumorigenesis.

Gain at both arms of the chromosome 7 has frequently been detected in prostate cancer in CGH analyses *(48,51)*. In fluorescence *in situ* hybridization analysis, at least one extra copy of the entire

chromosome 7 is often observed in prostate carcinoma samples *(45–47,49,50)*, and aneusomy of chromosome 7 has been shown to be associated with advanced tumor stage and poor prognosis of prostate cancer *(45,47,50,72)*. One of the target genes suggested is a receptor tyrosine kinase, *MET*, a well-known oncogene. Overexpression of the MET protein and mRNA has been shown in high-grade tumors and metastases, but, in some studies, MET expression has been detected also in a high number of PIN lesions *(101–104)*. Another candidate target gene for the gain of chromosome 7 has been the caveolin gene (*CAV1*), which, paradoxically, has also been the candidate tumor suppressor gene for the 7q31 LOH region, as described in Subheading 2.1. In various studies, *CAV1* has been reported to display both tumor suppressive and oncogenic potential, which is why it is not very clear whether it is a tumor suppressor gene or an oncogene. However, no high-level amplifications of *CAV1* have been found in prostate cancer *(52)*.

A third putative target gene for gain of chromosome 7 is the enhancer of zeste homolog 2 (*EZH2*). It is a component of the polycomb repressive complex 2 (PRC2), which is required for silencing of the *HOX* genes during embryogenesis. Recent studies have shown that EZH2 is highly expressed in metastatic prostate cancer, and the increased expression of EZH2 in primary prostate carcinomas is associated with poor prognosis and recurrence of the disease *(105–107)*. Inhibition of the EZH2 expression has been shown to decrease proliferation rate of prostate cancer as well as other cancer cells *(105,108)*, whereas its ectopic expression gives a growth advantage to cells *(108)*, demonstrating the oncogenic potential of EZH2. Amplifications of the *EZH2* locus at the chromosomal region 7q35 have been detected in various cancer types *(108)*. In addition, one prostate cancer xenograft has been found to contain *EZH2* gene amplification *(157)*, but the frequency of the *EZH2* amplification in prostate cancer is not known.

2.3. Array-Based CGH Studies

As a genome-wide molecular genetic method, CGH has been successfully used to give an overview of chromosomal aberrations in tumors. However, the resolution of the conventional chromosomal CGH is only approx 10 to 20 Mb, which is why small regions of deletion or amplification cannot be detected. In addition, some alterations, such as the gain of the 8q region found by conventional CGH, can be discontinuous, meaning that not all the genes within the gained area are equally amplified *(109)*. On the other hand, aCGH has higher resolution. The exact resolution of aCGH has not been thoroughly studied, however, it seems that with oligo-arrays, the resolution could be as high as tens of base pairs. There are many different types of arrays available for aCGH, such as large genomic clones (bacterial artificial chromosome, P1 artificial chromosome, etc.), cDNA, and oligo arrays.

A few high-resolution, genome-wide aCGH studies with prostate cancer have, thus far, been published *(110–114)*. They have mainly confirmed the previous findings of chromosomal CGH, but some novel regions of amplifications and deletions have also been presented. Using bacterial artificial chromosome arrays, Paris et al. *(114)* found that gain of 11q13.1, a region not previously implicated in prostate cancer, predicts the recurrence of prostatectomy-treated patients. One of the advantages of using cDNA arrays for aCGH is that expression analysis can be easily combined with the copy number analysis, thus, enabling the identification of genes whose expression is affected by copy number changes, as expected if the gene is significant in cancer development. The commonly available prostate cancer cell lines have already been analyzed with aCGH to cDNA arrays *(111,115)*. Wolf et al. *(111)* found a strong correlation between copy number and expression levels in four prostate cancer cell lines (PC-3, DU145, LNCaP, and 22Rv1) using a 16,000 cDNA microarray.

We have recently analyzed 18 prostate cancer cell lines and xenografts for DNA sequence copy number and expression alterations using a cDNA microarray for 16,000 genes *(158)*. The aCGH data was very similar to conventional CGH of the same samples, with the agreement level close to 90%. aCGH detected a few small regions of gain and deletion that were missed by conventional CGH. This

Table 1
Genes Showing Somatic Alterations in Prostate Cancer[a]

Gene	Identified function	Alteration detected in prostate cancer	Frequency of the alteration		References
TP53	Transcription factor, cell cycle regulator	Mutation	20–40%	(advanced tumors)	*(116–118)*
PTEN	Lipid/tyrosine phosphatase	Mutation, hz deletion	60%	(cell lines, xenografts and metastatic tumors)	*(122,123)*
AR	Androgen receptor	Amplification	30%	(HR tumors)	*(134,135)*
		Mutation	10–20%	(HR tumors)	*(131,132)*
GSTP1	Detoxifying enzyme	Hypermethylation	90–95%		*(140–142)*
KLF6	Transcription factor	Mutation	0–55%		*(143–145)*
EPHB2	Receptor tyrosine kinase	Mutation	8%		*(146)*
ATBF1	Transcription factor	Mutation	40%		*(32)*

[a]Hz, homozygous; HR, hormone-refractory.

suggests, first, that aCGH is a reliable tool for DNA sequence copy number analysis and, second, that the majority of gains and losses covers genomic regions that are so large that they are detected by conventional CGH. Our study confirmed the finding by Wolf et al. *(111)* that DNA sequence copy number is strongly associated with gene expression.

3. ALTERATIONS OF INDIVIDUAL GENES IN PROSTATE CANCER

The genes harboring mutations or epigenetic changes in prostate cancer are summarized in Table 1.

3.1. TP53 *and* PTEN *Mutations*

As discussed in Subheadings 2.1. and 2.2. of this chapter, many of the putative target genes of the chromosomal regions showing LOH or deletions, that is, the putative tumor suppressor genes, are still not known. This is mainly because mutations in the remaining alleles at the deleted regions have not been detected. Two extensively studied and frequently mutated tumor suppressor genes in prostate cancer are *TP53* and *PTEN*.

TP53 is the most commonly mutated gene in human cancers. It encodes for the tumor protein p53, which is a key regulator of the cell cycle, controlling the transition from the G1 phase to the S phase. Mutated TP53 protein accumulates in the nucleus, enabling an indirect detection of *TP53* mutations by immunohistochemistry. Both the immunohistochemical detection of the protein and direct sequencing of the gene have been used to study the frequency of *TP53* mutations in prostate cancer. According to most studies, mutations in the *TP53* gene are rare in early, localized prostate cancer, whereas in advanced (metastatic and/or hormone-refractory) prostate carcinomas, they are found in approx 20 to 40% of cases *(116–118)*. Nuclear accumulation of the TP53 protein has also been shown to be associated with poor prognosis *(117)*.

The *PTEN* gene encodes for a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that negatively regulates the phosphoinositide 3-kinase (protein kinase B)/Akt signaling pathway. According to most studies, mutations of *PTEN* are infrequent in primary prostate carcinomas *(119–121)*, but in

cell lines, xenografts, and metastatic lesions of prostate, the rate of mutations and deletions detected is high (60–100%) *(36,37,122,123)*. Because LOH at the *PTEN* locus has been reported to be much more frequent than mutations in the *PTEN* gene, which is the only tumor suppressor gene identified from this locus thus far *(124)*, it has been suggested that *PTEN* could be targeted by haploinsufficiency. In support of this hypothesis, a heterozygous $Pten^{+/-}$ transgenic adenocarcinoma mouse prostate model demonstrates that the loss of one allele of *PTEN* results in an increased rate of prostate cancer progression *(125)*. Mouse models also suggest a gene dosage dependent cooperation between *NKX3.1* and *PTEN (126)*. Interestingly, it was recently shown that the downstream effector of PTEN, Akt, is commonly phosphorylated in prostate cancer, and the phosphorylation is associated with poor prognosis *(127)*. One mechanism for the Akt phosphorylation is most likely the loss of PTEN function. PTEN is also involved in downregulation of the mammilian target of Rapamycin (mTOR) pathway, which can be inhibited by a drug called rapamycin. It has been shown that prostate cancer xenografts with inactive PTEN are more sensitive to rapamycin than xenografts with wild-type *PTEN (128)*.

3.2. *AR Mutations and Amplifications*

Because of the androgen-dependent growth of prostate cancer, it is natural that prostate carcinomas have been screened for *AR* mutations in many studies. The vast majority of the studies have indicated that *AR* mutations are rare in untreated prostate cancers (reviewed in ref. *129*) and in tumors treated by castration alone *(130)*. However, in patients treated with anti-androgens, such as flutamide and bicalutamide, *AR* mutations have been detected in approx 10 to 20% of cases *(131,132)*. It has been demonstrated that at least some of these mutations alter the ligand-specificity of the AR, and that the mutated receptor may paradoxically be activated by the anti-androgens *(132,133)*.

We originally demonstrated that the *AR* gene is amplified in 30% of hormone-refractory prostate carcinomas from patients treated with androgen withdrawal *(134)*. The finding has subsequently been confirmed by several studies (reviewed in ref. *135*). No amplifications have been found in untreated tumors, suggesting that the gene amplification is selected as a consequence of the androgen withdrawal *(136)*. This, in turn, suggests that tumors with *AR* gene amplification may be androgen hypersensitive instead of independent. Indeed, we have also shown that patients with *AR* gene amplification respond better to the second-line maximal androgen blockade than patients without the amplification *(136)*. The *AR* gene amplification leads to the overexpression of the gene, as expected. However, somewhat surprisingly, we and others have now shown that almost all hormone-refractory prostate carcinomas express high levels of *AR (137,138)*. The mechanisms for *AR* overexpression in tumors not containing the gene amplification still remain unknown. However, in their recent work, Chen et al. *(139)* showed that even a modest overexpression of *AR* is capable of converting androgen-dependent growth of prostate cancer cells into independent one. *AR* overexpression also transformed an AR antagonist to an agonist. Thus, it seems that overexpression of *AR* is truly a common mechanism for androgen-independence of prostate cancer.

3.3. *Hypermethylation of* GSTP1

The most commonly altered gene in prostate cancer detected thus far is the glutathione-*S*-transferase class π gene (*GSTP1*) (reviewed in ref. *1*). Glutathione-*S*-transferases are detoxifying enzymes that catalyze conjunction of glutathione with harmful, electrophilic molecules, either endogenously or exogenously produced, thereby protecting cells from carcinogenic factors. Silencing of the *GSTP1* expression by hypermethylation of the promoter region has been detected in 90 to 95% of prostate carcinoma, 70% of PIN, and 6% of proliferative inflammatory atrophy samples *(140,141)*, suggesting that it is an early event in prostate tumorigenesis. It has been suggested that detection of the hypermethylated *GSTP1* could be used as a diagnostic marker for prostate cancer. This has already been shown to be feasible by studies in which hypermethylation of *GSTP1* has been detected by methylation-specific PCR from body fluids *(142)*.

3.4. Other Gene Alterations

A few years ago, Narla et al. *(143)* reported that the Kruppel-like factor 6 (*KLF6*), a transcription factor regulating cell growth and differentiation, is deleted in 77% of primary prostate carcinomas. In 71% of the cases showing the deletion, the remaining allele was found to be mutated, thus, suggesting biallelic inactivation of KLF6 in these tumors. The finding was extremely intriguing, because the mutation rate of *KLF6* seemed to be one of the highest detected in prostate cancer thus far. However, in two other studies published soon after, the frequencies of *KLF6* alterations were reported to be much lower, with only 28% of prostate carcinoma samples showing loss, and 0 to 9% showing missense mutations of the gene *(144,145)*. This discrepancy may be caused by different handling of the sample material or techniques used for mutation detection, both of which can significantly affect the results in mutation screening of clinical samples. Therefore, further studies are needed to fully ascertain the frequency of *KLF6* alterations in prostate cancer.

Quite recently, mutations of the receptor tyrosine kinase gene, *EPHB2*, in prostate cancer were detected with nonsense-mediated decay microarray analysis *(146)*. EPHB2 belongs to the large family of Eph receptors that regulate intracellular signaling pathways involved in cell growth, migration, adhesion, and polarity *(147)*. Mutations in *EPHB2* were found in 8% of late stage clinical prostate carcinoma samples, a fraction of which also showed a concomitant loss of the wild-type allele. Because forced expression of *EPHB2* also suppressed the growth and colony formation of prostate cancer cells *(146)*, it was suggested that *EPHB2* might be a tumor suppressor gene in prostate cancer. Although the reported mutation frequency was not very high and it is not clear whether all of these mutations affect the function of the protein, *EPHB2* is still one of the few genes in which mutations have been detected, and, thus, should be studied further.

4. CONCLUSIONS AND FUTURE DIRECTIONS

Studies on novel drugs, such as trastuzumab, imatinib, and gefitinib, have clearly demonstrated that tumors containing alterations in genes encoding the targets of drugs are susceptible to these compounds *(148–151)*. Thus, it seems that these genetic alterations indicate truly critical mechanisms for tumor progression. This suggestion is further supported by findings showing that tumor cells that have become resistant to these drugs also acquired additional mutations abolishing the inhibitory effects of the drugs. Similarly, in prostate cancer, it has been shown that tumors acquire androgen-independence through either point mutation or amplification of the *AR* gene.

Thus, the critical point in developing novel therapeutics is to identify mutated genes in cancer. Unfortunately, not many such genes are known in prostate cancer. AR signaling clearly is one of the pathways that should be considered as a prime target for therapy. The currently available hormonal therapies, including anti-androgens, are not efficient enough in inhibiting the AR-transactivation, and, therefore, novel drugs targeting AR in prostate cancer tissue should be developed. *TP53* and *PTEN* are relatively commonly altered in late stage prostate cancers. Because both are tumor suppressor genes, it may be difficult to develop a drug affecting them directly, although small molecules rescuing the function of mutated *TP53* have been described *(152)*. Another option for affecting tumor suppressor pathways is to target some of the downstream effectors. For example, targeting phosphoinositide 3-kinase/Akt pathway in tumors with *PTEN* inactivation could be potentially effective *(153,154)*.

However, it is clear that we still need to find more genes that are commonly altered in this disease. Although the common chromosomal alterations are known, the individual genes that are targeted by these aberrations are still mostly unidentified. Thus, systematic genome-wide screening of mutations in prostate tumors should be carried out. While waiting for better sequencing tools allowing such an enormous task, the screening could be started with the 300 known cancer associated genes, the so called “Cancer Gene Census” *(155)* (http://www.sanger.ac.uk/genetics/CGP/Census/).

REFERENCES

1. DeMarzo, A. M., Nelson, W. G., Isaacs, W. B., and Epstein, J. I. (2003). Pathological and molecular aspects of prostate cancer. *Lancet* **361**, 955–964.
2. Arnold, J. T. and Isaacs, J. T. (2002). Mechanisms involved in the progression of androgen-independent prostate cancers: it is not only the cancer cell's fault. *Endocr. Relat. Cancer* **9**, 61–73.
3. Sandberg, A. A. (1992). Chromosomal abnormalities and related events in prostate cancer. *Hum. Pathol.* **23**, 368–380.
4. Kunimi, K., Bergerheim, U. S., Larsson, I. L., Ekman, .P, and Collins, V. P. (1991). Allelotyping of human prostatic adenocarcinoma. *Genomics* **11**, 530–536.
5. Cunningham, J. M., Shan, A., Wick, M. J., et al. (1996). Allelic imbalance and microsatellite instability in prostatic adenocarcinoma. *Cancer Res.* **56**, 4475–4482.
6. Dumur, C. I., Dechsukhum, C., Ware, J. L., et al. (2003). Genome-wide detection of LOH in prostate cancer using human SNP microarray technology. *Genomics* **81**, 260–269.
7. Lieberfarb, M. E., Lin, M., Lechpammer, M., et al. (2003). Genome-wide loss of heterozygosity analysis from laser capture microdissected prostate cancer using single nucleotide polymorphic allele (SNP) arrays and a novel bioinformatics platform dChipSNP. *Cancer Res.* **63**, 4781–4785.
8. Karan, D., Lin, M. F., Johansson, S. L., and Batra, S. K. (2003). Current status of the molecular genetics of human prostatic adenocarcinomas. *Int. J. Cancer.* **103**, 285–293.
9. Bova, G. S., Carter, B. S., Bussemakers, M. J., et al. (1993). Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. *Cancer Res.* **53**, 3869–3873.
10. Kagan, J., Stein, J., Babaian, R. J., et al. (1995). Homozygous deletions at 8p22 and 8p21 in prostate cancer implicate these regions as the sites for candidate tumor suppressor genes. *Oncogene* **11**, 2121–2126.
11. Oba, K., Matsuyama, H., Yoshihiro, S., et al. (2001). Two putative tumor suppressor genes on chromosome arm 8p may play different roles in prostate cancer. *Cancer Genet. Cytogenet.* **124**, 20–26.
12. Emmert-Buck, M. R., Vocke, C. D., Pozzatti, R. O., et al. (1995). Allelic loss on chromosome 8p12-21 in microdissected prostatic intraepithelial neoplasia. *Cancer Res.* **55**, 2959–2962.
13. He, W. W., Sciavolino, P. J., Wing, J., et al. (1997). A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. *Genomics* **43**, 69–77.
14. Voeller, H. J., Augustus, M., Madike, V., Bova, G. S., Carter, K. C., and Gelmann, E. P. (1997). Coding region of NKX3.1, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. *Cancer Res.* **57**, 4455–4459.
15. Abdulkadir, S. A., Magee, J. A., Peters, T. J., et al. (2002). Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. *Mol. Cell. Biol.* **22**, 1495–1503.
16. Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., et al. (1999). Roles for Nkx3.1 in prostate development and cancer. *Genes Dev.* **13**, 966–977.
17. Xu, J., Zheng, S. L., Komiya, A., et al. (2002). Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Nat. Genet.* **32**, 321–325.
18. Xu, J., Zheng, S. L., Komiya, A., et al. (2003). Common sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Am. J. Hum. Genet.* **72**, 208–212.
19. Miller, D. C., Zheng, S. L., Dunn, R. L., et al. (2003). Germ-line mutations of the macrophage scavenger receptor 1 gene: association with prostate cancer risk in African-American men. *Cancer Res.* **63**, 3486–3489.
20. Nupponen, N. N., Wallen, M. J., Ponciano, D., et al. (2004). Mutational analysis of susceptibility genes RNASEL/HPC1, ELAC2/HPC2, and MSR1 in sporadic prostate cancer. *Genes Chromosomes Cancer* **39**, 119–125.
21. Li, C., Berx, G., Larsson, C., et al. (1999). Distinct deleted regions on chromosome segment 16q23-24 associated with metastases in prostate cancer. *Genes Chromosomes Cancer* **24**, 175–182.
22. Elo, J. P., Harkonen, P., Kyllonen, A. P., et al. (1997). Loss of heterozygosity at 16q24.1-q24.2 is significantly associated with metastatic and aggressive behavior of prostate cancer. *Cancer Res.* **57**, 3356–3359.
23. Elo, J. P., Harkonen, P., Kyllonen, A. P., Lukkarinen, O., and Vihko, P. (1999). Three independently deleted regions at chromosome arm 16q in human prostate cancer: allelic loss at 16q24.1-q24.2 is associated with aggressive behaviour of the disease, recurrent growth, poor differentiation of the tumour and poor prognosis for the patient. *Br. J. Cancer* **79**, 156–160.
24. Suzuki, H., Komiya, A., Emi, M., et al. (1996). Three distinct commonly deleted regions of chromosome arm 16q in human primary and metastatic prostate cancers. *Genes Chromosomes Cancer* **17**, 225–233.
25. Latil, A., Cussenot, O., Fournier, G., Driouch, K., and Lidereau, R. (1997). Loss of heterozygosity at chromosome 16q in prostate adenocarcinoma: identification of three independent regions. *Cancer Res.* **57**, 1058–1062.
26. Frixen, U. H., Behrens, J., Sachs, M., et al. (1991). E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell. Biol.* **113**, 173–185.
27. Umbas, R., Schalken, J. A., Aalders, T. W., et al. (1992). Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. *Cancer Res.* **52**, 5104–5109.

28. Umbas, R., Isaacs, W. B., Bringuier, P. P., et al. (1994). Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res.* **54**, 3929–3933.
29. Richmond, P. J., Karayiannakis, A. J., Nagafuchi, A., Kaisary, A. V., and Pignatelli, M. (1997). Aberrant E-cadherin and alpha-catenin expression in prostate cancer: correlation with patient survival. *Cancer Res.* **57**, 3189–3193.
30. Rubin, M. A., Mucci, N. R., Figurski, J., Fecko, A., Pienta, K. J., and Day, M. L. (2001). E-cadherin expression in prostate cancer: a broad survey using high-density tissue microarray technology. *Hum. Pathol.* **32**, 690–697.
31. Murant, S. J., Rolley, N., Phillips, S. M., Stower, M., and Maitland, N. J. (2000). Allelic imbalance within the E-cadherin gene is an infrequent event in prostate carcinogenesis. *Genes Chromosomes Cancer* **27**, 104–109.
32. Sun, X., Frierson, H. F., Chen, C., et al. (2005). Frequent somatic mutations of the transcription factor ATBF1 in human prostate cancer. *Nat. Genet.* **37**, 407–412.
33. Hyytinen, E. R., Frierson, H. F. Jr., Boyd, J.C., Chung, L. W., and Dong, J. T. (1999). Three distinct regions of allelic loss at 13q14, 13q21-22, and 13q33 in prostate cancer. *Genes Chromosomes Cancer* **25**, 108–114.
34. Gray, I. C., Phillips, S. M., Lee, S. J., Neoptolemos, J. P., Weissenbach, J., and Spurr, N. K. (1995). Loss of the chromosomal region 10q23-25 in prostate cancer. *Cancer Res.* **55**, 4800–4803.
35. Lacombe, L., Orlow, I., Reuter, V. E., et al. (1996). Microsatellite instability and deletion analysis of chromosome 10 in human prostate cancer. *Int. J. Cancer* **69**, 110–113.
36. Li, J., Yen, C., Liaw, D., et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943–1947.
37. Steck, P. A., Pershouse, M. A., Jasser, S. A., et al. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* **15**, 356–362.
38. Li, D. M. and Sun H. (1997). TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res.* **57**, 2124–2129.
39. Kawamata, N., Park, D., Wilczynski, S., Yokota, J., and Koeffler, H. P. (1996). Point mutations of the Mxil gene are rare in prostate cancers. *Prostate* **29**, 191–193.
40. Kuczyk, M. A., Serth, J., Bokemeyer, C., et al. (1998). The MXI1 tumor suppressor gene is not mutated in primary prostate cancer. *Oncol. Rep.* **5**, 213–216.
41. Zenklusen, J. C., Thompson, J. C., Troncoso, P., Kagan, J., and Conti, C. J. (1994). Loss of heterozygosity in human primary prostate carcinomas: a possible tumor suppressor gene at 7q31.1. *Cancer Res.* **54**, 6370–6373.
42. Latil, A., Cussenot, O., Fournier, G., Baron, J. C., and Lidereau, R. (1995). Loss of heterozygosity at 7q31 is a frequent and early event in prostate cancer. *Clin. Cancer Res.* **1**, 1385–1389.
43. Takahashi, S., Shan, A. L., Ritland, S. R., et al. (1995). Frequent loss of heterozygosity at 7q31.1 in primary prostate cancer is associated with tumor aggressiveness and progression. *Cancer Res.* **55**, 4114–4119.
44. Zenklusen, J. C., Hodges, L. C., LaCava, M., Green, E. D., and Conti, C. J. (2000). Definitive functional evidence for a tumor suppressor gene on human chromosome 7q31.1 neighboring the Fra7G site. *Oncogene* **19**, 1729–1733.
45. Alcaraz, A., Takahashi, S., Brown, J. A., et al. (1994). Aneuploidy and aneusomy of chromosome 7 detected by fluorescence in situ hybridization are markers of poor prognosis in prostate cancer. *Cancer Res.* **54**, 3998–4002.
46. Visakorpi, T., Hyytinen, E., Kallioniemi, A., Isola, J., and Kallioniemi, O. P. (1994). Sensitive detection of chromosome copy number aberrations in prostate cancer by fluorescence in situ hybridization. *Am. J. Pathol.* **145**, 624–630.
47. Bandyk, M. G., Zhao, L., Troncoso, P., et al. (1994). Trisomy 7: a potential cytogenetic marker of human prostate cancer progression. *Genes Chromosomes Cancer* **9**, 19–27.
48. Visakorpi, T., Kallioniemi, A. H., Syvanen, A. C., et al. (1995). Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. *Cancer Res.* **55**, 342–347.
49. Wang, R. Y., Troncoso, P., Palmer, J. L., El-Naggar, A. K., and Liang, J. C. (1996). Trisomy 7 by dual-color fluorescence in situ hybridization: a potential biological marker for prostate cancer progression. *Clin. Cancer Res.* **2**, 1553–1558.
50. Cui, J., Deubler, D. A., Rohr, L. R., et al. (1998). Chromosome 7 abnormalities in prostate cancer detected by dual-color fluorescence in situ hybridization. *Cancer Genet. Cytogenet.* **107**, 51–60.
51. Alers, J. C., Krijtenburg, P. J., Vis, A. N., et al. (2001). Molecular cytogenetic analysis of prostatic adenocarcinomas from screening studies: early cancers may contain aggressive genetic features. *Am. J. Pathol.* **158**, 399–406.
52. Nupponen, N. N., Kakkola, L., Koivisto, P., and Visakorpi, T. (1998). Genetic alterations in hormone-refractory recurrent prostate carcinomas. *Am. J. Pathol.* **153**, 141–148.
53. Galbiati, F., Volonte, D., Engelman, J. A., et al. (1998). Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO J.* **17**, 6633–6648.
54. Zhang, W., Razani, B., Altschuler, Y., et al. (2000). Caveolin-1 inhibits epidermal growth factor-stimulated lamellipod extension and cell migration in metastatic mammary adenocarcinoma cells (MTLn3). Transformation suppressor effects of adenovirus-mediated gene delivery of caveolin-1. *J. Biol. Chem.* **275**, 20717–20725.
55. Bender, F. C., Reymond, M. A., Bron, C., and Quest, A. F. (2000). Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. *Cancer Res.* **60**, 5870–5878.

56. Wiechen, K., Diatchenko, L., Agoulnik, A., et al. (2001). Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. *Am. J. Pathol.* **159**, 1635–1643.
57. Wiechen, K., Sers, C., Agoulnik, A., et al. (2001). Down-regulation of caveolin-1, a candidate tumor suppressor gene, in sarcomas. *Am. J. Pathol.* **158**, 833–839.
58. Yang, G., Truong, L. D., Wheeler, T. M., and Thompson, T. C. (1999). Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. *Cancer Res.* **59**, 5719–5723.
59. Nasu, Y., Timme, T. L., Yang, G., et al. (1998). Suppression of caveolin expression induces androgen sensitivity in metastatic androgen-insensitive mouse prostate cancer cells. *Nat. Med.* **4**, 1062–1064.
60. Tahir, S. A., Yang, G., Ebara, S., et al. (2001). Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. *Cancer Res.* **61**, 3882–3885.
61. Li, L., Yang, G., Ebara, S., et al. (2001). Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells. *Cancer Res.* **61**, 4386–4392.
62. Cooney, K. A., Wetzel, J. C., Consolino, C. M., and Wojno, K. J. (1996). Identification and characterization of proximal 6q deletions in prostate cancer. *Cancer Res.* **56**, 4150–4153.
63. Srikantan, V., Sesterhenn, I. A., Davis, L., et al. (1999). Allelic loss on chromosome 6Q in primary prostate cancer. *Int. J. Cancer.* **84**, 331–335.
64. Hyytinen, E. R., Saadut, R., Chen, C., et al. (2002). Defining the region(s) of deletion at 6q16-q22 in human prostate cancer. *Genes Chromosomes Cancer* **34**, 306–312.
65. Konishi, N., Nakamura, M., Kishi, M., et al. (2003). Genetic mapping of allelic loss on chromosome 6q within heterogeneous prostate carcinoma. *Cancer Sci.* **94**, 764–768.
66. Ueda, T., Komiya, A., Emi, M., et al. (1997). Allelic losses on 18q21 are associated with progression and metastasis in human prostate cancer. *Genes Chromosomes Cancer* **20**, 140–147.
67. Padalecki, S. S., Troyer, D. A., Hansen, M. F., et al. (2000). Identification of two distinct regions of allelic imbalance on chromosome 18Q in metastatic prostate cancer. *Int. J. Cancer* **85**, 654–658.
68. Latil, A., Pesche, S., Valeri, A., Fournier, G., Cussenot, O., and Lidereau, R. (1999). Expression and mutational analysis of the MADR2/Smad2 gene in human prostate cancer. *Prostate* **40**, 2225–2231.
69. Yin, Z., Babaian, R. J., Troncoso, P., et al. (2001). Limiting the location of putative human prostate cancer tumor suppressor genes on chromosome 18q. *Oncogene* **20**, 2273–2280.
70. Nupponen, N. N. and Visakorpi, T. (2000). Molecular cytogenetics of prostate cancer. *Microsc. Res. Tech.* **51**, 456–463.
71. Cher, M. L., Bova, G. S., Moore, D. H., et al. (1996). Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Res.* **56**, 3091–3102.
72. Alers, J. C., Rochat, J., Krijtenburg, P. J., et al. (2000). Identification of genetic markers for prostatic cancer progression. *Lab. Invest.* **80**, 931–942.
73. van Dekken, H., Alers, J. C., Damen, I. A., et al. (2003). Genetic evaluation of localized prostate cancer in a cohort of forty patients: gain of distal 8q discriminates between progressors and nonprogressors. *Lab. Invest.* **83**, 789–796.
74. Ellwood-Yen, K., Graeber, T. G., Wongvipat, J., et al. (2003). Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer Cell* **4**, 223–238.
75. Jenkins, R. B., Qian, J., Lieber, M. M., and Bostwick, D. G. (1997). Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in situ hybridization. *Cancer Res.* **57**, 524–531.
76. Nupponen, N. N., Porkka, .K, Kakkola, L., et al. (1999). Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. *Am. J. Pathol.* **154**, 1777–1783.
77. Sato, K., Qian, J., Slezak, J. M., et al. (1999) Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma. *J. Natl. Cancer. Inst.* **91**, 1574–1580.
78. Savinainen, K. J., Linja, M. J., Saramaki, O. R., et al. (2004). Expression and copy number analysis of TRPS1, EIF3S3 and MYC genes in breast and prostate cancer. *Br. J. Cancer* **90**, 1041–1046.
79. Porkka, K. P., Tammela, T. L., Vessella, R. L., and Visakorpi, T. (2004). RAD21 and KIAA0196 at 8q24 are amplified and overexpressed in prostate cancer. *Genes Chromosomes Cancer* **39**, 1–10.
80. Saramaki, O., Willi, N., Bratt, O., et al. (2001). Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. *Am. J.Pathol.* **159**, 2089–2094.
81. Asano, .K, Vornlocher, H. P., Richter-Cook, N. J., Merrick, W. C., Hinnebusch, A. G., and Hershey, J. W. (1997). Structure of cDNAs encoding human eukaryotic initiation factor 3 subunits. Possible roles in RNA binding and macromolecular assembly. *J. Biol. Chem.* **272**, 27,042–27,052.
82. Nasmyth, K., Peters, J. M., and Uhlmann, F. (2000). Splitting the chromosome: cutting the ties that bind sister chromatids. *Science* **288**, 1379–1385.
83. Hirano, T. (2000). Chromosome cohesion, condensation, and separation. *Annu. Rev. Biochem.* **69**, 115–144.
84. Chen, F., Kamradt, M., Mulcahy, M., et al. (2002). Caspase proteolysis of the cohesin component RAD21 promotes apoptosis. *J. Biol. Chem.* **277**, 16,775–16,781.

85. Pati, D., Zhang, N., and Plon, S. E. (2002). Linking sister chromatid cohesion and apoptosis: role of Rad21. *Mol. Cell. Biol.* **22**, 8267–8277.
86. Reiter, R. E., Gu, Z., Watabe, T., et al. (1998). Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc. Natl. Acad. Sci. USA* **95**, 1735–1740.
87. Chang, G. T., Steenbeek, M., Schippers, E., et al. (2000). Characterization of a zinc-finger protein and its association with apoptosis in prostate cancer cells. *J. Natl. Cancer. Inst.* **92**, 1414–1421.
88. Gu, Z., Thomas, G., Yamashiro, J., et al. (2000). Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene* **19**, 1288–1296.
89. Ross, S., Spencer, S. D., Holcomb, I., et al. (2002). Prostate stem cell antigen as therapy target: tissue expression and in vivo efficacy of an immunoconjugate. *Cancer Res.* **62**, 2546–2553.
90. Chang, G. T., Blok, L. J., Steenbeek, M., et al. (1997). Differentially expressed genes in androgen-dependent and -independent prostate carcinomas. *Cancer Res.* **57**, 4075–4081.
91. Porkka, K., Saramaki, O., Tanner, M., and Visakorpi, T. (2002). Amplification and overexpression of Elongin C gene discovered in prostate cancer by cDNA microarrays. *Lab. Invest.* **82**, 629–637.
92. Wang, R., Xu, J., Saramaki, O., et al. (2004). PrLZ, a novel prostate-specific and androgen-responsive gene of the TPD52 family, amplified in chromosome 8q21.1 and overexpressed in human prostate cancer. *Cancer Res.* **64**, 1589–1594.
93. Rubin, M. A., Varambally, S., Beroukhim, R., et al. (2004). Overexpression, amplification, and androgen regulation of TPD52 in prostate cancer. *Cancer Res.* **64**, 3814–3822.
94. Aso, T., Lane, W. S., Conaway, J. W., and Conaway, R. C. (1995). Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II. *Science* **269**, 1439–1443.
95. Duan, D. R., Pause, A., Burgess, W. H., et al. (1995). Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science* **269**, 1402–1406.
96. Kibel, A., Iliopoulos, O., DeCaprio, J. A., and Kaelin, W. G., Jr. (1995). Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. *Science* **269**, 1444–1446.
97. Stebbins, C. E., Kaelin, W. G., Jr, and Pavletich, N. P. (1999). Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science* **284**, 455–461.
98. Kaelin, W.G., Jr. (2002). Molecular basis of the VHL hereditary cancer syndrome. *Nat. Rev. Cancer* **2**, 673–682.
99. Zhong, H., Agani, F., Baccala, A. A., et al. (1998) Increased expression of hypoxia inducible factor-1alpha in rat and human prostate cancer. *Cancer Res.* **58**, 5280–5284.
100. Kamura, T., Sato, S., Haque, D., et al. (1998). The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev.* **12**, 3872–3881.
101. Pisters, L. L., Troncoso, P., Zhau, H. E., Li, W., von Eschenbach, A. C., and Chung, L. W. (1995). c-met protooncogene expression in benign and malignant human prostate tissues. *J. Urol.* **154**, 293–298.
102. Humphrey, P. A., Zhu, X., Zarnegar, R., et al. (1995). Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. *Am. J. Pathol.* **147**, 386–396.
103. Watanabe, M., Fukutome, K., Kato, H., et al. (1999). Progression-linked overexpression of c-Met in prostatic intraepithelial neoplasia and latent as well as clinical prostate cancers. *Cancer Lett.* **141**, 173–178.
104. Knudsen, B. S., Gmyrek, G. A., Inra, J., et al. (2002). High expression of the Met receptor in prostate cancer metastasis to bone. *Urology* **60**, 1113–1117.
105. Varambally, S., Dhanasekaran, S. M., Zhou, M., et al. (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**, 624–629.
106. Rhodes, D. R., Sanda, M. G., Otte, A. P., Chinnaiyan, A. M., and Rubin, M. A. (2003). Multiplex biomarker approach for determining risk of prostate-specific antigen-defined recurrence of prostate cancer. *J. Natl. Cancer Inst.* **95**, 661–668.
107. Foster, C. S., Falconer, A., Dodson, A. R., et al. (2004). Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. *Oncogene* **23**, 5871–5879.
108. Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003). EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J.* **22**, 5323–5335.
109. Nupponen, N. N., Isola, J., and Visakorpi, T. (2000). Mapping the amplification of EIF3S3 in breast and prostate cancer. *Genes Chromosomes Cancer* **28**, 203–210.
110. Clark, J., Edwards, S., Feber, A., et al. (2003). Genome-wide screening for complete genetic loss in prostate cancer by comparative hybridization onto cDNA microarrays. *Oncogene* **22**, 1247–1452.
111. Wolf, M., Mousses, S., Hautaniemi, S., et al. (2004). High-resolution analysis of gene copy number alterations in human prostate cancer using CGH on cDNA microarrays: impact of copy number on gene expression. *Neoplasia* **6**, 240–247.
112. van Dekken, H., Paris, P. L., Albertson, D. G., et al. (2004). Evaluation of genetic patterns in different tumor areas of intermediate-grade prostatic adenocarcinomas by high-resolution genomic array analysis. *Genes Chromosomes Cancer* **39**, 249–256.

113. Paris, P. L., Albertson, D. G., Alers, J. C., et al. (2003). High-resolution analysis of paraffin-embedded and formalin-fixed prostate tumors using comparative genomic hybridization to genomic microarrays. *Am. J. Pathol.* **162**, 763–770.
114. Paris, P. L., Andaya, A., Fridlyand, J., et al. (2004). Whole genome scanning identifies genotypes associated with recurrence and metastasis in prostate tumors. *Hum. Mol. Genet.***13**, 1303–1313.
115. Zhao, H., Kim, Y., Wang, P., et al. (2005). Genome-wide characterization of gene expression variations and DNA copy number changes in prostate cancer cell lines. *Prostate* **63**, 187–197.
116. Bookstein, R., MacGrogan, D., Hilsenbeck, S. G., Sharkey, F., and Allred, D. C. (1993). p53 is mutated in a subset of advanced-stage prostate cancers. *Cancer Res.* **53**, 3369–3373.
117. Visakorpi, T., Kallioniemi, O. P., Heikkinen, A., Koivula, T., and Isola, J. (1992). Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. *J. Natl. Cancer Inst.* **84**, 883–887.
118. Navone, N. M., Troncoso, P., Pisters, L. L., et al. (1993). p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. *J. Natl. Cancer Inst.* **85**, 1657–1669.
119. Feilotter, H. E., Nagai, M. A., Boag, A. H., Eng, C., and Mulligan, L. M. (1998). Analysis of PTEN and the 10q23 region in primary prostate carcinomas. *Oncogene* **16**, 1743–1748.
120. Pesche, S., Latil, A., Muzeau, F., et al. (1998). PTEN/MMAC1/TEP1 involvement in primary prostate cancers. *Oncogene* **16**, 2879–2883.
121. Dong, J. T., Sipe, T. W., Hyytinen, E. R., et al. (1998). PTEN/MMAC1 is infrequently mutated in pT2 and pT3 carcinomas of the prostate. *Oncogene* **17**, 1979–1982.
122. Vlietstra, R. J., van Alewijk, D. C., Hermans, K. G., van Steenbrugge, G. J., and Trapman, J. (1998). Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. *Cancer Res.* **58**, 2720–2723.
123. Suzuki, H., Freije, D., Nusskern, D. R., et al. (1998). Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res.* **58**, 204–209.
124. Fernandez, M. and Eng, C. (2002). The expanding role of PTEN in neoplasia: a molecule for all seasons? *Clin. Cancer Res.* **8**, 1695–1698.
125. Kwabi-Addo, B., Giri, D., Schmidt, K., et al. (2001). Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression. *Proc. Natl. Acad. Sci. USA* **98**, 11,563–11,568.
126. Kim, M. J., Cardiff, R. D., Desai, N., et al. (2002). Cooperativity of Nkx3.1 and Pten loss of function in a mouse model of prostate carcinogenesis. *Proc. Natl. Acad. Sci. USA* **99**, 2884–2889.
127. Ayala, G., Thompson, T., Yang, G., et al. (2004). High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. *Clin. Cancer Res.* **10**, 6572–6578.
128. Neshat, M. S., Mellinghoff, I. K., Tran, C., et al. (2001). Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc. Natl. Acad. Sci. USA* **98**, 10,314–10,319.
129. Culig, Z., Klocker, H., Bartsch, G., and Hobisch, A. (2001). Androgen receptor mutations in carcinoma of the prostate: significance for endocrine therapy. *Am. J. Pharmacogenomics* **1**, 241–249.
130. Wallen, M. J., Linja, M., Kaartinen, K., Schleutker, J., and Visakorpi, T. (1999). Androgen receptor gene mutations in hormone-refractory prostate cancer. *J. Pathol.* **189**, 559–563.
131. Taplin, M. E., Bubley, G. J., Shuster, T. D., et al. (1995). Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.* **332**, 1393–1398.
132. Haapala, K., Hyytinen, E. R., Roiha, M., et al. (2001). Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide. *Lab. Invest.* **81**, 1647–1651.
133. Hara, T., Miyazaki, J., Araki, H., et al. (2003). Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. *Cancer Res.* **63**, 149–153.
134. Visakorpi, T., Hyytinen, E., Koivisto, P., et al. (1995). In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat.Genet.* **9**, 401–406.
135. Elo, J. P. and Visakorpi, T. (2001). Molecular genetics of prostate cancer. *Ann. Med.* **33**, 130–141.
136. Palmberg, C., Koivisto, P., Kakkola, L., Tammela, T. L. J., Kallioniemi, O. P., and Visakorpi, T. (2000). Androgen receptor gene amplification at the time of primary progression predicts response to combined androgen blockade as a second-line therapy in advanced prostate cancer. *J. Urology* **164**, 1992–1995.
137. Linja, M. J., Savinainen, K. J., Saramäki, O. R., Tammela, T. L. J., Vessella, R. L., and Visakorpi, T. (2001). Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res.* **61**, 3550–3555.
138. Latil, A., Bieche, I., Vidaud, D., et al. (2001). Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. *Cancer Res.* **61**, 1919–1926.
139. Chen, C. D., Welsbie, D. S., Tran, C., et al. (2004). Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* **10**, 33–39.
140. Nakayama, M., Bennett, C. J., Hicks, J. L., et al. (2003). Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am. J. Pathol.* **163**, 923–933.

141. Lee, W. H., Morton, R. A., Epstein, J. I., et al. (1994). Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc Natl. Acad. Sci. USA* **91**, 11,733–11,737.
142. Goessl, C., Krause, H., Muller, M., et al. (2000). Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res.* **60**, 5941–5945.
143. Narla, G., Heath, K. E., Reeves, H. L., et al. (2001). KLF6, a candidate tumor suppressor gene mutated in prostate cancer. *Science* **294**, 2563–2566.
144. Chen, C., Hyytinen, E. R., Sun, X., et al. (2003). Deletion, mutation, and loss of expression of KLF6 in human prostate cancer. *Am. J. Pathol.* **162**, 1349–1354.
145. Muhlbauer, K. R., Grone, H. J., Ernst, T., et al. (2003). Analysis of human prostate cancers and cell lines for mutations in the TP53 and KLF6 tumour suppressor genes. *Br. J. Cancer* **89**, 687–990.
146. Huusko, P., Ponciano-Jackson, D., Wolf, M., et al. (2004). Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer. *Nat. Genet.* **36**, 979–983.
147. Kullander, K. and Klein, R. (2002). Mechanisms and functions of Eph and ephrin signalling. *Nat. Rev. Mol. Cell. Biol.* **3**, 475–486.
148. Druker, B. J., Talpaz, M., Resta, D. J., et al. (2001). Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N. Engl. J. Med.* **344**, 1031–1037.
149. Slamon, D. J., Leyland-Jones, B., Shak, S., et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**, 783–792.
150. Lynch, T. J., Bell, D. W., Sordella, R., et al. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* **350**, 2129–2139.
151. Paez, J. G., Janne, P. A., Lee, J. C., et al. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497–1500.
152. Bykov, V. J., Issaeva, N., Shilov, A., et al. (2002). Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat. Med.* **8**, 282–288.
153. Neshat, M. S., Mellinghoff, I. K., Tran, C.,et al. (2001). Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc. Natl. Acad. Sci. USA* **98**, 10,314–10,319.
154. Podsypanina, K., Lee, R. T., Politis, C., et al. (2001). An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/– mice. *Proc. Natl. Acad. Sci. USA* **98**, 10,320–10,325.
155. Futreal, P. A., Coin, L., Marshall, M., et al. (2004). A census of human cancer genes. *Nat. Rev. Cancer* **4**, 177–183.
156. Asatiani, E., Huang, W. X., Wang, A., et al. (2005). Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. *Cancer Res.* **65**, 1164–1173.
157. Saramäki, O. R., Tammela, T. L., Martikainen, P. M., et al. (2006). The gene for polycomb group protein enhancer of zesle homolog 2 (EZH2) is amplified in late stage prostate cancer. *Genes Chromosomes Cancer* **45**, 639–645.
158. Saramäki, O. R., Porkka, K. P., Vessella, R. L., et al. (2006). Genetic aberrations in prostate cancer by microarray analysis. *Int. J. Cancer* **119**, 1322–1329.

16

Linkage Studies of Prostate Cancer Families to Identify Susceptibility Genes

William B. Isaacs and Jianfeng Xu

Summary

Identification of cancer susceptibility genes through the study of site-specific cancer families has resulted in tremendous insight into the molecular mechanisms underlying carcinogenesis in organs such as the breast, colon, and kidney. Although progress in this area for prostate cancer has been slow, extensive evidence exists to support an important genetic component of prostate cancer susceptibility. This chapter will review the progress and difficulties presented in the search for prostate cancer susceptibility alleles in prostate cancer families. Two genes identified in family studies, *RNASEL* and *MSR1*, although showing relatively weak associations with prostate cancer overall, implicate genetic variation in the inflammatory response as a possibly critical pathway in modifying risk for this common cancer. As with many other cancers, inherited defects in DNA repair pathways seem to result in some familial clustering of prostate cancer. Studies underway and planned that emphasize potentially more homogeneous subsets of prostate cancer, and that take advantage of methodological and technological advances to detect weaker genetic influences should prove valuable in accelerating the rate of progress in this area.

Key Words: Familial; genetics; inflammation; linkage; susceptibility gene.

1. INTRODUCTION—CANCER SUSCEPTIBILITY GENES, EARLY DETECTION AND LINKAGE STUDIES: IS THERE A PROSTATE "GATEKEEPER"?

In 2005 in the United States alone, an estimated 232,000 new prostate cancer cases will be diagnosed, accounting for more than 30% of all cancers affecting men, although, fortunately, only a fraction of these men (~1 in 8; or ~30,000 annually) will die from this disease *(1)*. By 2025, the number of people older than 65 years of age worldwide will have risen from 390 million in 1997 to 800 million, substantially increasing the number of men at increased risk for this disease *(2)*.

Despite these figures, the understanding of the molecular genetics of prostate cancer is still in an embryonic stage. Although mutations have been observed in a variety of tumor suppressor genes and oncogenes in prostate cancer at varying frequencies (*see* Chapter 15), a site-specific model for prostate cancer has not emerged. In the parlance of Kinzler and Vogelstein *(3)*, the prostate "gatekeeper," or gene(s) that controls the central growth regulatory pathway(s) in the prostate has not been identified. Indeed, little is known regarding the genes that control prostate cancer susceptibility in general. Linkage analysis and associated positional cloning efforts have resulted in the identification of both gatekeeper and susceptibility genes for other common cancers, including colorectal (*APC*, DNA mismatch repair genes), renal (*VHL*), and breast (*BRCA1* and *BRCA2*) cancer.

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

In virtually every instance in which a major cancer susceptibility gene has been identified, the critical studies that led to the discovery have been linkage studies of cancer families, providing both concrete evidence of such genes and critical positional information regarding the location of the gene *(4–12)*. In fact, most tumor suppressor genes characterized to date have been identified as a result of linkage studies in families that provided the chromosomal location from which positional cloning methods were initiated. The study of inherited cancers using linkage analysis has been the most efficient and the only reliable way to identify and characterize the genes responsible for organ-specific carcinogenesis for both inherited and sporadic forms of the disease. This point emphasizes the reason to study inherited prostate cancer even if this form accounts for less than 10% of the total cases diagnosed.

Of the three most common cancers in the United States, prostate cancer is the only one for which a major susceptibility gene has not been identified. Does this mean that prostate cancer development does not involve such genes? This is unlikely; a more likely explanation would be that extensive prostate cancer heterogeneity makes the identification of these genes particularly difficult (*see* Subheading 4.).

The potential impact of the discoveries of cancer susceptibility genes in terms of cancer prevention, early detection, and increased likelihood of cure is great *(13)*. In the case of prostate cancer, early localized disease is highly curable *(14)*. Identification of men at high risk is clearly a critical aspect to efficient and cost-effective screening strategies. In high-risk individuals, prevention of prostate cancer may be possible if the etiological factors underlying the disease were understood. Intriguingly, as better methods are developed to ablate prostate tissue, including numerous nonsurgical techniques (see refs. *15* and *16*), prophylactic prostatectomy in high-risk individuals may, in fact, provide a viable treatment option because these procedures are associated with increasingly lower morbidity. Indeed, as no requisite physiological function has been defined for the gland, its removal, *per se*, is associated with no known deleterious effects *(17)*.

2. PROSTATE CANCER AS A FAMILIAL CANCER—EVIDENCE FOR A GENETIC CONTRIBUTION

The clustering of prostate cancer in families was noted by researchers as early as the mid 1950s *(18)*. Over 40 years ago, Woolf *(19)* reported that deaths caused by prostate cancer were three times higher among the fathers and brothers of men dying from prostate cancer than among deceased relatives of men dying from other causes. Since then, numerous studies have consistently documented the increased risk of prostate cancer associated with family history. Johns and Houlston *(20)* recently reviewed more than 22 reported studies and summarized these data by meta-analysis, and concluded that having a first-degree relative increases risk by more than twofold (odds ratio [OR], 2.5; 95% confidence interval [CI], 2.2–2.8). This risk increased to even higher levels if more than one first-degree relative was affected (Fig. 1).

Familial clustering can be caused by both genetic components and common environmental risk factors shared within a family. An estimation of the relative contributions of these factors can be determined through twin studies, familiality analyses, and complex segregation analyses. Several twin studies of prostate cancer reported higher concordance rates in monozygotic twins than among dizygotic twins, implicating a genetic contribution for familial aggregation of prostate cancer *(21–23)*. Importantly, Lichtenstein et al. *(23)*, in an analysis of 44,788 pairs of twins from Scandinavian twin registries concluded that 42% (95% CI, 2950%) of prostate cancer risk may be accounted for by heritable factors. Cannon-Albright et al. *(24)* studied prostate cancer in the Utah Population Database and established elevated risk in close and distant relatives, also consistent with a genetic component.

By fitting patterns of familial aggregation of a disease in the general population with several alternative modes of inheritance (e.g., a major gene model, an environmental model, and/or a polygene model), segregation analysis can provide inferences regarding the specific model that best

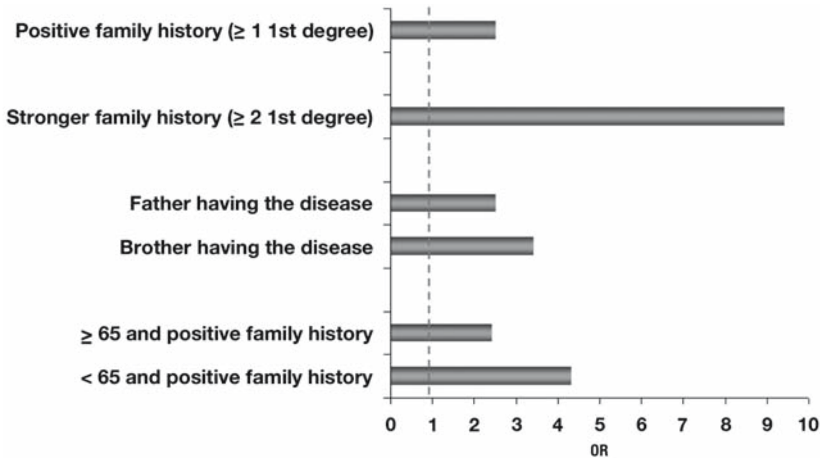


Fig. 1. Estimates of relative risk for prostate cancer from a meta-analysis *(20)*.

describes the transmission of the disease in families. Multiple segregation analyses have been performed for prostate cancer and each has indicated that familial clustering was consistent with the action of one or more high-risk genes (*25–27*, reviewed by Schaid, *ref. 28*), although some evidence for recessive or X-linked genes has also been reported *(29)*. Carter et al. *(25)* suggested that familial aggregation of prostate cancer could be best explained by autosomal dominant inheritance of a rare (disease gene frequency q = 0.003) high-risk allele leading to an early onset of prostate cancer. The estimated cumulative risk of prostate cancer by age 85 years was 88% for carriers vs 5% for noncarriers. This inherited form of prostate cancer was estimated to account for a significant proportion of early onset disease, and, overall, to be responsible for approx 9% of all prostate cancer occurrences. Grönberg et al. *(26)* also indicated that the observed familial aggregation of prostate cancer is best explained by a high-risk allele inherited in a dominant mode, although this model suggests a gene(s) with a higher population frequency (1%) and a moderate lifetime penetrance (63%). Schaid et al. *(27)* reported that although no single-gene model of inheritance clearly explained the familial clustering observed in men undergoing radical prostatectomy for clinically localized disease at the Mayo Clinic, the best-fitting model was also that of a rare autosomal dominant susceptibility gene, with the best fit observed in probands diagnosed at younger than 60 years of age. The frequency of the rare allele was estimated to be 0.006 in the population and the penetrances were 89% by age 85 years for the carriers and 3% for the noncarriers.

The most recent segregation analysis of prostate cancer studied 1476 men diagnosed at age younger than 70 years and ascertained through population registries in Melbourne, Sydney, and Perth, Australia *(29)*. Interestingly, the best fitting models included a dominantly inherited increased risk that was greater, in multiplicative terms, at younger ages; and a recessively inherited or X-linked increased risk that was greater, in multiplicative terms, at older ages. Equally interestingly, all two-locus models gave better fits than did single-locus models, suggesting that more than one major gene is involved.

3. CANDIDATE PROSTATE CANCER SUSCEPTIBILITY GENES IDENTIFIED THROUGH LINKAGE STUDIES

As described under Subheading 2, overwhelming evidence now exists to support a strong genetic influence in the etiology of at least some fraction of prostate cancer cases. However, the definition and characterization of this genetic influence is far more nebulous. Several major approaches have

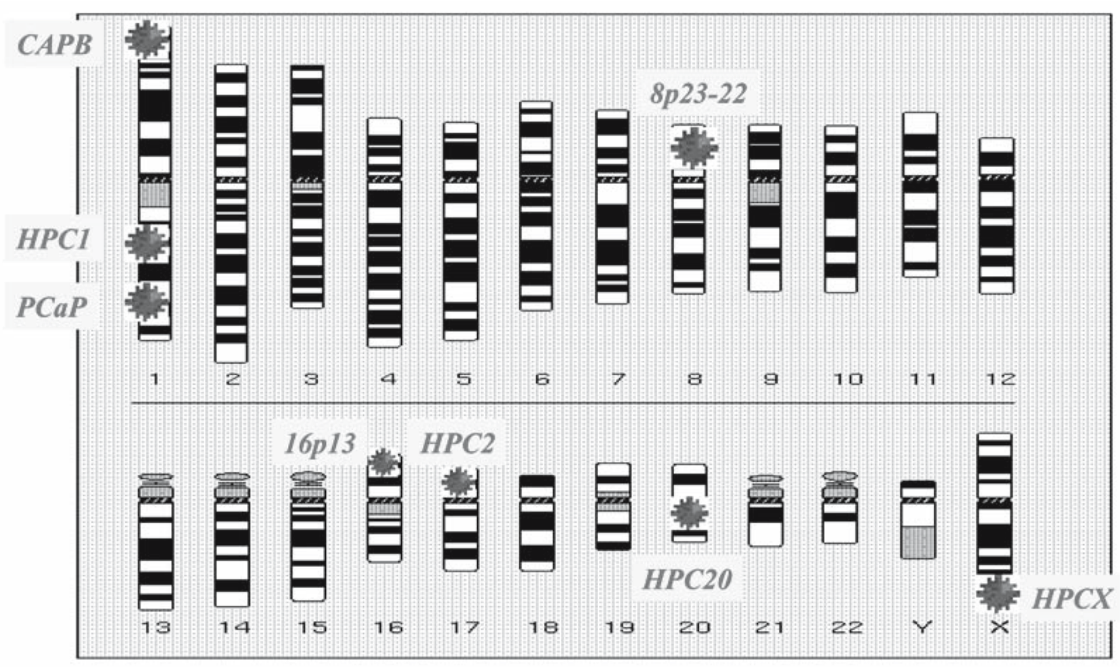


Fig. 2. Regions of implicated to harbor prostate cancer susceptibility genes from linkage studies of prostate cancer families. (Adapted from Simard et al. 2003).

been taken to define the genes involved in inherited susceptibility for prostate cancer. Based on the foundation laid by segregation analyses, many groups worldwide have pursued linkage studies of families with multiple men affected with prostate cancer to map genes affecting prostate cancer susceptibility. Systematic searches using family-based linkage scans with genetic markers genotyped at approx 10-cM intervals along each chromosome can provide powerful statistical evidence of the existence and location of disease predisposing genes. Hereditary prostate cancer 1 (*HPC1*) on chromosome 1 was the first locus implicated in prostate cancer using this approach *(30)*, and numerous other loci have now been suggested to contain prostate cancer susceptibility genes (Fig. 2). *ELAC2* at *17q (31)*, *RNASEL* at *1q24-25* (*HPC1*) *(32)*, and *MSR1* at *8p22 (33)* are genes located in regions implicated by linkage analysis, and which contain inactivating mutations in affected members of at least one or more prostate cancer families.

3.1. ELAC2/HPC2

A genome-wide scan of extended high-risk pedigrees from Utah provided evidence for linkage to a locus on chromosome arm *17p (31)*. Positional cloning and mutation screening within the defined region allowed identification of a gene, *ELAC2*, harboring mutations that segregate with prostate cancer in two pedigrees; one mutation is a frame shift and the other a nonconservative missense change. In addition, two common missense variants, *Ala541Thr* and *Ser217Leu*, in the gene were reported to be associated with the occurrence of prostate cancer *(31,34)*. Follow-up studies have been carried out by multiple independent groups. Although some positive associations of these two mis-

sense variants have been found, several null findings have been reported as well *(35–42)*. A meta-analysis of six independent studies estimated that risk genotypes in *ELAC2* may cause 2% of prostate cancer in the general population *(43)*.

3.2. RNASEL

The *RNASEL* gene encodes the 2'-5'-oligoadenylate-dependant RNase L, a mediator of the interferon-induced RNA degradation pathway mediating defense against viral infection. Its tumor suppressor potential has been postulated because the introduction of truncated RNase L protein in murine cells abolished the antiproliferative effect of interferon *(44)*. The *RNASEL* gene locus was implicated in prostate cancer susceptibility as a result of the first genome-wide linkage scan, which observed evidence of linkage at *1q25* in 91 hereditary prostate cancer pedigrees from North America and Sweden *(30)*, and the subsequent identification of a nonsense mutation *E265X* in four affected brothers in a European-American family, and a second deleterious variation, *M1I*, in affected brothers in a family of African descent *(32)*. In a follow-up study by Finnish investigators *(45)* of 116 index cases with hereditary prostate cancer, the truncating mutation, *E262X*, was found in five (4.3%) cases; four of these five index cases came from families with at least three men with prostate cancer. A study by Chen et al. *(46)* of 95 men with prostate cancer from 75 families found the *E262X* mutation in one family, with two of three affected brothers being heterozygous carriers. In a study of Ashkenazi Jews, a novel frameshift mutation (471delAAAG) in *RNASEL* was detected, which leads to premature truncation of the protein, and was estimated to be as frequent as 4% in this population *(47)*. A subsequent study by Kotar et al. *(48)* confirmed the presence of this founder mutation in an Ashkenazi Jewish population in Montreal, but found no significant association with prostate cancer risk.

Using families from the United States, Casey et al. *(49)* evaluated a common missense variant of *RNASEL*, *Arg462Gln,* which codes for a protein with decreased enzymatic activity. From 423 cases and 454 sibling controls, it was estimated that heterozygous carriers of the Gln-containing allele had an OR of 1.46, and homozygous carriers had an OR of 2.12, giving a population attributable fraction for this variant of 13%. A study conducted at the Mayo Clinic found an opposite trend, however, with ORs of 0.83 for heterozygotes and 0.54 for homozygotes *(50)*. Furthermore, a study from Japan based on 101 familial prostate cancers and 105 controls did not find the *Arg462Gln* variant among any cases, but found that 7.6% of the controls carried it *(51)*. Nonetheless, this study did find an increased prostate cancer risk for a different *RNASEL* variant, *Asp541Glu*, with an OR of 3.07. Subsequent studies in both the German *(52)* and Swedish *(53)* family populations failed to find significant evidence for *RNASEL* as a prostate cancer susceptibility gene. Thus, although a number of studies provide support, both functional and epidemiological, that *RNASEL* plays a role in hereditary prostate cancer, evidence to the contrary has also been presented.

3.3. MSR1

The *MSR1* gene is located on 8p22 and encodes a homotrimeric class A scavenger receptor. This receptor is macrophage specific and is able to bind a wide range of polyanionic ligands, which includes gram-negative and gram-positive bacteria, oxidized low-density lipoprotein, and certain polynucleotides. As part of a systematic evaluation of genes in a region of linkage on 8p, Xu et al. *(33)* found multiple carriers of nonsense and missense mutations in *MSR1* in a collection of prostate cancer families. To date, seven studies have been published regarding the role of *MSR1* mutations and variants with prostate cancer risk *(33,54–60)*. These studies investigated the *R293X*-null mutation and prostate cancer risk, as well the association of the rare mutations, S41T and *D174Y*, which were

only detected in African-American men *(33,55,60)*, and five common sequence variants: *PRO3, INDEL1, IVS5-59, P275A*, and *INDEL7*. As with *ELAC2* and *RNASEL*, however, the results were inconsistent across different reports, and it has been difficult to draw definitive conclusions from the individual studies. To address this issue, Sun et al. performed a meta-analysis that included all of the published studies on *MSR1* up to September 2005 *(61)*. This analysis indicated that several variants were significantly associated with sporadic prostate cancer risk, including *R293X* in white men (fixed effect OR, 1.35; $p = 0.04$; and random effect OR, 1.34; $p = 0.09$) and *D174Y* in black men (fixed effect OR, 2.39; $p = 0.01$; and random effect OR, 2.41; $p = 0.04$). When the initial study was excluded from the meta-analysis, the associations with prostate cancer risk were not significant in the remaining replication studies. However, the frequency of the *D174Y* mutation was consistently higher among cases in all three studies that examined African-American men. Overall, this meta-analysis suggests the *MSR1* gene does not independently confer a major risk to prostate cancer but may confer a moderate risk to prostate cancer, especially in African-American men.

4. PROSTATE CANCER LINKAGE STUDIES AND GENE IDENTIFICATION—WHY SO DIFFICULT?

As can be seen by the preceding discussion, although various candidate genes have been identified in genomics regions using linkage analysis, a gene that reproducibly accounts for more than a small percentage of familial prostate cancer cases has not been found. Indeed, the number of linkages reported by individual groups and the difficulty in reproducing linkage results from study to study has resulted in a less than ideal platform from which to pursue additional gene identification *(91)*.

Why is this so? There are a number of possibilities to consider. In general, prostate cancer is a very heterogeneous disease, with a complex etiology involving both genetic influences and strong, well-documented environmental influences, and inevitably interactions between the two. In this regard, it is interesting to note that, of the three genes implicated as prostate cancer susceptibility genes from the family studies described under Subheading 3, two (*MSR1* and *RNASEL)* play important roles in the innate immune system protecting against infectious agents. It is possible that, in addition to assessing mutational status, pathogen exposure information will be needed to understand the role for genetic variation in these genes in modifying prostate cancer susceptibility.

Another factor complicating the identification of reproducible genetic risk factors for prostate cancer is the prevalence of this disease. Current estimates from the Prostate Cancer Prevention Trial suggest that as many as one in four American men older than age 63 years, if biopsied, would be diagnosed as having prostate cancer *(62)*. At this frequency in the population, many familial clusters of prostate cancer, particularly pairs of affected brothers are caused simply by chance alone. Thus, in any collection of prostate cancer families, there will be families resulting from shared genetics, shared environmental factors, shared genetics interacting with shared environmental factors, as well as those caused by chance alone, and some (perhaps most) caused by some combination of these factors. Only when we restrict the phenotype under study can we reasonably hope to simplify this situation. For example, having large enough study populations so that well-defined subsets of prostate cancer can be emphasized is a possible solution. As opposed to families in which two or more relatives have prostate cancer, families with larger numbers of first-degree relatives affected (e.g., 5 or more) or multiple members with prostate cancers having poor prognostic features (e.g., Gleason Score 7 or higher, and/or non-organ-confined disease) are relatively rare. Will focusing on heavily loaded families or families with aggressive disease reduce the heterogeneity and enrich for genetic effects enough to simplify the picture? Only in a study population that is large enough and well-enough characterized can such a question be addressed. The collaborative effort undertaken by the International Consortium for Prostate Cancer Genetics (ICPCG) has this capacity (see Subheading 5.). Will focusing on only a subset of prostate cancer decrease the relevance of any discoveries made as a consequence, with respect to prostate cancer in general? It is of

note that it was the study of families with a familial adenomatous polyposis, a syndrome which accounts for only a very small fraction of all colon cancers, or even familial colon cancer, that led to the identification of the *APC* gene, a gene that is inactivated in the majority of colon cancers, both familial and sporadic *(3)*.

5. THE ICPCG STUDY OF PROSTATE CANCER SUSCEPTIBILITY

To increase the power to discover prostate cancer susceptibility genes, the ICPCG, a multicenter collaborative research group (www.icpcg.org), increased sample size by combining genome-wide screen linkage data from 1233 prostate cancer families, and used dominant, recessive, and nonparametric models to analyze the data *(63)*. Analysis of the complete family collection revealed "suggestive" evidence of linkage (logarithm of odds [LOD] > 1.86) at five regions: *5q12, 8p21, 15q11, 17q21*, and *22q12*; Table 1). The highest overall LOD score in the genome was 2.28 from the nonparametric analysis, found near marker *D5S2858* on 5q12 (77 cM from *pter*). The linkage results by group for each of these five chromosomal regions are shown in Table 2. As seen in this table, with the exception of *17q21*, the LOD scores for each of the highlighted regions are higher in the combined analysis than observed in any individual group, only reaching a level of "suggestive" evidence in the combined family data. This finding emphasizes the unique results that emerge from this combined approach.

Also performed were genome-wide screens in two subsets of families that may be enriched for stronger genetic effects. Among the 269 families with at least five affected members, "suggestive" evidence for linkage was found at *1q25, 8q13, 13q14, 16p13,* and *17q21*, and "significant" evidence of linkage (LOD = 3.57) was found at *22q12*. LOD scores greater than 1.0 at *22q12* were observed in four different family collections. Among the 606 families with early age at diagnosis, "suggestive" evidence for linkage was found at *3p24, 5q35, 11q22*, and *Xq12* (Table 1).

To summarize this study:

1. No single major locus clearly emerged from this large study of prostate cancer families.
2. The finding of numerous "suggestive" linkages is consistent with the hypothesis of multiple prostate cancer susceptibility genes with modest effects, or several major genes segregating in small subsets of families.
3. The most significant evidence of linkage was observed in the subset of families with five or more affected members.
4. If major prostate cancer susceptibility genes exist, they are likely located in one or more of the regions implicated in this study.

5.1. GENOME-WIDE SCAN FOR LINKAGE IN PROSTATE CANCER FAMILIES WITH AGGRESSIVE DISEASE

To examine the hypothesis that the tendency to develop *aggressive* prostate cancer may have an inherited component, and that linkage analysis of an appropriately chosen subset of prostate cancer families could be used to map the genes responsible, the ICPCG recently carried out a genome-wide linkage scan on pedigrees that had at least three men with clinically aggressive prostate cancer as defined by high-grade and/or non-organ-confined disease *(64)*. Pedigrees of this type are rare—of 1233 prostate cancer pedigrees, 166 (13%) met these criteria, making such a study possible only by pooling data from multiple family collections. Among the 166 pedigrees with aggressive prostate cancer, the strongest linkage signals were on chromosome 6p (LOD = 3.0), chromosome 11q (LOD = 2.4), and chromosome 20q (LOD = 2.5). For chromosome 11, the linkage signal was largest among pedigrees with an average at diagnosis of 65 years or younger. The results from the analysis of this unique collection of families implicate several loci as harboring genes that may predispose men to develop aggressive prostate cancer, and provide a basis for identification of such genes.

Table 1
Summary of Genome-Wide Scan Linkage Data from 1233 Prostate Cancer Families[a]

Characteristics	Region	cM	Nearest marker	Analysis	LOD	1-LOD drop interval	
						Genetic (cM)	Physical (Mb)
Primary analysis:	5q12	77	D5S2858	Nonparametric	2.28	66–96	43–78
entire set of families	8p21	46	D8S1047	Dominant	1.97	39–52	22–32
(n = 1233)	15q11	1	D15S817	Recessive	2.10	0–14	0–25
	17q21	77	D17S1820	Dominant	1.99	66–85	35–54
	22q12	42	D22S283	Dominant	1.95	35–47	35–47
Secondary analysis:	1q25	184	D1S2818	Nonparametric	2.62	170–198	195–196
subset of families with >	8q13	81	D8S543	Recessive	2.41	75–90	66–75
5 affected family members	13q14	56	D13S1807	Recessive	2.27	42–67	39–71
(n = 269)	16p13	34	D16S764	Nonparametric	1.88	19–46	9–23
	17q21	77	D17S1820	Dominant	2.04	66–83	39–53
	22q12	42	D22S283	Dominant	3.57	32–50	27–42
Secondary analysis:	3p24	57	D3S2432	Dominant	2.37	47–69	28–49
subset of families with mean	5q35	179	D5S1456	Dominant	2.05	166–193	162–174
age at diagnosis < 65 years	11q22	102	D11S898	Recessive	2.20	89–112	81–111
(n = 606)	Xq12	80	DXS7132	Dominant	2.30	62–90	40–85

[a] LOD, logarithm of odds.

Table 2
Support for Linkage From Each Group at Chromosomal Regions With "Suggestive" Linkage

	Chromosomal regions with "suggestive" likage				
Group	5q12 (77 cM) Nonparametric	8p21 (46 cM) Dominant	15q11 (1 cM) Recessive	17q21 (77 cM) Dominant	22q12 (42 cM) Dominant
All groups (n = 1233)	2.28	1.97	2.10	1.99	1.95
ACTANE (n = 64)	0.00	0.00	0.00	0.00	0.00
BC/CA/HI (n = 98)	1.17	0.00	0.26	0.76	0.00
Johns Hopkins University (n = 188)	0.29	0.16	0.95	0.72	1.28
Mayo Clinic (n = 159)	0.32	0.09	0.00	0.00	1.10
PROGRESS (n = 254)	0.25	1.64	0.64	0.01	0.00
University of Michigan (n = 176)	0.01	0.28	1.06	3.07	0.13
University of Tampere (n = 10)	0.00	0.19	0.00	0.42	0.02
University of Ulm (n = 139)	0.38	0.77	0.04	0.00	0.00
University of Umea (n = 50)	1.62	0.00	0.87	0.00	0.00
University of Utah (n = 95)	0.27	0.17	0.00	0.03	1.47

6. ALTERNATIVE APPROACHES TO FIND PROSTATE CANCER SUSCEPTIBILITY GENES

Another important approach to find prostate cancer susceptibility genes has been to perform association studies in populations of men with and without prostate cancer, typically without regard to family history. These case control studies have been greatly aided by the increased understanding of the variability of the human genome sequence among different individuals and the concept that common diseases may be caused by common genetic variants in the population *(65)*. Thus, by simply examining the frequency of polymorphic alleles, typically single-nucleotide polymorphisms, among cases and controls, associations between genes and disease risk can be rapidly assessed. A large number of genes involved in critical processes that occur in prostate cells, such as androgen action and metabolism, growth factor signaling, carcinogen detoxification, and DNA repair and inflammation, are being systematically evaluated in this fashion. Additionally, genome-wide association studies of prostate cancer involving thousands to millions of genetic markers are being contemplated and initiated (see http://cgems.nci.nih.gov/). These are likely to implicate a large number of genes as affecting prostate cancer risk.

A third approach has been to study the possible roles of genes responsible for other cancer-predisposing syndromes as prostate cancer susceptibility genes. This approach has revealed some interesting leads. The *BRCA2* gene has been implicated in early onset prostate cancers *(66)*, and both *CHEK2* and *NBS1*, genes involved in breast cancer susceptibility and the Nijmegen breakage syndrome, have been found to be mutated in prostate cancer families in different study populations *(67–69)*. That these three genes are all involved in DNA repair indicates that defects in the ability to repair damage to DNA and maintain genomic integrity predispose prostate epithelial cells, similar to many other cells in the body, to malignant transformation.

6.1. BRCA1 *and* BRCA2

As a result of various epidemiological studies during the past four decades, a link between prostate and breast cancer etiology has been suspected for many years *(70–72)*. However, an examination of a large number of prostate cancer families for other cancers *(73)* found only tumors of the central nervous system to be in significant excess; the number of breast cancer cases was not significantly elevated. Multiple studies have demonstrated an association between *BRCA1* and *BRCA2* mutations and increased risk of prostate cancer in mutation carriers *(74–77)*. The most direct evidence for a role of *BRCA2* comes from a study by Edwards et al. *(66)* showing that, in 263 men with diagnoses of prostate cancer who were at most 55 years of age, protein-truncating mutations were found in 2.3% and, notably, all of these mutations were clustered outside the ovarian cancer cluster region. The relative risk of developing prostate cancer by age 56 years from a deleterious germline *BRCA2* mutation was 23-fold. This establishes *BRCA2* as a *bona fide* prostate cancer susceptibility gene.

Furthermore, in an extensive study of other cancers in *BRCA2* carriers from the Breast Cancer Linkage Consortium *(78)*, a strong association with prostate cancer was seen (estimated relative risk [RR], 4.65; 95% CI, 3.48–6.22) for mutation carriers, particularly for men younger than 65 years of age (RR, 7.33). In support of both these findings, Warner et al. *(79)* determined the *BRCA1* and *BRCA2* status of 412 Ashkenazi Jewish women with breast cancer. The cumulative incidence of various cancers was assessed in first-degree relatives. The cumulative incidence of prostate cancer in the first-degree relatives of Ashkenazi Jewish women with breast cancer was 14.8% compared with 3.6% in the first-degree relatives of Ashkenazi Jewish women without breast cancer ($p = 0.002$). Interestingly, when the affected Ashkenazi Jewish women were divided into mutation carriers and noncarriers, the cumulative incidence rose to 34% in carriers and was 12.6% in noncarriers ($p = 0.05$). This cannot be caused by recall bias, because the family histories were recorded before the women knew of their mutation status. Although a study of multiplex Ashkenazi Jewish prostate cancer families did not find elevated rates of common mutations in either *BRCA1* or *BRCA2 (80)*, this

study did not include early onset cases. Overall, although it seems likely that mutations in *BRCA1* and *BRCA2* increase risk for prostate cancer, particularly in the case of *BRCA2* for early onset disease, the contribution of germline mutations in these genes to familial clustering of prostate cancer in general remains to be more fully determined. For example, mutation studies following up from recent linkage studies implicating chromosome 17q12–21 do not suggest that *BRCA1* is responsible for the positive LOD scores *(81,82)*. Further studies, most likely involving large combined data sets, will be required to more definitively assess the role of these important breast cancer susceptibility genes in prostate cancer risk.

6.2. CHEK2

The *CHEK2* gene on 22q12.1, an upstream regulator of p53, functions in the ATM-dependent DNA damage signaling pathways, such as *BRCA1* and *BRCA*2. It was first identified in Li-Fraumeni families, in which the observed mutations, most importantly *1100delC*, were shown to result in truncated protein *(83)*. Dong et al. *(69)* provided the first evidence of *CHEK2* mutations in prostate cancer. They analyzed a total of 578 prostate cancer patients, of which a total of 28 (4.8%) had germline *CHEK2* mutations (16 unique). In additional screenings in 149 families with familial prostate cancer, 11 mutations were revealed (5 unique) in 9 families. These mutations included two frameshifts and three missense mutations. Sixteen of the 18 unique mutations were seen in both sporadic and familial cases but not among 423 unaffected men. However, when all mutations were pooled together, an association was seen only with sporadic cases. A Finnish study analyzed 537 unselected prostate cancer patients, 120 patients from hereditary prostate cancer families and 480 controls *(67)*. Altogether, eight sequence variants were detected, including the frameshift mutation *1100delC* and a novel *D438Y* missense mutation. Unlike the study of Dong et al. *(69)*, an association was seen only in patients with a strong family history. More recently, additional studies on *CHEK2* and prostate cancer have been published by a Polish group. Cybulski et al. *(68)* sequenced *CHEK2* in 140 patients and then genotyped the three detected variants in a larger series of prostate cancer cases and controls (98 familial patients, 690 unselected patients, and 1921 controls). Truncating mutations (*IVS2+1G* and *1100delC*) were identified in 0.5% of the controls, in 1.6% of the unselected patients (OR, 3.4; p = 0.004) and in 4.1% of familial cases (OR, 9.0; p = 0.0002). The missense variant *I157T* was also detected and associated with prostate cancer, the effect being the strongest in men with familial disease (OR, 3.8; p = 0.00002).

7. SUMMARY AND CONCLUSION

Where is the study of prostate cancer susceptibility taking us? Hopefully, by extensive study of prostate cancer pedigrees, we can more effectively optimize the predictive information of family history. It will most likely be important to provide more clinical detail of the prostate cancers that compose the family history, rather than making all cases with a positive biopsy of equal significance. In addition, defining genes that affect prostate cancer risk in families as well as sporadic disease can potentially provide otherwise unattainable insights into the mechanisms of prostate carcinogenesis and the identification of novel therapeutic targets. For example, the fact that multiple genes involved in the inflammatory process (e.g., *MSR1 [54]*, *RNASEL [32]*, *MIC1 [84]*, *TRL4 [85]*, *TLR1-TLR6-TLR10 [86]*, *IL1-RN [87]*, and *IL10 [88]*) are implicated through genetic studies, provides a unique and compelling impetus for further study of this process in the development of prostate cancer *(89)*.

Progress has been made in identifying genes associated with familial prostate cancer, but there is much left to be learned, and many findings in this area have been difficult to replicate. Unfortunately, despite extensive efforts and a number of promising leads, a major gene that can be used to identify individuals at high risk for prostate cancer has not been identified. Because this disease stands to become an even larger medical burden as the world population ages, it is imperative that a better understanding of the molecular genetics of prostate cancer be acquired. Indeed, if the promise of the

revolution in molecular medicine is to be realized with respect to prostate cancer, increased efforts with better methodologies and technologies and large, well-characterized clinical study populations are urgently needed to effectively confront this research problem.

ACKNOWLEDGMENTS

The authors thank Dan Schaid, Nicola Camp, Johanna Schleutker, and Will Foulkes for their contributions.

REFERENCES

1. Jemal, A., Murray, T., Ward, E., et al. (2005). Cancer statistics, 2005. *CA Cancer J. Clin.* **55(1)**, 10–30.
2. World Health Organization. (1998). Executive Summary. Assessment of the health risk of dioxins: re-evaluation of the Tolerable Daily Intake (TDI). WHO Consultation, May 25–29, Geneva, Switzerland.
3. Kinzler, K. W. and Vogelstein, B., (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* **386**, 761–763.
4. Sparkes, R. S., Murphree, A. L., Lingua, R. W., et al. (1983). Gene for hereditary retinoblastoma assigned to human chromosome 13 by linkage to esterase D. *Science* **219**, 971–973.
5. Seizinger, B. R., Rouleau, G. A., Ozelius, L. J., et al. (1987). Genetic linkage of von Recklinghausen neurofibromatosis to the nerve growth factor receptor gene. *Cell* **49**, 589–594.
6. Barker, D., Wright, E., Nguyen, K., et al. (1987). Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science* **236**, 1100–1102.
7. Larsson, C., Skogseid, B., Oberg, K., Nakamura, Y., and Nordenskjold, M. (1988). Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. *Nature* **332**, 85–87.
8. Seizinger, B. R., Rouleau, G. A., Ozelius, L. J., et al. (1988). Von Hippel-Lindau disease maps to the region of chromosome 3 associated with renal cell carcinoma. *Nature* **332**, 268–269.
9. Hall, J. M., Lee, M. K., Newman, B., et al. (1990). Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* **250**, 1684–1689.
10. Peltomaki, P., Aaltonen, L. A., Sistonen, P., et al. (1993). Genetic mapping of a locus predisposing to human colorectal cancer [see comments]. *Science* 260, 810–812.
11. Wooster, R., Neuhausen, S. L., Mangion, J., et al. (1994). Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science* **265**, 2088–2090.
12. Cannon-Albright, L. A., Goldgar, D. E., Neuhausen, S., et al. (1994). Localization of the 9p melanoma susceptibility locus (MLM) to a 2-cM region between D9S736 and D9S171. *Genomics* **23(1)**, 265–268.
13. Chen, Y. C. and Hunter, D. J. (2005). Molecular epidemiology of cancer. *CA Cancer J. Clin.* **55(1)**, 45–54.
14. Brendler, C. B. and Walsh, P. C. (1992). The role of radical prostatectomy in the treatment of prostate cancer. *CA Cancer J. Clin.* **42**, 212–222.
15. Pardoll, D. M. (1999). Inducing autoimmune disease to treat cancer. Proc. Natl. Acad. Sci. USA **96**, 5340–5342.
16. Onik, G. M., Cohen, J. K., Reyes, G. D., Rubinsky, B., Chang, Z., and Baust, J. (1993). Transrectal ultrasound-guided percutaneous radical cryosurgical ablation of the prostate. *Cancer* **72**, 1291–1299.
17. Coffey, D. S. (1992). The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. In: *Cambell's Urology* (Walsh, P. C., Retik, A. B., Stamey, T. A., and Vaughan, E. D., eds.). WB Saunders, Philadelphia, PA, pp. 221–301.
18. Morganti, G., Gianferrari, L., Cresseri, A., Arrigoni, A., and Lovati, G. (1956–1957). Clinico-statistical and genetic research on neoplasms of the prostate. *Acta Genet. Stat. Med.* **6(2)**, 304–305.
19. Woolf, C. M. (1960). An investigation of the familial aspects of carcinoma of the prostate. *Cancer* **13**, 739–744.
20. Johns, L. E. and Houlston, R. S. (2003). A systematic review and meta-analysis of familial prostate cancer risk. *BJU Int.* **91(9)**, 789–794.
21. Grönberg, H., Damber, L., and Damber, J. E., (1994). Studies of genetic factors in prostate cancer in a twin population. *J. Urol.* **152**, 1484–1487; discussion 1487–1489.
22. Page, W. F., Braun, M. M., Partin, A. W., Caporaso, N., and Walsh, P. (1997). Heredity and prostate cancer: a study of World War II veteran twins. *Prostate* **33**, 240–245.
23. Lichtenstein, P., Holm, N. V., Verkasalo, P. K., et al. (2000). Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.* **343**, 78–85.
24. Cannon-Albright, L. A., Thomas, A., Goldgar, D. E., et al. (1994). Familiality of cancer in Utah. *Cancer Res.* **54(9)**, 2378–2385.
25. Carter, B. S., Beaty, T. H., Steinberg, G. D., Childs, B., and Walsh, P. C. (1992). Mendelian inheritance of familial prostate cancer. *Proc. Natl. Acad. Sci. USA* **89**, 3367–3371.
26. Grönberg, H., Damber, L., Damber, J. E., and Iselius, L. (1997). Segregation analysis of prostate cancer in Sweden: support for dominant inheritance. *Am. J. Epidemiol.* **146**, 552–557.

27. Schaid, D. J., McDonnell, S. K., Blute, M. L., and Thibodeau, S. N. (1998). Evidence for autosomal dominant inheritance of prostate cancer. *Am. J. Hum. Genet.* **62**, 1425–1438.
28. Schaid DJ. (2004). The complex genetic epidemiology of prostate cancer. *Hum. Mol. Genet.* **13(Spec No 1)**, R103–121.
29. Cui, J., Staples, M. P., Hopper, J. L., English, D. R., McCredie, M. R., and Giles, G. G. (2001). Segregation analyses of 1,476 population-based Australian families affected by prostate cancer. *Am. J. Hum. Genet.* **68(5)**, 1207–1218.
30. Smith, J. R., Freije, D., Carpten, J. D., et al. (1996). Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search [see comments]. *Science* **274**, 1371–1374.
31. Tavtigian, S. V., Simard, J., Teng, D. H., et al. (2001). A candidate prostate cancer susceptibility gene at chromosome 17p. *Nat. Gen.* **27**, 172–180.
32. Carpten, J., Nupponen, N., Isaacs, S., et al. (2002). Germline mutations in the ribonuclease L (*RNase L*) Gene in hereditary prostate cancer 1 (HPC1)-linked families. *Nat. Genet.* **30(2)**, 181–184.
33. Xu, J., Zheng, S. L., Komiya, A., et al. (2002). Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Nat. Genet.* **32(2)**, 321–325.
34. Rebbeck, T. R., Walker, A. H., Zeigler-Johnson, C., et al. (2001). Association of HPC2/ELAC2 genotypes and prostate cancer. *Am. J. Hum. Genet.* **67**, 1014–1019.
35. Xu, J., Zheng, S. L., Carpten, J. D., et al. (2001). Evaluation of linkage and association of HPC2/ELAC2 in patients with familial or sporadic prostate cancer. *Am. J. Hum. Genet.* **68(4)**, 901–911.
36. Wang L., McDonnell S. K., Elkins D. A., et al. (2001). Role of HPC2/ELAC2 in hereditary prostate cancer. *Cancer Res.* **61(17)**, 6494–6499.
37. Rokman, A., Ikonen, T., Mononen, N., et al. (2001). ELAC2/HPC2 involvement in hereditary and sporadic prostate cancer. *Cancer Res.* **61(16)**, 6038–6041.
38. Stanford, J. L., Sabacan, L. P., Noonan, E. A., et al. (2003). Association of HPC2/ELAC2 polymorphisms with risk of prostate cancer in a population-based study. *Cancer Epidemiol. Biomarkers Prev.* **12(9)**, 876–881.
39. Suarez, B. K., Gerhard, D. S., Lin, J., et al. (2001). Polymorphisms in the prostate cancer susceptibility gene HPC2/ELAC2 in multiplex families and healthy controls. *Cancer Res.* **61(13)**, 4982–4984.
40. Adler, D., Kanji, N., Trpkov, K., Fick, G., and Hughes, R. M. (2003). HPC2/ELAC2 gene variants associated with incident prostate cancer. *J. Hum. Genet.* **48(12)**, 634–638.
41. Takahashi, H., Lu, W., Watanabe, M., et al. (2003). Ser217Leu polymorphism of the HPC2/ELAC2 gene associated with prostatic cancer risk in Japanese men. *Int. J. Cancer* **107(2)**, 224–228.
42. Noonan-Wheeler, F. C., Wu, W., Roehl, K. A., et al. (2006). Association of hereditary prostate cancer gene polymorphic variants with sporadic aggressive prostate carcinoma. *Prostate* **66**, 49–56.
43. Camp, N. J. and Tavtigian, S. V. (2002). Meta analysis of associations of the ser217leu and ala541thr variants in *ELAC2* (*HPC2*) and prostate cancer. *Am. J. Hum. Genet.* **71**, 1475–1478.
44. Hassel, B. A., Zhou, A., Sotomayor, C., Maran, A., and Silverman, R. H. (1993). A dominant negative mutant of 2-5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon. *EMBO J.* **12(8)**, 3297–3304.
45. Rokman, A., Ikonen, T., Seppälä, E. H., et al. (2002). Germline alterations of the RNASEL gene, a candidate HPC1 gene at 1q25, in patients and families with prostate cancer. *Am. J. Hum. Genet.* **70(5)**, 1299–1304.
46. Chen, H., Griffin, A. R., Wu, Y. Q., et al. (2003). RNASEL mutations in hereditary prostate cancer. *J. Med. Genet.* **40**, e21.
47. Rennert, H., Bercovich, D., Hubert, A., et al. (2002). A novel founder mutation in the RNASEL gene, 471delAAAG, is associated with prostate cancer in Ashkenazi Jews. *Am. J. Hum. Genet.* **71**, 981–984.
48. Kotar, K., Hamel, N., Thiffault, I., and Foulkes, W. D. (2003). The RNASEL 471delAAAG allele and prostate cancer in Ashkenazi Jewish men. *J. Med. Genet.* **40(3)**, e22.
49. Casey, G., Neville, P. J., Plummer, S. J., et al. (2002) RNASEL Arg462Gln variant is implicated in up to 13% of prostate cancer cases. *Nat. Genet.* **32**, 581–583.
50. Wang, L., McDonnell, S., Elkins, D., et al. (2002) Analysis of the RNASEL gene in familial and sporadic prostate cancer. *Am. J. Hum. Genet.* **71**, 116–123.
51. Nakazato, H., Suzuki, K., Matsui, H., Ohtake, N., Nakata, S., and Yamanaka, H. (2003) Role of genetic polymorphisms of the RNASEL gene on familial prostate cancer risk in a Japanese population. *Br. J. Cancer* **89**, 691–696.
52. Maier, C., Haeusler, J., Herkommer, K., et al. (2005). Mutation screening and association study of RNASEL as a prostate cancer susceptibility gene. *Br. J. Cancer* **92(6)**, 1159–1164.
53. Wiklund, F., Jonsson, B. A., Brookes, A. J., et al. (2004). Genetic analysis of the RNASEL gene in hereditary, familial, and sporadic prostate cancer. *Clin. Cancer Res.* **10(21)**, 7150–7156.
54. Xu, J., Zheng, S. L., Komiya, A., et al. (2003). Common sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Am. J. Hum. Genet.* **72(1)**, 208–212.
55. Miller, D. C., Zheng, S. L., Dunn, R. L., et al. (2003). Germ-line mutations of the macrophage scavenger receptor 1 gene: association with prostate cancer risk in African-American men. *Cancer Res.* **63(13)**, 3486–3489.
56. Wang, L., McDonnell, S. K., Cunningham, J. M., et al. (2003). No association of germline alteration of MSR1 with prostate cancer risk. *Nat. Genet.* **35(2)**, 128–129.

57. Seppälä, E. H., Ikonen, T., Autio, V., et al. (2003). Germ-line alterations in MSR1 gene and prostate cancer risk. *Clin. Cancer Res.* **9(14)**, 5252–5256.
58. Lindmark, F., Jonsson, B. A., Bergh, A., et al. (2004). Analysis of the macrophage scavenger receptor 1 gene in Swedish hereditary and sporadic prostate cancer. *Prostate* **59(2)**, 132–140.
59. Hope, Q., Bullock, S., Evans, C., et al. (2005. The Cancer Research UK/British Association of Urological Surgeons' Section of Oncology Collaborators.Macrophage scavenger receptor 1 999C>T (R293X) mutation and risk of prostate cancer. *Cancer Epidemiol. Biomarkers Prev.* **14(2)**, 397–402.
60. Rennert, H., Zeigler-Johnson, C. M., Addya, K., et al. (2005). Association of susceptibility alleles in ELAC2/HPC2, RNASEL/HPC1, and MSR1 with prostate cancer severity in European American and African American men. *Cancer Epidemiol. Biomarkers Prev.* **14(4)**, 949–957.
61. Sun, J., Hsu, F.-C., Turner, A., et al. (2006). Meta-analysis of association of rare mutations and common sequence variances in the *MSR1* gene and prostate cancer risk. *Prostate* **66(7)**, 728–737
62. Thompson, I. M., Goodman, P. J., Tangen, C. M., et al. (2003). The influence of finasteride on the development of prostate cancer. *N. Engl. J. Med.* **349(3)**, 215–224.
63. Xu, J., Dimitrov, L., Chang, B. L., et al. (2005). A combined genomewide linkage scan of 1,233 families for prostate cancer-susceptibility genes conducted by the international consortium for prostate cancer genetics. *Am. J. Hum. Genet.* **77**, 219–229.
64. Schaid, D. J., McDonnell, S. K., Zarfas, K. E., et al. (2006).Pooled genome linkage scan of aggressive prostate cancer: Results from the International Consortium for Prostate Cancer Genetics. *Hum. Genet.* **120(4)**, 471–485.
65. Lohmueller, K. E., Pearce, C. L., Pike, M., Lander, E. S., and Hirschhorn, J. N. (2003). Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat. Genet.* **33(2)**, 177–182.
66. Edwards, S. M., Kote-Jarai, Z., Meitz, J., et al. (2003). Two percent of men with early-onset prostate cancer harbor germline mutations in the BRCA2 gene. *Am. J. Hum. Genet.* **72**, 1–12.
67. Seppälä, E. H. Ikonen, T., Mononen, N., et al. (2003). CHEK2 variants associate with hereditary prostate cancer. *Br. J. Cancer* **89(10)**, 1966–1970.
68. Cybulski, C., Gorski, B., Huzarski ,T., et al. (2004). CHEK2 is a multiorgan cancer susceptibility gene. *Am. J. Hum. Genet.* **75(6)**, 1131–1135.
69. Dong, X., Wang, L., Taniguchi, K., et al. (2003). Mutations in CHEK2 associated with prostate cancer risk. *Am. J. Hum. Genet.* **72(2)**, 270–280.
70. McCahy, P. J., Harris, C. A., and Neal, D. E. (1996). Breast and prostate cancer in the relatives of men with prostate cancer. *Br. J. Urol.* **78**, 552–556.
71. Thiessen, E. U. (1974). Concerning a familial association between breast cancer and both prostatic and uterine malignancies. *Cancer* **34**, 1102–1107.
72. Ekman, P., Pan, Y., Li, C., and Dich, J. (1997). Environmental and genetic factors: a possible link with prostate cancer. *Br. J. Urol.* **79(Suppl 2)**, 35–41.
73. Isaacs, S. D., Kiemeney, L. A., Baffoe-Bonnie, A., Beaty, T. H., and Walsh, P. C. (1995). Risk of cancer in relatives of prostate cancer probands. *J. Natl. Cancer Inst.* **87**, 991–996.
74. Ford, D., Easton, D. F., Bishop, D. T., Narod, S. A., and Goldgar, D. E. (1994). Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Lancet* **343**, 692–695.
75. Easton, D. F., Steele, L., Fields, P., et al. (1997). Cancer risks in two large breast cancer families linked to BRCA2 on chromosome 13q12-13. *Am. J. Hum. Genet.* **61**, 120–128.
76. Sigurdsson, S., Thorlacius, S., Tomasson, J., et al. (1997). BRCA2 mutation in Icelandic prostate cancer patients. *J. Mol. Med.* **75**, 758–761.
77. Struewing, J. P., Hartge, P., Wacholder, S., et al. (1997). The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews [see comments]. *N. Engl. J. Med.* **336**, 1401–1408.
78. The Breast Cancer Linkage Consortium. (1999). Cancer risks in BRCA2 mutation carriers. *J. Natl. Cancer Inst.* **91**, 1310–1316.
79. Warner, E., Foulkes, W., Goodwin, P., et al. (1999). Prevalence and penetrance of BRCA1 and BRCA2 gene mutations in unselected Ashkenazi Jewish women with breast cancer. *J. Natl. Cancer Inst.* **91**, 1241–1247.
80. Wilkens, E. P., Freije, D., Xu, J., et al. 1999. No evidence for a role of BRCA1 or BRCA2 mutations in Ashkenazi Jewish families with hereditary prostate cancer. *Prostate* **39**, 280–284.
81. Lange, E. M., Gillanders, E. M., Davis, C. C., et al. (2003). Genome-wide scan for prostate cancer susceptibility genes using families from the University of Michigan prostate cancer genetics project finds evidence for linkage on chromosome 17 near BRCA1. *Prostate* **57**, 326–334.
82. Zuhlke, K. A., Madeoy, J. J., Beebe-Dimmer, J., et al. (2004). Truncating BRCA1 mutations are uncommon in a cohort of hereditary prostate cancer families with evidence of linkage to 17q markers. *Clin Cancer Res* 10, 5975–5980.
83. Bell, D. W., Varley, J. M., Szydlo, T. E., et al. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science* **286(5449)**, 2528–2531.

84. Lindmark, F., Zheng, S. L., Wiklund, F., et al. (2004). H6D polymorphism in macrophage-inhibitory cytokine-1 gene associated with prostate cancer. *J. Natl. Cancer Inst.* **96(16)**, 1248–1254.
85. Zheng, S. L., Augustsson-Balter, K., Chang, B., et al. (2004). Sequence variants of toll-like receptor 4 are associated with prostate cancer risk: results from the CAncer Prostate in Sweden Study. *Cancer Res.* **64(8)**, 2918–2922.
86. Sun, J., Wiklund, F., Zheng, S. L., et al. (2005). Sequence variants in Toll-like receptor gene cluster (TLR6-TLR1-TLR10) and prostate cancer risk. *J. Natl. Cancer Inst.* **97(7)**, 525–532.
87. Lindmark, F., Zheng, S. L., Wiklund, F., et al. (2005). Interleukin-1 receptor antagonist haplotype associated with prostate cancer risk. *Br. J. Cancer.* **93(4)**, 493–497.
88. McCarron, S. L., Edwards, S., Evans, P. R., et al. (2002) Influence of cytokine gene polymorphisms on the development of prostate cancer. *Cancer Res.* **62(12)**, 3369–3372.
89. Nelson, W. G., De Marzo, A. M., and Isaacs, W. B. (2003). Prostate cancer. *N. Engl. J. Med.* **349(4)**, 366–381
90. Easton, D. F., Schaid, D. J., Wittemore, A. S., and Isaacs, W. J. (2003). International Consortium for Prostate Cancer Genetics. Where are the prostate cancer gene? A summary of eight genome wide searches. *Prostate* **57(4)**, 261–269.
91. Simard, J., Dumont, M., Labuda, D., et al. (2003). Prostate cancer susceptibility genes: lessons learned and challenges posed. *Endocr. Relat. Cancer.* **10(2)**, 225–259.

17
Somatic DNA Methylation Changes and Prostatic Carcinogenesis

William G. Nelson, Srinivasan Yegnasubramanian, Patrick J. Bastian, Masashi Nakayama, and Angelo M. De Marzo

Summary

Prostatic carcinogenesis proceeds via the acquisition of both genetic and epigenetic alterations. The epigenetic changes, principally in DNA methylation patterns and in chromatin structure, are equivalent to genetic changes, and often lead to defects in the functions of critical genes, which contribute to malignant cell behaviors. Epigenetic alterations also tend to appear at the earliest stages of prostate cancer development. As such, analysis of epigenetic genome changes has not only led to a new understanding of how prostate cancers likely arise, but has also provided new translational research opportunities, both for molecular biomarkers likely to aid in prostate cancer detection and diagnosis and for strategies to prevent and treat the disease.

Key Words: Chromatin structure; DNA methylation; DNA methyltransferase; epigenetics; glutathione-*S*-transferase; prostate cancer.

1. INTRODUCTION

The progressive acquisition of genomic alterations is a defining feature of all human cancers, including prostate cancer. Cancer cells are known to carry a variety of genetic defects, including gene mutations, deletions, translocations, and amplifications, that endow the cells with new capabilities for dysregulated proliferation, inappropriate survival, tissue invasion and destruction, immune system evasion, and metastasis *(1)*. More recently, it has become apparent that cancer cells also carry epigenetic defects, including changes in cytosine methylation patterns and in chromatin structure and organization, which are equivalent to genetic changes in effecting and maintaining neoplastic and malignant phenotypes *(2)*. For human prostate cancer, evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development than genetic changes, as well as more commonly and consistently. As a consequence, the study of somatic epigenetic changes has provided new insights into the mechanism(s) of prostatic carcinogenesis, new molecular biomarkers to aid in prostate cancer detection and diagnosis, and new opportunities for prostate cancer prevention and treatment.

2. DNA METHYLATION, DNA METHYLTRANSFERASES, AND CANCER DEVELOPMENT

The major DNA manifestations of the epigenetic alterations in cancer cells are changes in cytosine methylation. The self-complementary nucleotide sequence CpG, under-represented in mammalian genomes, most often carries cytosine bases with a methyl group at the 5 position ($^{5\text{-me}}$C). DNA

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

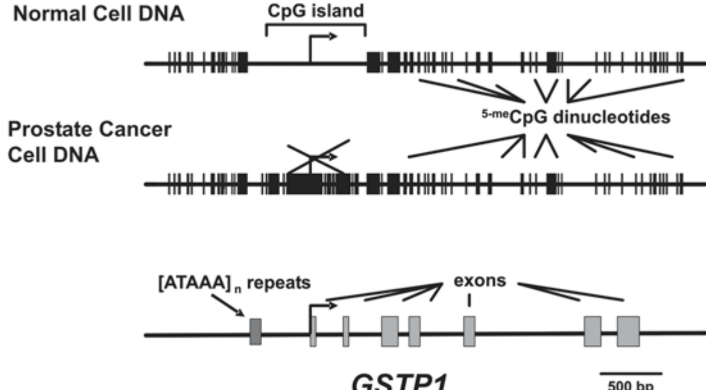


Fig. 1. *GSTP1* CpG island hypermethylation in prostate cancer cells. The *GSTP1* CpG island region, containing the transcriptional regulatory region, is unmethylated in normal cells, but carries extensive CpG dinucleotide methylation in prostate cancer cells, resulting in epigenetic gene silencing.

methyltransferases (DNMTs) are responsible for catalyzing the transfer of a methyl group from *S*-adenosyl-methionine to cytosine bases in CpG dinucleotides and maintaining patterns of CpG dinucleotide methylation across the genome through DNA replication and mitosis. Dense clusters of unmethylated CpG dinucleotides, termed "CpG islands," are present at the transcriptional regulatory region of many genes *(3)*; abnormal increases in cytosine methylation in these regions in cancer cells are associated with transcriptional silencing and heterochromatinization *(2)* (Fig. 1). Somatic CpG island hypermethylation is, thus, functionally equivalent in carcinogenesis to somatic gene deletion, or to somatic mutations leading to loss of gene or gene product function. Many cancer cells, despite containing hypermethylated CpG island sequences at the loci of several key genes, also contain sequences with hypomethylated CpG dinucleotides. Somatic CpG dinucleotide hypomethylation has been proposed to result in inappropriate gene activation or regulation, in increased gene recombination, and in de-repression of endogenous retrovirus genes *(4–6)*.

Although it is clear that the fidelity of CpG methylation pattern maintenance must be somehow corrupted in cancer cells, the means by which acquired increases and decreases in CpG dinucleotide methylation appear during the development of cancer have not been fully established. Proposed mechanisms for somatic alterations in CpG dinucleotide methylation feature:

1. The direct result of inappropriate expression or activity of DNMTs *(7)*.
2. The indirect consequences of targeted transcriptional *trans*-repression or *trans*-activation *(8)*.
3. The coordinated action of regulatory RNAs and DNMTs *(9,10)*.

Regardless of how the abnormal DNA methylation patterns arise in cancer cells, the resultant changes in gene function are subject to selection for cell growth and/or survival. For example, Luria-Delbruck fluctuation analyses have suggested that a small minority of human mammary epithelial cells spontaneously develop CpG island hypermethylation at the locus of the gene encoding the p16/INK4a cell cycle regulatory protein; these cells enjoy a growth advantage as the remaining human mammary epithelial cells undergo cell senescence *(11)*. Similarly, a reduction in CpG island hypermethylation at *MDR1*, encoding P-glycoprotein, provides a survival advantage to MCF-7 breast cancer cells in the face of treatment with the anti-neoplastic drugs doxorubicin and paclitaxel *(12)*. Finally, in HCT-116 colorectal cancer cells, which contain one mutant gene encoding p16/INK4a, with a frameshift mutation in the coding sequence, and one wild-type gene, the absence of p16/INK4a function is attributable to CpG hypermethylation, which is selectively present at the wild type, but not at the mutant, gene locus *(13)*. In these cells, there was no accumulation of CpG island

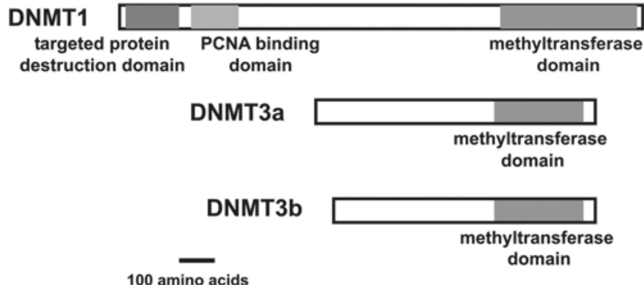


Fig. 2. Mammalian DNA methyltransferases (DNMTs). Of the three DNMTs, DNMT1 is the candidate maintenance methyltransferase, containing a binding domain for proliferating cell nuclear antigen (PCNA), that normally permits localization of the enzyme to the site of DNA replication, and a targeted protein destruction domain, that fine tunes expression of the enzyme to the S-phase of the replicative cell cycle.

hypermethylation changes at the gene encoding a mutant p16/INK4a, which would not have offered any selective cell growth advantage.

The most compelling mechanisms for the appearance of abnormal DNA methylation patterns in cancer cells described thus far focus on inappropriately regulated DNMT function. There are three known mammalian DNMTs responsible for CpG dinucleotide methylation, DNMT1, with preferential activity on hemimethylated DNA substrates, and DNMT3a and DNMT3b, both capable of *de novo* CpG dinucleotide methylation on unmethylated DNA substrates *(14)* (Fig. 2). Of the three, DNMT1, targeted to the replication fork during the *S*-phase of the cell cycle, is the likely enzyme responsible for maintaining methylation patterns through genome duplication and segregation at mitosis *(15)*. In contrast, DNMT3a and DNMT3b serve critical *de novo* DNA methylation functions during embryonic development, because mouse embryos carrying disrupted genes for these enzymes exhibit defects in gene imprinting associated with an inability to establish appropriate CpG dinucleotide methylation patterns *(16)*. In cancer cells, this strict division of labor between DNMT1 vs DNMT3a and DNMT3b may not always be followed. For example, although HCT-116 human colon cancer cells carrying targeted disruptions of DNMT1 or DNMT3b genes lose only 20% and 3% of total genomic cytosine methylation levels, respectively, cells with both genes disrupted lose some 95% of the methylated cytosine bases, indicating that DNMT3b can cooperate with and/or complement DNMT1 in the maintenance of genomic DNA methylation patterns *(17,18)*.

How these enzymes might produce CpG dinucleotide methylation changes in cancer cells is not well understood. However, significant data support a major role for DNMT1 in cancer development. First, forced overexpression of DNMT1 in normal cells causes increases in DNA methylation and epigenetic gene silencing *(7,19,20)*. Second, inadequate DNMT1 function can result in decreases in DNA methylation, and in cancer development, because mice carrying one disrupted *Dnmt1* allele and one hypomorphic *Dnmt1* allele, resulting in 10% of normal DNMT activity, have been reported to exhibit genomic instability and to develop T-cell lymphomas *(5,6)*. Finally, *Dnmt1* seems to be required for *c-fos* transformation of rodent fibroblasts in vitro, as well as for intestinal polyp development in *Apc*$^{Min/+}$ mice and tobacco carcinogen-induced murine lung cancer development in vivo *(21–24)*. Despite the well-demonstrated associations between too much and too little DNMT1 function and tumorigenesis in experimental models, abnormalities in DNMT1 regulation have been difficult to demonstrate in human cancers. Specifically, messenger RNA (mRNA) encoding DNMT1 does not seem to be commonly overexpressed or underexpressed, when normalized to proliferative activity, in human cancer cells *(25)*. However, a recent report has suggested that DNMT1 polypeptides levels are extensively regulated via targeted ubiquitin conjugation and destruction by the proteasome, and that cancer cells may exhibit marked defects in DNMT1 degradation even if

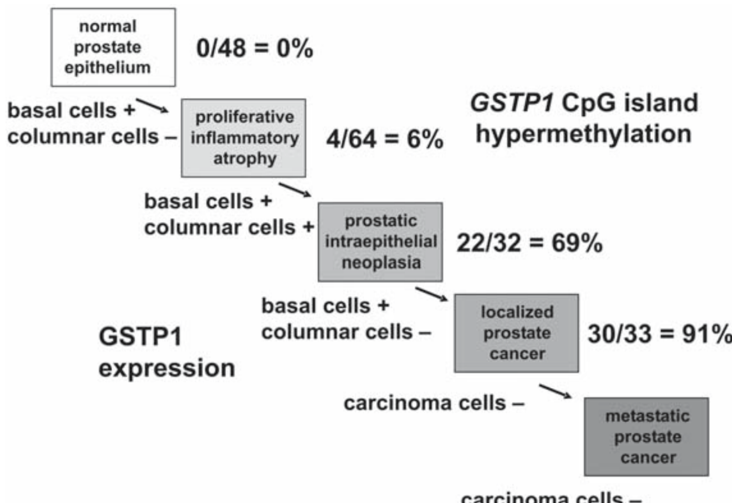


Fig. 3. The *GSTP1* CpG island hypermethylation during prostatic carcinogenesis: hypermethylation changes appear in prostate cancer precursors. *GSTP1* CpG island hypermethylation, accompanied by loss of GSTP1 expression, first appears in rare proliferative inflammatory atrophy (PIA) lesions, more commonly in prostatic intraepithelial neoplasia lesions, and then nearly ubiquitously in prostatic carcinomas *(36)*.

DNMT1 mRNA levels are not abnormal *(26)*. With these data in mind, the findings of heterogeneously increased and decreased DNMT1 levels in different cells in colorectal cancers by immunohistochemical staining hint that abnormal posttranslational regulation of DNMT1 polypeptide levels might contribute both to DNA hypermethylation and to DNA hypomethylation, promoting cancer development *(27)*.

3. ABNORMAL DNA METHYLATION CHANGES IN PROSTATE CANCER

The first gene found to be silenced via somatic CpG island hypermethylation in prostate cancer was *GSTP1*, encoding the π-class glutathione-*S*-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogens *(28)* (Fig. 1). This genome change remains the most common somatic genome abnormality of any kind (>90% of cases) reported thus far for prostate cancer, appearing earlier and more frequently than other gene defects that arise during prostate cancer development *(29)*. The associated loss of π-class GST function likely sensitizes prostatic epithelial cells to cell and genome damage inflicted by dietary carcinogens and inflammatory oxidants, perhaps explaining the well-documented contribution of diet and lifestyle factor to prostatic carcinogenesis *(29–31)*. Mice carrying disrupted *Gstp1/2* genes are more prone to develop skin tumors after exposure to a topical carcinogen than wild-type mice *(32)*. Provocatively, *GSTP1* CpG island hypermethylation, which is not present in normal prostatic epithelial cells (nor any other normal cells), seems to arise first in proliferative inflammatory atrophy (PIA) lesions, the earliest prostate cancer precursors, which are characterized by simultaneous inflammatory epithelial damage and regeneration *(33–36)* (Fig. 3). Epigenetic silencing of *GSTP1* expression persists in prostatic intraepithelial neoplasia lesions, later prostate cancer precursors, and in prostatic carcinomas, hinting at some sort of selective growth or survival advantage *(31,36–38)*. The recognition that DNA hypermethylation changes characteristic of prostate cancer cells first appear in PIA lesions suggests that chronic or recurrent inflammation may play some role in the *de novo* acquisition of abnormal DNA methylation patterns. Of interest in this regard is a report of interleukin 1β-triggered silencing of *FMR1* and *HPRT* in cells via a mechanism featuring nitric oxide generation *(39)*. Activated mac-

rophages, expressing high levels of the inducible form of nitric oxide synthetase, have been detected near PIA lesions in human prostate tissues (B. H. Lee, W. G. Nelson, and A. M. DeMarzo, Personal Communication).

Since the recognition that the *GSTP1* CpG island was frequently hypermethylated in prostate cancers, more than 40 genes have been reported to be targets of DNA hypermethylation-associated epigenetic gene silencing in prostate cancer cells (Table 1) *(40)*. From all the studies reported thus far, it seems likely that CpG island hypermethylation changes appear in at least two waves, first in prostate cancer precursor lesions, as the genome changes that initiate neoplastic transformation, and then later in transformed cells, as the genome changes that drive malignant progression. For example, in one case series, hypermethylation of CpG islands at *GSTP1*, *APC*, *RASSF1a*, *COX2*, and *MDR1* was present in the majority of localized prostate cancers and persistent in the majority of advanced metastatic cancers, whereas hypermethylation at the *ER a*, *hMLH1*, and *p14/INK4a* CpG islands was rare in primary cancers and more common in metastatic cancer deposits *(41)*. Finally, in a study of men with lethal prostate cancer who underwent autopsies, analysis of CpG island hypermethylation profiles from different metastatic deposits in different anatomic sites from different men revealed that the gene targets of epigenetic silencing were fivefold more variable case-to-case than site-to-site ($p < 0.0001$), providing statistical evidence that abnormal DNA methylation patterns may arise before prostate cancer cell growth and expansion at metastatic sites *(41)*.

Somatic DNA hypomethylation has also been described in prostate cancer cell DNA, but has not been studied in as great a detail, thus far, as somatic hypermethylation. An early analysis of total $^{5\text{-me}}$C base levels suggested that DNA hypomethylation might be rare in primary prostate cancers, but more common in prostate cancer metastases *(42)*. A subsequent study revealed decreased CpG dinucleotide methylation at *LINE-1* sequences in 53% of all the prostate cancer cases analyzed, with *LINE-1* hypomethylation changes present in 67% of cases with lymph node metastases, but only 8% of cases without lymph node metastases *(43)*. When DNA hypomethylation has been assessed along with CpG island hypermethylation changes at *GSTP1*, *RAR b 2*, *RASSF1a*, and *APC* in prostate cancers, the hypermethylation changes seemed likely to have preceded the hypomethylation changes, which were generally detected in cancers of higher stage and histological grade *(44)*. In addition, a provocative correlation between DNA hypomethylation and losses or gains of sequences on chromosome 8 has been described in prostate cancers, consistent with a possible contribution of decreased methylation to genetic instability *(6,45)*.

4. SENSITIVE DETECTION OF ABNORMALLY METHYLATED CPG ISLANDS AS A PROSTATE CANCER BIOMARKER

The use of serum assays for prostate-specific antigen (PSA) for prostate cancer screening has drastically changed the natural history of prostate cancer. Most men now typically diagnosed with nonpalpable, localized, prostate cancer are amenable to treatment with surgery or radiation therapy *(46)*. However, PSA screening is far from perfect. Findings of the Prostate Cancer Prevention Trial, in which men who entered the study with a “normal” serum PSA level were subjected to prostate biopsy at the end of the trial, revealed prostate cancer in 24.4% of the men treated with a placebo, with cancer in 6.6% of men who had a serum PSA of less than 0.5 ng/mL *(47,48)*. Prostate biopsies are also less than perfect. The current ultrasound-guided biopsy strategy features random sampling of prostate tissue, rather than some sort of image-directed approach to detect prostate cancer. The optimal number of random tissue samples needed for accurate detection of clinically significant prostate cancer remains controversial *(49,50)*. This tendency toward under-diagnosis, that is, missing prostate cancer when present, also seems to be accompanied by a general fear of over-diagnosis, that is, discovering and treating non-life-threatening prostate cancer and causing detrimental effects on quality of life (Fig. 4). Finally, although histological examination of prostate tissues enables accurate diagnosis of prostate cancer, distinct from other microscopic prostate abnormalities, and

Table 1
Genes Assessed for CpG Island Hypermethylation Changes in Prostate Cancer Tissues [a]

Gene	Number of cases studied	Frequency of hypermethylation (%)	Range (if more than one study)
GSTP1	1071	81.6	36–100
CDH1	367	16.6	0–100
MGMT	352	11.1	0–88
DAPK	320	13.8	0–36
p16/CDKN2a	313	16.0	0–66
RASSF1a	274	72.6	53–96
RAR b	234	68.0	53–79
APC	211	54.0	27–90
CD44	191	49.2	32–78
HIC1	182	99.5	99–100
TIMP3	182	3.3	0–6
AR	181	13.8	0–15
p14	158	3.8	0–11
EDNRB	147	65.3	49–100
PTGS2	110	65.5	22–88
Cyclin D2	101	31.7	
CDH13	101	30.7	
FHIT	101	14.9	
MDR1	73	88.0	
ESR1	73	19.2	
ER a –A	70	92.9	90–95
ER a –B	70	91.4	90–92
ER a –C	70	0.0	0
ER b	61	86.9	79–100
PR–A	70	0.0	0
PR–B	70	0.0	0
p15/CDKN2b	73	0.0	0
hMLH1	73	0.0	
VEGFR1	63	38.1	
p27	61	16.4	6–38
TIG1	50	52.0	
RB1	48	8.3	6–13
p21	48	8.3	6–13
RUNX3	37	40.5	
THBS1	37	27.0	
TNFRSF6	32	12.5	
17p	26	96.0	
ZNF185	25	44.0	
Inhibin a subunit	24	29.2	
Caveolin–1	22	90.9	
TSLC1	22	31.8	
NEP	22	14.3	
PTEN	16	0.0	
p73	16	0.0	

[a] From ref. *40*.

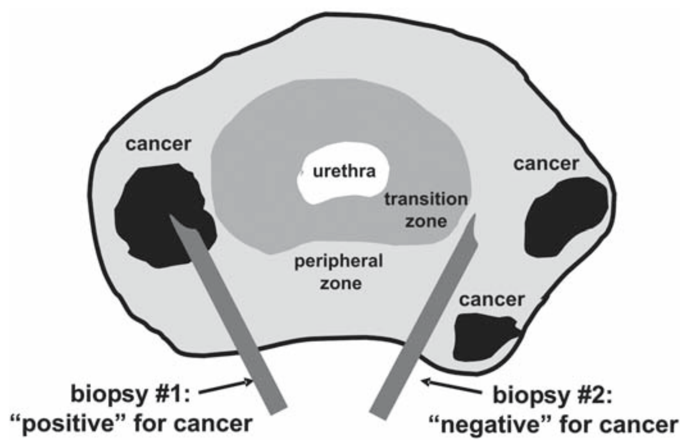


Fig. 4. Random sampling core needle biopsies of the prostate peripheral zone region can miss prostatic carcinomas. In a gland with multifocal prostate cancer, biopsy #1 detects a carcinoma lesion whereas biopsy #2 misses two carcinomas.

permits prognostic assessment of prostate cancer natural history, the use of minute core needle biopsy tissue fragments presents considerable challenges to diagnostic surgical pathologists *(35)*. For these reasons, new molecular biomarkers, capable of increasing the sensitivity and specificity of prostate cancer screening, improving the accuracy of prostate cancer diagnosis, and providing stratification for treatment recommendations, are needed.

Can somatic changes in DNA methylation patterns serve as useful prostate cancer molecular biomarkers? Clearly, with PCR technologies, nucleic acid markers are detectable with extraordinary sensitivity, often in the range of a single molecule, and, of the nucleic acids, DNA may be superior to RNA in terms of stability through specimen collection and sample handling. Thus, both genetic and epigenetic genome alterations have been pursued as molecular biomarkers for all human cancers *(51,52)*. DNA methylation changes are particularly attractive for this purpose for most human cancers, because unlike point mutations, for example, CpG island hypermethylation changes seem more consistent from case to case, permitting a single assay to be used to detect all cases *(2)*. Prostate cancer is no exception; there have not been any common point mutations in any genes yet described, however, several consistent CpG island hypermethylation changes have been reported *(40,41)*.

Currently, two major strategies for the detection of CpG dinucleotide methylation changes are most commonly used, although new strategies are under active development. The first detection method features the use of restriction endonucleases that cut recognition sites differently if the sites contain $^{5\text{-me}}$CpG. This method has been used along with Southern blot analysis and with PCR to discriminate DNA methylation changes at particular genome sites *(53)*. The second detection approach uses sodium bisulfite modification to facilitate the selective deamination of C, but not of $^{5\text{-me}}$C, to U, creating a DNA sequence difference at $^{5\text{-me}}$C vs C after PCR amplification. This approach has been used for mapping and sequencing of $^{5\text{-me}}$C at particular genome sites, and along with PCR, featuring primers specific for bisulfite-converted $^{5\text{-me}}$C-containing or C-containing sequences *(54,55)*. Each of these approaches has strengths and weaknesses as biomarker detection assays. Assays using $^{5\text{-me}}$CpG-sensitive restriction enzymes and PCR are spectacularly sensitive, capable of detecting single hypermethylated CpG island sequences, but are prone to false-positive results, attributable to inefficient cutting of unmethylated sequences. In contrast, bisulfite modification and PCR (MS-PCR) is somewhat less sensitive, because the bisulfite modification procedure can damage DNA sequence targets, but is often more specific *(40)*.

For prostate cancer, the greatest amount of attention has been afforded to *GSTP1* CpG island hypermethylation, the first alteration reported and a change present in greater than 90% of cases that have been carefully evaluated *(28,31,53)*. The somatic genome change has proven remarkably robust as a candidate molecular biomarker. Using a variety of different detection strategies, in some 24 studies with 1071 cases, *GSTP1* CpG island hypermethylation has been detected in DNA from prostate tissues in more than 81% of cases analyzed, with the sensitivity of detection varying somewhat, depending on assay *(40)*. *GSTP1* CpG island methylation changes are conspicuously absent from all normal human tissues, including normal prostatic epithelial cells isolated by laser capture microdissection, but are commonly present in prostate cancer precursor lesions, such as PIA and prostatic intraepithelial neoplasia *(33,36,37)*. Thus, assays capable of detecting *GSTP1* CpG island hypermethylation in DNA from prostate tissues have the potential of discriminating prostate cancer and its precursors from other prostate abnormalities, and of detecting neoplastic cells even when not readily evident by microscopy *(56)*. Furthermore, such assays have been found to detect prostate cancer DNA in prostatic secretions, permitting the use of urine specimens for prostate detection and diagnosis *(57–59)*.

In addition to the *GSTP1* CpG island, CpG island sequences at more than 40 other gene sites have been evaluated for hypermethylation changes in prostate cancer DNA (Table 1). By examining many CpG islands for each prostate cancer case, CpG island hypermethylation "profiles," distinct from other cancers, have emerged *(40,41)*. Using the quantitative MS-PCR technology, the detection of CpG island hypermethylation at combinations of sites, including *GSTP1*, *APC*, *RASSF1a*, *PTGS2*, and *MDR1*, has been reported to distinguish prostate cancer from noncancerous tissue with sensitivities of 97.3 to 100% and specificities of 92 to 100% *(41)*. It is likely that more somatic targets of CpG island hypermethylation will be discovered in the future; many such genes may offer new opportunities for molecular biomarkers that can be used in prostate cancer detection and diagnosis. Will some sort of DNA methylation assay become a prostate cancer screening tool? The best opportunity seems to be sensitive detection of somatic DNA methylation changes in urine, if the urine can be collected in such a way that it contains prostate secretions *(57)*.

In addition to aiding in prostate cancer detection and diagnosis, somatic DNA methylation changes may provide prognostic information. Both similarities and differences have been reported for patterns of DNA methylation in primary vs metastatic cancers *(41)*. Perhaps the somatic DNA methylation changes characteristic of metastatic prostate cancers can be detected in a fraction of primary prostate cancers at high risk for metastatic progression. Thus far, hypermethylation of CpG island sequences at *EDNRB*, *RAR*β, *RASSF1a*, *ER*β, and *TIG1* have been correlated with primary prostate cancer stage and/or grade, known prognostic markers *(41,60–63)*. Furthermore, differences in *PTGS2* CpG island hypermethylation in DNA from primary prostate cancers have been found to independently predict prostate cancer recurrence after radical prostatectomy, even when tumor grade and stage were considered *(41)* (Fig. 5). In a conceptually different approach, sensitive detection of somatic changes in DNA methylation may add great sensitivity to conventional prostate cancer staging. As described above, *GSTP1* CpG island hypermethylation changes are so common in prostate cancer cells, and so rare elsewhere, that detection of DNA with such changes in blood, lymph nodes, bone marrow, and so on, from a man known to have prostate cancer likely indicates the presence of cancer at these sites. In an example of this strategy, serum DNA showing *GSTP1* CpG island hypermethylation is more readily detected in men with known metastatic prostate cancer than without. Furthermore, for men with localized prostate cancer, the presence of prostate cancer DNA, identified by assays of *GSTP1* CpG island hypermethylation, portends prostate cancer recurrence after radical prostatectomy *(64)*.

Ultimately, the use of detection strategies for somatic DNA methylation changes can be imagined for prostate cancer screening, detection, diagnosis, prognosis, and treatment stratification. To best exploit the opportunity presented, a more complete knowledge of DNA methylation changes in prostate cancer cases, and the correlations of such changes with neoplastic transformation, with prostate

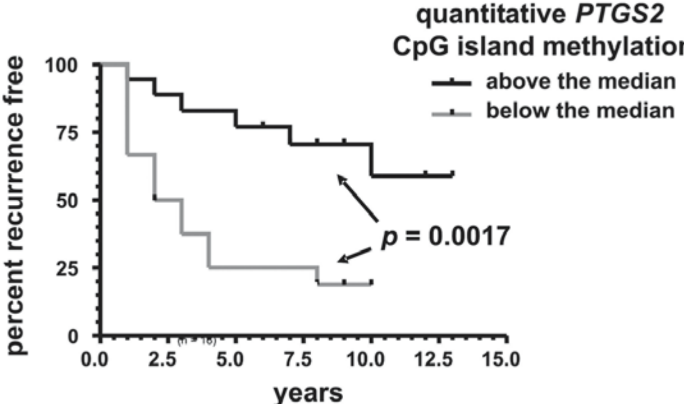


Fig. 5. *PTGS2* CpG island hypermethylation as a prognostic molecular biomarker for recurrence risk after radical prostatectomy. Quantitative MS-PCR detection of *PTGS2* CpG island hypermethylation stratifies men into low (below the median) and high (above the median) risk for prostate cancer recurrence after radical prostatectomy ($p = 0.0017$). Multivariate analyses indicated that quantitative *PTGS2* CpG island hypermethylation predicted prostate cancer recurrence independently of tumor stage and grade *(41)*.

cancer grade and stage, with malignant progression and metastasis, and with response to treatment, will need to be determined. In addition, assay strategies for detecting such changes will need to be refined. Finally, new approaches to biospecimen collection and handling, such as the recovery of prostate secretions, may also prove helpful.

5. THERAPEUTIC TARGETING OF EPIGENETIC GENE SILENCING FOR PROSTATE CANCER PREVENTION AND TREATMENT

Because the somatic changes in DNA methylation that drive epigenetic gene silencing in cancer cells, although functionally equivalent to gene deletion, do not involve irretrievable loss of DNA sequence information, such changes present an attractive "rational" therapeutic target for cancer treatment and prevention. As such, several approaches to reversal of CpG island hypermethylation-associated gene silencing have emerged. One strategy, now in late-stage clinical development, features the use of inhibitors of DNMTs, such as 5-aza-cytidine (5-aza-C) (recently approved by the US Food and Drug Administration [FDA] for the treatment of myelodysplastic disorders *[65]*), 5-aza-deoxycytidine (5-aza-dC), zebularine, procainamide, or hydralazine, to reduce $^{5\text{-m}}$CpG density at the CpG island sequences in dividing cancer cells *(66–69)* (Fig. 6). Another approach, also under active clinical scrutiny, has been the use of inhibitors of histone deacetylases (HDACs), such as sodium phenylbutyrate, valproic acid, suberoylanilide hydroxamic acid, and many others, to limit the formation of repressive chromatin conformation near the genes carrying abnormally methylated CpG islands *(70–73)*. Finally, the targeting of other CpG island methylation-associated chromatin regulators, such as the $^{5\text{-me}}$C-binding domain (MBD) family proteins, histone methyltransferases, and ATP-dependent remodeling enzymes, is in its earliest stages.

Nucleoside analog inhibitors of DNMTs, such as 5-aza-C and 5-aza-dC, have been widely used in attempts to reverse abnormal DNA methylation changes in cancer cells in vitro for the purpose of restoring "silenced" gene expression *(74)*. Unfortunately, despite some apparent successes using preclinical models and some promising results in early clinical trials, the clinical usefulness of these compounds for cancer has not yet been fully realized, even though 5-aza-C (Vidaza®, Pharmion Corporation) has gained an FDA-approved indication for myelodysplastic disorders *(65)*. In a phase II study ($n = 14$ men) of 5-aza-dC (decitabine) for androgen-independent, progressive, metastatic

Nucleoside Analog DNMT Inhibitors

5-aza-C (Vidaza®)
5-aza-dC (decitabine)
5,6-dihydro-5-aza-C
zebularine

Non-Nucleoside DNMT Inhibitors

Fig. 6. DNA methyltransferase (DNMT) inhibitors. Available DNMT inhibitors include nucleoside analogs and a non-nucleoside, procainamide.

prostate, with the drug given at a dose of 75 mg/m^2 intravenously every 8 hours for three doses, and treatment cycles repeated every 5 to 8 weeks to allow for resolution of toxicity, 2 of 12 men evaluable for response had stable disease with a time to progression of more than 10 weeks *(75)*. However, this may not be the best dose and dose-schedule for reversal of epigenetic gene silencing *(74)*. One of the most significant limitations of the nucleoside analog DNMT inhibitors in clinical trials, especially at the doses and dose-schedules used thus far, has been treatment-associated side effects, such as myelotoxicity with resultant neutropenia and thrombocytopenia, which are characteristic of other nucleoside analogs in general, including nucleoside analogs that are not DNMT inhibitors *(64)*. Another concern regarding the use of nucleoside analogs as DNMT inhibitors has been that incorporation of the nucleoside analogs into genomic DNA might lead to mutations and/or cancer development *(76)*.

Procainamide, a drug approved by the FDA for the treatment of cardiac arrhythmias, and hydralazine, a drug approved for the treatment of hypertension, are non-nucleoside analogs that both also seem to inhibit DNMT activity *(77–79)*. Procainamide may be selective for DNMT1, the enzyme most consistently associated with tumorigenesis in genetic studies in mouse models; nucleoside analogs seem to inhibit each of the DNMTs *(79)*. However, long-term use of procainamide (or of hydralazine) carries a risk of drug-induced lupus, more common in women than in men. In animal models, both 5-aza-C and procainamide seem to trigger autoimmunity, although whether or not autoimmunity is an unavoidable side effect of DNMT inhibition is not known *(78,80)*. Finally, as mentioned earlier, mice carrying one disrupted *Dnmt1* allele and one hypomorphic *Dnmt1* allele, resulting in 10% of normal DNMT activity, have been reported to exhibit genomic instability and to develop T-cell lymphomas, hinting that therapeutic reductions in $^{5\text{-m}}$CpG dinucleotides might promote the appearance of certain cancers (e.g., lymphomas) while attenuating the appearance of others (e.g., epithelial tumors; see refs. *5* and *6*). Thus, the clinical use of DNMT inhibitors is likely to be limited by both mechanism-based and mechanism-independent side effects.

Similar to DNMT inhibitors, HDAC inhibitors have also exhibited promising preclinical activity in cancer models. HDAC inhibitors under clinical development include sodium phenylbutyrate (and other butyrates), valproic acid, suberoylanilide hydroxamic acid, pyroxamide, *N*-acetyl dinaline (CI-994), and depsipeptide. However, the early clinical experience with these agents suggests that side

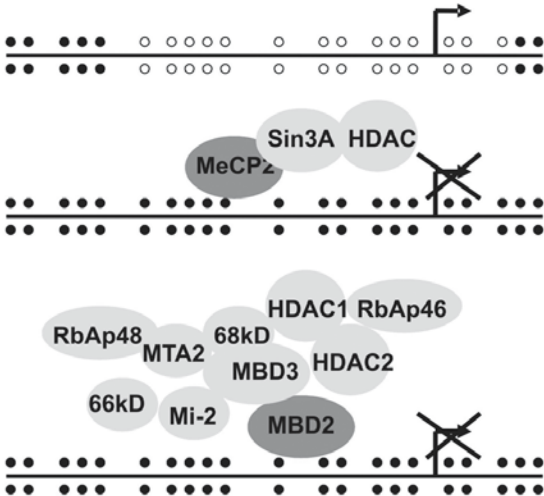


Fig. 7. Transcriptional *trans*-repression at hypermethylated CpG islands mediated by $^{5\text{-me}}$C-binding domain (MBD) proteins. MBD family proteins MeCP2 and MBD2 can recruit complexes of proteins that catalyze the heterochromatin formation at genes carrying hypermethylated CpG islands.

effects, such as nausea, vomiting, diarrhea, fatigue, edema, and so on, can occur, although severe adverse events seem rare *(70,71,73)*. In addition to DNMT inhibitors and HDAC inhibitors administered as single agents, combinations of DNMT inhibitors and HDAC inhibitors also seem to have intriguing activity in preclinical models *(24,81)*. Whether combinations of the currently available collection of DNMT inhibitors and HDAC inhibitors can reactivate silenced cancer genes, without unacceptable toxicity, in human clinical trials against prostate cancer, has not yet been determined.

Two MBD family proteins have been implicated in the silencing of critical genes in cancer cells carrying abnormally hypermethylated CpG island sequences, and may define pathways amenable to targeting for reversal of epigenetic gene silencing (Fig. 7). One of the MBD family proteins, MeCP2, contains an approx 70-amino acid minimal region that mediates selective binding to DNA containing $^{5\text{-m}}$CpG (an MBD motif), and a transcriptional repression domain that permits interaction with the transcriptional repressor Sin3 and associated HDACs *(82)*. MeCP2 can, thus, act as a CpG island hypermethylation-dependent transcriptional repressor by binding transcriptional regulatory sequences carrying $^{5\text{-m}}$CpG and recruiting HDACs. For this reason, MeCP2-mediated inhibition of $^{5\text{-m}}$CpG-containing promoter activity can usually be alleviated by treatment with trichostatin A, an inhibitor of HDACs *(82)*. Another MBD family protein, MBD2, which can also bind selectively to DNA containing $^{5\text{-m}}$CpG, has been found to be a component of a mega dalton (MD) transcription repression complex, MeCP1, that also contains the Mi-2/NuRD chromatin remodeling complex subunits MBD3, HDAC1, and HDAC2, the histone-binding proteins RbAp46 and RbAp48, the SWI/SNF helicase/ATPase domain-containing protein Mi2, MTA2, and two uncharacterized polypeptides of 66- and 68-kDa *(83)*. Although present in the Mi-2/NuRD complex, the MBD family protein MBD3 does not seem to recognize $^{5\text{-m}}$CpG-containing DNA. As a result, in the absence of MBD2, Mi-2/NuRD complexes, capable of catalyzing ATP-dependent chromatin remodeling, are incapable of selectively binding hypermethylated transcriptional regulatory sequences. In the MeCP1 complex, MBD2 acts to recruit the Mi-2/NuRD chromatin remodeling complex to $^{5\text{-m}}$CpG-containing DNA *(83)*. Of interest, although MeCP2-mediated transcriptional repression can typically be alleviated by treatment with HDAC inhibitors, MeCP1-mediated inhibition of $^{5\text{-m}}$CpG-containing promoter activity is often not affected by HDAC inhibitor exposure.

MBD family proteins have not yet been systematically targeted for the reactivation of critical genes in cancer cells that have been silenced by CpG island hypermethylation. However, MBD family proteins, particularly MBD2, make attractive therapeutic targets for two key reasons. First, the anticipated efficacy of drugs that interfere with MBD family protein activity is provocative, and second, the likely side effects may be minimal. As for efficacy, MBD2 is a validated target for new drugs. MBD2 selectively binds the *GSTP1* CpG island when it is methylated, as occurs ubiquitously and early during prostatic carcinogenesis, and siRNA-mediated reduction in MBD2 levels activates *GSTP1* expression despite CpG island hypermethylation *(31,84,85)*. Similarly, cells from $Mbd2^{-/-}$ mice are unable to repress transcription from exogenously hypermethylated promoters in transient transfection assays *(86)*. Also, $Apc^{Min/+}Mbd2^{-/-}$ mice develop far fewer intestinal adenomas, and survive longer, than do $Apc^{Min/+}Mbd^{2+/-}$ or $Apc^{Min/+}Mbd2^{+/+}$ mice *(87)*. As for toxicity, other than a maternal behavior defect, the $Mbd2^{-/-}$ mice seem fairly unremarkable, and have maintained normal gene imprinting, repression of endogenous retroviral sequences, and no obvious ectopic gene expression *(86)*. In contrast, $Dnmt1^{-/-}$, $Dnmt3a^{-/-}$, and $Dnmt3b^{-/-}$ mice are not viable. Furthermore, unlike mice with defective *Dnmt1* genes, $Mbd2^{-/-}$ mice do not seem prone to increased lymphomagenesis when crossed to mice carrying disrupted *p53* genes *(88)*. These observations hint that drugs targeted at MBD2 might be able to reactivate epigenetically silenced genes in cancer cells, or in precancerous lesions, with a significant margin of safety. For this reason, chemical biology approaches, featuring high-throughput screening of chemical libraries, have been applied to the search for such compounds (X. Lin, Z. Reichardt, and W. G. Nelson, Personel Communication). Provocatively, antisense inhibitors directed at *MBD2* mRNA have been reported to attenuate human cancer cell growth in vitro and in vivo *(89,90)*.

To fully exploit the therapeutic opportunities presented by targeting epigenetic gene silencing in prostate cancer, both new drugs and new clinical trial strategies for developing and evaluating such treatments will be needed. Similar to other rational "targeted" treatments for human cancers, the expectation is that epigenetic modulation might be accomplished with chronic or prolonged treatments that have minimal side effects. If used to prevent cancers, the safety demands of such treatments will be even greater. For these reasons, early clinical studies will seek to ascertain the "optimal biologic dose" presumably a dose that restores the expression and function of epigenetically silenced genes, rather than the "maximally tolerated dose." Also, because combinations of inhibitors (e.g., DNMT inhibitor plus HDAC inhibitor) may be more effective at reactivating silenced genes than individual inhibitors, the design of clinical trials needed to establish dose and dose-schedule may prove very complex. Almost certainly, the new technologies permitting sensitive detection of epigenetically silenced genes, such as MS-PCR, will be required as pharmacodynamic end points for such clinical development strategies.

6. SUMMARY AND CONCLUSIONS

Somatic changes in DNA methylation patterns seem to be the most common acquired genome alterations in prostate cancer cells, leading to epigenetic defects in critical gene function. The origin of these changes is not yet known, although likely involves some dysfunction or dysregulation of DNMT activity early during prostatic carcinogenesis. Sensitive detection of abnormally methylated DNA sequences has great promise for improving prostate cancer screening, detection, diagnosis, and assessment of prognosis. Therapeutic reversal of DNA methylation, and/or the associated gene silencing, has potential for prostate cancer prevention and treatment.

REFERENCES

1. Hanahan, D. and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100**, 57–70.
2. Herman, J. G. and Baylin, S. B. (2003). Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* **349**, 2042–2054.

3. Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. *Nature* **321**, 209–213.
4. Feinberg, A. P. and Tycko, B. (2004). The history of cancer epigenetics. *Nat. Rev. Cancer* **4**, 143–153.
5. Gaudet, F., Hodgson, J. G., Eden, A., et al. (2003). Induction of tumors in mice by genomic hypomethylation. *Science* **300**, 489–492.
6. Eden, A., Gaudet, F., Waghmare, A., and Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* **300**, 455.
7. Vertino, P. M., Yen, R. W., Gao, J., and Baylin, S. B. (1996). De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase. *Mol. Cell Biol.* **16**, 4555–4565.
8. Di Croce, L., Raker, V. A., Corsaro, M., et al. (2002). Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* **295**, 1079–1082.
9. Kawasaki, H. and Taira, K. (2004). Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. *Nature* **431**, 211–217.
10. Morris, K. V., Chan, S. W., Jacobsen, S. E., and Looney, D. J. (2004). Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**, 1289–1292.
11. Holst, C. R., Nuovo, G. J., Esteller, M., et al. (2003). Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. *Cancer Res.* **63**, 1596–1601.
12. David, G. L., Yegnasubramanian, S., Kumar, A., et al. (2004). MDR1 promoter hypermethylation in MCF-7 human breast cancer cells: changes in chromatin structure induced by treatment with 5-Aza-cytidine. *Cancer Biol. Ther.* **3**, 540–548.
13. Myohanen, S. K., Baylin, S. B., and Herman, J. G. (1998). Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. *Cancer Res.* **58**, 591–593.
14. Jeltsch, A. (2002). Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. *Chembiochem.* **3**, 274–293.
15. Leonhardt, H., Page, A. W., Weier, H. U., and Bestor, T. H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71**, 865–873.
16. Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**, 247–257.
17. Rhee, I., Jair, K. W., Yen, R. W., et al. (2000). CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature* **404**, 1003–1007.
18. Rhee, I., Bachman, K. E., Park, B. H., et al. (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* **416**, 552–556.
19. Graff, J. R., Herman, J. G., Myohanen, S., Baylin, S. B., and Vertino, P. M. (1997). Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. *J. Biol. Chem.* **272**, 22,322–22,329.
20. Feltus, F. A., Lee, E. K., Costello, J. F., Plass, C., and Vertino, P. M. (2003). Predicting aberrant CpG island methylation. *Proc. Natl. Acad. Sci. USA* **100**, 12,253–12,258.
21. Bakin, A. V. and Curran, T. (1999). Role of DNA 5-methylcytosine transferase in cell transformation by fos. *Science* **283**, 387–390.
22. Laird, P. W., Jackson-Grusby, L., Fazeli, A., et al. (1995). Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* **81**, 197–205.
23. Eads, C. A., Nickel, A. E., and Laird, P. W. (2002). Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. *Cancer Res* **62**, 1296–1299.
24. Belinsky, S. A., Klinge, D. M., Stidley, C. A., et al. (2003). Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res.* **63**, 7089–7093.
25. Eads, C. A., Danenberg, K. D., Kawakami, K., Saltz, L. B., Danenberg, P. V., and Laird, P. W. (1999). CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res.* **59**, 2302–2306.
26. Agoston, A. T., Argani, P., Yegnasubramanian, S., et al. (2005). Increased protein stability causes DNA methyltransferase 1 dysregulation in breast cancer. *J. Biol. Chem.* **280**, 18,302–18,310.
27. De Marzo, A. M., Marchi, V. L., Yang, E. S., Veeraswamy, R., Lin, X., and Nelson, W. G. (1999). Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. *Cancer Res.* **59**, 3855–3860.
28. Lee, W. H., Morton, R. A., Epstein, J. I., et al. (1994). Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc. Natl. Acad. Sci. USA* **91**, 11,733–11,737.
29. Nelson, W. G., De Marzo, A. M., and Isaacs, W. B. (2003). Prostate cancer. *N. Engl. J. Med.* **349**, 366–381.
30. Nelson, C. P., Kidd, L. C., Sauvageot, J., et al. (2001). Protection against 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- b]pyridine cytotoxicity and DNA adduct formation in human prostate by glutathione S-transferase P1. *Cancer Res.* **61**, 103–109.
31. Lin, X., Tascilar, M., Lee, W. H., et al. (2001). GSTP1 CpG island hypermethylation is responsible for the absence of GSTP1 expression in human prostate cancer cells. *Am. J. Pathol.* **159**, 1815–1826.

32. Henderson, C. J., Smith, A. G., Ure, J., Brown, K., Bacon, E. J., and Wolf, C. R. (1998). Increased skin tumorigenesis in mice lacking pi class glutathione S- transferases. *Proc. Natl. Acad. Sci. USA* **95**, 5275–5280.
33. Millar, D. S., Paul, C. L., Molloy, P. L., and Clark, S. J. (2000). A distinct sequence (ATAAA)n separates methylated and unmethylated domains at the 5'-end of the GSTP1 CpG island. *J. Biol. Chem.* **275**, 24,893–24,899.
34. De Marzo, A. M., Marchi, V. L., Epstein, J. I., and Nelson, W. G. (1999). Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. *Am. J. Pathol.* **155**, 1985–1992.
35. DeMarzo, A. M., Nelson, W. G., Isaacs, W. B., and Epstein, J. I. (2003). Pathological and molecular aspects of prostate cancer. *Lancet* **361**, 955–964.
36. Nakayama, M., Bennett, C. J., Hicks, J. L., et al. (2003). Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am. J. Pathol.* **163**, 923–933.
37. Brooks, J. D., Weinstein, M., Lin, X., et al. (1998). CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. *Cancer Epidemiol. Biomarkers Prev.* **7**, 531–536.
38. DeWeese, T. L. and Nelson, W. G. (2003). Inadequate "caretaker" gene function and human cancer development. *Methods Mol. Biol.* **222**, 249–268.
39. Hmadcha, A., Bedoya, F. J., Sobrino, F., and Pintado, E. (1999). Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. *J. Exp. Med.* **190**, 1595–1604.
40. Bastian, P. J., Yegnasubramanian, S., Palapattu, G. S., et al. (2004). Molecular biomarker in prostate cancer: the role of CpG island hypermethylation. *Eur. Urol.* **46**, 698–708.
41. Yegnasubramanian, S., Kowalski, J., Gonzalgo, M. L., et al. (2004). Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res.* **64**, 1975–1986.
42. Bedford, M. T. and van Helden, P. D. (1987). Hypomethylation of DNA in pathological conditions of the human prostate. *Cancer Res.* **47**, 5274–5276.
43. Santourlidis, S., Florl, A., Ackermann, R., Wirtz, H. C., and Schulz, W. A. (1999). High frequency of alterations in DNA methylation in adenocarcinoma of the prostate. *Prostate* **39**, 166–174.
44. Florl, A. R., Steinhoff, C., Muller, M., et al. (2004). Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. *Br. J. Cancer* **91**, 985–994.
45. Schulz, W. A., Elo, J. P., Florl, A. R., et al. (2002). Genomewide DNA hypomethylation is associated with alterations on chromosome 8 in prostate carcinoma. *Genes Chromosomes Cancer* **35**, 58–65.
46. Soh, S., Kattan, M. W., Berkman, S., Wheeler, T. M., and Scardino, P. T. (1997). Has there been a recent shift in the pathological features and prognosis of patients treated with radical prostatectomy? *J. Urol.* **157**, 2212–2218.
47. Thompson, I. M., Pauler, D. K., Goodman, P. J., et al. (2004). Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *N. Engl. J. Med.* **350**, 2239–2246.
48. Thompson, I. M., Goodman, P. J., Tangen, C. M., et al. (2003). The influence of finasteride on the development of prostate cancer. *N. Engl. J. Med.* **349**, 215–224.
49. Makhlouf, A. A., Krupski, T. L., Kunkle, D., and Theodorescu, D. (2004). The effect of sampling more cores on the predictive accuracy of pathological grade and tumour distribution in the prostate biopsy. *BJU Int.* **93**, 271–274.
50. de la Taille, A., Antiphon, P., Salomon, L., et al. (2003). Prospective evaluation of a 21-sample needle biopsy procedure designed to improve the prostate cancer detection rate. *Urology* **61**, 1181–1186.
51. Sidransky, D. (2002). Emerging molecular markers of cancer. *Nat. Rev. Cancer* **2**, 210–219.
52. Laird, P. W. (2003). The power and the promise of DNA methylation markers. *Nat. Rev. Cancer* **3**, 253–266.
53. Lee, W. H., Isaacs, W. B., Bova, G. S., and Nelson, W. G. (1997). CG island methylation changes near the GSTP1 gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. *Cancer Epidemiol. Biomarkers Prev.* **6**, 443–450.
54. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* **22**, 2990–2997.
55. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* **93**, 9821–9826.
56. Harden, S. V., Sanderson, H., Goodman, S. N., et al. (2003). Quantitative GSTP1 methylation and the detection of prostate adenocarcinoma in sextant biopsies. *J. Natl. Cancer Inst.* **95**, 1634–1637.
57. Gonzalgo, M. L., Nakayama, M., Lee, S. M., De Marzo, A. M., and Nelson, W. G. (2004). Detection of GSTP1 methylation in prostatic secretions using combinatorial MSP analysis. *Urology* **63**, 414–418.
58. Goessl, C., Muller, M., Heicappell, R., Krause, H., and Miller, K. (2001). DNA-based detection of prostate cancer in blood, urine, and ejaculates. *Ann. NY Acad. Sci.* **945**, 51–58.
59. Gonzalgo, M. L., Pavlovich, C. P., Lee, S. M., and Nelson, W. G. (2003). Prostate cancer detection by GSTP1 methylation analysis of postbiopsy urine specimens. *Clin. Cancer Res.* **9**, 2673–2677.
60. Jeronimo, C., Henrique, R., Hoque, M. O., et al. (2004). Quantitative RARbeta2 hypermethylation: a promising prostate cancer marker. *Clin. Cancer Res.* **10**, 4010–4014.
61. Liu, L., Yoon, J. H., Dammann, R., and Pfeifer, G. P. (2002). Frequent hypermethylation of the RASSF1A gene in prostate cancer. *Oncogene* **21**, 6835–6840.

62. Zhang, J., Liu, L., and Pfeifer, G. P. (2004). Methylation of the retinoid response gene TIG1 in prostate cancer correlates with methylation of the retinoic acid receptor beta gene. *Oncogene* **23**, 2241–2249.
63. Zhu, X., Leav, I., Leung, Y. K., et al. (2004). Dynamic regulation of estrogen receptor-beta expression by DNA methylation during prostate cancer development and metastasis. *Am. J. Pathol.* **164**, 2003–2012.
64. Bastian, P. J., Palapattu, G. S., Lin, X., et al. (2005). Preoperative serum DNA GSTP1 CpG island hypermethylation and the risk of early prostate-specific antigen recurrence following radical prostatectomy. *Clin. Cancer Res.* **11**, 4037–4043.
65. Kaminskas, E., Farrell, A., Abraham, S., et al. (2005). Approval summary: azacitidine for treatment of myelodysplastic syndrome subtypes. *Clin. Cancer Res.* **11**, 3604–3608.
66. Jones, P. A. and Taylor, S. M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**, 85–93.
67. Cheng, J. C., Matsen, C. B., Gonzales, F. A., et al. (2003). Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J. Natl. Cancer Inst.* **95**, 399–409.
68. Lin, X., Asgari, K., Putzi, M. J., et al. (2001). Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. *Cancer Res.* **61**, 8611–8616.
69. Segura-Pacheco, B., Trejo-Becerril, C., Perez-Cardenas, E., et al. (2003). Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. *Clin. Cancer Res.* **9**, 1596–1603.
70. Kelly, W. K., Richon, V. M., O'Connor, O., et al. (2003). Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. *Clin. Cancer Res.* **9**, 3578–3588.
71. Carducci, M. A., Gilbert, J., Bowling, M. K., et al. (2001). A phase I clinical and pharmacological evaluation of sodium phenylbutyrate on an 120-h infusion schedule. *Clin. Cancer Res.* **7**, 3047–3055.
72. Carducci, M. A., Nelson, J. B., Chan-Tack, K. M., et al. (1996). Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clin. Cancer Res.* **2**, 379–387.
73. Gilbert, J., Baker, S. D., Bowling, M. K., et al. (2001). A phase I dose escalation and bioavailability study of oral sodium phenylbutyrate in patients with refractory solid tumor malignancies. *Clin. Cancer Res.* **7**, 2292–2300.
74. Santini, V., Kantarjian, H. M., and Issa, J. P. (2001). Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. *Ann. Intern. Med.* **134**, 573–586.
75. Thibault, A., Figg, W. D., Bergan, R. C., et al. (1998). A phase II study of 5-aza-2'deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori* 84, 87–89.
76. Jackson-Grusby, L., Laird, P. W., Magge, S. N., Moeller, B. J., and Jaenisch, R. (1997). Mutagenicity of 5-aza-2'-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc. Natl. Acad. Sci. USA* **94**, 4681–4685.
77. Scheinbart, L. S., Johnson, M. A., Gross, L. A., Edelstein, S. R., and Richardson, B. C. (1991). Procainamide inhibits DNA methyltransferase in a human T cell line. *J. Rheumatol.* **18**, 530–534.
78. Cornacchia, E., Golbus, J., Maybaum, J., Strahler, J., Hanash, S., and Richardson, B. (1988). Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *J. Immunol.* **140**, 2197–2200.
79. Lee, B. H., Yegnasubramanian, S., and Nelson, W. G. (2005). Procainamide is a specific inhibitor of DNA methyltransferase 1. *J. Biol. Chem.* **280**, 40,749–40,756
80. Quddus, J., Johnson, K. J., Gavalchin, J., et al. (1993). Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice. *J. Clin. Invest.* **92**, 38–53.
81. Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.* **21**, 103–107.
82. Jones, P. L., Veenstra, G. J., Wade, P. A., et al. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* **19**, 187–191.
83. Feng, Q. and Zhang, Y. (2001). The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. *Genes Dev.* **15**, 827–832.
84. Bakker, J., Lin, X., and Nelson, W. G. (2002). Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells. *J. Biol. Chem.* **277**, 22,573–22,580.
85. Lin, X. and Nelson, W. G. (2003). Methyl-CpG-binding domain protein-2 mediates transcriptional repression associated with hypermethylated GSTP1 CpG islands in MCF-7 breast cancer cells. *Cancer Res.* **63**, 498–504.
86. Hendrich, B., Guy, J., Ramsahoye, B., Wilson, V. A., and Bird, A. (2001). Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. *Genes Dev.* **15**, 710–723.
87. Sansom, O. J., Berger, J., Bishop, S. M., Hendrich, B., Bird, A., and Clarke, A. R. (2003). Deficiency of Mbd2 suppresses intestinal tumorigenesis. *Nat. Genet.* **34**, 145–147.
88. Sansom, O. J., Bishop, S. M., Bird, A., and Clarke, A. R. (2005). MBD2 deficiency does not accelerate p53 mediated lymphomagenesis. *Oncogene* **24**, 2430–2432.
89. Campbell, P. M., Bovenzi, V., and Szyf, M. (2004). Methylated DNA-binding protein 2 antisense inhibitors suppress tumourigenesis of human cancer cell lines in vitro and in vivo. *Carcinogenesis* **25**, 499–507.
90. Slack, A., Bovenzi, V., Bigey, P., et al. (2002). Antisense MBD2 gene therapy inhibits tumorigenesis. *J. Gene Med.* **4**, 381–389.

18

Telomeres, Telomerase, Chromosome Stability, and Prostate Cancer

Alan K. Meeker

Summary

Prostate cancer exhibits diverse clinical behavior, a phenotypic reflection of the heterogeneous genetic and epigenetic aberrations underlying this disease. Recent work indicates that short, dysfunctional telomeres may be responsible for much of this genetic damage. Telomeres are essential DNA elements located at the ends of each chromosome. Telomeres shorten because of cell division and damage from reactive oxygen species, such as those elaborated by the inflammatory response. Recent studies indicate that abnormal telomere shortening occurs in the earliest phase of prostate tumorigenesis, at the pre-invasive prostatic intraepithelial neoplasia (PIN) stage, and that telomeres remain short in the majority of prostate adenocarcinomas. Thus, it is likely that telomere dysfunction plays a key role in both the initiation and progression of prostate cancer and evidence indicates that telomere length may have prognostic value.

Unlike normal prostate cells, prostate cancer cells activate the telomere maintenance enzyme telomerase, providing at least a minimal amount of stability to the shortened chromosomal ends. However, telomerase activity is subject to hormonal regulation, being downregulated during hormonal withdrawal; therefore, during androgen ablation therapy, the already shortened telomeres in prostate cancer cells may once again become unstable, leading to further genetic changes contributing to the development of androgen-independent disease. Apart from androgens, telomerase is also regulated in the prostate by the oncogene *c-Myc*, a gene frequently deregulated in prostate cancer.

The dependence of prostate cancer on telomerase activity makes this enzyme an attractive therapeutic target, and several strategies aimed at exploiting this dependence are currently under development.

In summary, short dysfunctional telomeres are likely to be major players in prostate cancer initiation and progression, as well as the development of hormone-refractory disease after androgen ablation. Measurement of telomere lengths or telomerase activity may provide valuable diagnostic and/or prognostic information. It is hoped that a thorough understanding of prostate cancer telomere biology will lead to effective strategies for disease prevention and treatment.

Key Words: Chromosome stability; prostate cancer; telomerase; telomeres.

1. INTRODUCTION

Prostate cancers exhibit marked genetic, morphological, and behavioral heterogeneity, likely a reflection of an underlying genetic instability. Similar to other cancers, prostate cancer is, in large part, a problem of inappropriate, unrestrained, and unbalanced cell proliferation and displays a strong positive association with increasing age. A common thread linking genetic instability, cellular proliferation, and aging, is telomere biology. This chapter outlines the basics of what we currently know regarding telomeres, the telomere maintenance enzyme telomerase, and the potential roles they may play in prostate cancer.

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

Both genetics and poorly characterized environmental factors have been broadly implicated in prostate cancer causation *(1–8)*. However, there are only three key risk factors currently known for prostate cancer: a positive family history of the disease, African ancestry, and increasing age *(6)*. With respect to age, the populations of most industrialized nations are aging at an accelerating pace. Prostate cancer will likely remain a significant clinical problem for the foreseeable future. Despite decades of research, a detailed understanding of the precise cellular and molecular mechanisms under lying prostate carcinogenesis is still lacking. Understanding of the disease at this level of detail is needed to facilitate the development of improved techniques for assessing an individual's risk of developing prostate cancer, as well as the design of effective preventive and therapeutic interventions.

2. PROSTATE CANCER IS A HETEROGENEOUS DISEASE DRIVEN BY GENETIC INSTABILITY

Prostate cancer shows remarkable variability at the genetic and histological levels, as well as in its clinical course *(9–11)*. This heterogeneity is likely a manifestation of the numerous chromosomal changes that typify prostate cancer cells; a reflection of ongoing or episodic genetic instability.

It is widely accepted that cancer is, to a large extent, a disease driven by the accumulation of multiple somatic alterations in the expression and/or sequence of cancer-associated genes (oncogenes and tumor suppressor genes) *(12)*. Phenotypically, cancer is largely a disorder of improper and unbalanced cellular proliferation. Thus, it is not surprising that many oncogenes and tumor suppressor genes are members of growth factor signaling pathways that positively or negatively affect cell division *(13–16)*. Unlike several other cancers in which one or a few of these key genes are frequently lost or mutated, no such predominant patterns have emerged for prostate cancer. Rather, analyses of prostate cancers have revealed a plethora of genetic changes across the entire genome *(11,17–24)*. In addition, the number of genetic changes increases with disease stage, implying a link between genetic instability and disease progression *(25)*.

The process of DNA replication in humans is incredibly accurate, representing an important line of defense against the development of cancer-causing mutations. Loeb has argued persuasively that an increase over this extremely low baseline mutation rate (a "mutator phenotype") is needed for accrual of sufficient mutations to bring about malignant transformation *(26)*. In support of this, numerous syndromes exist in which inherited mutations in genes whose protein products are involved in maintaining genomic stability result in an increased cancer risk *(27)*. Well-known examples include defects in the *BRCA1* and *BRCA2* genes associated with hereditary breast and ovarian cancers. These same genes have also been found altered in subsets of sporadic cancer cases *(28)*. To date, however, defective DNA maintenance and repair genes do not seem to be major contributors to the development of prostate cancer, thus, leaving open the question of the source of the observed heterogeneous genetic changes observed in prostate cancer.

3. GENETIC INSTABILITY IN HUMAN CANCERS

Two general types of genetic instability have been described in human tumors *(29)*. The first type, microsatellite instability, manifests as simple DNA sequence changes, resulting from defects in genes such as *hMLH1* or *hMSH2* that function in DNA mismatch repair. This type of genetic instability seems to play a role in only a small fraction of prostate cancer cases.

The second type of genetic instability is chromosomal instability (CIN), encompassing both numerical and structural chromosomal abnormalities. In contrast to microsatellite instability, structural chromosomal aberrations are nearly universal features of prostate carcinomas. Methods such as fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization have revealed evidence for both losses and gains of chromosomal material in prostate cancer cells. Such abnormalities are detectable in the earliest histologically identifiable stage of prostate tumorigenesis—prostatic intraepithelial neoplasia (PIN)—supporting an early role for genetic lesions in the development of prostate cancer *(19,30–32)*. Although alterations in chromosome number may arise via defects in

• • • TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG
AATCCCAATCCCAATCCCAATCCCAATCCCAATCCCAATCCCAATCC

• • • TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG
AATCCCAATCCCAATCCCAATCCCAATCCCAATCCCAAT

• • • TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTA
AATCCCAATCCCAATCCCAATCCCAATCCCA

• • • TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTA
AATCCCAATCCCAATCCCAATC

• • • TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTT
AATCCCAATCCCAAT

• • • TTAGGGTTAGGGTTAGGGTTAGGGTTAGG
AATCCCAA

• • • TTAGGGTTAGGGTTAGGGTT
AATCC

Cell division
Oxidative stress

Fig. 1. Telomeres shorten as a result of cell division and oxidative stress. In the absence of compensatory mechanisms, telomeres shorten with each cell division and/or because of unrepaired oxidative DNA damage. Note that the G-rich single-strand overhang is, in reality, much longer (approx 35–600 nucleotides), and that telomere losses are larger in magnitude than depicted here in this conceptual diagram (approx 100 base pairs lost per cell division, hundreds of base pairs lost for oxidative damage).

centrosomes or the mitotic spindle checkpoint, both of which have been implicated in prostate cancer, very little information exists regarding the origins of structural chromosomal abnormalities *(33,34)*.

Because CIN, in particular, seems to be a major contributor to prostate tumorigenesis, and appears early in the process, it is important to identify the defects responsible for CIN. Filling this major gap in our understanding of the etiology of prostate cancer will allow the design of new strategies for cancer treatment and prevention. An attractive candidate for the instigator of CIN in prostate cancer is telomere dysfunction.

4. THE LINK BETWEEN TELOMERES AND CIN

Telomeres are essential structures composed of specialized terminal DNA sequence repeats complexed with telomere-binding proteins, located at the ends of every human chromosome *(35)*. These structures stabilize and protect the ends of the chromosomes. Telomeric DNA is noncoding and consists of tandem repeats of the hexanucleotide sequence, TTAGGG *(36,37)*. The lengths of telomeric repeats vary from cell to cell and chromosome to chromosome, typically ranging from approx 1000 to 2000 repeats (i.e., 6–12 kb) of telomeric DNA per chromosome. To function properly, telomeres must be of sufficient length and possess the proper repertoire and amounts of telomere-associated binding proteins. Mutation or loss of either can lead to dysfunctional or "uncapped" telomeres, which destabilize the chromosomes *(38,39)*.

Importantly, telomeric DNA is dynamic. Telomeres shorten by approx 100 base pairs each time a cell divides because of the inability of DNA polymerases to completely replicate terminal DNA sequences during S-phase *(40,41)*. Thus, telomere length can serve as a proxy for the number of times a cell has divided *(42,43)*. Telomere shortening may also occur because of unrepaired single-strand breaks caused by oxidative damage to telomeric DNA (Fig. 1) *(44,45)*. Conversely, telomeres may be lengthened through the action of the telomere synthetic enzyme telomerase or, uncommonly, via a poorly characterized telomerase-independent genetic recombination mechanism *(46,47)*.

The requirement for a minimal amount of telomeric DNA to maintain telomere function likely reflects the need for sufficient binding sites for telomere-binding proteins to form a stable telomeric complex. Short telomeres become dysfunctional, leading to the formation of fused, dicentric chromosomes that mis-segregate or break during mitosis. Newly created chromosomal breaks may themselves fuse, thus, perpetuating a cycle of chromosome fusion and breakage *(48,49)*. It is in this way that critically short telomeres initiate CIN *(50–52)*. Numerous studies support the link between telomere dysfunction and CIN in human cancers. For example, in a study of head and neck tumors, Gisselsson et al. found correlations between chromosomes bearing severely short telomeres, chromosomal fusions, rearrangements, anaphase bridges, and multipolar mitoses *(53)*.

5. TELOMERE SHORTENING NORMALLY TRIGGERS TUMOR SUPPRESSIVE CELLULAR RESPONSES

Normally, cells that experience moderate telomere shortening enter an irreversible cell cycle arrest, termed replicative senescence, or initiate programmed cell death, responses to telomere shortening that have evolved as tumor suppressive barriers against abnormal clonal expansion and the development of excessive telomere shortening that would accompany the ensuing cell division *(54)*. Thus, progressive telomere shortening acts as a "mitotic clock," counting down cell divisions and signaling cell cycle exit once one or more telomeres reaches a threshold length *(40)*. As predicted, forced expression of the enzyme telomerase (which counteracts telomere shortening) in pre-senescent cells can prevent replicative senescence and endow the cells with unlimited replicative potential or "immortalization" *(55,56)*. In normal somatic human cells, telomerase activity is repressed, thus, telomere length will decrease in proliferating cells and can therefore be used as a signal to exit the cell cycle. Although the precise mechanism(s) by which short telomeres trigger senescence and apoptosis are still being worked out, evidence to date implicates the tumor suppressor p53 in the response to shortened telomeres that are perhaps recognized as a form of DNA damage. There is also evidence for a p53-independent response to dysfunctional telomeres involving the cell cycle inhibitory tumor suppressor protein, p16 *(57)*. Importantly, abrogation of these checkpoints allows continued cell division and, in the absence of telomerase, eventual severe telomere shortening, beyond the minimum threshold length required for proper telomere function, therefore, causing telomere uncapping and chromosomal destabilization *(58)*.

6. PROSTATE CANCER CELLS POSSESS ABNORMALLY SHORT TELOMERES

The majority of human cancer tissues and cancer-derived cell lines examined to date have been found to contain abnormally short telomeres *(42,59–61)*. One interpretation of this fact is that cancers arise from progenitor cells lacking the means to compensate for the telomere loss that accompanies cell division. Another, although not mutually exclusive, interpretation is that incipient tumor cells experience, or are more susceptible to, oxidative stress that leads to telomere loss.

Similar to other cancers, prostate adenocarcinomas have shorter than normal telomeres. Using a Southern blot technique for bulk telomere length assessment, Sommerfeld et al. observed telomere shortening in primary prostate cancers when compared with matched adjacent or nearby normal-appearing and benign proliferative (BPH) areas in radical prostatectomy specimens *(62)*. Using the same technique, Koeneman et al. found prostate cancers to have an average telomere length 2 kb shorter than normal-appearing prostate tissues, in excellent agreement with the values obtained by Sommerfeld et al. *(63)*.

Recently, a new FISH technique for direct telomere length assessment in archival formalin-fixed tissue samples was developed *(64)*. In this method, the intensity of each telomeric FISH signal is linearly related to that telomere's length. Because tissue architecture is maintained and single-cell resolution is achieved, confounding contributions from the multiple intermingled cell types present in such tissue samples is avoided. In addition, cancer cells can be directly compared with adjacent or

nearby normal epithelial cells. In agreement with the previous telomere length analyses, this method revealed shorter telomeres in primary prostate cancer cells, when compared with normal epithelial cells, in 15 (88%) of 17 cases *(64)*. This analysis is currently being extended to include more primary tumors as well as local and distant metastatic lesions. To date, 86% of 50 primary tumors examined contained exclusively or predominately shorter-than-normal telomeres, whereas only 6% had telomeres in the normal range. In no case was abnormal telomere *lengthening* observed in these primary tumors. Similarly, 84% of local metastases (pelvic lymph nodes) displayed telomere shortening, whereas a small subset (5%) displayed normal or longer-than-normal telomeres, and 11% exhibited notable telomere length heterogeneity. A smaller proportion of distant metastases, 57% (56 of 98 metastases from 28 cases), had short telomeres. However, in 11 cases in which distant metastases could be matched to their respective primary tumors, the metastases typically had retained the telomere length phenotype of the corresponding primary tumor or had undergone even further telomere shortening. As with local metastases, abnormal telomere lengthening was only observed in a small subset (6%) of distant metastases (A. K. Meeker, unpublished data).

In summary, the vast majority of primary prostate cancers possess abnormally short telomeres, supporting the hypothesis that telomere shortening is a contributory factor to prostate carcinogenesis. Although small minorities of both local and distant metastases display elongated telomeres, abnormal telomere lengthening is not a requirement for either carcinogenesis or progression to advanced disease.

7. POTENTIAL PROGNOSTIC VALUE OF TELOMERE LENGTHS IN PROSTATE CANCER

Telomere shortening has been found to be predictive of poor prognosis in several cancers, including those of the lung, endometrium, breast, and neuroblastoma *(65–68)*. A potential link between telomere length and prostate cancer prognosis was first reported by Donaldson et al., using a slot blot method devised by Bryant et al., which measures relative telomeric DNA content as a surrogate for telomere length *(69)*. In this retrospective case–control study, both lack of biochemical recurrence and overall survival were statistically significantly correlated with tumor telomere length as measured by this assay. Specifically, all seven prostatectomy patients whose tumor telomeric DNA contents were less than that of control samples (placenta) showed evidence of biochemical recurrence (elevated prostate-specific antigen [PSA] levels) within 10 years after surgery *(70)*. Of nine patients with short tumor telomeres, seven died within 10 years, in contrast to 100% 10-year survival for patients with normal-to-long tumor telomeres; these patients also showed no evidence of disease recurrence. Potential drawbacks of this study include a small sample size (18 cases; only 7 of 9 men in the short telomere category underwent surgery), and the fact that it was not known whether the deaths observed were specifically caused by prostate cancer.

In a more recent retrospective study using 77 prostatectomy samples and a more sensitive chemiluminescent slot blot assay, Fordyce et al. reported that less-than-normal telomere content in primary prostate cancers was associated with recurrence, independent of patient age, pathologic grade (Gleason sum), and regional lymph node status *(71)*. The magnitude of the relative hazard for disease recurrence associated with low telomere content was on par with that of Gleason grade and nodal status. Interestingly, a positive correlation was found between the telomere content of the tumor and that of the surrounding normal-appearing prostate tissue within the same prostatectomy samples. An association was also found between telomere content of the normal-appearing prostate tissues and 72-month recurrence-free survival. The authors postulated that telomere loss in morphologically normal tissue may represent areas at heightened risk of experiencing genetic instability. This is reminiscent of the so-called “field effect” phenomenon that has long been discussed in the cancer literature *(72–75)*. The authors further proposed that cancers arising in such areas may show greater genotypic and phenotypic heterogeneity, and, thus, be more prone to behave aggressively because of a greater level of CIN caused by short telomeres *(71)*.

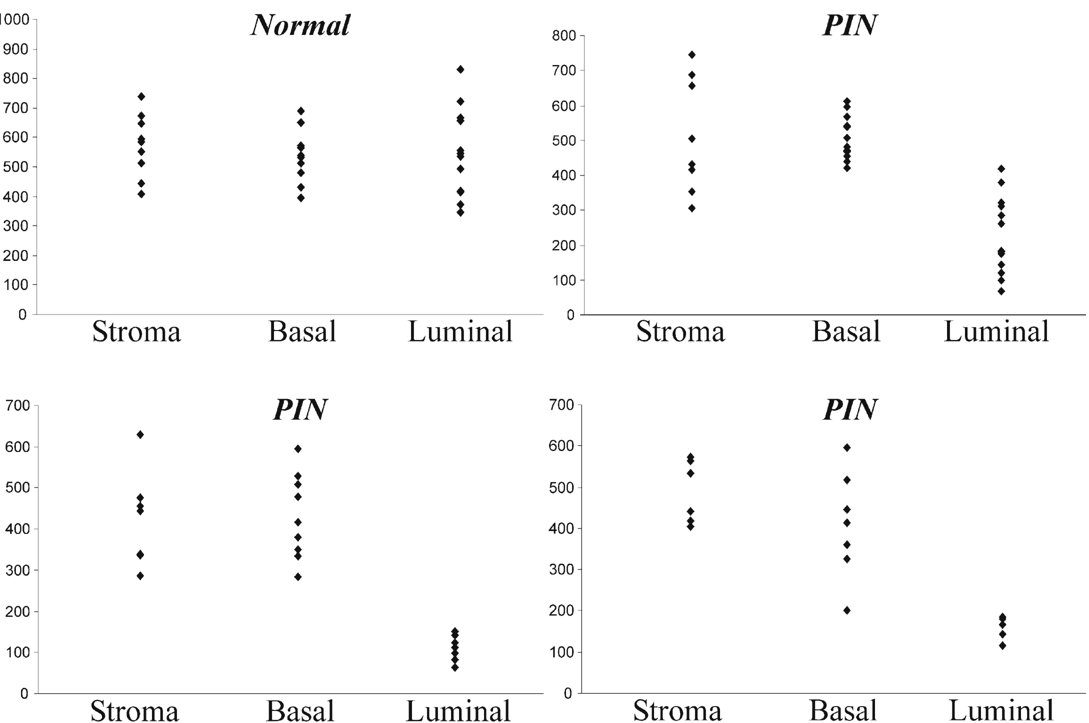


Fig. 2. Telomere shortening occurs early in prostate tumorigenesis and is limited to luminal epithelial cells. Image analysis was used to quantify telomere lengths in specific prostatic cell types within histologically normal tissue (upper left) and prostatic intraepithelial neoplasia (PIN) lesions. Each point plotted represents the telomere content of an individual cell. Note that telomere shortening is pronounced in the luminal epithelial cells in the three PIN lesions *(77)*.

8. TELOMERE SHORTENING OCCURS EARLY DURING PROSTATE TUMORIGENESIS

Although telomeres are short in primary prostate cancers, this gives no information regarding when telomere shortening occurs. It could be argued, for instance, that the observed telomere shortening is merely a reflection of the large amount of cell division that takes place during the process of tumor expansion, in which case, it might have little if anything to do with the actual initiating events causing the disease. For telomere shortening to have a causal role in prostate carcinogenesis one would expect to find evidence of it at the earliest recognizable, pre-invasive stage of malignant transformation, which, in the prostate, is high-grade PIN (HGPIN) *(76)*. In fact, use of the telomere FISH method to probe HGPIN has revealed that telomeres are abnormally short in the majority of HGPIN foci, with significant telomere shortening found in 93% of HGPIN lesions examined in radical prostatectomy specimens (n = 30) *(77)*. In addition, telomere shortening was also found in HGPIN lesions in needle biopsy samples from 20 patients without histopathological evidence of concurrent prostate cancer. Notably, in this study, telomere shortening in HGPIN foci was restricted to the *luminal* secretory epithelial cells only, although the underlying basal epithelial cells and surrounding stromal cells displayed normal telomere lengths (Fig. 2). In a separate independent study, Vukovic et al. reported significant shortening in 63% of HGPIN overall (n = 30), with a higher rate of telomere shortening (80%) reported for foci situated near (within 2 mm) adenocarcinoma within the same tissue sample *(78)*.

The level of telomere shortening observed in PIN likely exceeds that which would normally elicit a cell cycle checkpoint response, thus, implying that this key tumor suppression system is defective in PIN lesions. Similar levels of telomere shortening have been correlated with markers of CIN in other organ systems *(79–82)*. One candidate element of an abrogated checkpoint response in PIN is 14-3-3a sigma. This protein is normally induced by p53 in response to DNA damage and leads to G2/M cell cycle arrest *(83)*. Lodygin et al. found the promoter of this gene to be hypermethylated in prostate cancer cell lines and in 100% of primary prostate cancers ($n = 41$). Decreased protein expression was also observed in 91% of prostate cancers, whereas normal, atrophic, and BPH areas showed high levels of expression in both luminal and basal epithelial cells. Interestingly, 14-3-3a sigma protein expression levels were also low in the luminal cells of PIN lesions, whereas basal cells retained robust expression, matching the pattern of telomere lengths observed in PIN *(77,84)*. Because the majority of PIN lesions are not thought to progress to invasive cancers, intact telomere-based replicative senescence or apoptosis checkpoints may represent a critical bottleneck restraining the outgrowth of most PIN lesions.

9. TELOMERASE ACTIVITY RE-STABILIZES CHROMOSOMES AND ALLOWS UNLIMITED REPLICATION

Although dysfunctional telomeres may help initiate cancer formation, if left unchecked, continued telomere shortening in premalignant lesions and cancers would cause increasing levels of genetic instability, ultimately becoming lethal to the tumor. Cancer cells overcome this problem by re-stabilizing their telomeres, primarily through activation of the enzyme telomerase, a specialized reverse transcriptase that adds back telomere DNA repeats to chromosome ends *(85)*. Telomerase provides at least two critical functions to the tumor cell; namely, quelling CIN and supplying the capacity for unlimited replication (immortalization) *(86,87)*. Research has revealed that telomere length maintenance seems to be a necessary step for human cells to become malignant; confirming the long-held belief that cellular immortalization is a prerequisite for carcinogenesis *(88,89)*. In keeping with this, at least 85% of human epithelial cancers have telomerase activity *(90–92)*.

Human telomerase is minimally composed of two essential subunits, a catalytic protein subunit (hTERT) and an RNA subunit (hTR or hTERC) that contains the template for telomere repeat addition *(93–98)*. As mentioned in Section 5, telomerase activity is stringently repressed in most normal human somatic cells *(99,100)*. There are notable exceptions, however, including activated lymphocytes, subpopulations of highly proliferative tissues (such as the hematopoietic system), and proliferative zones in renewal tissues (such as the intestinal crypts and the basal layer of the skin and cervix), plus hormonally responsive cells of the female breast and endometrium *(101–109)*. Notably, the activity detected in these normal tissues is typically much weaker than that found in malignant tumors of the same tissue of origin. Whereas the RNA subunit, hTR, displays a widespread expression pattern, expression of the catalytic subunit, hTERT, seems to be the rate-limiting factor for telomerase activity in many (e.g., ovary, skin, and cervix) but not all (e.g., kidney, colon, and endometrium) tissues *(56,93,94,96,98,110–119)*.

Although telomerase activity seems to be the preferred way cancer cells stabilize their telomeres, approx 15% of human cancer cases lack detectable telomerase activity. At least a subset of these, particularly certain tumors of mesenchymal origin, maintain their telomeres via a telomerase-independent pathway known as alternative lengthening of telomeres (ALT), the details of which are still being uncovered *(120–124)*.

10. TWO SIDES TO THE TELOMERE COIN

A consideration of telomere biology, as outlined, leads to the conclusion that telomeres can play opposing roles in the development of cancer. On the one hand, cellular responses to telomere shortening caused by cell division place strict limits on the proliferative capacity of a given cell lineage, thus,

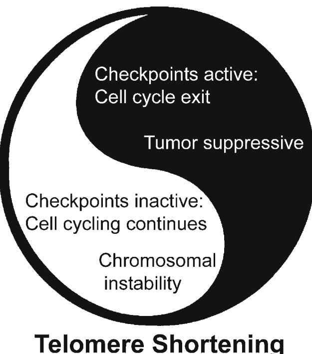


Fig. 3. Telomere shortening can either promote or suppress tumorigenesis. If tumor-suppressive telomere length-sensitive cell cycle checkpoints are intact, then telomere shortening results in cell cycle exit before the telomeres become critically short and unstable. Checkpoint abrogation allows continued cell cycling with concomitant telomere shortening, eventually leading to chromosomal instability caused by telomere dysfunction.

serving to prevent the expansion of potentially malignant cells. On the other hand, critically short, dysfunctional telomeres can promote carcinogenesis by initiating CIN *(50,51)*. The deciding factor in determining which of these opposing outcomes wins out seems to be the status of the cellular checkpoint pathways that monitor telomere length (Fig. 3). Thus, telomerase-deficient mouse models support a tumor suppressive role for telomere shortening only if cellular checkpoints are intact; particularly the p53 tumor suppressor pathway. Research on mice doubly deficient for telomerase and p53 has revealed a key role for dysfunctional telomeres in the development of epithelial cancers in the setting of defective checkpoints, and murine tumors arising in a background of dysfunctional telomeres display the types of complex karyotypic abnormalities typically observed in human carcinomas *(125,126)*.

11. TELOMERASE ACTIVITY AND EXPRESSION OF TELOMERASE COMPONENTS IN PROSTATE CANCER

At some point after the onset of genetic instability caused by telomere shortening, the developing tumor must re-stabilize its telomeres to prevent runaway genetic damage that would ultimately accumulate to lethal levels. Prostate cancers, similar to most other human cancers, typically accomplish this by activating the enzyme telomerase.

Telomerase can be assayed in a number of different ways. Expression of the RNA species encoding either the catalytic protein subunit or the template RNA can be determined by RT-PCR or *in situ* hybridization techniques, whereas telomerase enzymatic activity can be assayed in tissue lysates using a highly sensitive PCR-based assay *(90)*.

Several studies have assayed telomerase activity in clinical prostate samples, all of which have found detectable activity in tissue lysates from cancerous regions *(62,63,90,127–133)*. The proportion of telomerase positive cases ranges from 47 to 100%, with most reports finding activity in the majority of cases (Table 1). When tested, activity is found less frequently in adjacent normal-appearing regions (no activity in three of five studies) or areas of BPH associated with cancer (10–50% of samples), and, in such cases, the level of activity is typically much less than that found in cancer. Being PCR-based, the telomerase activity assay is highly sensitive, capable of detecting activity as few as 10 cancer cells. Therefore, it remains unknown whether the activity detected in cancer-adjacent areas represents true positivity in normal cells, transformed cells (e.g., PIN), or if it originates

Table 1
Telomerase Activity in Surgically Removed Prostate Tissues [a]

Primary prostate cancer	Normal adjacent	BPH adjacent	Normal w/o cancer	BPH w/o cancer	PIN	Clinicopathological correlations	References
47% (30)	ND	ND	ND	ND	ND	ND	*127*
69% (13)	0% (2)	0% (1)	ND	ND	16% (25)	ND	*63*
70% (46)	ND	10% (10)	ND	ND	ND	Yes: grade [b]	*128*
78% (18)	ND	13% (16)	ND	ND	ND	No: grade, stage, ploidy [c]	*129*
84% (25)	0% (25)	12% (25) [d]	ND	0% (10)	ND	No [c]	*62*
90% (31)	ND	10% (10) [d]	0% (10)	ND	ND	Yes: grade[c]	*130*
90% (50)	33% (9)	46% (37)	ND	25% (8)	ND	No: grade, stage, PSA[c]	*131*
92% (87)	36% (11)	50% (26)	ND	0% (16)	73% (15)	No: grade, PSA [c]	*132*
100% (2)	0% (8)	10% (10)	ND	ND	60% (5)	ND	*90*
ND	ND	ND	ND	0% (46) [e]	ND	NA	*133*

[a] Percent of samples with detectable telomerase activity (No. samples). BPH, benign prostatic hyperplasia; W/o, without; PIN, prostatic intraepithelial neoplasia; ND, not determined; PSA, prostate-specific antigen; NA, not applicable.

[b] Correlation relates to telomerase activity level.

[c] Correlation relates to telomerase activity status (activity + vs activity –).

[d] Weak activity compared with that observed in cancer samples.

[e] Samples obtained from transurethral resection.

from a few occult cancer cells. In support of the latter, normal and BPH tissues taken from prostates without evidence of cancer are typically negative for telomerase activity (Table 1). Furthermore, Engelhardt et al. found a positive correlation between telomerase activity and degree of tumor cell infiltration, implying that, for a given tissue sample, tumor cell content is an important factor in the detection of telomerase activity *(127)*. In addition, because activity is detected in activated lymphocytes, inflammation, a common histological finding in the prostate, is another potential source of the activity observed in noncancerous tissues.

What are we to make of the approx 10–20% of prostate cancer cases that are telomerase negative? Activity may have been missed in some of these cases because of technical issues, such as assay failure or focal intratumoral heterogeneity *(131)*. However, it is plausible that some of these tumors truly lack telomerase activity, although it is highly unlikely that these cancers are maintaining their telomeres by the ALT mechanism described at the end of Section 9, because this is virtually never observed in epithelial cancers. Thus, it is likely that at least a portion of telomerase-negative prostate cancers may be mortal and, therefore, might be expected to have a more favorable clinical course than their telomerase-positive, immortal counterparts.

In six of these studies, associations were sought between telomerase activity and established prognostic indicators. Although two studies found a positive correlation between either the presence or level of telomerase activity and tumor grade, four other studies found no correlation with grade, stage, or preoperative PSA levels. However, the number of cases in many of these studies was small. Furthermore, a tumor initially lacking telomerase could still acquire it at a later time.

Regarding the telomerase subunits hTR and hTERT, several studies have examined these in clinical prostate specimens (Table 2) *(128,134–140)*. By RT-PCR, hTR, the template RNA, is detected in 100% of prostate cancers, 100% of BPH samples, and 56% of stromal samples. The detection of hTR in normal and benign prostate tissues is not surprising, given the previously mentioned widespread expression pattern observed for hTR. As seen in Table 2, hTERT is detectable in the majority of cancer samples but not in the surrounding stroma. In the study by Kamradt et al., hTERT was also detected in 35% of BPH samples, but the expression levels were much lower than those found in cancer *(128)*. In this study, cancer samples also exhibited a wide range of hTERT expression levels, and hTERT levels were positively correlated with hTR levels, implying coordinate regulation of these two genes.

Previous studies examining *hTERT* messenger RNA (mRNA) in bladder, colorectal, lung, and breast cancer, as well as liposarcoma and Wilm's tumor, found expression levels correlated with prognosis *(141–147)*. However, in other studies (gastrointestinal cancer and uterine cervix) telomerase was not related to prognosis *(148,149)*. In the studies on prostate by Liu et al. and Kamrdt et al., neither hTR nor hTERT, as detected by RT-PCR, correlated with prognostic indicators *(128, 138)*. In contrast, in a study by de Kok et al., hTERT levels were found to correlate with stage but not tumor grade, although grade did not vary widely in this series *(150)*.

In several instances in which both hTR and hTERT were expressed, the tumor lacked detectable telomerase activity. Such findings indicate that caution is warranted in making inferences regarding telomerase activity status based on RT-PCR results. For example, Kamradt et al. found a significant discordance between *hTERT* mRNA expression and telomerase activity. Although the majority of cancers scored positive for telomerase activity (70%), fully one-third of cases lacking activity were nonetheless hTERT and hTR positive *(128)*. In addition, hTERT message was also detected in one-third of BPH tissues, although activity was observed in only 10% of these cases and was much weaker than that found in cancer. Such discordances between the presence of *hTERT* mRNA and enzymatic activity are perhaps not altogether surprising because the presence of a given mRNA does not guarantee production of the corresponding protein. In addition, there is evidence for a minimum amount of *hTERT* mRNA required for telomerase enzymatic activity *(151)*. Issues of mRNA and protein production aside, there are additional layers of telomerase regulation that could affect whether or not catalytic activity is detected in a given specimen, including mRNA splicing, posttranslational protein

Table 2
Analysis of Telomerase Component Expression by *In Situ* Hybridization, RT-PCR, and Immunohistochemistry [a]

Adenocarcinoma	Stroma	Normal	BPH	PIN	Clinicopathological correlations	Reference
hTERT mRNA *in situ* hybridization						
• 100% cases + homogeneous, cytoplasmic	Endothelial cells and lymphocytes +	Basal cells +	Basal cells +	100% cases + homogeneous, cytoplasmic	ND	*128*
• 89% cases + (RRP touch preparations)	ND	ND	ND	ND	Yes: grade, PSA but not stage	*134*
• ND	–	Basal cells +	ND	ND	ND	*135*
hTR RNA *in situ* hybridization						
• 100% cases + heterogeneous	ND	Basal cells – Luminal cells +	ND	Both basal and luminal cells +	Not correlated with grade (small study)	*136*
• 78% cases +	Lymphocytes +	Basal cells + Luminal cells –	ND	87% + both basal and luminal cells	ND	*137*
hTR, *hTERT* RT-PCR						
• hTR: 100% + • hTERT: 98% +	ND	ND	100%+ 35% +	ND	No No	*128*
• hTR: 100% + • hTERT: 94% +	56% + 0% +	ND	ND	ND	No (grade) No (grade)	*138*
• hTERT: 67% +	ND	ND	ND	ND	ND	*139*
hTERT protein by immunohistochemistry						
• 64% (Gleason <7) + 100% (Gleason 7)	Lymphocytes +	Basal cells + (strong nuclear)	ND	38% foci + (discontinuous nuclear stain in basal cells)	Yes: proportion + nuclei w/ Gleason grade	*140*
• 100% cases heterogeneous staining	ND	Basal cells – Luminal cells + in 40% of cases	ND	Basal cells – Luminal cells + in 83% of cases	No correlation with grade (small study)	*136*

[a] BPH, benign prostatic hyperplasia; PIN, prostatic intraepithelial neoplasia; ND, not determined; RRP, radical retropubic prostatectomy; PSA, prostate-specific antigen; NA, not applicable.

modifications, proper assembly of the protein and template RNA components, association with other protein molecules, correct localization of the active complex, and accessibility of the telomeric substrate *(152–159)*.

In contrast to RT-PCR, *in situ* hybridization studies provide information not only on gene expression status, but also on the specific cellular and subcellular location of the expressed RNA species. In agreement with the results from RT-PCR studies, *in situ* hybridization studies also found *hTR* and *hTERT* expression in the majority of cancer cases (Table 2). Infiltrating lymphocytes were also positive for both hTR and hTERT, in keeping with the fact that they have detectable telomerase activity, whereas stromal cells were generally negative for hTERT, with the exception of the study by Kamradt et al., which found hTERT staining of endothelial cells. PIN lesions were found to be positive for hTERT and hTR in both basal and luminal epithelial cells, in agreement with multiple reports of telomerase activity in subsets of PIN lesions *(63,90,132)*. Regarding *in situ* hybridization studies of normal prostatic epithelium, two studies described hTERT positivity in normal basal cells, however, conflicting results have been published on hTR expression patterns. Bettendorf et al. found basal cells to be hTR negative and luminal cells to be hTR positive whereas Paradis et al. reported the opposite finding (Table 2). Lastly, in BPH tissues, basal cells were reported by Kamradt et al. to be positive for hTERT.

There are few reports of staining for hTERT protein with antibodies to telomerase, perhaps reflecting the poor performance of antibodies to hTERT and/or low levels of hTERT protein expression making its detection difficult. Iczkowski et al. reported the presence of telomerase immunoreactivity in lymphocytes, most prostate cancers, normal basal cells, and discontinuous basal cell staining in approximately one-third of PIN lesions *(140)*. In this study, an association was found between the proportion of cancers staining positive for telomerase and Gleason grade, with 64% of tumors of Gleason sum less than 7 having positive staining, and 100% of cases with Gleason sum at least 7 being positive for hTERT. In addition, the proportion of positive nuclei within a given tumor increased with increasing Gleason grade. In another immunohistochemistry (IHC) study, Bettendorf et al. found antibody staining in 100% of cancer cases, positivity in PIN luminal cells in 83% of PIN lesions, and positive reactivity in the luminal cells in 40% of normal-appearing epithelia *(136)*. As with the *in situ* hybridization studies, it is not clear why these studies disagree with respect to the specific cell types in normal prostate epithelia (basal vs luminal) that stain positively for hTERT. Further study will be needed to resolve these questions.

12. TELOMERASE ACTIVITY AS A POTENTIAL DIAGNOSTIC MARKER

Although there are potential confounding factors when assaying telomerase activity (e.g., activity in activated lymphocytes and intratumoral heterogeneity of activity), the high prevalence and relatively strong activity found in prostate cancers compared with normal and BPH, plus the very high sensitivity of the standard telomerase activity assay justifies the consideration of using telomerase activity as a diagnostic marker for prostate cancer. To date, eight studies have been performed evaluating telomerase activity in prostate needle biopsy samples (Table 3) *(160–167)*. In these studies, activity was detected in more than half of all cancer cases (range, 58–91%) with most reporting telomerase in greater than 80% of cases with cancer. In two of five studies that also tested normal prostate tissue, no activity was detected in normal samples. However, the remaining three studies found activity in 11% to 17% of samples lacking prostate cancer. Likewise, two studies that included BPH tissues detected activity in 12% and 38% of cases, although, in the former, it was noted that the activity observed was very weak and cancer was later detected in these patients by repeat biopsy *(160)*. This study also found a trend for increased activity levels in cancers of higher Gleason grade, although this did not reach statistical significance in this small study. Lin et al. and Wang et al. also noted a positive correlation between either activity levels or telomerase detection rates and pathological grade. Using a split biopsy approach, Lin et al. showed that differences in activity levels they

Table 3
Telomerase Activity in Needle Biopsies [a]

Primary prostate cancer	Normal	BPH	PIN	Clinicopathological correlations	References
58% (19)	ND	12% (17) [b]	ND	Trend: grade [c]	*160*
66% (38)	11% (19)	ND	ND	ND	*161*
70% (26) [d]	ND	ND	25% (12) [d]	ND	*162*
84% (25) [d]	17% (12)[d]	ND	50% (4) [d]	ND	*163*
89% (9)	0% (11)	38% (16)	ND	ND	*164*
90% (19)	0% (44) [e]	ND	ND	Yes: grade [f]	*165*
90% (20) [g]	ND	ND	ND	ND	*166*
91% (35)	12% (8)	ND	ND	Yes: grade [h]	*167*

[a] Percent samples with telomerase activity (No. samples). BPH, benign prostatic hyperplasia; PIN, prostatic intraepithelial neoplasia; ND, not determined.
[b] Positive BPH samples had very low levels of activity and cancer was detected after repeat biopsy.
[c] Trend for higher activity levels in higher Gleason grades.
[d] Samples were obtained as touch imprints on microscope slides from needle biopsies.
[e] Includes both normal and BPH specimens.
[f] Correlation between Gleason grade and semiquantitative telomerase activity levels determined by dilution.
[g] 81% of fine-needle aspirates positive for cancer by cytology were also telomerase positive.
[h] Higher detection rates for cases with higher Gleason scores.

observed were not simply caused by different amounts of cancer in the needle biopsy samples *(165)*. Similarly, using a touch imprint preparation method, Chieco et al. found no association between activity detected and either area of cancer or extent of inflammation on the needle biopsy *(162)*.

Regarding precancer, two of the studies reported finding telomerase activity in 25% and 50% of biopsy samples containing PIN.

In addition to needle core biopsy samples, detection of telomerase components and activity has been attempted in expressed prostatic fluid samples. Such noninvasive approaches would have obvious advantages over the use of needle biopsies. Examining freshly expressed prostatic fluids from 30 prostate cancer patients, Wang et al. detected telomerase activity in 83% of cases compared with an 11% (1 in 9) detection rate for samples from men without clinical evidence of prostate cancer *(168)*. These values are in excellent agreement with the radical prostatectomy tissue-based studies described in Table 1. A positive correlation between activity status and serum PSA level was also noted in this study. Vicentini et al. obtained similar results in their study on pelleted cells obtained from prostatic massage fluids collected during digital rectal examination (DRE). Here, activity was detected in 90% of samples from men with histologically proven prostate cancer; whereas 13% of samples from men with BPH were positive *(169)*. Notably, all telomerase-positive BPH samples also had evidence of acute inflammation. A lower cancer detection rate of 58% was observed in the study of Meid et al. in which activity was assayed in samples from either voided urine or urethral washings after prostate massage *(170)*. In this study, the sensitivity of detection was greater in more poorly differentiated cancers and no activity was detected in samples from 12 individuals lacking evidence of prostate cancer. In contrast to the other studies using prostatic fluids, Suh et al. were unable to detect any activity in cells isolated from ejaculated fluids from nine prostate cancer patients, although glutathione-*S*-transferase-π promoter hypermethylation, a nearly universal feature of prostate cancer, was detectable in four of the nine samples *(171)*.

Overall, the potential usefulness of either telomerase activity assays or measures of hTERT or hTR expression in aiding prostate cancer diagnosis seems limited primarily because of problems with

specificity. Certainly this is the case for hTR, because its expression is commonly detected in normal prostate, prostatic stroma, and BPH, in addition to prostate cancer (Table 2). Likewise, hTERT is also detectable in an appreciable number of nonmalignant prostate tissue samples, either by *in situ* hybridization techniques, RT-PCR, or by IHC. In terms of telomerase enzymatic activity, most studies examining samples obtained from radical prostatectomies find activity in the majority of prostate cancers, but vary widely in their detection rates of activity in cancer-associated normal and BPH tissues (Table 3). However, purely normal or BPH tissues without associated cancer or normal prostate epithelial cells in culture typically lack detectable activity. Thus, telomerase activity may be a useful indicator of the presence of prostate cancer. Particularly encouraging is the fact that similarly high detection rates for cancer are also observed in studies using needle biopsy material and, in two of four studies, expressed prostatic fluids.

Unfortunately, the standard activity assay itself is technically demanding, requires unfixed tissue, and is susceptible to both false negative and false positive results *(91)*. Thus, further testing needs to be performed before firm conclusions can be drawn regarding the ultimate clinical value of the telomerase activity assay.

13. TELOMERASE REGULATION

Telomerase regulation is complex and occurs at multiple levels, including gene transcription, mRNA splicing, posttranslational modifications, proper assembly of enzyme subunits, and recruitment and access to the telomere itself *(172–178)*. Known transcriptional regulators of hTERT include the positive regulator, c-Myc, and the negatively acting regulators, menin, Rak, SIP1, and the c-Myc antagonist, Mad1 *(179)*. *c-Myc* mRNA is reportedly increased in prostate cancer, is correlated with a worse prognosis, and is a candidate gene in a region of chromosome 8p that is often found to be amplified in prostate cancer *(180)*. In a study of 33 prostate cancers, Latil et al. found that hTERT expression levels were significantly correlated with c-Myc expression. Interestingly, c-Myc was not overexpressed in three samples of hormone-refractory disease or in the androgen-insensitive prostate cancer cell lines, DU-145 and PC3 *(139)*.

One recently discovered regulatory mechanism involves the subnuclear distribution of telomerase. Previously, it was shown that hTR associates with the nucleolus. Recently, three reports following the fate of fluorescently tagged hTERT protein indicate that hTERT may either be preferentially associated with, or excluded from, the nucleolus. These observations imply that the nucleolus is involved in telomerase assembly and/or sequestration, and this process is perturbed in cancer cells *(181–183)*.

Another pathway likely involved in proper folding and assembly of the telomerase holoenzyme involves chaperones, specifically the hsp90 and p23 proteins *(157)*. Akalin et al. have shown that telomerase activity increases during tumorigenic conversion of prostate cells in an SV40 T-antigen transformation model, with concomitant increases in hsp90 and p23, but without changes in hTR or hTERT levels *(184)*. In addition, when examined by IHC, increased staining was observed for hsp90 and p23 in prostate cancer. Such findings may give further rationale for the therapeutic potential of hsp90 blocking agents currently being considered for use in cancer clinical trials.

The gene *PinX1*, found in chromosomal region 8p22–23, which shows frequent loss of heterozygosity in cancers, including prostate cancer, has been shown to negatively regulate telomerase activity *(185,186)*. Hawkins et al. sought evidence of a link between *PinX1* mutations and hereditary prostate cancer but found no evidence for such an association *(185)*. However, it is possible that this gene could be playing a role in sporadic prostate cancer cases, perhaps caused by haploinsufficiency leading to increased telomerase activity in cases in which loss of heterozygosity has occurred. This hypothesis requires further testing.

The La autoantigen, a multifunctional protein important in RNA stabilization and ribonucleoprotein (RNP) maturation, has been shown to associate with telomerase. Forced overexpression of this

gene in DU-145 prostate cancer cells leads to progressive telomere shortening, presumably caused by telomerase inhibition *(187)*.

The well-known tumor suppressor, *BRCA1*, also affects telomerase. Overexpression of *BRCA1* (but not cancer-associated mutant *BRCA1*s) in DU-145 cells strongly inhibits telomerase gene transcription but not the transcription of other telomere/telomerase related genes, including *c-Myc (188,189)*. The main BRCA1-mediated block in hTERT transcription seems to occur via inhibition of *c-Myc* activation through a c-Myc response element (E-box) in the hTERT gene promoter. Telomerase activity was significantly decreased in these BRCA1-expressing cells and fairly rapid telomere shortening was also observed. The rapidity of the telomere loss implies that there is more involved here than simply inhibition of telomerase, and such observations have also been reported in other telomerase inhibition studies *(190,191)*. In keeping with a negative regulatory role, knockdown of endogenous BRCA1 expression by small interfering RNA treatment was shown to increase hTERT promoter activity in DU-145 cells *(188)*.

Retinoic acid and vitamin D3 have both been shown to decrease hTERT levels, and their combined use was shown to decrease both *hTERT* mRNA and telomerase activity in PC3 and LNCaP prostate cancer cells. Tumor growth in a PC3 xenograft model was also slowed by this treatment *(192–195)*.

Nerve growth factor (NGF) production is decreased in prostate cancer, and NGF treatment decreases telomerase activity in PC3, DU-145, but not LNCaP cells, which lack NGF receptor expression *(196)*. The insulin-like growth factor (IGF) signaling axis also seems to be involved in telomerase regulation in the prostate. Wetterau and colleagues discovered that IGF-1 stimulated telomerase activity in PC3, DU-145, and LAPC4 cells *(197)*. Another member of the IGF signaling axis, IGF binding protein (IGFBP)-2 is growth inhibitory to normal prostate but stimulates growth of prostate cancer. In DU-145 and LAPC-4 cancer cells, IGFBP2 increased the levels of telomerase activity, however, the effects of IGFBP2 are dependent on context. For instance, in the presence of 10% fetal bovine serum, IGFBP2 was observed to be growth-inhibitory in LAPC-4 cells *(198)*.

Several other proteins have been shown to interact with the telomerase RNP. Many of these are known RNA-binding proteins with functions in RNA maturation, stabilization, and assembly of RNPs, thus, it is likely they are playing similar roles in assembly reactions for telomerase. These proteins include hStau, L22, TEP1, dyskerin, hnRNP binding proteins, and snoRNA binding proteins *(152–156,158,159)*. Little is currently known regarding their relevance, if any, to prostate cancer.

14. STEROID HORMONE REGULATION OF TELOMERASE IN THE PROSTATE

In the prostate, telomerase activity is subject to regulation by steroid hormones, particularly androgens. Normal prostate tissues free of cancer, as well as cultured normal prostate epithelial cells, are typically negative for telomerase activity in the presence of androgen (Table 1). However, androgen withdrawal leads to the activation of telomerase. This was first observed in the involuted residual prostate and seminal vesicles of the Copenhagen rat, in which telomerase activity becomes strongly positive after castration *(199)*. This activity remains undiminished even at 2 years after castration, despite the near total lack of ongoing cell proliferation in the quiescent, involuted glands. These residual glands are highly enriched for prostatic stem cells, as evidenced by the rapid induction of cell division and regrowth of these glands after restoration of testosterone. During androgen-induced glandular regeneration, telomerase activity subsides *(199)*. Based on the kinetics of the appearance and disappearance of activity as glands undergo involution and regrowth, respectively, and the results of mixing experiments between extracts of intact and involuted glands, it was concluded that telomerase activity was negatively regulated by androgens rather than being constitutively active in a small subpopulation of normal stem cells. These studies were later replicated in a non-human primate by Ravindranath et al., who found similar results to those observed in the rodent model *(200)*. Thus,

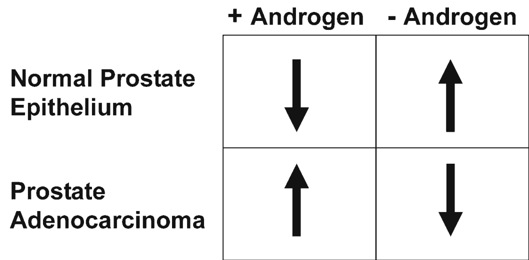


Fig. 4. Telomerase regulation by androgens in the prostate: healthy vs cancer. Androgens act to repress telomerase in the normal prostate and this repression is relieved after androgen withdrawal. Prostate cancer behaves in a manner opposite to that of normal prostate epithelial cells.

the overall picture for the normal prostate is that telomerase is actively repressed in an androgen-dependent manner, and that activity is upregulated after androgen withdrawal. In sharp contrast, this normal regulation of telomerase is lost in prostate cancer, because the majority of primary tumors display robust telomerase activity despite the presence of normal levels of circulating androgens. In fact, with respect to telomerase regulation, the response of prostate cancer cells to androgen seems to be *exactly the opposite* that of normal cells. Not only are cancer cells telomerase positive in the presence of androgen but telomerase activity is suppressed after androgen withdrawal (Fig. 4) *(201)*. For example, Guo et al. found that *hTERT* mRNA levels increased in the hormone-responsive prostate cancer cell line, LNCaP, when treated with the nonmetabolizable synthetic androgen, R1881 *(202)*. This effect was considered to be indirect, based on the kinetics of the response and also the fact that R1881 failed to activate a reporter gene construct under the control of the hTERT promoter, in keeping with the known response elements in the telomerase promoter region. Although there is an androgen response element in the hTR promoter region, none is apparent in the hTERT promoter *(203–206)*. Guo et al. also observed that, in the hormone-responsive CWR22 prostate cancer xenograft model, both telomerase activity and *hTERT* mRNA decreased after castration, and this effect was reversed after re-introduction of androgen. A similar pattern was observed by Soda et al., Thelen et al., and Bosland et al., with androgen deprivation causing a decrease in telomerase activity and dihydrotestosterone treatment causing an increase in activity in LNCaP cells, but not in normal prostate epithelial cells or the hormone-insensitive prostate cancer cell line, DU-145 *(207–209)*. In contrast, Bouchal et al. did not observe a positive effect in LNCaP with dihydrotestosterone, but did see suppression of telomerase activity after treatment with the anti-androgen, bicalutamide, in LNCaP but not DU-145 cells *(210,211)*. Bicalutamide also decreased *hTERT* mRNA and *c-Myc* mRNA but did not change expression of a host of other telomerase modulators, including dyskerin, hsp90, p23, SIP1, Mad1, and menin. In clinical specimens, Iczkowski et al. reported that telomerase expression in cancer, as measured by antibody staining, was significantly suppressed after complete androgen ablation *(212)*. It is worth noting that inhibition of telomerase by androgen withdrawal observed in prostate cancer, although substantial, is typically not 100% complete.

The precise details of how androgens regulate telomerase in the prostate remain to be elucidated. As mentioned previously, a potential mediator of androgen's effects on telomerase in the prostate is c-Myc. It has been shown that androgen is capable of upregulating c-Myc and that hTERT expression is positively correlated with c-Myc expression in prostate cancer *(139,213)*. Another candidate regulator is the polycomb protein CBX7, which was recently highlighted in a screen for genes upregulated in human prostate epithelial cells that had bypassed senescence *(214)*.

In addition to androgens, estrogen has also been implicated in telomerase regulation in the prostate. Examining normal prostate epithelial cells and BPH tissue explants in culture, Nanni et al. found

them lacking both *hTERT* mRNA and telomerase activity. Estrogen treatment led to induction of hTERT message and telomerase activity in these cultures, as well as increases in activity (over baseline levels) in cancer cell lines and cancer tissue explants *(215)*. No such effects were observed in estrogen receptor-negative cells. Unlike androgens, estrogen *was* able to activate an hTERT promoter expression construct. Thus, in contrast to androgen action; estrogens seem to directly activate hTERT transcription, likely via interaction with the known estrogen response element located in the hTERT promoter. In support of this, chromatin immunoprecipitation experiments indicate that both estrogen receptor (ER)-α and ER-β bind to this estrogen response element *(215)*. It was also found that 4-hydroxy tamoxifen inhibited telomerase activity in both LNCaP and DU-145 cells, perhaps via preferential recruitment of ER-β to the hTERT promoter. In addition, the selective aromatase inhibitor letrozole also decreased telomerase activity in LNCaP cells in the presence of testosterone but not the non-aromatizable synthetic androgen, R1881, thus, raising the possibility that local conversion of testosterone to estrogen may play a role in regulating telomerase in the prostate. Finally, Bouchal et al. observed a slight increase in telomerase activity in LNCaP cells after a 3-day treatment with 17-β estradiol, but not with a shorter 1-day treatment *(210,211)*.

15. TELOMERASE-BASED OPPORTUNITIES FOR THERAPY

There are three overall strategic paradigms for therapeutic targeting of telomeres or telomerase in cancer. The first involves taking advantage of the tumor's dependence on telomerase enzymatic activity for survival. This strategy includes approaches aimed at directly inhibiting telomerase enzymatic activity or blocking expression of either hTERT or hTR. The second approach attempts to exploit the fact that the hTERT promoter is active in most cancer cells, for example, by specifically targeting oncolytic viruses or gene therapy to tumor cells, or by directing immunotherapy against cells expressing hTERT. The third approach involves attempts at altering the telomeres themselves. Many of these approaches have undergone preclinical testing using prostate cancer cell lines and xenografts.

One concern regarding anti-telomerase therapy stems from an anticipated delay in effect after treatment. For approaches based on telomerase inhibition, it had previously been thought that after enzyme inhibition there would, of necessity, be a lag period during which telomeres would progressively shorten before any treatment effect would be observed, and that such a lag would be related to the starting telomere lengths of the cells undergoing treatment. However, several studies have observed earlier than expected negative effects on cell growth and survival, hinting that telomerase may have other functions in the cell besides telomere length maintenance. In support of this, several studies indicate that telomerase may be playing other roles that support cell growth and survival *(216–223)*. If true, then telomerase inhibitors may also prove useful in treating the minority of tumors possessing normal-to-long telomere lengths.

Another concern regards the question of selectivity of action against tumor cells over normal telomerase-positive cells, such as the stem cells of the hematopoietic system and those within tissues with high turnover rates. This is of particular importance for those approaches in which telomerase-positive cells are actively targeted for destruction, including immunotherapy, gene therapy, and oncolytic viral therapies. Encouragingly, work to date describing the treatment of human tumor xenografts in mice have, in general, not produced major toxicity in normal tissues.

16. DIRECT INHIBITION OF TELOMERASE

Various small molecules have been used in attempts to find suitable inhibitors of the telomerase enzyme *(224)*. Because telomerase is a reverse transcriptase, nucleoside analogs (e.g., AZT) were among the first agents tested. Unfortunately, these showed only weak inhibition *(225,226)*. Non-nucleoside inhibitors have also been tried with limited success *(227)*. Natural compounds, including genistein, silibinin, and tea catechins have also been assayed for potential anti-telomerase effects

(208,228,229). Genistein decreased hTERT levels and telomerase activity in LNCaP and DU-145 cells, and, when assessed in LNCaP, decreased *c-Myc* mRNA and cellular proliferation *(228)*. Likewise, silibinin was also found to decrease *hTERT* mRNA and telomerase activity in LNCaP cells *(208)*.

Several groups have explored telomerase inhibition by use of short oligonucleotides of varying chemistries, and this general approach has been reviewed by Corey *(230)*. This approach was first attempted with peptide nucleic acid (PNA) oligonucleotides having sequences targeted against the template RNA sequence. Here, oligonucleotide binding acts to directly block enzymatic activity (template antagonist), leading to telomere shortening, growth arrest, and apoptosis *(231–234)*. Treatment of DU-145 prostate cancer cells with PNA oligonucleotides led to decreased cell growth but incomplete cell kill. Antisense PNAs targeted against the *hTERT* mRNA have also shown antiproliferative effects *(235)*.

Herbert et al. developed a thiophosphoramidite DNA oligonucleotide template antagonist that was further optimized to give a 13-mer oligonucleotide with improved potency, stability, and bioavailability *(236–238)*. Treatment of telomerase-positive cancer cells with the new molecule, GRN163, caused telomerase inhibition, progressive telomere shortening, and eventual senescence or apoptosis. As was predicted, growth inhibition correlated inversely with the starting telomere lengths of the cell lines used. GRN163 also showed significant antitumor effects in vivo in a DU-145 prostate cancer xenograft model without evidence of host toxicity during an 8-week treatment period.

2'-O-(2-methoxy ethyl) RNA oligonucleotides have also been studied, producing telomere shortening and eventual total cell killing in LNCaP and DU-145 cells treated in vitro *(239,240)*. Importantly, these effects were reversible after drug withdrawal and were not observed with a control mismatched sequence oligonucleotide. The match oligonucleotide was found to have significant negative effects on cell proliferation, anchorage-independent growth, and colony formation relatively early during treatment. Thus, it should be borne in mind that a strict focus on end points such as complete cell cycle arrest or apoptosis may lead to an underestimate of potentially significant clinical effects from telomerase inhibitors. Antitumor activity was also observed in a xenograft model and combined efficacy with various chemotherapeutic agents was observed in vitro with these oligonucleotides, however, this effect was complex, being dependent on the particular agent and cell line used. For example, oligonucleotide treatment sensitized DU-145 cells to cisplatin and carboplatin, but not to doxorubicin, etoposide, or paclitaxel; whereas LNCaP cells were not sensitized to any of these agents.

17. INDIRECT INHIBITION OF TELOMERASE

Antisense approaches have been used aimed at degrading either the *hTERT* or *hTR* RNAs. Hammerhead ribozymes against both of these critical telomerase components have been produced *(223,241–245)*. 2'-5' Oligoadenylate chimeric antisense oligonucleotides targeting hTR for destruction by RNAseL have shown effect against various cancer cell types, including prostate cancer, both in vitro and in vivo *(246)*. Schindler et al., found phosphorothioate antisense oligonucleotides targeting hTERT effective in inhibiting DU-145 cell viability *(247)*. The use of anti-hTERT small interfering RNAs have also been reported *(248)*.

Telomerase inhibition has also been accomplished by using dominant negative (DN) forms of the telomerase catalytic subunit; either by introduction of a catalytically dead *hTERT* mutant gene into cells, or by the use of oligonucleotide-mediated alternate splicing of endogenous *hTERT* mRNA. DN mutant hTERT was shown to inhibit telomerase activity, shorten telomeres, and lead to senescence or apoptosis in various tumor cells *(249,250)*. Guo et al. examined the effects of expressing DN hTERT in three prostate cancer cell lines: PC3, DU-145, and LNCaP. In this study, negative effects on PC3 tumor cells, including telomere shortening, growth inhibition, decreased colony formation, and decreased tumor growth in nude mice, were dependent on the degree of DN

hTERT expression *(251)*. Interestingly, although high level DN hTERT expression restored mortality to PC3 and LNCaP cells, this was not true for DU-145 cells. Even at high levels of expression, no growth arrest was observed in vitro, and only a slowing of tumor growth was observed in vivo in DU-145 xenografts. These findings are reminiscent of results observed in another study using antisense against *hTR* in DU-145 *(233)*. On the other hand, a study using oligonucleotide-mediated alternate splicing of hTERT to generate DN hTERT in DU-145 found decreased telomerase activity and accompanying inhibition of cell growth and promotion of apoptosis *(252)*. Such findings underscore the apparent need for thorough telomerase inhibition and imply that factors intrinsic to the specific tumor cell population may affect the ultimate response to specific approaches to inhibit telomerase. Likewise, sensitization to antitumor drugs is often observed, but only for certain agents *(253–256)*.

Acetylation and deacetylation of histones is linked to changes in chromatin structure that can dramatically affect gene expression, and inhibitors of histone deacetylases are currently being evaluated as anticancer agents. Two such agents, sodium butyrate and trichostatin A, have been show to decrease *hTERT* mRNA levels and telomerase activity in LNCaP and PC3 prostate cancer cells *(257)*. Interestingly, in one study, trichostatin A was found to activate *hTERT* mRNA production and telomerase activity in both normal and transformed telomerase-negative human cells *(258)*.

Another prominent mechanism affecting gene silencing is methylation of cytosines in the CpG islands in the promoter region of genes. Guilleret et al., have reported a *positive* correlation between hypermethylation of the hTERT promoter and *hTERT* mRNA expression and telomerase activity in several cell lines, including PC3 *(177)*. This group has also reported that treatment of telomerase-positive tumor cells with the demethylating agent 5-aza-2'-deoxycytidine led to *repression* of hTERT (without changes in c-Myc expression) and decreased telomerase activity *(259)*. These results are unusual in that promoter methylation typically acts to repress gene expression, whereas demethylation typically reactivates gene expression, although it is possible that the observed effects are indirect, perhaps caused by reactivation of expression of a telomerase repressor. Other groups have also observed instances in which the hTERT promoter is unmethylated in telomerase negative cells and partially or totally methylated in telomerase-positive cells, however, in at least one study, no correlation was found between methylation status of the hTERT promoter and *hTERT* gene expression, and, in this case, the demethylating agent 5-aza-2'-deoxycytidine induced telomerase expression in telomerase-negative cells *(260–262)*. More work will thus be required to clarify the apparently complex relationship between telomerase gene expression and promoter methylation status.

There is evidence that cellular kinase and phosphates may modulate telomerase activity. Kang et al. reported that there are two potential Akt phosphorylation sites on hTERT and that the Akt inhibitor, wortmannin, decreased both Akt-dependent phosphorylation of telomerase peptides in vitro and telomerase activity in a human melanoma cell line, whereas treatment with okadaic acid, an inhibitor of protein phosphatase 2A, which counteracts Akt, produced the opposite effect *(263)*. In another study, protein phosphatase 2A was shown to abolish telomerase activity in breast cancer cells, whereas okadaic acid treatment stimulated telomerase activity *(264)*. Protein kinase C activity also seems to enhance telomerase activity, and some protein kinase C inhibitors have been shown to inhibit telomerase *(111,265–267)*.

Retinoic acid and vitamin D3 have both been reported to have growth suppressive effects in cancer cells, including prostate cancer, and anti-telomerase effects have been observed with these agents *(192–194)*. Ikeda et al. have identified a putative vitamin D receptor binding sequence within the hTERT promoter *(195)*. Furthermore, combined treatment of retinoic acid and vitamin D3 resulted in decreased hTERT expression and telomerase activity in PC3 and LNCaP cells, and slowed tumor growth in PC3 xenografts. However, the effect of these agents on telomerase is apparently cell type dependent because combined treatment with retinoic acid and vitamin D3 did not suppress hTERT in DU-145 prostate cancer cells.

18. EXPLOITING TELOMERASE EXPRESSION IN CANCER CELLS—GENE THERAPY

Because telomerase expression is activated in most cancer cells, attempts have been made to make use of the active hTERT promoter to drive the expression of pro-apoptotic genes, suicide transgenes, or genes that facilitate selective drug uptake, in tumor cells. Pro-apoptotic and suicide genes that have been used in this approach include: caspases 6 and 8, *FADD*, *Bax*, *TNF*, and *TRAIL* and *HSV-TK* *(268–273)*. Genes used for selective drug uptake include *Escherichia coli* nitroreductase and the noradrenaline transporter *(274–278)*. Several of these studies have shown decreased growth and/or apoptosis in tumor cells, both in vitro and in vivo. For example, the noradrenaline transporter allows uptake of a ^{131}I-labeled drug in transfected telomerase-positive cells. This approach is advantageous in that there is a bystander effect of the drug such that neighboring cells will also be killed, thus, 100% efficiency of transgene delivery to the tumor is not necessary. In experiments using LNCaP and DU-145 cells, the gene is expressed, cells take up the drug and exhibit dose-dependent toxicity at doses showing negligible killing of control parental cells that lack the transgene. A comparison between transporter expression constructs using the hTERT promoter or the PSA promoter showed that the telomerase promoter resulted in superior drug uptake. In addition, testing in cell spheroids confirmed that a bystander effect was operating.

19. EXPLOITING TELOMERASE EXPRESSION IN CANCER CELLS—ONCOLYTIC VIRAL THERAPY

The hTERT promoter has been used in attempts to achieve selective replication of lytic viruses in cancer cells. The general approach was pioneered in the prostate by Rodriguez et al., who made use of the *PSA* promoter to restrict viral replication to prostate epithelial cells *(279)*. For telomerase targeting, early viral genes essential for viral replication are placed under the control of the hTERT promoter, thus, limiting viral replication to hTERT-positive tumor cells while sparing the surrounding normal cells. Productive viral infection in tumor cells leads to death (lysis) of the infected cells and release of infective virions, which can then spread to other nearby susceptible cells. This allows amplification of the killing effect of the initial virus, which should increase antitumor efficacy and overcome the problem of tumor cells that were uninfected during the initial treatment phase.

The human adenovirus has been used by several groups to create selectively replicating viruses. Here, the adenovirus *E1A* early gene is placed under control of the hTERT promoter sequence *(280–282)*. Using this approach, Lanson et al. saw good selectivity of tumor cell killing, including DU-145 prostate cancer cells, over normal control cells *(283)*. Likewise, Irving et al. found that viral replication and cell lysis was restricted to several telomerase-positive tumor cell types when tested in vitro *(284)*. In vivo, activity was observed in LNCaP xenograft tumors after intratumoral viral injection, with some complete regressions noted in this study. In tests of systemic delivery in mice, no toxicity was observed for hTERT-selective virus in contrast to other adenoviral constructs driven by the CMV promoter that caused hepatotoxicity *(284)*. Ruan et al. recently reported the addition of further restriction on viral replication by placing the adenoviral gene *E4* under control of the E2F-1 transcription factor in addition to having *E1* under hTERT promoter control *(285)*. This construct demonstrated improved selectivity and decreased toxicity compared with constructs lacking this additional constraint on viral replication and was tested in LNCaP, PC3, and VCap prostate cancer cells. In LNCaP xenografts, a single intravenous injection led to complete regression in 83% of preestablished tumors (n = 12). Furthermore, when used in combination, viral infection was shown to enhance the antitumor activity of doxorubicin *(285)*.

The preclinical results for hTERT-selective oncolytic virus seem promising. However, there is a critical need for studies evaluating the safety of these viral constructs in humans, especially because the mouse is only a semipermissive host for adenovirus *(286)*. Thus, although useful, human tumor xenografts in mice will not inform us fully regarding the potential toxicities we may see in patients.

There is also the potential for such viruses to replicate in normal tissue stem cells that are currently thought to express telomerase. However, these cells may be difficult to infect and typically display low levels of telomerase or intermittent activity, therefore, the desired selectivity of this approach may be maintained. A further concern specific to prostate cancer is the fact that hTERT expression is dramatically reduced after androgen withdrawal, thus, potentially reducing treatment efficacy in patients who have undergone androgen ablative therapy, but this will only be known after this approach has been refined and tested in the clinic.

20. EXPLOITING TELOMERASE EXPRESSION IN CANCER CELLS—IMMUNOTHERAPY

Because of its widespread and fairly selective expression, telomerase has been touted as a potential "universal" tumor-associated antigen for use in immunotherapy approaches. Indeed, immune effector cells recognizing hTERT do exist and can be raised experimentally *(287,288)*. For example, peripheral blood lymphocytes from both healthy individuals and cancer patients respond to HLA-A2-restricted hTERT peptides, providing evidence for endogenous hTERT-specific cytotoxic T-Lymphocyte (CTL) precursors. In addition, CTL obtained from three of four hormone-refractory prostate cancer patients were shown capable of lysing tumor cells in vitro, including LNCaP and PC3 *(288)*.

In a phase I vaccination trial using *ex vivo*-generated telomerase peptide-pulsed autologous dendritic cells, hTERT-specific T lymphocytes were induced in more than half of patients with advanced prostate or breast cancer, without significant toxicity *(289)*. These cells could be expanded in vitro and were competent for tumor cell killing. In addition, some partial tumor regressions were observed in association with the presence of tumor-infiltrating lymphocytes in this study. Careful choice of telomerase peptides seems necessary, because not all peptides are able to produce CTL with the ability to recognize tumor cells expressing telomerase *(290)*.

Nair et al. have shown that a CTL response can be induced in mice immunized with dendritic cells (DC) transfected with human *hTERT* RNA *(291)*. These CTL were able to lyse human tumor cells, including prostate cancer cells. In addition, it was determined that total tumor RNA was superior to *hTERT* RNA alone. Similarly, Heiser et al. found that the use of amplified total prostate tumor RNA to pulse DC cells induced polyclonal CTL that, although they included anti-PSA and anti-hTERT subpopulations, were more effective at tumor cell lysis than CTL induced by pulsing DC with *PSA* RNA or *hTERT* RNA alone *(292)*.

hTERT would seem an especially attractive target for immunotherapy because, presumably, it would be difficult for tumor cells to develop resistance by antigen loss because telomerase is likely to be essential for tumor cell survival (although the telomerase-independent telomere maintenance ALT pathway has been demonstrated in certain tumors of mesenchymal origin, it does not seem to be operative in carcinomas, including prostate cancer). Of course, tumor cells may find other ways of avoiding detection by the immune system, such as loss of antigen presentation via major histocompatibility complex or transporter for antigen presentation downregulation. Finally, although autoimmunity caused by the breaking of self-tolerance is a concern with immunotherapy approaches, thus far, there has been no evidence of this, perhaps because normal cells that express hTERT express it at only very low levels.

21. TELOMERE-TARGETED THERAPIES

Targeting the telomeres themselves has also been explored as a potential anticancer approach. G-rich DNA single-stranded overhangs present at the telomeres are able to adopt a structural motif known as a G-quartet or G-quadruplex. G-quartets are folded in such a way that sets of four guanine nucleotide bases associate with one another via non-Watson and Crick hydrogen bonds. This peculiar structure restricts the access of telomerase, thereby inhibiting telomere elongation. Several compounds have been developed with the aid of computer modeling designed to interact with and stabi-

lize G-quartets *(293–297)*. It is thought that such agents will act to inhibit telomerase, thereby leading to senescence or apoptosis of cancer cells. Testing G-quartet interactive cationic porphyrins on DU-145, PC3, and LNCaP cells, Izbicka et al. noted relatively rapid effects on cell growth and proposed that structural changes at the telomere, in addition to telomerase inhibition, likely contributed to the observed effects *(298)*. Likewise, Incles et al. noted senescence induction in DU-145 at rates faster than predicted based on the inhibition of telomerase alone *(299)*. The observation of extensive end-to-end chromosome fusions in this study strongly implied that widespread telomere uncapping had occurred, thus, the effects observed were likely the result of structural changes induced at the telomeres by the agent. Such results highlight the need for inclusion of appropriate normal cell controls in studies of G-quartet-promoting agents to ensure that any effects observed are selective for cancer cells.

One other approach based on targeting the telomere itself involves the introduction of a construct coding for a mutated version of the telomerase template RNA *(248,300)*. Mutations in the template region of hTR lead to incorporation of mutant telomere repeats by telomerase in telomerase-positive cells. These, in turn, disrupt the interaction between the telomere and telomere-specific binding proteins, causing uncapping of one or more telomeres. Expression of mutant hTR in LNCaP and MCF-7 cancer cells causes decreased growth and colony formation and increased apoptosis, as well as decreased tumor growth in an MCF-7 xenograft model, even if expression of the mutated hTR was low. This is in sharp contrast to telomerase inhibition strategies, which seem to require high levels of enzyme inhibition to produce significant effects.

22. CONCLUSION—INTEGRATING TELOMERE BIOLOGY INTO THE PROCESS OF PROSTATE CARCINOGENESIS

Telomere shortening can initiate CIN and occurs early during prostate tumorigenesis; it is, therefore, a likely contributor to the complex genetic changes underlying the phenotypic diversity of prostate cancer. If true, this helps resolve the puzzle of why prostate cancers seem to lack prevalent genetic changes in specific tumor suppressors, oncogenes, or genome stability genes. Instead, the source of CIN may stem from defects in fundamental structural components of the chromosomes themselves, namely, the telomeres.

Early observations of abnormally short telomeres plus the nearly universal activation of telomerase in primary prostate cancers strongly suggested that the majority of prostate cancers undergo critical telomere shortening during their development. Recently, it was found that this occurs early in the disease process, by the pre-invasive PIN stage, in which telomeres are abnormally short and telomerase activity first becomes evident. Notably, basal epithelial cells, which express genome protective genes, such as *GSTP1*, retain normal telomere lengths in PIN and, thus, may not be the ultimate target cells for prostate carcinogenesis. Other cells with phenotypes intermediate between basal and luminal epithelial cells have been proposed as potential transformation targets in the prostate *(301–303)*. These putative transit amplifying/transient proliferating (TA/TP) cells, which are thought to lie along the differentiation pathway between basal stem cells and luminal secretory cells, possess features of the basal stem cell compartment, such as protection from apoptosis and proliferative competence—features also typically found in prostate cancer cells *(304)*.

The root cause of the telomere shortening that occurs during malignant transformation of the prostate is unknown. Because telomeres shorten during cell division, one possibility is that telomeres are progressively lost through normal division as cells are replaced during aging. This would help explain the strong dependence of prostate cancer on age *(6)*. On the other hand, the normal rate of cell division in the prostate is relatively low, thus, it may be that other processes serve to accelerate cell turnover. One such process may be inflammation, a common histological finding in the prostate that also increases with age *(9,305)*. Locally, the inflammatory response generates genotoxic chemical species, such as oxy-radicals as part of the host defense mechanism against pathogens. Exposure to

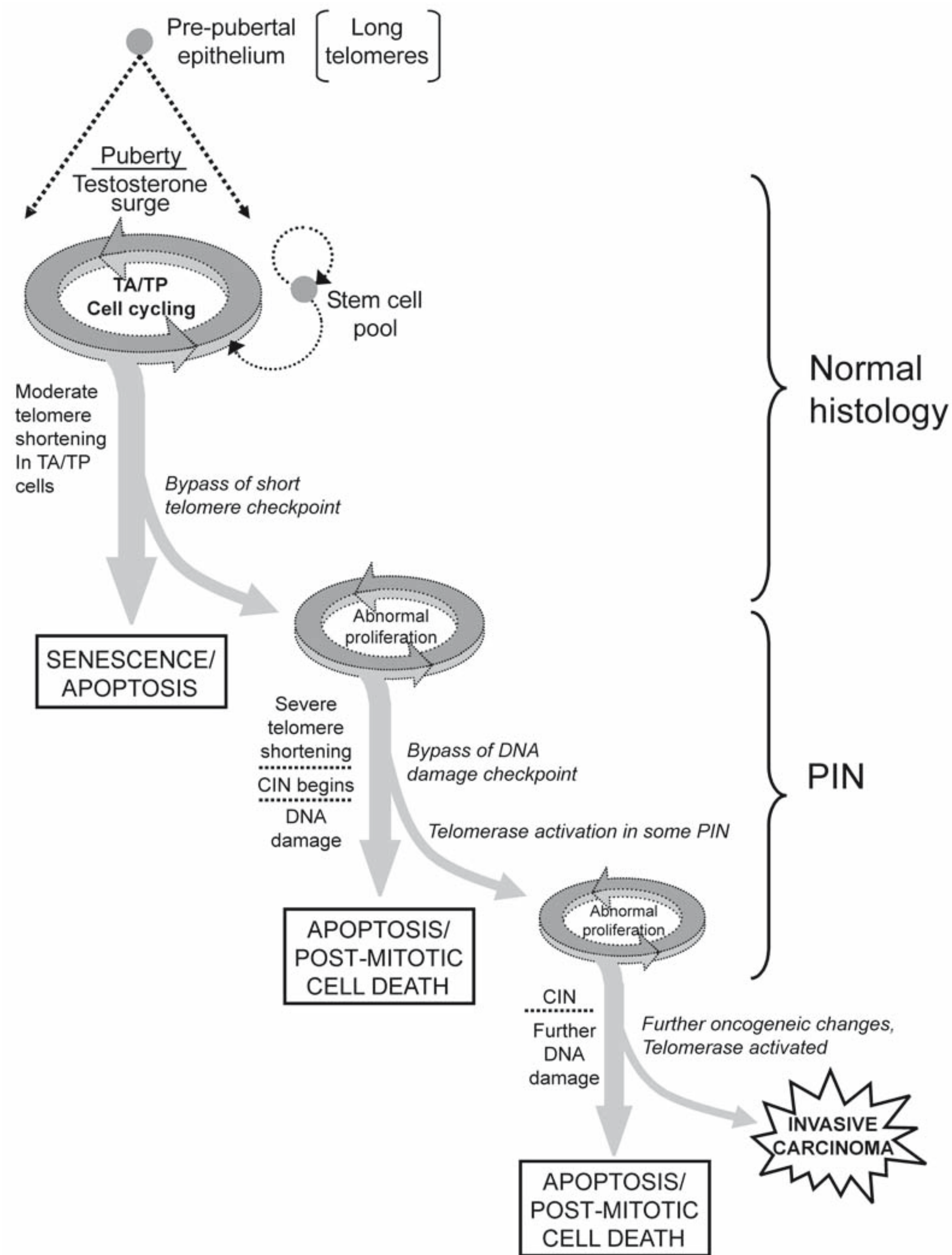


Fig. 5. Changes in telomere length, telomerase activity, and chromosomal instability (CIN) during prostate tumorigenesis and prostate cancer progression. Telomeres (*solid line*) shorten early in the disease process, driving CIN and the development of prostatic intraepithelial neoplasia (PIN). At this stage, telomerase becomes active, partially stabilizing the shortened telomeres and reducing CIN. Telomerase activity allows unlimited replication, thus, fostering the progression from PIN to invasive cancer and, ultimately, metastatic disease. Androgen ablative therapy (*arrow*) results in suppression of telomerase activity reigniting CIN in surviving prostate cancer cells, thus, promoting the development of the hormone-refractory lethal cancer phenotype.

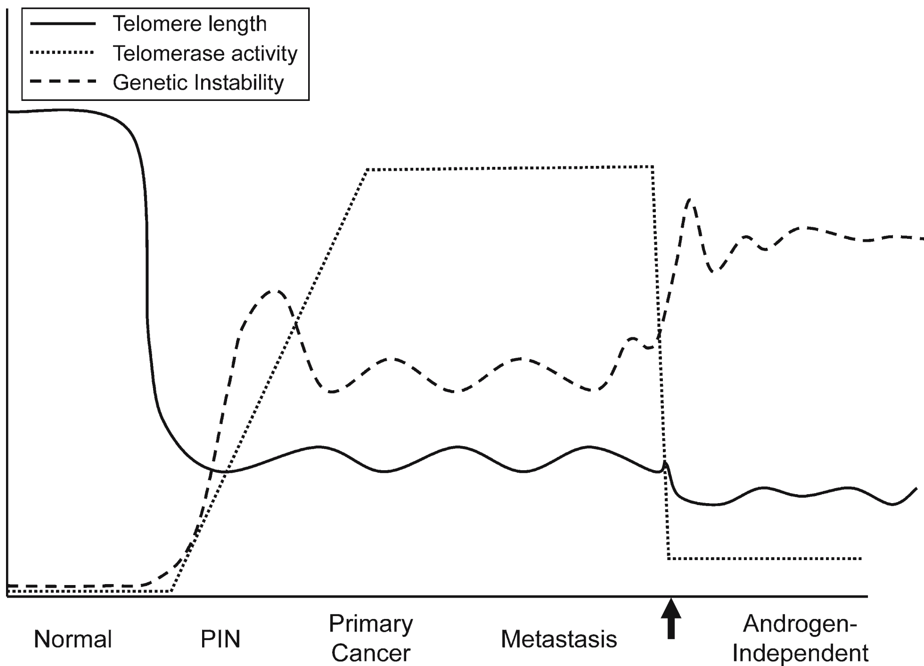


Fig. 6. Model of telomere length changes and telomerase activity in prostate carcinogenesis. PIN, prostatic intraepithelial neoplasia. TA/TP, transit amplifying/transient proliferating.

these chemicals causes co-lateral host cell killing, leading to proliferation to replace the lost cells, and oxidative stress itself is able to cause rapid telomere loss *(306)*. Chronic inflammation not only increases cell turnover and telomere loss, but the accompanying genotoxic stress may impair telomere-sensitive senescence/apoptosis checkpoints, as well as DNA-damage sensitive checkpoints, perhaps in cells whose genomic protection is impaired, for instance, because of epigenetic downregulation of glutathione-*S*-transferase-π *(17,307)*.

Figure 5 outlines a model of the potential involvement of telomeres and telomerase in prostate carcinogenesis. After puberty, proliferation of telomerase negative transit amplifying/transient proliferating cells during normal aging, or driven by chronic inflammation, leads to moderate telomere shortening that engages tumor suppressive cell cycle checkpoints. If, however, there is genetic or epigenetic inactivation of cell senescence checkpoints (such as the *p16/pRB* checkpoint), proliferating telomerase-negative cells continue to divide, despite their shortened telomeres *(308,309)*. With time, rare intermediate cells that also have experienced mutations in key growth regulatory pathways, such as the Hedgehog signaling pathway, undergo abnormal clonal expansion *(310)*. During this time, telomeres continue to shorten until one or more telomeres become dysfunctional, thereby initiating CIN, culminating in the emergence of PIN. CIN involves DNA damage, therefore DNA damage checkpoints should act to limit a PIN lesion from progressing further. A subset of PIN lesions may bypass this second checkpoint (DNA damage checkpoint), leading to further oncogenic mutations, resulting in invasive cancer. Finally, immortalization by telomere length stabilization, most commonly through telomerase activation, allows unlimited tumor expansion (although all chromosomes in all cells may not achieve complete stabilization).

Presumably, the chance that initiated cells and premalignant lesions will abrogate *both* telomere length checkpoints and DNA damage checkpoints is small, explaining why most initiated cells and lesions apparently fail to fully progress to cancer. Even cells that have overcome these tumor-suppressive barriers must still eventually re-stabilize their telomeres to prevent runaway lethal genetic

instability (postmitotic cell death) *(311)*. Thus, the acquisition of telomerase activity likely represents one of the final hurdles on the road to cancer.

Lastly, telomeres may play an important role in the development of advanced hormone-refractory prostate cancer. Because telomerase activity is significantly suppressed in prostate cancer cells after androgen withdrawal, androgen ablative therapies may reignite CIN in the surviving prostate cancer cells (Fig. 6). Such cells represent a source of cancer cell variation from which a more aggressive, hormone-refractory lethal tumor may evolve.

In summary, short dysfunctional telomeres may be critically involved in the initiation and progression of prostate cancer, as well as the emergence of hormone-refractory cancer after androgen ablation. If so, a thorough understanding of telomere biology as it relates to prostate cancer should provide new opportunities for effective disease prevention and treatment.

REFERENCES

1. Carter, B. S., Bova, G. S., Beaty, T. H., et al. (1993). Hereditary prostate cancer: epidemiologic and clinical features. *J. Urol.* **150**, 797–802.
2. Carter, B. S., Beaty, T. H., Steinberg, G. D., Childs, B. and Walsh, P. C. (1992). Mendelian inheritance of familial prostate cancer. *Proc. Natl. Acad. Sci. USA* **89**, 3367–3371.
3. Risch, N. (2001). The genetic epidemiology of cancer: interpreting family and twin studies and their implications for molecular genetic approaches. *Cancer Epidemiol. Biomarkers Prev.* **10**, 733–741.
4. Page, W. F., Braun, M. M., Partin, A. W., Caporaso, N. and Walsh, P. (1997). Heredity and prostate cancer: a study of World War II veteran twins. *Prostate* **33**, 240–245.
5. Lichtenstein, P., Holm, N. V., Verkasalo, P. K., et al. (2000). Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.* **343**, 78–85.
6. Hsing, A. W., Tsao, L. and Devesa, S. S. (2000). International trends and patterns of prostate cancer incidence and mortality. *Int. J. Cancer* **85**, 60–67.
7. Shimizu, H., Ross, R. K., Bernstein, L., Yatani, R., Henderson, B. E. and Mack, T. M. (1991). Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br. J. Cancer* **63**, 963–966.
8. Kolonel, L. N. (1980). Cancer patterns of four ethnic groups in Hawaii. *J. Natl. Cancer Inst.* **65**, 1127–1139.
9. DeMarzo, A. M., Nelson, W. G., Isaacs, W. B. and Epstein, J. I. (2003). Pathological and molecular aspects of prostate cancer. *Lancet* **361**, 955–964.
10. Shah, R. B., Mehra, R., Chinnaiyan, A .M., et al. (2004). Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **64**, 9209–9216.
11. Visakorpi, T. (2003). The molecular genetics of prostate cancer. *Urology* **62**, 3–10.
12. Fearon, E. R. and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* **61**, 759–767.
13. Hartwell, L. (1992) Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* **71**, 543–546.
14. Vogelstein, B. and Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nat. Med.* **10**, 789–799.
15. Weinberg, R. A. (1989). Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. *Cancer Res.* **49**, 3713–3721.
16. Kinzler, K. W. and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* **386**, 761–763.
17. Nelson, W. G., De Marzo, A. M. and Isaacs, W.B. (2003). Prostate cancer. *N. Engl. J. Med.* **349**, 366–381.
18. Wolf, M., Mousses, S., Hautaniemi, S., et al. (2004). High-resolution analysis of gene copy number alterations in human prostate cancer using CGH on cDNA microarrays: impact of copy number on gene expression. *Neoplasia* **6**, 240–247.
19. Al-Maghrabi, J., Vorobyova, L., Chapman, W., Jewett, M., Zielenska, M. and Squire, J. A. (2001). p53 Alteration and chromosomal instability in prostatic high-grade intraepithelial neoplasia and concurrent carcinoma: analysis by immunohistochemistry, interphase in situ hybridization, and sequencing of laser-captured microdissected specimens. *Mod. Pathol.* **14**, 1252–1262.
20. Beheshti, B., Park, P. C., Sweet, J. M., Trachtenberg, J., Jewett, M. A. and Squire, J. A. (2001). Evidence of chromosomal instability in prostate cancer determined by spectral karyotyping (SKY) and interphase fish analysis. *Neoplasia* **3**, 62–69.
21. Cher, M. L., MacGrogan, D., Bookstein, R., Brown, J. A., Jenkins, R. B. and Jensen, R. H. (1994). Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. *Genes Chromosomes Cancer* **11**, 153–162.
22. Joos, S., Bergerheim, U. S., Pan, Y., et al. (1995). Mapping of chromosomal gains and losses in prostate cancer by comparative genomic hybridization. *Genes Chromosomes Cancer* **14**, 267–276.

23. Nupponen, N. N., Kakkola, L., Koivisto, P., and Visakorpi, T. (1998). Genetic alterations in hormone-refractory recurrent prostate carcinomas. *Am. J. Pathol.* **153**, 141–148.
24. Isaacs, W. B., Bova, G. S., Morton, R. A., Bussemakers, M. J., Brooks, J. D. and Ewing, C. M. (1994). Molecular biology of prostate cancer. *Semin. Oncol.* **21**, 514–521.
25. Elo, J. P. and Visakorpi, T. (2001). Molecular genetics of prostate cancer. *Ann. Med.* **33**, 130–141.
26. Loeb, L. A. (1991). Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res.* **51**, 3075–3079.
27. Heinen, C. D., Schmutte, C., and Fishel, R. (2002). DNA repair and tumorigenesis: lessons from hereditary cancer syndromes. *Cancer Biol. Ther.* **1**, 477–485.
28. Sherr, C. J. (2004). Principles of tumor suppression. *Cell* **116**, 235–246.
29. Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature* **396**, 643–649.
30. Qian, J., Jenkins, R. B., and Bostwick, D. G. (1999). Genetic and chromosomal alterations in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence in situ hybridization. *Eur. Urol.* **35**, 479–483.
31. Macoska, J. A., Trybus, T. M., Sakr, W. A., et al. (1994). Fluorescence in situ hybridization analysis of 8p allelic loss and chromosome 8 instability in human prostate cancer. *Cancer Res.* **54**, 3824–3830.
32. Erbersdobler, A., Gurses, N., and Henke, R. P. (1996). Numerical chromosomal changes in high-grade prostatic intraepithelial neoplasia (PIN) and concomitant invasive carcinoma. *Pathol. Res. Pract.* **192**, 418–427.
33. Roh, M., Gary, B., Song, C., et al. (2003). Overexpression of the oncogenic kinase Pim-1 leads to genomic instability. *Cancer Res.* **63**, 8079–8084.
34. Pihan, G. A., Wallace, J., Zhou, Y. and Doxsey, S. J. (2003). Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res.* **63**, 1398–1404.
35. Blackburn, E.H. (1991) Telomeres. *Trends Biochem. Sci.* **16**, 378–381.
36. Moyzis, R. K., Buckingham, J. M., Cram, L. S., et al. (1988). A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. USA* **85**, 6622–6626.
37. Meyne, J., Ratliff, R. L., and Moyzis, R. K. (1989). Conservation of the human telomere sequence (TTAGGG)n among vertebrates. *Proc. Natl. Acad. Sci. USA* **86**, 7049–7053.
38. Saldanha, S. N., Andrews, L. G., and Tollefsbol, T. O. (2003). Assessment of telomere length and factors that contribute to its stability. *Eur. J. Biochem.* **270**, 389–403.
39. Karlseder, J. (2003). Telomere repeat binding factors: keeping the ends in check. *Cancer Lett.* **194**, 189–197.
40. Harley, C. B., Futcher, A. B., and Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature* **345**, 458–460.
41. Lindsey, J., McGill, N. I., Lindsey, L. A., Green, D. K., and Cooke, H. J. (1991). In vivo loss of telomeric repeats with age in humans. *Mutation Research* **256**, 45–48.
42. Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K., and Allshire, R. C. (1990). Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346**, 866–868.
43. Levy, M. Z., Allsopp, R. C., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). Telomere end-replication problem and cell aging. *J. Mol. Biol.* **225**, 951–960.
44. Kruk, P. A., Rampino, N. J., and Bohr, V. A. (1995). DNA damage and repair in telomeres: relation to aging. *Proc. Natl. Acad. Sci. USA* **92**, 258–262.
45. von Zglinicki, T., Pilger, R., and Sitte, N. (2000). Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radic. Biol. Med.* **28**, 64–74.
46. Greider, C. W. and Blackburn, E. H. (1985). Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* **43**, 405–413.
47. Reddel, R. R., Bryan, T. M., Colgin, L. M., Perrem, K. T., and Yeager, T. R. (2001). Alternative lengthening of telomeres in human cells. *Radiat. Res.* **155**, 194–200.
48. McClintock, B. (1941) The stability of broken ends of chromosomes in Zea mays. *Genetics* **26**, 234–282.
49. Lo, A. W., Sabatier, L., Fouladi, B., Pottier, G., Ricoul, M., and Murnane, J. P. (2002). DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. *Neoplasia* **4**, 531–538.
50. Artandi, S. E. and DePinho, R. A. (2000). A critical role for telomeres in suppressing and facilitating carcinogenesis. *Curr. Opin. Genet. Dev.* **10**, 39–46.
51. Feldser, D.M., Hackett, J.A., and Greider, C.W. (2003). Telomere dysfunction and the initiation of genome instability. *Nat. Rev. Cancer* **3**, 623–627.
52. Gisselsson, D., Pettersson, L., Hoglund, M., et al. (2000). Chromosomal breakage-fusion-bridge events cause genetic intratumor heterogeneity. *Proc. Natl. Acad. Sci. USA* **97**, 5357–5362.
53. Gisselsson, D., Jonson, T., Yu, C., et al. (2002). Centrosomal abnormalities, multipolar mitoses, and chromosomal instability in head and neck tumours with dysfunctional telomeres. *Br. J. Cancer* **87**, 202–207.
54. Wright, W. E. and Shay, J. W. (2001). Cellular senescence as a tumor-protection mechanism: the essential role of counting. *Curr. Opin. Genet. Dev.* **11**, 98–103.
55. Bodnar, A. G., Ouellette, M., Frolkis, M., et al. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352.

56. Vaziri, H. and Benchimol, S. (1998). Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol.* **8**, 279–282.
57. Jacobs, J. J. and de Lange, T. (2004). Significant Role for p16(INK4a) in p53-Independent Telomere-Directed Senescence. *Curr. Biol.* **14**, 2302–2308.
58. Counter, C., Avilion, A., LeFeuvre, C., et al. (1992). Telomere shortening associated with chrosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11**, 1921–1929.
59. Mehle, C., Ljungberg, B., and Roos, G. (1994). Telomere shortening in renal cell carcinoma. *Cancer Res.* **54**, 236–241.
60. Furugori, E., Hirayama, R., Nakamura, K. I., Kammori, M., Esaki, Y., and Takubo, K. (2000). Telomere shortening in gastric carcinoma with aging despite telomerase activation. *J. Cancer Res. Clin. Oncol.* **126**, 481–485.
61. Takagi, S., Kinouchi, Y., Hiwatashi, N., et al. (1999). Telomere shortening and the clinicopathologic characteristics of human colorectal carcinomas. *Cancer* **86**, 1431–1436.
62. Sommerfeld, H. J., Meeker, A. K., Piatyszek, M. A., Bova, G. S., Shay, J. W. and Coffey, D. S. (1996). Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res.* **56**, 218–222.
63. Koeneman, K. S., Pan, C. X., Jin, J. K., et al. (1998). Telomerase activity, telomere length, and DNA ploidy in prostatic intraepithelial neoplasia (PIN). *J. Urol.* **160**, 1533–1539.
64. Meeker, A. K., Gage, W. R., Hicks, J. L., et al. (2002). Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. *Am. J. Pathol.* **160**, 1259–1268.
65. Hiyama, K., Ishioka, S., Shirotani, Y., et al. (1995). Alterations in telomeric repeat length in lung cancer are associated with loss of heterozygosity in p53 and Rb. *Oncogene* **10**, 937–944.
66. Smith, J. K. and Yeh, G. (1992). Telomere reduction in endometrial adenocarcinoma. *Am. J. Obstet. Gynecol.* **167**, 1883–1887.
67. Griffith, J. K., Bryant, J. E., Fordyce, C. A., Gilliland, F. D., Joste, N. E. and Moyzis, R. K. (1999). Reduced telomere DNA content is correlated with genomic instability and metastasis in invasive human breast carcinoma. *Breast Cancer Res. Treat.* **54**, 59–64.
68. Hiyama, E., Hiyama, K., Yokoyama, T., Ichikawa, T., and Matsuura, Y. (1992). Length of telomeric repeats in neuroblastoma: correlation with prognosis and other biological characteristics. *Jpn. J. Cancer Res.* **83**, 159–164.
69. Bryant, J. E., Hutchings, K. G., Moyzis, R. K., and Griffith, J. K. (1997). Measurement of telomeric DNA content in human tissues. *Biotechniques* **23**, 476–478, 480, 482, passim.
70. Donaldson, L., Fordyce, C., Gilliland, F., et al. (1999). Association between outcome and telomere DNA content in prostate cancer. *J. Urol.* **162**, 1788–1792.
71. Fordyce, C. A., Heaphy, C. M., Joste, N. E., Smith, A.Y., Hunt, W. C., and Griffith, J. K. (2005). Association between cancer-free survival and telomere DNA content in prostate tumors. *J. Urol.* **173**, 610–614.
72. Crissman, J. D., Sakr, W. A., Hussein, M. E. and Pontes, J. E. (1993). DNA quantitation of intraepithelial neoplasia and invasive carcinoma of the prostate. *Prostate* **22**, 155–162.
73. Foster, C. S., Bostwick, D. G., Bonkhoff, H., et al. (2000). Cellular and molecular pathology of prostate cancer precursors. *Scand. J. Urol. Nephrol. Suppl.* 19–43.
74. Bostwick, D. G., Shan, A., Qian, J., et al. (1998). Independent origin of multiple foci of prostatic intraepithelial neoplasia: comparison with matched foci of prostate carcinoma. *Cancer* **83**, 1995–2002.
75. Yu, Y. P., Landsittel, D., Jing, L., et al. (2004). Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. *J. Clin. Oncol.* **22**, 2790–2799.
76. Bostwick, D. G., Pacelli, A. and Lopez-Beltran, A. (1996). Molecular biology of prostatic intraepithelial neoplasia. *Prostate* **29**, 117–134.
77. Meeker, A. K., Hicks, J. L., Platz, E. A., et al. (2002). Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res.* **62**, 6405–6409.
78. Vukovic, B., Park, P. C., Al-Maghrabi, J., et al. (2003). Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. *Oncogene* **22**, 1978–1987.
79. Meeker, A. K., Hicks, J. L., Iacobuzio-Donahue, C. A., et al. (2004). Telomere length abnormalities occur early in the initiation of epithelial carcinogenesis. *Clin. Cancer Res.* **10**, 3317–3326.
80. Meeker, A. K., Hicks, J. L., Gabrielson, E., Strauss, W. M., De Marzo, A. M., and Argani, P. (2004). Telomere shortening occurs in subsets of normal breast epithelium as well as in situ and invasive carcinoma. *Am. J. Pathol.* **164**, 925–935.
81. Montgomery, E., Argani, P., Hicks, J. L., DeMarzo, A. M., and Meeker, A. K. (2004). Telomere lengths of translocation-associated and nontranslocation-associated sarcomas differ dramatically. *Am. J. Pathol.* **164**, 1523–1529.
82. O'Sullivan, J.N., Bronner, M.P., Brentnall, T.A., et al. (2002). Chromosomal instability in ulcerative colitis is related to telomere shortening. *Nat. Genet.* **32**, 280–284.
83. Hermeking, H. (2003). The 14-3-3 cancer connection. *Nat. Rev. Cancer* **3**, 931–943.
84. Lodygin, D., Diebold, J., and Hermeking, H. (2004). Prostate cancer is characterized by epigenetic silencing of 14-3-3sigma expression. *Oncogene* **23**, 9034–9041.
85. Greider, C. W. and Blackburn, E. H. (1987). The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* **51**, 887–898.

86. Greider, C. W. (1998). Telomerase activity, cell proliferation, and cancer. *Proc. Natl. Acad. Sci. USA* **95**, 90–92.
87. Shay, J. W. and Wright, W. E. (1996). The reactivation of telomerase activity in cancer progression. *Trends Genet.* **12**, 129–131.
88. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R.A. (1999). Creation of human tumour cells with defined genetic elements. *Nature* **400**, 464–468.
89. Elenbaas, B., Spirio, L., Koerner, F., et al. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev.* **15**, 50–65.
90. Kim, N.W., Piatyszek, M.A., Prowse, K.R., et al. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015.
91. Meeker, A. K. and Coffey, D. S. (1997). Telomerase: a promising marker of biological immortality of germ, stem, and cancer cells. A review. *Biochemistry (Mosc)* **62**, 1323–1331.
92. Shay, J. W. and Bacchetti, S. (1997). A survey of telomerase activity in human cancer. *Eur. J. Cancer* **33**, 787–791.
93. Nakamura, T. M., Morin, G. B., Chapman, K. B., et al. (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science* **277**, 955–959.
94. Meyerson, M., Counter, C. M., Eaton, E. N., et al. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* **90**, 785–795.
95. Harrington, L., Zhou, W., McPhail, T., et al. (1997). Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev.* **11**, 3109–3115.
96. Weinrich, S. L., Pruzan, R., Ma, L., et al. (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat. Genet.* **17**, 498–502.
97. Kilian, A., Bowtell, D. D., Abud, H. E., et al. (1997). Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum. Mol. Genet.* **6**, 2011–2019.
98. Feng, J., Funk, W. D., Wang, S. S., et al. (1995). The RNA component of human telomerase. *Science* **269**, 1236–1241.
99. Shay, J. W. (1997). Telomerase in human development and cancer. *J. Cell. Physiol.* **173**, 266–270.
100. Harley, C. B. (2002). Telomerase is not an oncogene. *Oncogene* **21**, 494–502.
101. Weng, N. P., Levine, B. L., June, C. H., and Hodes, R. J. (1996). Regulated expression of telomerase activity in human T lymphocyte development and activation. *J. Exp. Med.* **183**, 2471–2479.
102. Hiyama, K., Hirai, Y., Kyoizumi, S., et al. (1995). Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.* **155**, 3711–3715.
103. Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W., and Shay, J.W. (1996). Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.* **18**, 173–179.
104. Broccoli, D., Young, J. W., and de Lange, T. (1995). Telomerase activity in normal and malignant hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **92**, 9082–9086.
105. Hiyama, E., Hiyama, K., Tatumoto, N., Kodama, T., Shay, J., and Yokoyama, T. (1996). Telomerase activity in human intestine. *Int. J. Oncol.* **9**, 453–458.
106. Harle-Bachor, C. and Boukamp, P. (1996). Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes. *Proc. Natl. Acad. Sci. USA* **93**, 6476–6481.
107. Yasumoto, S., Kunimura, C., Kikuchi, K., et al. (1996). Telomerase activity in normal human epithelial cells. *Oncogene* **13**, 433–439.
108. Kyo, S., Takakura, M., Kohama, T., and Inoue, M. (1997). Telomerase activity in human endometrium. *Cancer Res.* **57**, 610–614.
109. Taylor, R. S., Ramirez, R. D., Ogoshi, M., Chaffins, M., Piatyszek, M. A., and Shay, J. W. (1996). Detection of telomerase activity in malignant and nonmalignant skin conditions. *J. Invest. Dermatol.* **106**, 759–765.
110. Avilion, A. A., Piatyszek, M. A., Gupta, J., Shay, J. W., Bacchetti, S., and Greider, C. W. (1996). Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res.* **56**, 645–650.
111. Bodnar, A. G., Kim, N. W., Effros, R. B., and Chiu, C. P. (1996). Mechanism of telomerase induction during T cell activation. *Exp. Cell. Res.* **228**, 58–64.
112. Nakamura, Y., Tahara, E., Tahara, H., Yasui, W., and Ide, T. (1999). Quantitative reevaluation of telomerase activity in cancerous and noncancerous gastrointestinal tissues. *Mol. Carcinog.* **26**, 312–320.
113. Counter, C. M., Meyerson, M., Eaton, E. N., et al. (1998). Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. *Oncogene* **16**, 1217–1222.
114. Kyo, S., Kanaya, T., Takakura, M., et al. (1999). Expression of human telomerase subunits in ovarian malignant, borderline and benign tumors. *Int. J. Cancer* **80**, 804–809.
115. Park, T. W., Riethdorf, S., Riethdorf, L., Loning, T., and Janicke, F. (1999). Differential telomerase activity, expression of the telomerase catalytic sub-unit and telomerase-RNA in ovarian tumors. *Int. J. Cancer* **84**, 426–431.
116. Wu, A., Ichihashi, M., and Ueda, M. (1999). Correlation of the expression of human telomerase subunits with telomerase activity in normal skin and skin tumors. *Cancer* **86**, 2038–2044.
117. Rohde, V., Sattler, H. P., Bund, T., et al. (2000). Expression of the human telomerase reverse transcriptase is not related to telomerase activity in normal and malignant renal tissue. *Clin. Cancer Res.* **6**, 4803–4809.

118. Tahara, H., Yasui, W., Tahara, E., et al. (1999). Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. *Oncogene* **18**, 1561–1567.
119. Ulaner, G. A., Hu, J. F., Vu, T. H., Oruganti, H., Giudice, L. C., and Hoffman, A. R. (2000). Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium. *Int. J. Cancer* **85**, 330–335.
120. Reddel, R. R. (2003). Alternative lengthening of telomeres, telomerase, and cancer. *Cancer Lett.* **194**, 155–162.
121. Naka, K., Ikeda, K., and Motoyama, N. (2002). Recruitment of NBS1 into PML oncogenic domains via interaction with SP100 protein. *Biochem. Biophys. Res. Commun.* **299**, 863–871.
122. Stavropoulos, D. J., Bradshaw, P. S., Li, X., et al. (2002). The Bloom syndrome helicase BLM interacts with TRF2 in ALT cells and promotes telomeric DNA synthesis. *Hum. Mol. Genet.* **11**, 3135–3144.
123. Shigeeda, N., Uchida, M., Barrett, J. C., and Tsutsui, T. (2003). Candidate chromosomal regions for genes involved in activation of alternative lengthening of telomeres in human immortal cell lines. *Exp. Gerontol.* **38**, 641–651.
124. Stewart, S. A. and Weinberg, R. A. (2000). Telomerase and human tumorigenesis. *Semin. Cancer Biol.* **10**, 399–406.
125. Artandi, S. E., Chang, S., Lee, S. L., et al. (2000). Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* **406**, 641–645.
126. O'Hagan, R. C., Chang, S., Maser, R. S., et al. (2002). Telomere dysfunction provokes regional amplification and deletion in cancer genomes. *Cancer Cell* **2**, 149–155.
127. Engelhardt, M., Albanell, J., Drullinsky, P., et al. (1997). Relative contribution of normal and neoplastic cells determines telomerase activity and telomere length in primary cancers of the prostate, colon, and sarcoma. *Clin. Cancer Res.* **3**, 1849–1857.
128. Kamradt, J., Drosse, C., Kalkbrenner, S., et al. (2003). Telomerase activity and telomerase subunit gene expression levels are not related in prostate cancer: a real-time quantification and in situ hybridization study. *Lab. Invest.* **83**, 623–633.
129. Kallakury, B. V., Brien, T. P., Lowry, C. V., et al. (1997). Telomerase activity in human benign prostate tissue and prostatic adenocarcinomas. *Diagn. Mol. Pathol.* **6**, 192–198.
130. Lin, Y., Uemura, H., Fujinami, K., Hosaka, M., Harada, M., and Kubota, Y. (1997) Telomerase activity in primary prostate cancer. *J. Urol.* **157**, 1161–1165.
131. Wullich, B., Rohde, V., Oehlenschlager, B., et al. (1999). Focal intratumoral heterogeneity for telomerase activity in human prostate cancer. *J. Urol.* **161**, 1997–2001.
132. Zhang, W., Kapusta, L. R., Slingerland, J. M., and Klotz, L. H. (1998) Telomerase activity in prostate cancer, prostatic intraepithelial neoplasia, and benign prostatic epithelium. *Cancer Res.* **58**, 619–621.
133. Caldarera, E., Crooks, N. H., Muir, G. H., Pavone-Macaluso, M., and Carmichael, P. L. (2000). An appraisal of telomerase activity in benign prostatic hyperplasia. *Prostate* **45**, 267–270.
134. Athanassiadou, P., Bantis, A., Gonidi, M., et al. (2003). Telomerase expression as a marker in prostate cancer: correlation to clinicopathologic predictors. *J. Exp. Clin. Cancer Res.* **22**, 613–618.
135. Kolquist, K. A., Ellisen, L. W., Counter, C. M., et al. (1998). Expression of TERT in early premalignant lesions and a subset of cells in normal tissues. *Nat. Genet.* **19**, 182–186.
136. Bettendorf, O., Heine, B., Kneif, S., et al. (2003). Expression-patterns of the RNA component (hTR)and the catalytic subunit (hTERT) of human telomerase in nonneoplastic prostate tissue, prostatic intraepithelial neoplasia, and prostate cancer. *Prostate* **55**, 99–104.
137. Paradis, V., Dargere, D., Laurendeau, I., et al. (1999). Expression of the RNA component of human telomerase (hTR) in prostate cancer, prostatic intraepithelial neoplasia, and normal prostate tissue. *J. Pathol.* **189**, 213–218.
138. Liu, B.C., LaRose, I., Weinstein, L. J., Ahn, M., Weinstein, M. H., and Richie, J.P. (2001) Expression of telomerase subunits in normal and neoplastic prostate epithelial cells isolated by laser capture microdissection. *Cancer* **92**, 1943–1948.
139. Latil, A., Vidaud, D., Valeri, A., et al. (2000). htert expression correlates with MYC over-expression in human prostate cancer. *Int. J. Cancer* **89**, 172–176.
140. Iczkowski, K. A., Pantazis, C. G., McGregor, D.H., Wu, Y., and Tawfik, O. W. (2002). Telomerase reverse transcriptase subunit immunoreactivity: a marker for high-grade prostate carcinoma. *Cancer* **95**, 2487–2493.
141. De Kok, J. B., Schalken, J. A., Aalders, T. W., Ruers, T. J., Willems, H. L., and Swinkels, D. W. (2000). Quantitative measurement of telomerase reverse transcriptase (hTERT) mRNA in urothelial cell carcinomas. *Int. J. Cancer* **87**, 217–220.
142. Gertler, R., Rosenberg, R., Stricker, D., et al. (2002). Prognostic potential of the telomerase subunit human telomerase reverse transcriptase in tumor tissue and nontumorous mucosa from patients with colorectal carcinoma. *Cancer* **95**, 2103–2111.
143. Wang, L., Soria, J. C., Kemp, B. L., Liu, D. D., Mao, L., and Khuri, F. R. (2002). hTERT expression is a prognostic factor of survival in patients with stage I non-small cell lung cancer. *Clin. Cancer Res.* **8**, 2883–2889.
144. Poremba, C., Heine, B., Diallo, R., et al. (2002). Telomerase as a prognostic marker in breast cancer: high-throughput tissue microarray analysis of hTERT and hTR. *J. Pathol.* **198**, 181–189.

145. Bieche, I., Nogues, C., Paradis, V., et al. (2000). Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. *Clin. Cancer Res.* **6**, 452–459.
146. Schneider-Stock, R., Emrich, T., Peters, B., Jaeger, V., and Roessner, A. (2001). Analysis of human telomerase reverse transcriptase mRNA (hTERT) expression in myxoid liposarcomas using LightCycler real-time quantitative reverse transcriptase-polymerase chain reaction. *Electrophoresis* **22**, 1098–1101.
147. Dome, J. S., Chung, S., Bergemann, T., et al. (1999). High telomerase reverse transcriptase (hTERT) messenger RNA level correlates with tumor recurrence in patients with favorable histology Wilms' tumor. *Cancer Res.* **59**, 4301–4307.
148. Wisman, G. B., Knol, A. J., Helder, M. N., et al. (2001). Telomerase in relation to clinicopathologic prognostic factors and survival in cervical cancer. *Int. J. Cancer* **91**, 658–664.
149. Nakamura, Y., Tahara, E., Tahara, H., Yasui, W., and Ide, T. (1999). Quantitative reevaluation of telomerase activity in cancerous and noncancerous gastrointestinal tissues. *Mol. Carcinog.* **26**, 312–320.
150. de Kok, J. B., Verhaegh, G. W., Roelofs, R. W., et al. (2002) DD3(PCA3), a very sensitive and specific marker to detect prostate tumors. *Cancer Res.* **62**, 2695–2698.
151. Snijders, P. J., van Duin, M., Walboomers, J. M., et al. (1998). Telomerase activity exclusively in cervical carcinomas and a subset of cervical intraepithelial neoplasia grade III lesions: strong association with elevated messenger RNA levels of its catalytic subunit and high-risk human papillomavirus DNA. *Cancer Res.* **58**, 3812–3818.
152. Mitchell, J. R., Cheng, J., and Collins, K. (1999). A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. *Mol. Cell. Biol.* **19**, 567–576.
153. Mitchell, J. R., Wood, E., and Collins, K. (1999). A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* **402**, 551–555.
154. Ford, L. P., Suh, J. M., Wright, W. E., and Shay, J. W. (2000). Heterogeneous nuclear ribonucleoproteins C1 and C2 associate with the RNA component of human telomerase. *Mol. Cell. Biol.* **20**, 9084–9091.
155. Dragon, F., Pogacic, V., and Filipowicz, W. (2000). In vitro assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs. *Mol. Cell. Biol.* **20**, 3037–3048.
156. LaBranche, H., Dupuis, S., Ben-David, Y., Bani, M. R., Wellinger, R. J., and Chabot, B. (1998). Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nat. Genet.* **19**, 199–202.
157. Holt, S. E., Aisner, D. L., Baur, J., et al. (1999). Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev.* **13**, 817–826.
158. Le, S., Sternglanz, R., and Greider, C. W. (2000). Identification of two RNA-binding proteins associated with human telomerase RNA. *Mol. Biol. Cell.* **11**, 999–1010.
159. Harrington, L., McPhail, T., Mar, V., et al. (1997). A Mammalian Telomerase-Associated Protein. *Science* **275**, 973–977.
160. Wymenga, L. F., Wisman, G. B., Veenstra, R., Ruiters, M. H., and Mensink, H. J. (2000). Telomerase activity in needle biopsies from prostate cancer and benign prostates. *Eur. J. Clin. Invest.* **30**, 330–335.
161. Takahashi, C., Miyagawa, I., Kumano, S., and Oshimura, M. (1997). Detection of telomerase activity in prostate cancer by needle biopsy. *Eur. Urol.* **32**, 494–498.
162. Chieco, P., Bertaccini, A., Giovannini, C., Stecca, B. A., and Martorana, G. (2001). Telomerase activity in touch-imprint cell preparations from fresh prostate needle biopsy specimens. *Eur. Urol.* **40**, 666–672.
163. Bertaccini, A., Chieco, P., Giovannini, C., Capra, I., and Martorana, G. (1999). Biopsying prostate gland: telomerase evaluation. *Prostate Cancer Prostatic Dis.* **2**, S6.
164. Scates, D. K., Muir, G. H., Venitt, S., and Carmichael, P. L. (1997). Detection of telomerase activity in human prostate: a diagnostic marker for prostatic cancer? *Br. J. Urol.* **80**, 263–268.
165. Lin, Y., Uemura, H., Fujinami, K., et al. (1998). Detection of telomerase activity in prostate needle-biopsy samples. *Prostate* **36**, 121–128.
166. Muller, M. (1999). Determination of the enzyme telomerase in needle biopsies and fine needle aspiration cytology samples of the prostate in the diagnosis of prostate cancer. *Prostate Cancer Prostatic Dis.* **2**, S23.
167. Wang, Z., Ramin, S. A., Tsai, C., et al. (2001). Telomerase activity in prostate sextant needle cores from radical prostatectomy specimens. *Urol. Oncol.* **6**, 57–62.
168. Wang, Z., Ramin, S. A., Tsai, C., et al. (2000). Detection of telomerase activity in prostatic fluid specimens. *Urol. Oncol.* **6**, 4–9.
169. Vicentini, C., Gravina, G. L., Angelucci, A., et al. (2004). Detection of telomerase activity in prostate massage samples improves differentiating prostate cancer from benign prostatic hyperplasia. *J. Cancer Res. Clin. Oncol.* **130**, 217–221.
170. Meid, F. H., Gygi, C. M., Leisinger, H. J., Bosman, F. T., and Benhattar, J. (2001). The use of telomerase activity for the detection of prostatic cancer cells after prostatic massage. *J. Urol.* **165**, 1802–1805.
171. Suh, C. I., Shanafelt, T., May, D. J., et al. (2000). Comparison of telomerase activity and GSTP1 promoter methylation in ejaculate as potential screening tests for prostate cancer. *Mol. Cell Probes* **14**, 211–217.
172. Horikawa, I. and Barrett, J. C. (2003). Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanisms. *Carcinogenesis* **24**, 1167–1176.
173. Armbruster, B. N., Etheridge, K. T., Broccoli, D., and Counter, C. M. (2003). Putative telomere-recruiting domain in the catalytic subunit of human telomerase. *Mol. Cell. Biol.* **23**, 3237–3246.

174. Huard, S., Moriarty, T. J., and Autexier, C. (2003). The C terminus of the human telomerase reverse transcriptase is a determinant of enzyme processivity. *Nucleic Acids Res.* **31**, 4059–4070.
175. Hisatomi, H., Ohyashiki, K., Ohyashiki, J. H., et al. (2003). Expression profile of a gamma-deletion variant of the human telomerase reverse transcriptase gene. *Neoplasia* **5**, 193–197.
176. Kugoh, H., Shigenami, K., Funaki, K., Barrett, J. C., and Oshimura, M. (2003). Human chromosome 5 carries a putative telomerase repressor gene. *Genes Chromosomes Cancer* **36**, 37–47.
177. Guilleret, I., Yan, P., Grange, F., Braunschweig, R., Bosman, F. T., and Benhattar, J. (2002). Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. *Int. J. Cancer* **101**, 335–341.
178. Guilleret, I., Yan, P., Guillou, L., Braunschweig, R., Coindre, J. M., and Benhattar, J. (2002). The human telomerase RNA gene (hTERC) is regulated during carcinogenesis but is not dependent on DNA methylation. *Carcinogenesis* **23**, 2025–2030.
179. Lin, S. Y. and Elledge, S. J. (2003). Multiple tumor suppressor pathways negatively regulate telomerase. *Cell* **113**, 881–889.
180. Sato, K., Qian, J., Slezak, J. M., et al. (1999). Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma. *J. Natl. Cancer Inst.* **91**, 1574–1580.
181. Wong, J. M., Kusdra, L., and Collins, K. (2002). Subnuclear shuttling of human telomerase induced by transformation and DNA damage. *Nat. Cell. Biol.* **4**, 731–736.
182. Yang, Y., Chen, Y., Zhang, C., Huang, H., and Weissman, S. M. (2002). Nucleolar localization of hTERT protein is associated with telomerase function. *Exp. Cell. Res.* **277**, 201–209.
183. Etheridge, K. T., Banik, S. S., Armbruster, B. N., et al. (2002). The nucleolar localization domain of the catalytic subunit of human telomerase. *J. Biol. Chem.* **277**, 24,764–24,770.
184. Akalin, A., Elmore, L. W., Forsythe, H. L., et al. (2001). A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. *Cancer Res.* **61**, 4791–4796.
185. Hawkins, G. A., Chang, B. L., Zheng, S. L., et al. (2004). Mutational analysis of PINX1 in hereditary prostate cancer. *Prostate* **60**, 298–302.
186. Zhou, X. Z. and Lu, K. P. (2001). The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. *Cell* **107**, 347–359.
187. Ford, L. P., Shay, J. W. and Wright, W. E. (2001). The La antigen associates with the human telomerase ribonucleoprotein and influences telomere length in vivo. *RNA* **7**, 1068–1075.
188. Xiong, J., Fan, S., Meng, Q., et al. (2003). BRCA1 inhibition of telomerase activity in cultured cells. *Mol. Cell. Biol.* **23**, 8668–8690.
189. Li, H., Lee, T. H., and Avraham, H. (2002). A novel tricomplex of BRCA1, Nmi, and c-Myc inhibits c-Myc-induced human telomerase reverse transcriptase gene (hTERT) promoter activity in breast cancer. *J. Biol. Chem.* **277**, 20,965–20,973.
190. Ancelin, K., Brunori, M., Bauwens, S., et al. (2002). Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. *Mol. Cell. Biol.* **22**, 3474–3487.
191. Zhang, J. X., Zhang, Z. K., Sheng, H. P., Tsao, S. W., and Loh, T. T. (2000). Telomerase activity is not related to apoptosis in leukemic cell lines. *Life Sci.* **66**, 1713–1723.
192. Choi, S. H., Kang, H. K., Im, E. O., et al. (2000). Inhibition of cell growth and telomerase activity of breast cancer cells in vitro by retinoic acids. *Int. J. Oncol.* **17**, 971–976.
193. Hisatake, J., Kubota, T., Hisatake, Y., Uskokovic, M., Tomoyasu, S., and Koeffler, H. P. (1999). 5,6-trans-16-ene-vitamin D3: a new class of potent inhibitors of proliferation of prostate, breast, and myeloid leukemic cells. *Cancer Res.* **59**, 4023–4029.
194. Jiang, F., Bao, J., Li, P., Nicosia, S. V., and Bai, W. (2004). Induction of ovarian cancer cell apoptosis by 1,25-dihydroxyvitamin D3 through the down-regulation of telomerase. *J. Biol. Chem.* **279**, 53,213–53,221.
195. Ikeda, N., Uemura, H., Ishiguro, H., et al. (2003). Combination treatment with 1alpha,25-dihydroxyvitamin D3 and 9-cis-retinoic acid directly inhibits human telomerase reverse transcriptase transcription in prostate cancer cells. *Mol. Cancer Ther.* **2**, 739–746.
196. Sigala, S., Faraoni, I., Botticini, D., et al. (1999). Suppression of telomerase, reexpression of KAI1, and abrogation of tumorigenicity by nerve growth factor in prostate cancer cell lines. *Clin Cancer Res*, **5**, 1211–1218.
197. Wetterau, L. A., Francis, M. J., Ma, L., and Cohen, P. (2003). Insulin-like growth factor I stimulates telomerase activity in prostate cancer cells. *J. Clin. Endocrinol. Metab.* **88**, 3354–3359.
198. Moore, M. G., Wetterau, L. A., Francis, M. J., Peehl, D. M., and Cohen, P. (2003). Novel stimulatory role for insulin-like growth factor binding protein-2 in prostate cancer cells. *Int. J. Cancer* **105**, 14–19.
199. Meeker, A. K., Sommerfeld, H. J., and Coffey, D. S. (1996). Telomerase is activated in the prostate and seminal vesicles of the castrated rat. *Endocrinology* **137**, 5743–5746.
200. Ravindranath, N., Ioffe, S. L., Marshall, G. R., Ramaswamy, S., Plant, T. M., and Dym, M. (2001). Androgen depletion activates telomerase in the prostate of the nonhuman primate, Macaca mulatta. *Prostate* **49**, 79–89.

201. Meeker, A. K. (2001). Telomere Dynamics and Androgen Regulation of Telomerase Enzymatic Activity in Normal and Pathological States of the Prostate. Pharmacology. Johns Hopkins University School of Medicine, Baltimore, MD, p. 265.
202. Guo, C., Armbruster, B. N., Price, D. T., and Counter, C. M. (2003). In vivo regulation of hTERT expression and telomerase activity by androgen. *J. Urol.* **170**, 615–618.
203. Zhao, J. Q., Hoare, S. F., McFarlane, R., et al. (1998). Cloning and characterization of human and mouse telomerase RNA gene promoter sequences. *Oncogene* **16**, 1345–1350.
204. Horikawa, I., Cable, P. L., Afshari, C., and Barrett, J. C. (1999). Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer Res.* **59**, 826–830.
205. Cong, Y. S., Wen, J., and Bacchetti, S. (1999). The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. *Hum Mol Genet*, **8**, 137–142.
206. Takakura, M., Kyo, S., Kanaya, T., et al. (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res.* **59**, 551–557.
207. Soda, H., Raymond, E., Sharma, S., et al. (2000). Effects of androgens on telomerase activity in normal and malignant prostate cells in vitro. *Prostate* **43**, 161–168.
208. Thelen, P., Wuttke, W., Jarry, H., Grzmil, M., and Ringert, R. H. (2004). Inhibition of telomerase activity and secretion of prostate specific antigen by silibinin in prostate cancer cells. *J. Urol.* **171**, 1934–1938.
209. Bosland, M. (2003) In vivo regulation of hTERT expression and telomerase activity by androgen. *Urologic Oncology: Seminars and Original Investigations* **21**, 416.
210. Bouchal, J., Kolar, Z., Mad'arova, J., Hlobilkova, A., and von Angerer, E. (2002). The effects of natural ligands of hormone receptors and their antagonists on telomerase activity in the androgen sensitive prostatic cancer cell line LNCaP. *Biochem. Pharmacol.* **63**, 1177–1181.
211. Bouchal, J., Baumforth, K. R., Svachova, M., Murray, P. G., von Angerer, E., and Kolar, Z. (2005). Microarray analysis of bicalutamide action on telomerase activity, p53 pathway and viability of prostate carcinoma cell lines. *J. Pharm. Pharmacol.* **57**, 83–92.
212. Iczkowski, K. A., Huang, W., Mazzucchelli, R., Pantazis, C .G., Stevens, G. R., and Montironi, R. (2004). Androgen ablation therapy for prostate carcinoma suppresses the immunoreactive telomerase subunit hTERT. *Cancer* **100**, 294–299.
213. Silva, I. S., Morsch, D. M., Urnauer, L., and Spritzer, P. M. (2001). Androgen-induced cell growth and c-myc expression in human non-transformed epithelial prostatic cells in primary culture. *Endocr. Res.* **27**, 153–169.
214. Gil, J., Bernard, D., Martinez, D., and Beach, D. (2004). Polycomb CBX7 has a unifying role in cellular lifespan. *Nat. Cell Biol.* **6**, 67–72.
215. Nanni, S., Narducci, M., Della Pietra, L., et al. (2002). Signaling through estrogen receptors modulates telomerase activity in human prostate cancer. *J. Clin. Invest.* **110**, 219–227.
216. Stewart, S. A., Hahn, W. C., O'Connor, B. F., et al. (2002) Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc. Natl. Acad. Sci. USA* **99**, 12,606–12,611.
217. Artandi, S. E., Alson, S., Tietze, M. K., et al. (2002). Constitutive telomerase expression promotes mammary carcinomas in aging mice. *Proc. Natl. Acad. Sci. USA* **99**, 8191–8196.
218. Smith, L. L., Coller, H. A., and Roberts, J. M. (2003). Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat. Cell. Biol.* **5**, 474–479.
219. Zhang, P., Chan, S .L., Fu, W., Mendoza, M., and Mattson, M.P. (2003). TERT suppresses apoptotis at a premitochondrial step by a mechanism requiring reverse transcriptase activity and 14-3-3 protein-binding ability. *Faseb J.* **17**, 767–769.
220. Cao, Y., Li, H., Deb, S., and Liu, J.P. (2002). TERT regulates cell survival independent of telomerase enzymatic activity. *Oncogene* **21**, 3130–3138.
221. Masutomi, K., Yu, E. Y., Khurts, S., et al. (2003). Telomerase maintains telomere structure in normal human cells. *Cell* **114**, 241–253.
222. Kondo, S., Kondo, Y., Li, G., Silverman, R. H., and Cowell, J. K. (1998). Targeted therapy of human malignant glioma in a mouse model by 2-5A antisense directed against telomerase RNA. *Oncogene* **16**, 3323–3330.
223. Ludwig, A., Saretzki, G., Holm, P.S., et al. (2001). Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase. *Cancer Res* **61**, 3053–3061.
224. Beltz, L. A. (2002). The 'other' telomerase inhibitors: non-G-quadruplex interactive agent, non-antisense, non-reverse transcriptase telomerase inhibitors. *Curr. Med. Chem. Anti-Canc. Agents* **2**, 589–603.
225. Strahl, C. and Blackburn, E. H. (1996) Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol. Cell. Biol.* **16**, 53–65.
226. Melana, S. M., Holland, J. F., and Pogo, B. G. (1998) Inhibition of cell growth and telomerase activity of breast cancer cells in vitro by 3'-azido-3'-deoxythymidine. *Clin. Cancer Res.* **4**, 693–696.
227. Damm, K., Hemmann, U., Garin-Chesa, P., et al. (2001). A highly selective telomerase inhibitor limiting human cancer cell proliferation. *Embo J.* **20**, 6958–6968.

228. Ouchi, H., Ishiguro, H., Ikeda, N., Hori, M., Kubota, Y., and Uemura, H. (2005). Genistein induces cell growth inhibition in prostate cancer through the suppression of telomerase activity. *Int. J. Urol.* **12**, 73–80.
229. Naasani, I., Seimiya, H., and Tsuruo, T. (1998). Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins. *Biochem. Biophys. Res. Commun.* **249**, 391–396.
230. Corey, D. R. (2002). Telomerase inhibition, oligonucleotides, and clinical trials. *Oncogene* **21**, 631–637.
231. Shammas, M. A., Simmons, C. G., Corey, D. R., and Reis, R. J. (1999). Telomerase inhibition by peptide nucleic acids reverses 'immortality' of transformed human cells. *Oncogene* **18**, 6191–6200.
232. Norton, J. C., Piatyszek, M. A., Wright, W. E., Shay, J. W., and Corey, D. R. (1996). Inhibition of human telomerase activity by peptide nucleic acids. *Nat. Biotech.* **14**, 615–619.
233. Herbert, B., Pitts, A. E., Baker, S I., et al. (1999). Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc. Natl. Acad. Sci. USA* **96**, 14,276–14,281.
234. Villa, R., Folini, M., Lualdi, S., Veronese, S., Daidone, M. G., and Zaffaroni, N. (2000). Inhibition of telomerase activity by a cell-penetrating peptide nucleic acid construct in human melanoma cells. *FEBS Lett.* **473**, 241–248.
235. Huard, S. and Autexier, C. (2002). Targeting human telomerase in cancer therapy. *Curr. Med. Chem. Anti-Canc. Agents.* **2**, 577–587.
236. Herbert, B. S., Pongracz, K., Shay, J. W., and Gryaznov, S. M. (2002). Oligonucleotide N3'—>P5' phosphoramidates as efficient telomerase inhibitors. *Oncogene* **21**, 638–642.
237. Pongracz, K., Li, S., Herbert, B. S., et al. (2003). Novel short oligonucleotide conjugates as inhibitors of human telomerase. *Nucleosides Nucleotides Nucleic Acids* **22**, 1627–1629.
238. Asai, A., Oshima, Y., Yamamoto, Y., et al. (2003). A novel telomerase template antagonist (GRN163) as a potential anticancer agent. *Cancer Res.* **63**, 3931–3939.
239. Elayadi, A. N., Demieville, A., Wancewicz, E. V., Monia, B. P., and Corey, D. R. (2001). Inhibition of telomerase by 2'-O-(2-methoxyethyl) RNA oligomers: effect of length, phosphorothioate substitution and time inside cells. *Nucleic Acids Res.* **29**, 1683–1689.
240. Chen, Z., Monia, B. P. and Corey, D. R. (2002). Telomerase inhibition, telomere shortening, and decreased cell proliferation by cell permeable 2'-O-methoxyethyl oligonucleotides. *J. Med. Chem.* **45**, 5423–5425.
241. Yokoyama, Y., Takahashi, Y., Shinohara, A., et al. (1998). Attenuation of telomerase activity by a hammerhead ribozyme targeting the template region of telomerase RNA in endometrial carcinoma cells. *Cancer Res.* **58**, 5406–5410.
242. Folini, M., Pennati, M., and Zaffaroni, N. (2002). Targeting human telomerase by antisense oligonucleotides and ribozymes. *Curr. Med. Chem. Anti-Canc. Agents* **2**, 605–612.
243. Wan, M. S., Fell, P. L., and Akhtar, S. (1998). Synthetic 2'-O-methyl-modified hammerhead ribozymes targeted to the RNA component of telomerase as sequence-specific inhibitors of telomerase activity. *Antisense Nucleic Acid Drug Dev.* **8**, 309–317.
244. Yokoyama, Y., Takahashi, Y., Shinohara, A., et al. (2000). The 5'-end of hTERT mRNA is a good target for hammerhead ribozyme to suppress telomerase activity. *Biochem. Biophys. Res. Commun.* **273**, 316–321.
245. Saretzki, G., Ludwig, A., von Zglinicki, T., and Runnebaum, I. B. (2001). Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells. *Cancer Gene Ther.* **8**, 827–834.
246. Kondo, Y., Koga, S., Komata, T., and Kondo, S. (2000). Treatment of prostate cancer in vitro and in vivo with 2-5A-anti- telomerase RNA component. *Oncogene* **19**, 2205–2211.
247. Schindler, A., Fiedler, U., Meye, A., et al. (2001). Human telomerase reverse transcriptase antisense treatment downregulates the viability of prostate cancer cells in vitro. *Int. J. Oncol.* **19**, 25–30.
248. Li, S., Rosenberg, J. E., Donjacour, A. A., et al. (2004). Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. *Cancer Res.* **64**, 4833–4840.
249. Hahn, W. C., Stewart, S. A., Brooks, M.W ., et al. (1999). Inhibition of telomerase limits the growth of human cancer cells. *Nat. Med.* **5**, 1164–1170.
250. Zhang, X., Mar, V., Zhou, W., Harrington, L., and Robinson, M. O. (1999). Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev.* **13**, 2388–2399.
251. Guo, C., Geverd, D., Liao, R., Hamad, N., Counter, C. M., and Price, D. T. (2001). Inhibition of telomerase is related to the life span and tumorigenicity of human prostate cancer cells. *J. Urol.* **166**, 694–698.
252. Brambilla, C., Folini, M., Gandellini, P., Daprai, L., Daidone, M. G., and Zaffaroni, N. (2004). Oligomer-mediated modulation of hTERT alternative splicing induces telomerase inhibition and cell growth decline in human prostate cancer cells. *Cell. Mol. Life Sci.* **61**, 1764–1774.
253. Misawa, M., Tauchi, T., Sashida, G., et al. (2002). Inhibition of human telomerase enhances the effect of chemotherapeutic agents in lung cancer cells. *Int. J. Oncol.* **21**, 1087–1092.
254. Tauchi, T., Nakajima, A., Sashida, G., et al. (2002). Inhibition of human telomerase enhances the effect of the tyrosine kinase inhibitor, imatinib, in BCR-ABL-positive leukemia cells. *Clin. Cancer Res.* **8**, 3341–3347.
255. Biroccio, A., Gabellini, C., Amodei, S., et al. (2003). Telomere dysfunction increases cisplatin and ecteinascidin-743 sensitivity of melanoma cells. *Mol. Pharmacol.* **63**, 632–638.

256. Tentori, L., Portarena, I., Barbarino, M., et al. (2003). Inhibition of telomerase increases resistance of melanoma cells to temozolomide, but not to temozolomide combined with poly (adp-ribose) polymerase inhibitor. *Mol. Pharmacol.* **63**, 192–202.
257. Suenaga, M., Soda, H., Oka, M., et al. (2002). Histone deacetylase inhibitors suppress telomerase reverse transcriptase mRNA expression in prostate cancer cells. *Int. J. Cancer* **97**, 621–625.
258. Cong, Y. S., and Bacchetti, S. (2000). Histone deacetylation is involved in the transcriptional repression of hTERT in normal human cells. *J. Biol. Chem.* **275**, 35,665–35,668.
259. Guilleret, I., and Benhattar, J. (2003). Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres. *Exp. Cell. Res.* **289**, 326–334.
260. Dessain, S.K., Yu, H., Reddel, R.R., Beijersbergen, R. L., and Weinberg, R. A. (2000) Methylation of the human telomerase gene CpG island. *Cancer Res.* **60**, 537–541.
261. Devereux, T. R., Horikawa, I., Anna, C. H., Annab, L. A., Afshari, C. A., and Barrett, J. C. (1999) DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res.* **59**, 6087–6090.
262. Shin, K. H., Kang, M. K., Dicterow, E., and Park, N. H. (2003). Hypermethylation of the hTERT promoter inhibits the expression of telomerase activity in normal oral fibroblasts and senescent normal oral keratinocytes. *Br. J. Cancer* **89**, 1473–1478.
263. Kang, S. S., Kwon, T., Kwon, D. Y., and Do, S. I. (1999). Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J. Biol. Chem.* **274**, 13,085–13,090.
264. Li, H., Zhao, L. L., Funder, J. W., and Liu, J. P. (1997). Protein phosphatase 2A inhibits nuclear telomerase activity in human breast cancer cells. *J. Biol. Chem.* **272**, 16,729–16,732.
265. Li, H., Zhao, L., Yang, Z., Funder, J. W., and Liu, J. P. (1998). Telomerase is controlled by protein kinase C alpha in human breast cancer cells. *J. Biol. Chem.* **273**, 33,436–33,442.
266. Ku, W. C., Cheng, A. J., and Wang, T. C. (1997). Inhibition of telomerase activity by PKC inhibitors in human nasopharyngeal cancer cells in culture. *Biochem. Biophys. Res. Commun.* **241**, 730–736.
267. Kim, Y. W., Hur, S. Y., Kim, T. E., et al. (2001). Protein kinase C modulates telomerase activity in human cervical cancer cells. *Exp. Mol. Med.* **33**, 156–163.
268. Komata, T., Kondo, Y., Kanzawa, T., et al. (2001). Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter. *Cancer Res.* **61**, 5796–5802.
269. Koga, S., Hirohata, S., Kondo, Y., et al. (2000). A novel telomerase-specific gene therapy: gene transfer of caspase-8 utilizing the human telomerase catalytic subunit gene promoter. *Hum. Gene Ther.* **11**, 1397–1406.
270. Gu, J., Kagawa, S., Takakura, M., et al. (2000). Tumor-specific transgene expression from the human telomerase reverse transcriptase promoter enables targeting of the therapeutic effects of the Bax gene to cancers. *Cancer Res.* **60**, 5359–5364.
271. Lin, T., Gu, J., Zhang, L., et al. (2002). Targeted expression of green fluorescent protein/tumor necrosis factor-related apoptosis-inducing ligand fusion protein from human telomerase reverse transcriptase promoter elicits antitumor activity without toxic effects on primary human hepatocytes. *Cancer Res.* **62**, 3620–3625.
272. Lin, T., Huang, X., Gu, J., et al. (2002). Long-term tumor-free survival from treatment with the GFP-TRAIL fusion gene expressed from the hTERT promoter in breast cancer cells. *Oncogene* **21**, 8020–8028.
273. Song, J. S., and Kim, H. P. (2004) Adenovirus-mediated HSV-TK gene therapy using the human telomerase promoter induced apoptosis of small cell lung cancer cell line. *Oncol. Rep.* **12**, 443–447.
274. Plumb, J. A., Bilsland, A., Kakani, R., et al. (2001). Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954. *Oncogene* **20**, 7797–7803.
275. Boyd, M., Mairs, R. J., Keith, W. N., et al. (2004). An efficient targeted radiotherapy/gene therapy strategy utilising human telomerase promoters and radioastatine and harnessing radiation-mediated bystander effects. *J. Gene Med.* **6**, 937–947.
276. Verdijk, R. M., Wilke, M., Beslier, V., et al. (2004). Escherichia coli-nitroreductase suicide gene control of human telomerase reverse transcriptase-transduced minor histocompatibility antigen-specific cytotoxic T cells. *Bone Marrow Transplant* **33**, 963–967.
277. Boyd, M., Mairs, R. J., Mairs, S. C., et al. (2001). Expression in UVW glioma cells of the noradrenaline transporter gene, driven by the telomerase RNA promoter, induces active uptake of [131I]MIBG and clonogenic cell kill. *Oncogene*, **20**, 7804–7808.
278. Fullerton, N.E., Boyd, M., Mairs, R.J., et al. (2004). Combining a targeted radiotherapy and gene therapy approach for adenocarcinoma of prostate. *Prostate Cancer Prostatic Dis.* **7**, 355–363.
279. Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., and Henderson, D. R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* **57**, 2559–2563.
280. Wirth, T., Zender, L., Schulte, B., et al. (2003). A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. *Cancer Res.* **63**, 3181–3188.

281. Kawashima, T., Kagawa, S., Kobayashi, N., et al. (2004). Telomerase-specific replication-selective virotherapy for human cancer. *Clin. Cancer Res.* **10**, 285–292.
282. Huang, Q., Zhang, X., Wang, H., et al. (2004). A novel conditionally replicative adenovirus vector targeting telomerase-positive tumor cells. *Clin. Cancer Res.* **10**, 1439–1445.
283. Lanson, N. A., Jr., Friedlander, P. L., Schwarzenberger, P., Kolls, J. K., and Wang, G. (2003). Replication of an adenoviral vector controlled by the human telomerase reverse transcriptase promoter causes tumor-selective tumor lysis. *Cancer Res.* **63**, 7936–7941.
284. Irving, J., Wang, Z., Powell, S., et al. (2004). Conditionally replicative adenovirus driven by the human telomerase promoter provides broad-spectrum antitumor activity without liver toxicity. *Cancer Gene Ther.* **11**, 174–185.
285. Ryan, P. C., Jakubczak, J. L., Stewart, D. A., et al. (2004). Antitumor efficacy and tumor-selective replication with a single intravenous injection of OAS403, an oncolytic adenovirus dependent on two prevalent alterations in human cancer. *Cancer Gene Ther.* **11**, 555–569.
286. Paielli, D. L., Wing, M. S., Rogulski, K. R., et al. (2000). Evaluation of the biodistribution, persistence, toxicity, and potential of germ-line transmission of a replication-competent human adenovirus following intraprostatic administration in the mouse. *Mol. Ther.* **1**, 263–274.
287. Vonderheide, R. H., Hahn, W. C., Schultze, J. L., and Nadler, L. M. (1999). The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity* **10**, 673–679.
288. Minev, B., Hipp, J., Firat, H., Schmidt, J. D., Langlade-Demoyen, P., and Zanetti, M. (2000). Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. *Proc. Natl. Acad. Sci. USA* **97**, 4796–4801.
289. Vonderheide, R. H., Domchek, S. M., Schultze, J. L., et al. (2004). Vaccination of cancer patients against telomerase induces functional antitumor CD8+ T lymphocytes. *Clin. Cancer Res.* **10**, 828–839.
290. Parkhurst, M. R., Riley, J. P., Igarashi, T., Li, Y., Robbins, P. F., and Rosenberg, S. A. (2004). Immunization of patients with the hTERT:540-548 peptide induces peptide-reactive T lymphocytes that do not recognize tumors endogenously expressing telomerase. *Clin. Cancer Res.* **10**, 4688–4698.
291. Nair, S. K., Heiser, A., Boczkowski, D., et al. (2000). Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. *Nat. Med.* **6**, 1011–1017.
292. Heiser, A., Maurice, M. A., Yancey, D. R., et al. (2001). Induction of polyclonal prostate cancer-specific ctl using dendritic cells transfected with amplified tumor rna. *J. Immunol.* **166**, 2953–2960.
293. Zahler, A. M., Williamson, J. R., Cech, T. R., and Prescott, D. M. (1991). Inhibition of telomerase by G-quartet DNA structures. *Nature* **350**, 718–720.
294. Mergny, J. L., Mailliet, P., Lavelle, F., Riou, J. F., Laoui, A., and Helene, C. (1999). The development of telomerase inhibitors: the G-quartet approach. *Anticancer Drug Des.* **14**, 327–339.
295. Gowan, S. M., Harrison, J. R., Patterson, L., et al. (2002). A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting in vitro and in vivo antitumor activity. *Mo.l Pharmacol.* **61**, 1154–1162.
296. Shammas, M. A., Shmookler Reis, R. J., Li, C., et al. (2004). Telomerase inhibition and cell growth arrest after telomestatin treatment in multiple myeloma. *Clin. Cancer Res.* **10**, 770–776.
297. Burger, A. M., Dai, F., Schultes, C. M., et al. (2005). The G-quadruplex-interactive molecule BRACO-19 inhibits tumor growth, consistent with telomere targeting and interference with telomerase function. *Cancer Res.* **65**, 1489–1496.
298. Izbicka, E., Wheelhouse, R. T., Raymond, E., et al. (1999). Effects of cationic porphyrins as G-quadruplex interactive agents in human tumor cells. *Cancer Res.* **59**, 639–644.
299. Incles, C. M., Schultes, C. M., Kempski, H., Koehler, H., Kelland, L. R., and Neidle, S. (2004). A G-quadruplex telomere targeting agent produces p16-associated senescence and chromosomal fusions in human prostate cancer cells. *Mol. Cancer Ther.* **3**, 1201–1206.
300. Kim, M. M., Rivera, M. A., Botchkina, I. L., Shalaby, R., Thor, A. D., and Blackburn, E. H. (2001). A low threshold level of expression of mutant-template telomerase RNA inhibits human tumor cell proliferation. *Proc. Natl. Acad. Sci. USA* **98**, 7982–7987.
301. Schalken, J. A. and van Leenders, G. (2003). Cellular and molecular biology of the prostate: stem cell biology. *Urology* **62**, 11–20.
302. Wang, Y., Hayward, S., Cao, M., Thayer, K., and Cunha, G. (2001). Cell differentiation lineage in the prostate. *Differentiation* **68**, 270–279.
303. De Marzo, A. M., Meeker, A. K., Epstein, J. I., and Coffey, D. S. (1998). Prostate stem cell compartments: expression of the cell cycle inhibitor p27Kip1 in normal, hyperplastic, and neoplastic cells. *Am. J. Pathol.* **153**, 911–919.
304. De Marzo, A. M., Nelson, W. G., Meeker, A. K., and Coffey, D. S. (1998). Stem cell features of benign and malignant prostate epithelial cells. *J. Urol.* **160**, 2381–2392.
305. De Marzo, A. M., DeWeese, T. L., Platz, E. A., et al. (2004). Pathological and molecular mechanisms of prostate carcinogenesis: implications for diagnosis, detection, prevention, and treatment. *J. Cell. Biochem.* **91**, 459–477.
306. von Zglinicki, T. (2002). Oxidative stress shortens telomeres. *Trends Biochem. Sci.* **27**, 339–344.
307. Nakayama, M., Bennett, C. J., Hicks, J. L., et al. (2003). Hypermethylation of the human glutathione S-transferase-pi

gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am. J. Pathol.* **163**, 923–933.

308. Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A., and Klingelhutz, A. J. (1998). Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* **396**, 84–88.
309. Jarrard, D. F., Sarkar, S., Shi, Y., et al. (1999). p16/pRb pathway alterations are required for bypassing senescence in human prostate epithelial cells. *Cancer Res.* **59**, 2957–2964.
310. Karhadkar, S. S., Bova, G. S., Abdallah, N., et al. (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* **431**, 707–712.
311. Okada, H. and Mak, T.W. (2004). Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat. Rev. Cancer* **4**, 592–603.

19
Racial Differences in Prostate Cancer Mortality

James L. Mohler

Summary

African Americans have a higher incidence of, and greater mortality from, prostate cancer than Caucasian Americans, and the reasons for racial disparity remain unclear. The racial differences in participation in prostate cancer early detection programs are narrowing. However, racial differences persist in interaction with the health care system. Many of these differences may be more socioeconomic than racial, but many African Americans lack trust in the American health care system that may be overcome by personal interaction with their health care provider. African Americans more often use alternative medicines and may more frequently decline potentially curative treatments. Racial differences in host and tumor biology are difficult to evaluate. African Americans more often consume higher fat diets and are more often obese, both of which may promote prostate carcinogenesis. Although there is no evidence for higher serum androgen levels in African Americans compared with Caucasian Americans, their prostates may be more sensitive to androgens because of a shortened CAG repeat within the androgen receptor gene. Studies involving hereditary prostate cancer and genetic polymorphisms that may affect prostate cancer risk have frequently not included high numbers of African Americans, and the interpretation of such studies are further complicated by issues regarding population stratification. Although there are no racial differences in prostate-specific antigen (PSA) and its derivatives, there is some evidence that African American prostate cancer may have higher tissue levels of sex hormone-binding globulin (SHBG) and increased androgen receptor protein expression. Although some studies suggest racial differences in growth factors and cell regulatory pathways, these studies require studies of larger numbers of men.

Key Words: Androgen access; early detection; healthcare interaction; host biology; population; race; stratification.

1. INTRODUCTION

In the United States a new patient is diagnosed with prostate cancer every 3 minutes and another dies from the disease every 17 minutes *(1)*. The worldwide incidence of prostate cancer is increasing an estimated 1.1% annually *(2)*. Although the frequency of incidental prostate cancer is similar between races *(3,4)*, African Americans have a higher incidence and greater mortality from prostate cancer than Caucasian Americans. In fact, African Americans have the highest incidence and mortality rates of prostate cancer in the world. Differences in prostate cancer incidence between men of different geographic origins is not unique to the United States; for example, in Sao Paulo, Brazil, prostate cancer is 1.8 times more common in Brazilians of African than European descent *(5)*.

Data from the Surveillance, Epipdemiology, and End Results (SEER) database (1993–1997) show that the incidence of invasive prostate cancer is greater in African Americans than Caucasian Americans: 1.9 times greater in men younger than 65 years of age and 1.6 times greater in men 65 years of age or older *(6)*. In men younger than 65 years of age, the prostate cancer mortality rate for African Americans is 3.1 times that of Caucasian Americans. In men 65 years of age or older, the prostate

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

cancer mortality rate for African Americans is 2.3 times that of Caucasian Americans *(6)*. These data suggest that there is no racial difference in the development of prostate cancer (initiation), but clinical prostate cancer progresses more frequently in African Americans than white Americans, and once prostate cancer presents clinically, prostate cancer is more lethal in African Americans than white Americans.

The reasons for the disproportionate mortality from prostate cancer in African Americans compared with white Americans can be categorized into three general areas:

1. Racial differences in interaction between men and the health care system.
2. Biological differences between the races that affect prostate cancer development and progression.
3. Biological differences within benign and malignant prostate tissue itself between African Americans and white Americans.

The relative contribution of each of these areas must be examined carefully to understand the racial differences in prostate cancer mortality and allocate most appropriately the public resources for research and intervention to minimize or eliminate the racial disparity in prostate cancer mortality. Fortunately, study of all aspects of this important health care problem has accelerated rapidly in the past decade. The number of citations in PubMed on prostate cancer and African Americans has increased from 65 in 1994 to 207 in 1999 to 405 in 2004. The National Cancer Institute, American Cancer Society, and Department of Defense (DoD) Prostate Cancer Research Program have given high priority to research proposals that address prostate cancer racial disparities. Thoughtful discussions regarding the definitions of race *(7,8)*, the usefulness of race in genomics *(9,10)* and medicine *(11)*, and "whether socioeconomic conditions represent a more pertinent cause of [health care] disparities than race" have begun *(12–14)*.

One such effort is the DoD funded North Carolina-Louisiana Prostate Cancer Project (PCaP), "Racial Differences in Prostate Cancer: Influence of Health Care and Host and Tumor Biology. Reference 15a (Figure 1)." This study will compartmentalize the reasons for the racial differences in prostate cancer aggressiveness into three areas: Racial differences in:

1. Interaction between men with prostate cancer and their family, friends, physicians, and health care system.
2. Characteristics of the host, including diet, frequency of prostate cancer susceptibility genes, and androgen axis.
3. Characteristics of the tumor itself, such as cellular apoptotic and proliferation rates and androgen receptor and androgen-regulated gene expression.

PCaP was funded August 1, 2003, and the first home visit occurred September 10, 2004. PCaP research subjects include 500 African Americans from North Carolina deemed high risk, 500 African Americans from Louisiana deemed moderate risk, and 1000 white Americans (500 of whom are from North Carolina and 500 of whom are from Louisiana) who are deemed low risk. PCaP is developing a repository of interview data, biological specimens, and tumor specimens from these 2000 men that will include a limitless supply of DNA because PCaP is immortalizing leukocytes from all research subjects. The reasons for racial difference in prostate cancer mortality remains unclear (Figure 2) and require careful study to define.

2. RACIAL DIFFERENCES IN PATIENT-HEALTH CARE SYSTEM INTERACTION

African Americans present with incurable prostate cancer more often than white Americans. Among men diagnosed with prostate cancer from 1983–1987 reported by the SEER program, 29% of African Americans presented with metastatic prostate cancer compared with 19% of white Americans. In contrast, among men diagnosed with prostate cancer in 1992, 22% of African Americans presented with metastatic prostate cancer compared with 12% of white Americans *(15)*. The decrease in the frequency of diagnosis of metastatic disease in both races coincided with the introduction of serum prostate-specific antigen (PSA) screening for early detection. Consequently, the persistent racial differences in frequency of metastatic prostate cancer may result from racial differences in access to healthcare because of socio-

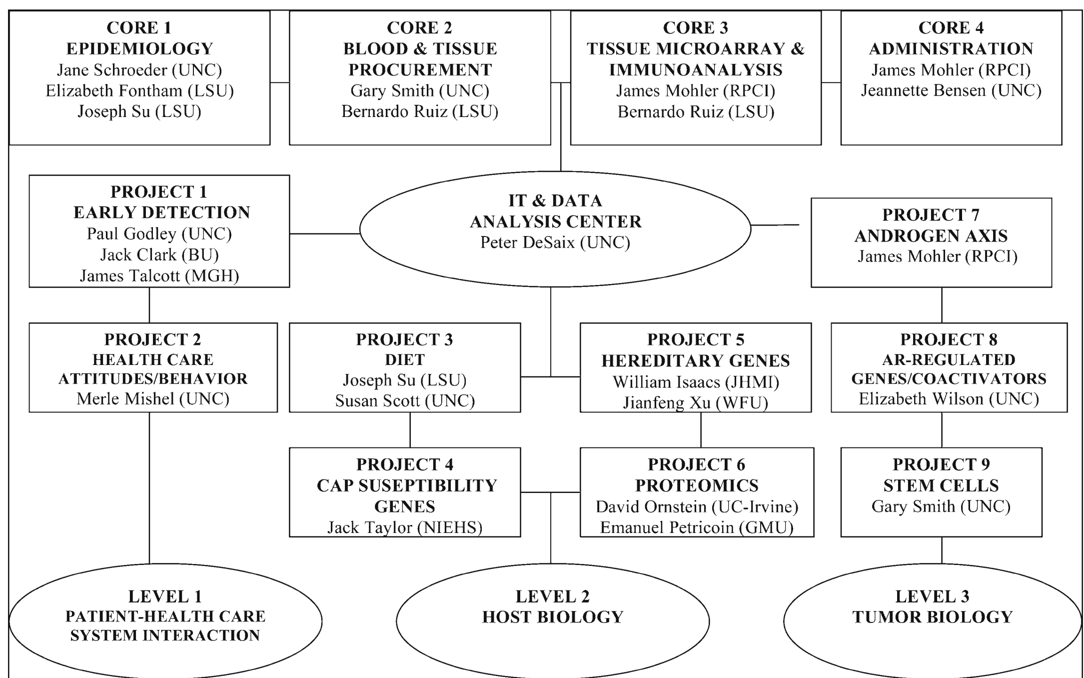


Fig. 1. The North Carolina-Louisiana Prostate Cancer Project (PCaP) is a multi-institutional population-based study of racial differences in prostate cancer aggressiveness. 2000 men with newly diagnosed prostate cancer of whom 50% are African Americans and 50% are Caucasion Americans are being enrolled in an attempt to discern the relative contribution of racial differences in interaction with the health care system and host and tumor biology to racial differences in prostate cancer mortality (UNC, University of North Carolina; LSU, Louisiana State University Health Sciences Center; RPCI, Roswell Park Cancer Institute; BU, Boston University, MGH, Massachusetts General Hospital; JHMI, John Hopkins Medical Institutions; WFU, Wake Forest University; NIEHS, National Institute of Environmental Health Sciences; UC-Irvine, University of California-Irvine; GMU, George Mason University; IT, Information Technology; AR, androgen receptor).

economic status *(16)* and participation in early detection programs *(17)*. Secondly, once diagnosed with potentially curable prostate cancer, African Americans may choose the most effective treatments less frequently; for example, African Americans have been reported more likely to choose to observe their prostate cancer *(3,15,18–21)*. Racial disparities persist in receipt of definitive primary care and androgen-deprivation therapy *(22,23)*. The consequences of the selection of observation by African Americans are difficult to evaluate because many studies of men selecting observation of prostate cancer have been conducted in Scandinavians *(24–28)* and, in studies of Americans, race has not been considered *(29–31)*. Consensus has been achieved recently that treatment outcome is similar by race if clinical parameters are used to adjust for prostate cancer volume and differentiation and patient age and comorbidity *(32)*. African Americans and white Americans have the same survival for clinically localized prostate cancer when treated with radical prostatectomy *(33–42)* or radiation therapy *(34,36,43–47)*, advanced prostate cancer *(48)*, or castration recurrent prostate cancer *(48–51)*. What barriers to earlier diagnosis persist in African Americans, and why does prostate cancer develop clinically at a younger age in African Americans than white Americans?

2.1. Early Detection Behavior

Lack of early detection behavior has been identified as one possible factor in the disparity of outcome, with African Americans reportedly less receptive to prostate cancer screening *(52–55)*. A

recent report *(56)* garnered a high level of national press attention regarding the contrast between the lower use of PSA for early detection and the higher rate of prostate cancer mortality in African Americans compared with white Americans. A case–control study of 67,245 New Jersey Medicare beneficiaries between 1991 and 1996 found that PSA use among African Americans was 65% of that of white Americans when controlled for socioeconomic status and comorbid conditions. However, racial differences in use of early detection for prostate cancer have narrowed; by 1998, PSA use was similar in African Americans and white Americans *(57)*. The benefits of PSA for early detection may be greater in African Americans than white Americans, and these benefits will be even greater yet in younger men. The factors influencing receptivity are unclear, with knowledge regarding prostate cancer, realization of personal risk, financial limitations, literacy level, and economic factors identified as explanatory factors *(54,55,58–60)*. African Americans are more likely than white Americans to live in rural areas and Americans living in rural areas generally have higher prostate cancer death rates, present more often with advanced stage, and use PSA for early detection less often *(61)*. Access to health care services has been suggested to account for 10–30% of the geographic variation in prostate cancer mortality *(61)*; for example, access and use of health care services was found to be deficient among rural African Americans in North Carolina *(62–65)*, a state where prostate cancer incidence and mortality is among the highest in the United States *(66)*. Unwillingness to be screened and to follow up with treatment was found to be related to cancer fatalism *(67)*, which is prevalent among older and lower-income African Americans *(68)*. Further research on health-related quality of life using preference-based measurement techniques *(69–73)* is needed to discern the factors that predict screening behavior and the role that screening behavior plays in explaining racial disparity in prostate cancer mortality.

2.2. *Interaction With the Health Care System*

Race may impact patient interaction with the health care system, and the extent of this may help explain the disparity in prostate cancer mortality by race. Socioeconomic status has been found to influence willingness to participate in clinical trials *(58)*. Much research has been flawed by using race as a proxy for socioeconomic variables and poverty in particular *(62)*. However, when corrected for socioeconomic status and health insurance status, African Americans remain less likely than white Americans to receive the most curative treatment for cancer in general *(74)*, and prostate cancer specifically *(23)*. Prostate cancer treatment decision-making may be impacted by racial differences in patient–physician communication and belief in traditional sources of healing and reliance on the church and religion, both of which have been reported to be prevalent in the African-American community, particularly among those with limited income and education *(75–80)*. Strategies to assist men with treatment decision-making have been developed and performed equally well in African Americans and white Americans *(81,82)*. Recent research has indicated that African Americans will participate fully in prostate cancer early detection and care when health information is presented as part of a culturally sensitive, clear, and respectful patient–health care provider relationship *(83)*.

Most patients receiving a diagnosis of cancer report that they are not sufficiently informed to make treatment decisions and, although they want physicians to make final decisions, patients want to be fully informed regarding their options *(84–89)*. The ability to communicate with the physician is complicated for African American cancer patients by a historical lack of trust in the health care system *(52–55,59,78–80,83,90–93)*. The consequences of the Tuskegee Syphilis Study continue to impact the African American community more than many people appreciate *(94,95)*. Trust, competence, and motive are viable issues for African American cancer patients *(58)*. Some data suggest that African Americans use other culturally relevant systems, such as family, religious community, and folk healers before seeking professional help *(78,96–98)*, and this may reduce patient communication with their physician *(82,99)*. A further issue is the perception by African American patients that they are treated differently than white Americans by physicians *(100,101)*. There has been little research on how these factors impact the doctor–patient relationship *(100,101)*, but men of both races respond

well to prostate cancer educational interventions, although white American men reportedly accept written information well whereas African American men prefer to receive similar information through personal communication *(83,99)*. Freeman has recommended universal insurance coverage, patient navigation programs, targeting resources to geographic areas of high cancer mortality, systems for monitoring equity of health care provision, and improved identification of those at increased cancer risk *(102)*.

2.3. Complementary and Alternative Medicines

Complementary and alternative medicines are used commonly by subjects at risk or diagnosed with cancer *(103)*. The overall prevalence of complementary and alternative medicine use by men with prostate cancer has been reported to range between 27.4 and 43% *(104–107)*. Five hundred forty-three men diagnosed with prostate cancer in Northern California in 1998 were interviewed by telephone to assess complementary and alternative medicine use among different racial/ethnic groups. Thirty percent of men used complementary and alternative medicines and usage did not differ between 218 white Americans, 109 Hispanic Americans, 107 African Americans, and 109 Asian Americans. An average of 9% used macrobiotics and megavitamins; 16.4% used herbal remedies; 3.1% used massage, acupuncture, and movement therapies; and 14.7 used counseling, relaxation, and spiritual treatment. Complementary and alternative medicine users were slightly younger and more likely to be college graduates but did not differ from nonusers by income level, country of birth, or marital status. Saw palmetto *(108)* is one of the most common herbals used for prostate disease, and PC-SPES *(109)* was used by many men, even those without prostate cancer, until concerns regarding safety, and eventually legal action, made it unavailable. Both products may have anti-androgenic activity.

3. RACIAL DIFFERENCES IN HOST BIOLOGY

3.1. Diet

Profound differences in diet exist among different racial, ethnic, and geographic areas that may impact development and progression of prostate cancer. In epidemiological studies, prostate cancer risk has been associated with diets high in saturated fats. African Americans consume more saturated fats than white Americans *(110,111)*. Kolonel conducted a large, multiethnic case–control study seeking evidence for a protective effect of vegetables, fruits, and legumes against prostate cancer. Home interviews were conducted of 1619 African-American, Caucasian, Japanese, or Chinese men identified from population-based tumor registries in Hawaii, San Francisco, and Los Angeles in the United States, and British Columbia and Ontario in Canada, and 1618 controls who were frequency matched to cases based on ethnicity, age, and region of residence of the case. African Americans and Caucasian Americans were diagnosed between 1989 and 1991 and Chinese and Japanese were diagnosed between 1987 and 1991, and every research subject was ethnically homogeneous for at least three grandparents. Within each ethnic group, cases and controls were similar in terms of body mass index (BMI). In all groups, cases consumed more calories and unsaturated fat than controls, although this difference disappeared in the African Americans and Caucasian Americans after adjusting for calories. Cases consumed more fruits and vegetables than controls in all groups except the Chinese. In contrast, cases in all groups had lower mean intake of legumes than controls and these differences persisted after adjustment for caloric intake. The results persisted after all men in the control group who had elevated PSA levels were eliminated and when the analyses were performed in cases with advanced disease only. Thus, an inverse association with prostate cancer risk was found for overall vegetable consumption, two categories of vegetables (yellow-orange and cruciferous) and two individual vegetables (corn and carrots). Corn consumption may warrant further investigation because of its relatively high content of lutein and small amounts of α-carotene, β-carotene, and folic acid. Inverse associations were also found for consumption of total legumes and soy products. The protec-

tive effects of legumes has been reported in three other studies *(112–114)* but not a fourth *(115)*. The value of lycopene for prostate cancer prevention and the proper form for consumption is controversial. This study found no association with intake of total tomatoes, cooked tomatoes, lycopene-containing fruits, or a combination of all lycopene-containing foods despite highly ethnic diets that resulted in a large range of intakes of lycopene and lycopene-containing foods.

The 3rd National Health and Nutrition Examination Survey reported that serum lycopene concentrations were significantly lower in African Americans than white Americans. A recent large case–control study reported that lycopene levels were inversely associated and other carotenoids were positively associated with prostate cancer risk *(116)*.

The overall role of diet for modulation of cancer risk has been questioned recently *(117)*. Data from 71,910 women and 37,725 men participating in the Nurses' Health Study and the Health Professionals' Follow-Up Study revealed that overall cancer incidence was not affected by the amount of fruits and vegetables in the diet. However, the study population is not representative of the general population and the incidence of cancer was too low to draw conclusions regarding diet and individual types of cancer. The impact of diet on prostate cancer risk and mortality is difficult to assess because diet is difficult to quantify, the accuracy of biomarkers of dietary intake are subject to debate, and diet studies are expensive to conduct. PCaP, the largest population-based and most comprehensive study of the impact of diet on prostate cancer aggressiveness, has begun in North Carolina and Louisiana and may help clarify the relationship between prostate cancer and diet.

Obesity is epidemic in America, and interest in the possibility that obesity impacts prostate carcinogenesis has increased. Two reports found no relationship between BMI and risk of prostate cancer *(118,119)*. Clarke and associates reported that inactivity, regardless of body habitus, was strongly associated with prostate cancer risk and that this association was stronger among African Americans than white Americans *(119)*. Freedland and associates *(120)* studied 1250 men with prostate cancer, newly diagnosed and treated by radical prostatectomy at five equal-access health centers. BMI was higher in African than white Americans, and increased BMI was associated with poor prognosis after radical prostatectomy. BMI may alter insulin levels and the insulin-like growth factor (IGF) axis, but serum levels of IGF1 and IGF-binding protein 3 (IGF-BP3) were similar in men of different races *(121)* or androgen metabolism. BMI did not correlate with pathological grade or stage, or tissue androgen levels *(122)*.

An interesting hypothesis regarding prostate cancer in African Americans *(123)* parallels the relationship between sickle cell anemia and malaria and examines the unfavorable overall health consequences of the relatively recent forced migration of Africans to North America *(124)*. Africans have genetically downregulated zinc absorption capacity because zinc levels in water and diet are high. If African Americans had zinc transporter levels equivalent to white Americans and still resided in Africa, most would suffer from zinc toxicity. Evidence suggests that low zinc levels in the prostate are associated with increased incidence of prostate cancer *(125)* and that zinc may inhibit growth of prostate cancer *(126,127)*. In prostate specimens from 30 white Americans and 28 African Americans, all prostate cancer specimens from white Americans had higher expression of two major zinc transporters (hZIP1 and hZIP2) than prostate cancer from African Americans *(128)*. The zinc transporters were also downregulated in benign prostate tissues from African-American men compared with aged-matched white Americans. An intervention as simple as zinc supplementation may prove useful for prostate cancer prevention in African Americans *(125)*.

In 1990, Schwartz and colleagues suggested that the descriptive epidemiology of prostate cancer (i.e., the increasing risk with age, African-American race, and residence at northern latitudes) resembled the descriptive epidemiology of vitamin D deficiency in the elderly *(129)*. Evidence in support of that hypothesis included that human prostate cells possess specific receptors for the hormonal form of vitamin D, 1,25(OH)2D, that produces differentiation when administered in vitro *(130,131)*, and that prostate cells synthesize 1,25(OH)2 D from its prohormonal precursor, 25-hydroxyvitamin D *(132)*. Autocrine synthesis of 1,25(OH)2D opens new possibilities for prostate cancer prevention and therapy *(133)*, because 25-hydroxyvitamin D is inactive until bioactivated by prostate cells, and can be administered more safely than 1,25(OH)2D *(134,135)*.

3.2. SERUM ANDROGENS AND ANDROGEN RECEPTOR TRINUCLEOTIDE REPEATS

Greater androgenic stimulation could contribute to increased aggressiveness of clinical disease in African Americans compared with white Americans and result from racial differences in serum or tissue androgens or tissue androgen receptor expression. Among college students in Los Angeles, mean testosterone levels of African Americans were 19% higher than white Americans, and free testosterone levels were 21% higher *(136)*. Studies that are more recent have found that racial differences in serum testosterone levels decrease as men reach 40 years of age *(137)*, and that serum testosterone levels are similar in men of both races undergoing prostate biopsies that prove positive or negative for prostate cancer *(138,139)*. Case–control studies provide evidence that prostate cancer can be associated with both higher *(140–142)* and lower *(143,144)* serum levels of testosterone. These conflicting results may result from small patient numbers, study of hospitalized patients, and inclusion of cases with advanced prostate cancer.

Early onset of puberty and late onset of menopause are well-established risk factors for breast cancer, whose growth is stimulated by estrogen *(145)*. If African Americans undergo puberty earlier than white Americans, the African-American prostate could receive a longer period of androgenic stimulation. Harlan et al. reported on physician-assigned Tanner stages of pubertal development collected by the United States Health Examination Survey of 3047 Caucasian American male youths and 499 African American male youths, ages 12 to 17 years *(146)*. The authors found no significant racial differences in genital stage or pubic hair stage at each age, except that Caucasian-American youths were slightly more advanced than African-American youths at age 13 years. Richards et al. *(147)* assessed Tanner stage of 902 Caucasian-American male youths and 485 African-American male youths from the Bogalusa Heart Study using multiple observers and reported statistically significant differences in the time of onset of Tanner stages I, II, and V of puberty between African-American and Caucasian-American youths. Although African-American children entered the early stages of puberty at younger ages, the Caucasian-American children entered the latter stages of pubertal development before the African-American children. Nankin et al. evaluated 232 caucasian-American children from an upper-middle class suburb of Pittsburgh, PA, and 166 African-American children from a Pittsburgh housing project who had applied for participation in a summer sports league *(148)*. They reported that African-American children experience sexual development earlier than Caucasian-American children; at all study times between 11 and 14 years of age, the mean Tanner stage of the African-American children was more advanced than the mean Tanner stage of the Caucasian-American children. Laska-Mierzejewska reported that sexual maturation occurred earlier in blacks vs whites based on 15 different body measurements of 4000 Cuban youths living in Havana in 1963–1964 *(149)*. Cameron et al. *(150)* studied 178 rural and 152 urban South African black children and reported that the urban children entered puberty at an earlier age than their rural counterparts *(150)*.

The Bogalusa Heart Study *(147)* and the United States Health Examination Survey *(146)* studies were large and representative of most US populations and should be considered more heavily than other smaller studies that have flaws in either their design or sampling methods, or studies that are based in other countries. From the current literature, one cannot draw a definitive conclusion regarding the timing of sexual development in African-American and Caucasian-American men, yet the current evidence points to there being little or no difference. In addition, today's youths may differ from those of the 1960s in nutrition, lifestyle, and socioeconomic status. Onset of puberty and length or exposure to serum androgens may not be a sufficient reason to explain differences in prostate cancer incidence and mortality between the races, but it may help explain why one individual has a greater chance of developing prostate cancer than another, regardless of race.

Exon A of the androgen receptor contains polymorphisms within the glutamine and glycine repeat regions. Shortened CAG repeat lengths result in increased expression of androgen receptor messenger RNA (mRNA) and protein that could make androgen responsive genes more sensitive to equivalent levels of androgen *(151–153)*. Evidence suggests that shortened CAG repeat lengths may play a role in the racial differences in prostate cancer *(154)*, because the CAG repeat length is lower in African Ameri-

cans. Four often-quoted studies suggested a relationship between CAG repeat length and risk of prostate cancer *(155–157)* and higher grade and stage of prostate cancer *(155)*. Studies that are more recent have failed to confirm these results *(158–165)*. Of particular interest are two subsequent studies from Stanford *(159)* and the paper by Stanford, which failed to confirm the initial report *(156)* from her group. Giovannucci *(166)* and Coetzee and Irvine *(167)*, experts in the field and authors of two of the four landmark studies, have proposed explanations for these conflicting results. Giovannucci thinks that reduced CAG repeat lengths foster development of aggressive cancers in younger men because of greater androgenic stimulation of their prostates. Population-based and case–control studies most likely to find a relationship between CAG repeat length and prostate cancer are those that were conducted in populations with minimal PSA screening; therefore, the results were not confounded by clinically insignificant prostate cancers. In addition, series that contain high percentages of highly aggressive cancers and younger men are more likely to reveal a relationship between CAG repeat length and prostate cancer than series of older men whose prostate cancers are more related to oxidative insults or other age-related conditions. He suggests that African Americans who suffer a higher incidence and mortality from prostate cancer and develop prostate cancer at a younger age than Caucasian Americans are the most likely group in which a relationship between CAG repeat length and prostate cancer will be found. However, Gilligan et al. *(168)* tested this hypothesis by evaluating the CAG repeat length in 685 African American men in Louisiana, South Carolina, and the District of Columbia who participated in a prostate cancer early detection program. The median CAG repeat length was 21 among 118 men diagnosed with prostate cancer, and 19 among 567 men who had normal serum PSA levels and benign digital rectal exams. After adjusting for age, no association was found between prostate cancer risk and CAG repeat length. This finding should not surprise Coetzee and Irvine *(167)*, who think that the relationship between CAG repeat length and prostate cancer has been confounded by population stratification. They write, "if an increased frequency of shorter AR-CAG repeat length is associated with Africans and if Africans have a higher risk of prostate cancer, then an association of shorter AR-CAG repeat length with prostate cancer might simply reflect the 'Africanness' of the African American population without having a direct cause–effect relationship." A similar effect was found when the relationship between CAG repeat length and advanced disease was examined by Bennett et al. *(169)* in the Veteran Affairs Health Care System. A relationship between shorter CAG repeat length and metastatic prostate cancer at presentation disappeared if controlled for race. Again quoting Coetzee and Irvine *(167)*, Bennett et al. *(169)* showed "that among prostate cancer patients, AR-CAG size does matter, albeit simply in their case to mark the racial-ethnic origin of the patient."

Two groups have produced a confusing finding in men who have undergone potentially curative radical prostatectomy *(170,171)*. Increased CAG repeat length was associated with increased risk of recurrence, although both studies used subset analysis and categorization of repeat length to find evidence of increased risk. However, increased CAG repeat length should decrease transactivation of androgen-regulated genes and, hence, prostate cancer cellular proliferation. Increased CAG repeat length protecting against recurrence does not make sense biologically. Further study of larger patient populations is required to confirm these results.

The effect of GGC repeat length on prostate cancer is less studied. GGC repeat lengths vary from 8 to 17, with repeat lengths of 16 to 18 accounting for 90% of androgen receptor alleles in white controls *(172)*. Longer GGC repeat lengths have been associated with lower levels of androgen receptor protein expression but did not affect the transactivation activity of androgen receptor-dependent genes *(172)*. Stanford *(156)* suggested an association between aggressive prostate cancer and GGC repeat lengths longer than 16, but this result was not confirmed in a larger study that included the previous study subset *(173)*.

3.3. Prostate Cancer Susceptibility Genes

The number of candidate polymorphisms available for study is growing with the advent of polymorphism discovery efforts. Microarrays, mass spectrometry, and other high-throughput genotyping

technologies make it feasible to analyze hundreds of polymorphisms in thousands of samples. A growing number of case–control studies on genetic susceptibility to prostate cancer test for relationships between polymorphisms and prostate cancer risk. Many of the polymorphisms implicated in prostate cancer susceptibility, including those involved in DNA repair and androgen metabolism, are reasonable candidates for affecting disease severity and prognosis.

Recently, a number of studies have been reported that examine racial differences in polymorphism frequency in candidate genes. Ethnic differences in polymorphism frequency are common and may account for some of the variation in cancer incidence across ethnic groups *(174–177)*. Prostate cancer susceptibility may be influenced by androgen metabolism genes *(176,178)* and genes involved in DNA repair *(179,180)*. Racial polymorphisms also occur within androgen metabolism genes, especially 5α-reductase, which converts testosterone to dihydrotestosterone (DHT), the preferred ligand for the androgen receptor. Increased frequency of polymorphisms in the androgen metabolism genes, *SRD5A2 (181,182)* and *CYP3A4 (183)*, may contribute to increased risk of cancer in African Americans. Africans and African Americans have the highest frequency of alleles previously associated with increased prostate cancer risk *(183)*, and some of these alleles correlated with poor prognosis *(184)*.

African Americans have more frequent homozygous alleles at the FOK1 site in the vitamin D receptor *(185)*, but vitamin D receptor had no impact on African-American prostate cancer outcome after treatment *(186)*. Sequence variants in the human 25-hydroxyvitamin D3 1-α-hydroxylase (*CYP27B1*) gene do not play a role as a prostate cancer susceptibility gene *(187)*. Glutathione-*S*-transferase polymorphisms were not related to prostate cancer risk *(188)*. Variation of the COX-2 promoter may influence risk and progression of prostate cancer in African Americans. SNP -297G was associated with decreased risk for prostate cancer (odds ratio [OR], 0.49) and SNPs -1265A and -899C were associated with increased risk for prostate cancer in African Americans (OR, 2.72 and 3.67, respectively) *(189)*. CYP3A is an enzyme important in drug metabolism and biotransformation. There are racial differences in the most common variant, CYP3A4*1B, which ranges from 0% (Chinese and Japanese) to 45% (African Americans). Several of the other polymorphisms in CYP3A also show racial variation *(190)*.

3.4. Familial Prostate Cancer Susceptibility Genes

Variation in genetic susceptibility may play a key role in the observed differences in the incidence of, and mortality from, prostate cancer in African Americans and Caucasian Americans *(191)*. Although the etiology of prostate cancer is complex, family and twin studies reproducibly indicate an important role for inherited genetic susceptibility as a critical risk factor *(192)*. Prostate cancer susceptibility genes have been mapped to 1q24–25 *(193)*, Xq27–28 *(194)*, and 8p22–23 *(195)*. Other loci, including *PCaP* and *CaPB (196)*, *HPC20 (197)*, and *HPC2/ELAC (198)*, have been examined. The frequency of these genes and an understanding of their importance in hereditary prostate cancer cases, sporadic white American cases, and unaffected controls continues to evolve, but almost all reported studies contain few African Americans. Recently, more groups have begun to examine affected African-American families. Familial aggregation rates are similar in African Americans and white Americans *(199)*. Race-specific penetrance estimates for carriers of deleterious genotypes were similar among African Americans and white Americans, but lower in Asian Americans *(200)*. The higher incidences of prostate cancer in African Americans may not be caused by a higher prevalence of germline mutations predisposing to the disease in African Americans. Men with more than six family members with prostate cancer had a higher chance of presenting with lymph node metastatic or widely metastatic prostate cancer than men with only four to six family members affected *(201)*. Linkage analysis of 33 African-American prostate cancer families from 2 independent research groups provided some evidence for clustering, especially at *HPC1*. Increased evidence of linkage was found in families with prostate cancer diagnosis younger than age 65 years, and male-to-male transmission *(202)*. These studies, though very preliminary, suggest that genetics could contribute to the increased aggressiveness of prostate cancer in African Americans.

3.5. Population Stratification

African Americans may be more genetically heterogeneous for three reasons. First, humans originated in Africa and hence, Africans have had more time to accumulate genetic variations than more recent emigrant groups. Second, Caucasian Americans share a common European geographic origin, whereas African Americans derive ancestry from more diverse geographic origins. African Americans derive ancestry from several different geographic areas within Africa whose people differ greatly. Genetic differences among Africans may contribute to the wide variation in prostate cancer incidence and mortality in the different regions of Africa that translate into differences among African Americans. For example, prostate cancer mortality is more than fivefold higher in Sub-Saharan Africa than Northern Africa *(2)*. Lastly, African Americans are genetically more heterogeneous than their African ancestors because of admixture with Caucasian Americans. African Americans in different geographical locations within the Americas, within the United States, and even within a single state, demonstrate markedly different levels of admixture with Caucasian Americans *(203,204)*.

Different levels of population stratification (admixture of African American and Caucasian American genotypes) may affect observed differences in prostate cancer mortality between population samples. The extent of admixture-linked disequilibrium can be evaluated using molecular analysis of a panel of short tandem repeats by PCR, restriction fragment length polymorphisms by Southern blot analysis, specific polymorphic alleles, or polymorphisms in mitochondrial DNA. Most analyses of admixture-linked disequilibria in African Americans include analysis of polymorphism at the *AT3* locus and the linked *FY* (Duffy) locus. There is nearly a fixed allele-frequency difference between European Americans and native Africans for these loci that may reflect a selective advantage of the *FY* –/– genotype in areas with endemic malaria *(205)*.

Despite the many different population-based epidemiological studies reported and many more that may have been performed but not reported, a polymorphism has not been identified that is strongly associated with prostate cancer risk or aggressiveness. This suggests that multiple gene–gene and gene–environment interactions are important if genetics are important at all. Furthermore, these studies are confounded by population stratification. Population stratification has little effect when risk differences are small, unless extreme differences exist in genotype frequency *(206)*. For example, high levels of population stratification accounted for differences in the distribution of CYP3A4-V found between Nigerians, African Americans, and Caucasian Americans *(207)*. The importance of population stratification in racial differences in prostate cancer incidence, aggressiveness, and mortality warrants careful study.

4. RACIAL DIFFERENCES IN THE PROSTATE AND ITS CANCER

The early literature (before 1995) reported that African Americans had higher mortality rates from prostate cancer than white Americans when matched for clinical stage, and access to healthcare was equal *(16,33,208–210)*. However, these differences disappeared in men diagnosed with prostate cancer after 70 years of age *(209)*, or even reversed *(210)*. More recently, men undergoing treatment for prostate cancer have shown no significant racial differences in treatment outcomes after radical prostatectomy *(33–42)*, radiation therapy *(34,36,43–47)*, or androgen deprivation therapy *(48–51)*. Prostate cancer may be biologically more aggressive in African Americans than Caucasian Americans because of racial differences in the prostate/prostate cancer microenvironment. Racial differences in maternal androgens during gestation and serum androgens and time of puberty during adolescence may imprint the African American prostate to respond more to similar levels of androgenic stimulation in adulthood. Subtle differences in the African American prostate microenvironment may enhance oxidative stress and promote the earlier transition of incidental to clinical prostate cancer. The prostate and its cancer may show more racial differences than many other organs.

4.1. Prostate-Specific Antigen

Early studies of large groups of men enrolling in prostate cancer early detection programs suggested that African-American men had higher serum PSA levels than Caucasian-American men and

that prostate cancer detection on systematic prostate biopsies was at least twice as common in African Americans compared with Caucasian Americans *(211a,211b)*. These findings prompted suggestion of race and age-specific guidelines for abnormal PSA *(211a)* that have not become widely used. Studies that are more recent have suggested that total PSA, the various types of PSA, and PSA derivatives are similar between races *(211–213)*. In frozen prostate cancer samples from radical prostatectomy specimens, tissue levels of PSA measured using radioimmunoassay were similar between races (mean [interquartile range]: African Americans, 74.2 [46.1–121.7] nmol/g tissue; Caucasian Americans, 92.1 [53.3–144.3] nmol/g tissue) *(122)*. The performance of PSA for early detection of prostate cancer may be improved by measurement of other members of the kallikrein family, such as human glandular kallikrein (hK)-2. hK2 improved the performance of PSA in Italian men; the ratio between hK2 and percent free PSA may enhance prostate cancer detection among African American men in Louisiana with serum PSA levels of 4 to 10 ng/mL *(212)*.

4.2. Tissue Androgens

Serum androgens may not accurately reflect the true androgenic environment within the prostate. Testosterone is the major circulating androgen, and DHT is 1/10 the concentration of testosterone in serum. Conversely, DHT is the major intraprostatic androgen, because the prostate contains 5α-reductase that converts testosterone to DHT. DHT is the preferred ligand for the androgen receptor because the androgen receptor–DHT complex is more stable than the androgen receptor–testosterone complex. Therefore, androgen levels were measured in prostate tissue from African Americans and Caucasian Americans who underwent radical prostatectomy for clinically localized prostate cancer *(122)*.

Steroid hormones were extracted from snap frozen tissue obtained intraoperatively from radical prostatectomy specimens from 36 African Americans and 59 Caucasian Americans and measured using radioimmunoassay. African Americans and Caucasian Americans had similar testosterone, DHT, dehydroepiandrosterone, dehydroepiandrosterone sulfate, and PSA levels. However, African Americans had higher androstenedione (ASD) ($p = 0.006$) and sex hormone-binding globulin (SHBG) ($p = 0.009$) levels. Racial differences in ASD ($p = 0.015$) and SHBG ($p = 0.008$) persisted after controlling for age, BMI, PSA, and pathologic Gleason sum and stage (nonparametric rank analysis of covariance). Higher ASD tissue levels in African Americans are unlikely to be important biologically because ASD conversion did not result in higher levels of testosterone in the prostates of African Americans.

Higher serum levels of SHBG have been postulated to be protective against prostate cancer because SHBG decreases bioavailable testosterone for androgen receptor ligand activation. African Americans have been reported to have higher serum levels of SHBG than white Americans *(214)*, but SHBG serum levels have not correlated significantly with risk of prostate cancer *(215,216)*. Our group was the first to report that tissue SHBG levels were 38% higher in African Americans than white Americans. Serum and tissue levels of SHBG may differ because, in 1989, SHBG was reported to be produced by prostate cells *(217)*. More recently, SHBG has been demonstrated to be produced in the human prostate cancer cell lines, LNCaP, DU-145, and PC, and in cultured human prostate epithelial and stromal cells *(218)*. SHBG binds a membrane receptor in prostate *(219)* that was reported to initiate an intracellular signal that increased cAMP levels and modulated androgen action in the prostate *(220)*. Immunostaining revealed heterogenous expression of SHGB protein primarily in the epithelium of both benign prostate *(218)* and prostate cancer *(221)*, and *in situ* hybridization of adjacent sections confirmed local SHBG synthesis *(218)*. Higher tissue levels of SHBG may enhance androgen action in prostate tissue *(222)* of African Americans through cAMP-dependent pathways. If this occurs in African-American men, androgen-regulated genes may be overexpressed in African Americans compared with Caucasian Americans.

4.3. Androgen Receptor

Androgen receptor protein expression was evaluated in malignant and benign prostate tissue from African Americans and Caucasian Americans who underwent radical prostatectomy for clini-

cally localized prostate cancer. Archived radical prostatectomy specimens obtained from 25 Caucasian Americans and 25 African Americans had androgen receptor protein antigen retrieved and immunostained. Androgen receptor protein expression from prostate cancer and benign tissue was assessed using two methods. Visual scoring suggested that androgen receptor immunostained more intensely in both malignant and benign epithelial nuclei in African Americans than Caucasian Americans. Automated digital color video image analysis was used to measure percent positive nuclei and the intensity of expression with each nucleus (mean optical density). In African Americans compared with Caucasian Americans, malignant nuclei were 27% more likely immunostained for androgen receptor ($p = 0.005$), and among immunoreactive nuclei, androgen receptor protein expression was 81% greater ($p = 0.002$). Among immunoreactive benign nuclei, androgen receptor protein expression was 22% greater in African Americans compared with Caucasian Americans ($p = 0.027$). Racial differences in androgen receptor protein expression were not explained by age or pathological grade and stage.

Prostate cancer may occur at a younger age and progress more rapidly in African Americans than Caucasian Americans because of racial differences in androgenic stimulation of the prostate. Increased androgenic stimulation may result from higher levels of androgen receptor protein and SHBG that are activated by racially similar serum and tissue levels of androgens. These findings must be confirmed, and tissue expression of androgen receptor-regulated genes must be evaluated in men of both races. Androgen-regulated genes identified using the CWR-22 human prostate cancer xenograft model include *CDK1* and *CDK2*, cyclin A and B1 *(223)*; α-enolase, α-tubulin, *IκBα*, *IGFBP-5 (224)*, *PSA*, *hK-2*, *Nkx3.1*, and *ARA-70*, which were also expressed at similar levels in recurrent and androgen-dependent tumors *(225)*; and *EF-1α*, tomoregulin, *TRX-R1*, and *Mxi-1*, which were also proliferation associated *(218,226)*.

4.4. Androgen Receptor Co-Regulators, Growth Factors, and Apoptotic Proteins

Little is known regarding racial differences in other molecules that may have biological relevance to prostate cancer aggressiveness. Candidates for study include androgen receptor co-regulators; growth factors, such as hepatocyte growth factor, IGF, and epidermal growth factor, and their cognate receptors; specific components of cell signaling pathways; levels of the vitamin D metabolizing system; angiogenesis/neovascularization; tumor stem-like cells; and macrophages, natural killer cells, and lymphocytes that may differ between races. Analyses are complicated by heterogeneity of tissue expression within prostate tissue (normal, benign, prostatic intraepithelial neoplasia, and prostate cancer) and among tissue compartments (stromal, epithelial, endothelial, and inflammation), and lack of availability of frozen prostate tissues and highly specific antibodies that perform well in archival tissue.

New methods facilitate racial comparisons of biomarkers of prostate cancer aggressiveness. Tissue microarray is a high-throughput method that enables the histological study of many small specimens processed under the same conditions *(227)* and serial sectioning of the microarrayed tissue block allows up to 200 replicas of the same slide. Quantitative immunohistochemistry *(228–230)* allows biomarker measurement and morphological correlation, and laser microdissection provides purified cell populations for molecular evaluation. Study of racial differences in prostate cancer biology may be facilitated by the recent establishment of epithelial and stromal cell lines from the radical prostatectomy specimen of an African American *(231)*. The epithelial cell line exhibits androgen-dependent growth, expresses PSA, and the androgen receptor contains 26 CAG repeats. The cell line does not form tumors in nude mice and has a hypertriploid karyotype, with additional gains in multiple chromosomes.

Series of relatively limited numbers of men suggest the possibility of racial differences in some biologically relevant growth factors and regulatory pathways. Nuclear receptor coactivators, such as transcriptional intermediary factor 2, steroid receptor coactivator 1, and p160 coactivator, should be

Health Care System Interaction > Host Biology ≈ Tumor Biology

Fig. 2. Reasons for racial differences in prostate cancer mortality remain unclear. A working hypothesis is and much data suggests, that racial differences in health care system interaction may be more important than host or tumor biology.

studied because coactivator overexpression can increase androgen receptor transactivation by other steroids, including adrenal androgens *(232,233)*.

Caveolin-1 is a structural protein found in caveolae that plays a role in cholesterol transport and signal transduction. Caveolin-1 suppresses c-myc-mediated apoptosis and is overexpressed in murine and human prostate cancer. Prostate cancers expressed higher levels of caveolin-1 in African Americans than Caucasian Americans *(234)*. Prostate cancer may grow more rapidly in African Americans because of reduced rates of apoptosis *(234)*, and African Americans could respond less to radiation or androgen-deprivation therapy because of altered regulation of apoptotic pathways *(235)*.

p53 levels may differ between races because mutations in p53 confer resistance to apoptosis and increased risk of relapse after prostatectomy or radiation therapy *(236–238)*; *Bcl-2* overexpression is associated with adverse prognosis and progression to androgen independence *(239–241)*. Reduced expression of *p27kip1* has been associated with poor prostate cancer-free survival *(242,243)*. *PTEN* expression is frequently lost in localized and advanced prostate cancer *(244–247)*. Constitutive activation of *NF-κB* is associated with resistance to apoptosis *(248)* and progression to androgen independence *(249)*. Activated Akt occurs progressively from normal to invasive prostate cancer *(250)*. Activated mitogen-activated protein kinase has been associated with progression to androgen-independent prostate cancer *(251)*.

African American prostate cancer from radical prostatectomy specimens demonstrated increased expression of epidermal growth factor receptor *(252)*. No racial differences were reported in *Skp2* expression; *Skp2* controls the abundance of p27, an inhibitor of cell proliferation *(253)*. N-myc downstream-regulated gene 1 protein is a recently discovered protein whose transcription is induced by androgens and hypoxia. The expression of this protein was greater in both malignant and benign epithelia from African Americans compared with white Americans. Racial differences in response of prostate epithelium to hypoxia and androgens may contribute to increased incidence and aggressiveness of prostate cancer in African Americans *(254)*. Finally, the existence of a stem cell in the adult prostate gland has been hypothesized by many investigators *(255,256)*. Stem cell-like populations have been identified from prostates *(257)*. The number of stem cell-like cells in the prostate may be correlated with aggressiveness of prostate cancer and duration of response to androgen-deprivation therapy.

REFERENCES

1. Jemal, A., Siegal, R., Ward, E., Murray, T., Xu, J., Smigal, C., and Thun, M. J. (2006). Cancer Statistics. *CA Cancer J. Clin.* **56**, 106–130.
2. Parkin, D. M., Bray, F., Ferlay, J., and Pisani, P. (2005). Global cancer statistics, 2002. *CA Cancer J. Clin.* **55**, 74–108.
3. Guileyardo, J. M., Johnson, W. D., Welsh, R. A., Akazaki, K., and Correa, P. (1980). Prevalence of latent prostate carcinoma in two U.S. populations. *J. Natl. Cancer Inst.* **65**, 311–316.
4. Sakr, W. A., Haas, G. P., Cassin, B. F., Pontes, J. E., and Crissman, J. D. (1993). The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. *J. Urol.* **150**, 379–385.
5. Bouchardy, C., Mirra, A. P., Khlat, M., Parkin, D. M., de Souza, J. M., and Gotlieb, S. L. (1991). Ethnicity and cancer risk in Sao Paulo, Brazil. *Cancer Epidemiol. Biomarkers Prev.* **1**, 21–27.

6. Ries, L., Eisner, M., Hankey, B., et al. (2000). SEER Cancer Statistics Review 1973–1997. Bethesda, MD National Cancer Institute.
7. Montagu, A., ed. (1964). The concept of race. Free Press of Glencoe, New York, NY.
8. Risch, N., Burchard, E., Ziv, E., and Tang, H. (2002). Categorization of humans in biomedical research: genes, race and disease. *Genome Biol.* 3, comment2007.
9. Cooper, R. S., Kaufman, J. S., and Ward, R. (2003). Race and genomics. *N. Engl. J. Med.* 348, 1166–1170.
10. Phimister, E. G. (2003). Medicine and the racial divide. *N. Engl. J. Med.* **348**, 1081–1082.
11. Bloche, M. G. (2004). Race-based therapeutics. *N. Engl. J. Med.* **351**, 2035–2037.
12. Woolf, S. H. (2004). Society's choice: the tradeoff between efficacy and equity and the lives at stake. *Am. J. Prev. Med.* **27**, 49–56.
13. Kawachi, I., and Kennedy, B. K. (2002). The health of nations: Why inequality is harmful to your health. New Press, New York, NY.
14. Woolf, S. H., Johnson, R. E., Fryer, G. E., Jr., Rust, G., and Satcher, D. (2004). The health impact of resolving racial disparities: an analysis of US mortality data. *Am. J. Public Health* **94**, 2078–2081.
15. Mettlin, C. J., Murphy, G. P., McGinnis, L. S., and Menck, H. R. (1995). The National Cancer Data Base report on prostate cancer. American College of Surgeons Commission on Cancer and the American Cancer Society. *Cancer* **76**, 1104–1112.
15a. Schroeder, J. C., Bensen, J. T., Su, L. J., Mishel, M., Ivanova, A., Smith, G. J., Godley, P. A., Fontham, E. T., and Mohler, J. L. (2006). The North Carolina-Louisiana Prostate Cancer Project (PCaP): Methods and design of a multidisciplinary population-based cohort study of racial differences in prostate cancer outcomes. *Prostate*.
16. Robbins, A. S., Whittemore, A. S., and Van Den Eeden, S. K. (1998). Race, prostate cancer survival, and membership in a large health maintenance organization. *J. Natl. Cancer Inst.* **90**, 986–990.
17. Demark-Wahnefried, W., Strigo, T., Catoe, K., Conaway, M., Brunetti, M., Rimer, B. K., and Robertson, C. N. (1995). Knowledge, beliefs, and prior screening behavior among blacks and whites reporting for prostate cancer screening. *Urology* **46**, 346–351.
18. Harlan, L., Brawley, O., Pommerenke, F., Wali, P., and Kramer, B. (1995). Geographic, age, and racial variation in the treatment of local/regional carcinoma of the prostate. *J. Clin. Oncol.* **13**, 93–100.
19. Schapira, M. M., McAuliffe, T. L., and Nattinger, A. B. (1995). Treatment of localized prostate cancer in African-American compared with Caucasian men. Less use of aggressive therapy for comparable disease. *Med. Care* **33**, 1079–1088.
20. Mettlin, C. J., Murphy, G. P., Cunningham, M. P., and Menck, H. R. (1997). The National Cancer Data Base report on race, age, and region variations in prostate cancer treatment. *Cancer* **80**, 1261–1266.
21. Fowler, J. E. and Bigler, S. A. (1998). Prostate cancer in black and white American Men. *Monogr. Urol.* **19**, 95–120.
22. Shavers, V. L. and Brown, M. L. (2002). Racial and ethnic disparities in the receipt of cancer treatment. *J. Natl. Cancer Inst.* **94**, 334–357.
23. Klabunde, C. N., Potosky, A. L., Harlan, L. C., and Kramer, B. S. (1998). Trends and black/white differences in treatment for nonmetastatic prostate cancer. *Med. Care* **36**, 1337–1348.
24. Adolfsson, J., Carstensen, J., and Lowhagen, T. (1992). Deferred treatment in clinically localised prostatic carcinoma. *Br. J. Urol.* **69**, 183–187.
25. Johansson, J. E., Adami, H. O., Andersson, S. O., Bergstrom, R., Holmberg, L., and Krusemo, U. B. (1992). High 10-year survival rate in patients with early, untreated prostatic cancer. *JAMA* **267**, 2191–2196.
26. Aus, G., Hugosson, J., and Norlen, L. (1995). Long-term survival and mortality in prostate cancer treated with noncurative intent. *J. Urol.* **154**, 460–465.
27. Johansson, J. E., Holmberg, L., Johansson, S., Bergstrom, R., and Adami, H. O. (1997). Fifteen-year survival in prostate cancer. A prospective, population-based study in Sweden. *JAMA* **277**, 467–471.
28. Brasso, K., Friis, S., Juel, K., Jorgensen, T., and Iversen, P. (1999). Mortality of patients with clinically localized prostate cancer treated with observation for 10 years or longer: a population based registry study. *J. Urol.* **161**, 524–528.
29. Warner, J. and Whitmore, W. F., Jr. (1994). Expectant management of clinically localized prostatic cancer. *J Urol* **152**, 1761–1765.
30. Albertsen, P. C., Fryback, D. G., Storer, B. E., Kolon, T. F., and Fine, J. (1995). Long-term survival among men with conservatively treated localized prostate cancer. *JAMA* **274**, 626–631.
31. Albertsen, P. C., Hanley, J. A., Gleason, D. F., and Barry, M. J. (1998). Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer. *JAMA* **280**, 975–980.
32. Bach, P. B., Schrag, D., Brawley, O. W., Galaznik, A., Yakren, S., and Begg, C. B. (2002). Survival of blacks and whites after a cancer diagnosis. *JAMA* **287,** 2106–2113.
33. Optenberg, S. A., Thompson, I. M., Friedrichs, P., Wojcik, B., Stein, C. R., and Kramer, B. (1995). Race, treatment, and long-term survival from prostate cancer in an equal-access medical care delivery system. *JAMA* **274**, 1599–1605.
34. Fowler, J. E., Jr. and Terrell, F. (1996). Survival in blacks and whites after treatment for localized prostate cancer. *J. Urol.* **156**, 133–136.

35. Iselin, C. E., Box, J. W., Vollmer, R. T., Layfield, L. J., Robertson, J. E., and Paulson, D. F. (1998). Surgical control of clinically localized prostate carcinoma is equivalent in African-American and white males. *Cancer* **83**, 2353–2360.
36. Hart, K. B., Wood, D. P., Jr., Tekyi-Mensah, S., Porter, A. T., Pontes, J. E., and Forman, J. D. (1999). The impact of race on biochemical disease-free survival in early-stage prostate cancer patients treated with surgery or radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys.* **45**, 1235–1238.
37. Eastham, J. A. and Kattan, M. W. (2000). Disease recurrence in black and white men undergoing radical prostatectomy for clinical stage T1-T2 prostate cancer. *J. Urol.* **163**, 143–145.
38. Freedland, S. J., Jalkut, M., Dorey, F., Sutter, M. E., and Aronson, W. J. (2000). Race is not an independent predictor of biochemical recurrence after radical prostatectomy in an equal access medical center. *Urology* **56**, 87–91.
39. Underwood, W., 3rd, Wei, J., Rubin, M. A., Montie, J. E., Resh, J., and Sanda, M. G. (2004). Postprostatectomy cancer-free survival of African Americans is similar to non-African Americans after adjustment for baseline cancer severity. *Urol. Oncol.* **22**, 20–24.
40. Grossfeld, G. D., Latini, D. M., Downs, T., Lubeck, D. P., Mehta, S. S., and Carroll, P. R. (2002). Is ethnicity an independent predictor of prostate cancer recurrence after radical prostatectomy? *J. Urol.* **168**, 2510–2515.
41. Banerjee, M., Powell, I. J., George, J., Biswas, D., Bianco, F., and Severson, R. K. (2002). Prostate specific antigen progression after radical prostatectomy in African-American men versus white men. *Cancer* **94**, 2577–2583.
42. Cross, C. K., Shultz, D., Malkowicz, S. B., et al. (2002). Impact of race on prostate-specific antigen outcome after radical prostatectomy for clinically localized adenocarcinoma of the prostate. *J. Clin. Oncol.* **20**, 2863–2868.
43. Roach, M., 3rd, Lu, J., Pilepich, M. V., Asbell, S. O., Mohiuddin, M., and Grignon, D. (2003). Race and survival of men treated for prostate cancer on radiation therapy oncology group phase III randomized trials. *J. Urol.* **169**, 245–250.
44. Lee, L. N., Barnswell, C., Torre, T., Fearn, P., Kattan, M., and Potters, L. (2002). Prognostic significance of race on biochemical control in patients with localized prostate cancer treated with permanent brachytherapy: multivariate and matched-pair analyses. *Int. J. Radiat. Oncol. Biol. Phys.* **53**, 282–289.
45. Young, C. D., Lewis, P., Weinberg, V., Lee, T. T., Coleman, C. W., and Roach, M., 3rd. (2000). The impact of race on freedom from prostate-specific antigen failure in prostate cancer patients treated with definitive radiation therapy. *Semin. Urol. Oncol.* **18**, 121–126.
46. Roach, M., 3rd, Krall, J., Keller, J. W., et al. (1992). The prognostic significance of race and survival from prostate cancer based on patients irradiated on Radiation Therapy Oncology Group protocols (1976-1985). *Int. J. Radiat. Oncol. Biol. Phys.* **24**, 441–449.
47. Roach, M., 3rd, Lu, J., Pilepich, M. V., Asbell, S. O., Mohiuddin, M., Terry, R., and Grignon, D. (1999). Long-term survival after radiotherapy alone: radiation therapy oncology group prostate cancer trials. *J. Urol.* **161**, 864–868.
48. Polednak, A. P. (2003). Black-white differences in survival from late-stage prostate cancer. *Ethn. Dis.* **13**, 220–225.
49. Fowler, J. E., Jr., Bigler, S. A., Kolski, J. M., and Yee, D. T. (1998). Early results of a prospective study of hormone therapy for patients with locally advanced prostate carcinoma. *Cancer* **82**, 1112–1117.
50. Wyatt, R. B., Sanchez-Ortiz, R. F., Wood, C. G., Ramirez, E., Logothetis, C., and Pettaway, C. A. (2004). Prognostic factors for survival among Caucasian, African-American and Hispanic men with androgen-independent prostate cancer. *J. Natl. Med. Assoc.* **96**, 1587–1593.
51. Halabi, S., Small, E. J., Vogelzang, N. J., Barrier, R. C., Jr., George, S. L., and Gilligan, T. D. (2004). Impact of race on survival in men with metastatic hormone-refractory prostate cancer. *Urology* **64**, 212–217.
52. Gefland, D., Parzuchowski, J., Cort, M., and Powell, I. (1995). Digital rectal examinations and prostate cancer screening: Attitudes of African American Men. *Oncol. Nurs. Forum* **22**, 1253–1255.
53. Freeman, H. P., Muth, B. J., and Kerner, J. F. (1995). Expanding access to cancer screening and clinical follow-up among the medically underserved. *Cancer Pract.* 3, 19–30.
54. Myers, R. E., Wolf, T. A., Balshem, A. M., Ross, E. A., and Chodak, G. W. (1994). Receptivity of African-American men to prostate cancer screening. *Urology* **43**, 480–487.
55. Weinrich, S. P., Weinrich, M. C., Boyd, M. D., and Atkinson, C. (1998). The impact of prostate cancer knowledge on cancer screening. *Oncol. Nurs. Forum* **25**, 527–534.
56. Gilligan, T., Wang, P. S., Levin, R., Kantoff, P. W., and Avorn, J. (2004). Racial differences in screening for prostate cancer in the elderly. *Arch. Intern. Med.* **164**, 1858–1864.
57. Etzioni, R., Berry, K. M., Legler, J. M., and Shaw, P. (2002). Prostate-specific antigen testing in black and white men: an analysis of medicare claims from 1991–1998. *Urology* **59**, 251–255.
58. Robinson, S. B., Ashley, M., and Haynes, M. A. (1996). Attitude of African-Americans regarding prostate cancer clinical trials. *J. Community Health* **21**, 77–87.
59. Anscher, M. S. (1999). Prostate cancer in African-American men. *NC Med. J.* **60**, 10–12.
60. Bennett, C. L., Ferreira, M. R., Davis, T. C., et al. (1998). Relation between literacy, race, and stage of presentation among low-income patients with prostate cancer. *J. Clin. Oncol.* **16**, 3101–3104.
61. Jemal, A., Ward, E., Wu, X., Martin, H. J., McLaughlin, C. C., and Thun, M. J. (2005). Geographic patterns of prostate cancer mortality and variations in access to medical care in the United States. *Cancer Epidemiol. Biomarkers Prev.* **14**, 590–595.

62. Mueller, K. J., Ortega, S. T., Parker, K., Patil, K., and Askenazi, A. (1999). Health status and access to care among rural minorities. *J. Health Care Poor Underserved* **10**, 230–249.
63. Ballard, D. J., Strogatz, D. S., Wagner, E. H., Siscovick, D. S., James, S. A., Kleinbaum, D. G., Cutchin, L. M., and Ibrahim, M. A. (1988). Hypertension control in a rural southern community: medical care process and dropping out. *Am. J. Prev. Med.* **4**, 133–139.
64. O'Connor, P. J., Wagner, E. H., and Strogatz, D. S. (1990). Hypertension control in a rural community. An assessment of community-oriented primary care. *J. Fam. Pract.* **30**, 420–424.
65. Strogatz, D. S. (1990). Use of medical care for chest pain: differences between blacks and whites. *Am. J. Public Health* **80**, 290–294.
66. Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. (2001). Cancer statistics, 2001. *CA Cancer J. Clin.* **51**, 15–36.
67. Powe, B. D. (1995). Cancer fatalism among elderly Caucasians and African Americans. *Oncol. Nurs. Forum* **22**, 1355–1359.
68. Ferrao, K. (1993). Are black older adults health-pessimistic. *J. Health Soc. Behav.* **34**, 201–214.
69. Clark, J. A., Rieker, P., Propert, K. J., and Talcott, J. A. (1999). Changes in quality of life following treatment for early prostate cancer. *Urology* **53**, 161–168.
70. Talcott, J. A., Clark, J. A., Stark, P. C., and Mitchell, S. P. (2001). Long-term treatment related complications of brachytherapy for early prostate cancer: a survey of patients previously treated. *J. Urol.* **166**, 494–499.
71. Betensky, R. A., Talcott, J. A., and Weeks, J. C. (2000). Binary data with two, non-nested sources of clustering: an analysis of physician recommendations for early prostate cancer treatment. *Biostatistics* **1**, 219–230.
72. Clark, J. Y. and Talcott, J. A. (2001). A symptom index to assess the outcomes of treatment for early prostate cancer. *Med. Care* in press.
73. Bokhour, B. G., Clark, J. A., Inui, T. S., Silliman, R. A., and Talcott, J. A. (2001). Sexuality after treatment for early prostate cancer: exploring the meanings of "erectile dysfunction". *J. Gen. Intern. Med.* **16**, 649–655.
74. (2003). Institute of Medicine. Unequal Treatment: Confronting racial and ethnic disparities in healthcare. National Academy Press, Washington, DC.
75. Fields, S. D. (2001). The health belief system of African-Americans: essential information for today's practicing nurses. *J. Multicult. Nurs. Health* **7**, 36–40.
76. Parks, F. M. (1998). Models of helping and coping: A transgenerational theory of African-American traditional healing. *Interamerican J. Psychol.* **32**, 95–110.
77. Mathews, H. F., Lannin, D. R., and Mitchell, J. P. (1994). Coming to terms with advanced breast cancer: black women's narratives from eastern North Carolina. *Soc. Sci. Med.* **38**, 789–800.
78. Cooley, M. E. and Jennings-Dozier, K. (1998). Cultural assessment of black American men treated for prostate cancer: clinical case studies. *Oncol. Nurs. Forum* **25**, 1729–1736.
79. Jenkins, R. A. and Pargament, K. I. (1995). Religion and spirituality as resources for coping with cancer. *J. Psychosoc. Oncol.* **13**, 51–74.
80. Krause, N. (1992). Stress, religiosity and psychological well-being among older blacks. *J. Aging Health* **4**, 412–439.
81. Germino, B. B., Mishel, M. H., Belyea, M., Harris, L., Ware, A., and Mohler, J. (1998). Uncertainty in prostate cancer. Ethnic and family patterns. *Cancer Pract.* **6**, 107–113.
82. Mishel, M. H., Belyea, M., Germino, B. B., et al. (2002). Helping patients with localized prostate carcinoma manage uncertainty and treatment side effects: nurse-delivered psychoeducational intervention over the telephone. *Cancer* **94**, 1854–1866.
83. Woods, V. D., Montgomery, S. B., Belliard, J. C., Ramirez-Johnson, J., and Wilson, C. M. (2004). Culture, black men, and prostate cancer: what is reality? *Cancer Control* **11**, 388–396.
84. Beisecker, A. E. and Beisecker, T. D. (1990). Patient information-seeking behaviors when communicating with doctors. *Med Care* **28**, 19–28.
85. Davison, B. J., Degner, L. F., and Morgan, T. R. (1995). Information and decision-making preferences of men with prostate cancer. *Oncol. Nurs. Forum* **22**, 1401–1408.
86. McPherson, K. (1994). The Cochrane Lecture. The best and the enemy of the good: randomised controlled trials, uncertainty, and assessing the role of patient choice in medical decision making. *J. Epidemiol. Community Health* **48**, 6–15.
87. Mishel, M. H. (1997). Uncertainty in acute illness. *Annu. Rev. Nurs. Res.* **15**, 57–80.
88. O'Rourke, M. E. (2001). Decision making and prostate cancer treatment selection: a review. *Semin. Oncol. Nurs.* **17**, 108–117.
89. Pautler, S. E., Tan, J. K., Dugas, G. R., et al. (2001). Use of the internet for self-education by patients with prostate cancer. *Urology* **57**, 230–233.
90. Fontham, E. T., Pickle, L. W., Haenszel, W., Correa, P., Lin, Y. P., and Falk, R. T. (1988). Dietary vitamins A and C and lung cancer risk in Louisiana. *Cancer* **62**, 2267–2273.
91. Correa, P., Fontham, E., Pickle, L. W., Chen, V., Lin, Y. P., and Haenszel, W. (1985). Dietary determinants of gastric cancer in south Louisiana inhabitants. *J. Natl. Cancer Inst.* **75**, 645–654.
92. Falk, R. T., Pickle, L. W., Fontham, E. T., Correa, P., and Fraumeni, J. F., Jr. (1988). Life-style risk factors for pancreatic cancer in Louisiana: a case–control study. *Am. J. Epidemiol.* **128**, 324–336.

93. Wu, A. H., Fontham, E. T., Reynolds, P., et al. (1996). Family history of cancer and risk of lung cancer among lifetime nonsmoking women in the United States. *Am. J. Epidemiol.* **143**, 535–542.
94. Freimuth, V. S., Quinn, S. C., Thomas, S. B., Cole, G., Zook, E., and Duncan, T. (2001). African Americans' views on research and the Tuskegee Syphilis Study. *Soc. Sci. Med.* **52**, 797–808.
95. Corbie-Smith, G., Thomas, S. B., and St George, D. M. (2002). Distrust, race, and research. *Arch. Intern. Med.* **162**, 2458–2463.
96. Mouton, C. P., Harris, S., Rovi, S., Solorzano, P., and Johnson, M. S. (1997). Barriers to black women's participation in cancer clinical trials. *J. Natl. Med. Assoc.* **89**, 721–727.
97. Neff, J. A., and Hoppe, S. K. (1993). Race/ethnicity, acculturation, and psychological distress: Fatalism and religiosity as cultural resources. *J. Community Psychol.* **21**, 3–20.
98. Neighbors, H. W. and Jackson, J. S. (1984). The use of informal and formal help: four patterns of illness behavior in the black community. *Am. J. Community Psychol.* **12**, 629–644.
99. Mishel, M. H., Germino, B. B., Belyea, M., et al. (2003). Moderators of an uncertainty management intervention: for men with localized prostate cancer. *Nurs. Res.* **52**, 89–97.
100. Anderson, K. O., Mendoza, T. R., Valero, V., et al. (2000). Minority cancer patients and their providers: pain management attitudes and practice. *Cancer* **88**, 1929–1938.
101. Cooper-Patrick, L., Gallo, J. J., Gonzales, J. J., et al. (1999). Race, gender, and partnership in the patient-physician relationship. *JAMA* **282**, 583–589.
102. Freeman, H. P. (2004). Poverty, culture, and social injustice: determinants of cancer disparities. *CA Cancer J. Clin.* **54**, 72–77.
103. Sparber, A., Bauer, L., Curt, G., et al. (2000). Use of complementary medicine by adult patients participating in cancer clinical trials. *Oncol. Nurs. Forum* **27**, 623–630.
104. Lee, M. M., Chang, J. S., Jacobs, B., and Wrensch, M. R. (2002). Complementary and alternative medicine use among men with prostate cancer in 4 ethnic populations. *Am. J. Public Health* **92**, 1606–1609.
105. Nam, R. K., Fleshner, N., Rakovitch, E., et al. (1999). Prevalence and patterns of the use of complementary therapies among prostate cancer patients: an epidemiological analysis. *J. Urol.* **161**, 1521–1524.
106. Kao, G. D. and Devine, P. (2000). Use of complementary health practices by prostate carcinoma patients undergoing radiation therapy. *Cancer* **88**, 615–619.
107. Lippert, M. C., McClain, R., Boyd, J. C., and Theodorescu, D. (1999). Alternative medicine use in patients with localized prostate carcinoma treated with curative intent. *Cancer* **86**, 2642–2648.
108. Wilt, T. J., Ishani, A., Stark, G., MacDonald, R., Lau, J., and Mulrow, C. (1998). Saw palmetto extracts for treatment of benign prostatic hyperplasia: a systematic review. *JAMA* **280**, 1604–1609.
109. Pfeifer, B. L., Pirani, J. F., Hamann, S. R., and Klippel, K. F. (2000). PC-SPES, a dietary supplement for the treatment of hormone refractory prostate cancer. *BJU Int.* **85**, 481–485.
110. Whittemore, A. S., Kolonel, L. N., Wu, A. H., et al. (1995). Prostate cancer in relation to diet, physical activity, and body size in blacks, whites, and Asians in the United States and Canada. *J. Natl. Cancer Inst.* **87**, 652–661.
111. Hayes, R. B., Ziegler, R. G., Gridley, G., et al. (1999). Dietary factors and risks for prostate cancer among blacks and whites in the United States. *Cancer Epidemiol. Biomarkers Prev.* **8**, 25–34.
112. Mills, P. K., Beeson, W. L., Phillips, R. L., and Fraser, G. E. (1989). Cohort study of diet, lifestyle, and prostate cancer in Adventist men. *Cancer* **64**, 598–604.
113. Jacobsen, B. K., Knutsen, S. F., and Fraser, G. E. (1998). Does high soy milk intake reduce prostate cancer incidence? The Adventist Health Study (United States). *Cancer Causes Control* **9**, 553–557.
114. Severson, R. K., Nomura, A. M., Grove, J. S., and Stemmermann, G. N. (1989). A prospective study of demographics, diet, and prostate cancer among men of Japanese ancestry in Hawaii. *Cancer Res.* **49**, 1857–1860.
115. Schuurman, A. G., Goldbohm, R. A., Dorant, E., and van den Brandt, P. A. (1998). Vegetable and fruit consumption and prostate cancer risk: a cohort study in The Netherlands. *Cancer Epidemiol. Biomarkers Prev.* **7**, 673–680.
116. Vogt, T. M., Mayne, S. T., Graubard, B. I., et al. (2002). Serum lycopene, other serum carotenoids, and risk of prostate cancer in US Blacks and Whites. *Am. J. Epidemiol.* **155**, 1023–1032.
117. Hung, H. C., Joshipura, K. J., Jiang, R., et al. (2004). Fruit and vegetable intake and risk of major chronic disease. *J. Natl. Cancer Inst.* **96**, 1577–1584.
118. Hsing, A. W., Deng, J., Sesterhenn, I. A., et al. (2000). Body size and prostate cancer: a population-based case–control study in China. *Cancer Epidemiol. Biomarkers Prev.* **9**, 1335–1341.
119. Clarke, G. and Whittemore, A. S. (2000). Prostate cancer risk in relation to anthropometry and physical activity: the National Health and Nutrition Examination Survey I Epidemiological Follow-Up Study. *Cancer Epidemiol. Biomarkers Prev.* **9**, 875–881.
120. Freedland, S. J., Terris, M. K., Presti, J. C., Jr., et al. (2004). Obesity and biochemical outcome following radical prostatectomy for organ confined disease with negative surgical margins. *J. Urol.* **172**, 520–524.
121. DeLellis, K., Rinaldi, S., Kaaks, R. J., Kolonel, L. N., Henderson, B., and Le Marchand, L. (2004). Dietary and lifestyle correlates of plasma insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3): the multiethnic cohort. *Cancer Epidemiol. Biomarkers Prev.* **13**, 1444–1451.

122. Mohler, J. L., Gaston, K. E., Moore, D. T., et al. (2004). Racial differences in prostate androgen levels in men with clinically localized prostate cancer. *J. Urol.* 171, 2277–2280.
123. Costello, L. C., Feng, P., Milon, B., Tan, M., and Franklin, R. B. (2004). Role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve. *Prostate Cancer Prostatic Dis.* **7**, 111–117.
124. Diamond, J. M. (1997). Guns, Germs, and Steel: The Fates of Human Societies, 1st Ed. WW Norton and Co, New York, NY.
125. Costello, L. C., Franklin, R. B., Feng, P., and Tan, M. (2004). Re: Zinc supplement use and risk of prostate cancer. *J. Natl. Cancer Inst.* **96**, 239–240; author reply 240–241.
126. Liang, J. Y., Liu, Y. Y., Zou, J., Franklin, R. B., Costello, L. C., and Feng, P. (1999). Inhibitory effect of zinc on human prostatic carcinoma cell growth. *Prostate* **40**, 200–207.
127. Costello, L. C. and Franklin, R. B. (1998). Novel role of zinc in the regulation of prostate citrate metabolism and its implications in prostate cancer. *Prostate* **35**, 285–296.
128. Rishi, I., Baidouri, H., Abbasi, J. A., et al. (2003). Prostate cancer in African American men is associated with down-regulation of zinc transporters. *Appl. Immunohistochem. Mol. Morphol.* **11**, 253–260.
129. Schwartz, G. G. and Hulka, B. S. (1990). Is vitamin D deficiency a risk factor for prostate cancer? (Hypothesis). *Anticancer Res.* **10**, 1307–1311.
130. Schwartz, G. G., Wang, M. H., Zang, M., Singh, R. K., and Siegal, G. P. (1997). 1 alpha,25-Dihydroxyvitamin D (calcitriol) inhibits the invasiveness of human prostate cancer cells. *Cancer Epidemiol. Biomarkers Prev.* **6**, 727–732.
131. Lokeshwar, B. L., Schwartz, G. G., Selzer, M. G., et al. (1999). Inhibition of prostate cancer metastasis in vivo: a comparison of 1,23-dihydroxyvitamin D (calcitriol) and EB1089. *Cancer Epidemiol. Biomarkers Prev.* **8**, 241–248.
132. Schwartz, G. G., Whitlatch, L. W., Chen, T. C., Lokeshwar, B. L., and Holick, M. F. (1998). Human prostate cells synthesize 1,25-dihydroxyvitamin D3 from 25-hydroxyvitamin D3. *Cancer Epidemiol. Biomarkers Prev.* 7, 391–395.
133. Osborn, J. L., Schwartz, G. G., Smith, D. C., Bahnson, R. R., Day, R., and Trump, D. L. (1995). Phase II trial of oral1,25-Dihydroxyvitamin D (Calcitriol) in hormone refractory prostate cancer. *Urol. Oncol.* **1**, 1995–1998.
134. Barreto, A. M., Schwartz, G. G., Woodruff, R., and Cramer, S. D. (2000). 25-Hydroxyvitamin D3, the prohormone of 1,25-dihydroxyvitamin D3, inhibits the proliferation of primary prostatic epithelial cells. *Cancer Epidemiol. Biomarkers Prev.* **9**, 265–270.
135. Chen, T. C., Schwartz, G. G., Burnstein, K. L., Lokeshwar, B. L., and Holick, M. F. (2000). The in vitro evaluation of 25-hydroxyvitamin D3 and 19-nor-1alpha,25-dihydroxyvitamin D2 as therapeutic agents for prostate cancer. *Clin. Cancer Res.* **6**, 901–908.
136. Ross, R. K., Shimizu, H., Paganini-Hill, A., Honda, G., and Henderson, B. E. (1987). Case–control studies of prostate cancer in blacks and whites in southern California. *J. Natl. Cancer Inst.* **78**, 869–874.
137. Ellis, L. and Nyborg, H. (1992). Racial/ethnic variations in male testosterone levels: a probable contributor to group differences in health. *Steroids* **57**, 72–75.
138. Gustafsson, O., Norming, U., Gustafsson, S., Eneroth, P., Astrom, G., and Nyman, C. R. (1996). Dihydrotestosterone and testosterone levels in men screened for prostate cancer: a study of a randomized population. *Br. J. Urol.* **77**, 433–440.
139. Kubricht, W. S., 3rd, Williams, B. J., Whatley, T., Pinckard, P., and Eastham, J. A. (1999). Serum testosterone levels in African-American and white men undergoing prostate biopsy. *Urology* **54**, 1035–1038.
140. Ghanadian, R., Puah, C. M., and O'Donoghue, E. P. (1979). Serum testosterone and dihydrotestosterone in carcinoma of the prostate. *Br. J. Cancer* **39**, 696–699.
141. Ahluwalia, B., Jackson, M. A., Jones, G. W., Williams, A. O., Rao, M. S., and Rajguru, S. (1981). Blood hormone profiles in prostate cancer patients in high-risk and low-risk populations. *Cancer* **48**, 2267–2273.
142. Drafta, D., Proca, E., Zamfir, V., Schindler, A. E., Neacsu, E., and Stroe, E. (1982). Plasma steroids in benign prostatic hypertrophy and carcinoma of the prostate. *J. Steroid Biochem.* **17**, 689–693.
143. Zumoff, B., Levin, J., Strain, G. W., et al. (1982). Abnormal levels of plasma hormones in men with prostate cancer: evidence toward a "two-disease" theory. *Prostate* **3**, 579–588.
144. Meikle, A. W. and Stanish, W. M. (1982). Familial prostatic cancer risk and low testosterone. *J. Clin. Endocrinol. Metab.* **54**, 1104–1108.
145. Rubin, J. L. and Farber, M. C. (1988). Pathology. WB Saunders, Philadelphia, PA.
146. Harlan, W. R., Grillo, G. P., Cornoni-Huntley, J., and Leaverton, P. E. (1979). Secondary sex characteristics of boys 12 to 17 years of age: the U.S. Health Examination Survey. *J. Pediatr.* **95**, 293–297.
147. Richards, R. J., Svec, F., Bao, W., Srinivasan, S. R., and Berenson, G. S. (1992). Steroid hormones during puberty: racial (black-white) differences in androstenedione and estradiol—the Bogalusa Heart Study. *J. Clin. Endocrinol. Metab.* **75**, 624–631.
148. Nankin, H. R., Sperling, M., Kenny, F. M., Drash, A. L., and Troen, P. (1974). Correlation between sexual maturation and serum gonadotropins: comparison of black and white youngsters. *Am. J. Med. Sci.* **268**, 139–147.
149. Laska-Mierzejewska, T. (1970). Morphological and developmental difference between Negro and white Cuban youths. *Hum. Biol.* **42**, 581–597.

150. Cameron, N., Grieve, C. A., Kruger, A., and Leschner, K. F. (1993). Secondary sexual development in rural and urban South African black children. *Ann. Hum. Biol.* **20**, 583–593.
151. Kazemi-Esfarjani, P., Trifiro, M. A., and Pinsky, L. (1995). Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the (CAG)n-expanded neuronopathies. *Hum. Mol. Genet.* **4**, 523–527.
152. Chamberlain, N. L., Driver, E. D., and Miesfeld, R. L. (1994). The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res.* **22**, 3181–3186.
153. Choong, C. S., Kemppainen, J. A., Zhou, Z. X., and Wilson, E. M. (1996). Reduced androgen receptor gene expression with first exon CAG repeat expansion. *Mol. Endocrinol.* **10**, 1527–1535.
154. O'Brien, T. G., Guo, Y., Visvanathan, K., et al. (2004). Differences in ornithine decarboxylase and androgen receptor allele frequencies among ethnic groups. *Mol. Carcinog.* **41**, 120–123.
155. Giovannucci, E., Stampfer, M. J., Krithivas, K., et al. (1997). The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc. Natl. Acad. Sci. USA* **94**, 3320–3323.
156. Stanford, J. L., Just, J. J., Gibbs, M., et al. (1997). Polymorphic repeats in the androgen receptor gene: molecular markers of prostate cancer risk. *Cancer Res.* **57**, 1194–1198.
157. Ingles, S. A., Ross, R. K., Yu, M. C., et al. (1997). Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. *J. Natl. Cancer Inst.* **89**, 166–170.
158. Chen, C., Lamharzi, N., Weiss, N. S., et al. (2002). Androgen receptor polymorphisms and the incidence of prostate cancer. *Cancer Epidemiol. Biomarkers Prev.* **11**, 1033–1040.
159. Miller, E. A., Stanford, J. L., Hsu, L., Noonan, E., and Ostrander, E. A. (2001). Polymorphic repeats in the androgen receptor gene in high-risk sibships. *Prostate* **48**, 200–205.
160. Correa-Cerro, L., Wohr, G., Haussler, J., et al. (1999). (CAG)nCAA and GGN repeats in the human androgen receptor gene are not associated with prostate cancer in a French-German population. *Eur. J. Hum. Genet.* **7**, 357–362.
161. Bratt, O., Borg, A., Kristoffersson, U., Lundgren, R., Zhang, Q. X., and Olsson, H. (1999). CAG repeat length in the androgen receptor gene is related to age at diagnosis of prostate cancer and response to endocrine therapy, but not to prostate cancer risk. *Br. J. Cancer* **81**, 672–676.
162. Beillin, J., Hawewood, L., Frydenberg, M., Mameghan, H., Martyres, R., Farish, S., Yue, C., Deam, D., Byron, K., and Zajac, J. (1992). A case-control study of the androgen receptor gene CAG repeat polymorphism in Australian prostate carcinoma subjects. *Cancer* **92,** 941–949.
163. Latil, A., Azzouzi, R., Cancel, G., Guillaume, E., Cochan-Priollet B., Berthon-, P., and Cussenot, O. (2001) Prostate carcinoma risk and allelic variants of genes involved in androgen biosynthesis and metabolism pahtways. *Cancer* **92,** 1130–1137.
164. Ross, R. K., Pike, M. C., Coetzee, G. A., et al. (1998). Androgen metabolism and prostate cancer: establishing a model of genetic susceptibility. *Cancer Res.* **58**, 4497–4504.
165. Edwards, S. M., Badzioch, M. D., Minter, R., et al. (1999). Androgen receptor polymorphisms: association with prostate cancer risk, relapse and overall survival. *Int. J. Cancer* **84**, 458–465.
166. Giovannucci, E. (2002). Is the androgen receptor CAG repeat length significant for prostate cancer? *Cancer Epidemiol. Biomarkers Prev.* **11**, 985–986.
167. Coetzee, G., and Irvine, R. (2002). Size of the androgen receptor CAG repeat and prostate cancer: does it matter? *J. Clin. Oncol.* **20**, 3572–3573.
168. Gilligan, T., Manola, J., Sartor, O., Weinrich, S. P., Moul, J. W., and Kantoff, P. W. (2004). Absence of a correlation of androgen receptor gene CAG repeat length and prostate cancer risk in an African-American population. *Clin. Prostate Cancer* **3**, 98–103.
169. Bennett, C. L., Price, D. K., Kim, S., et al. (2002). Racial variation in CAG repeat lengths within the androgen receptor gene among prostate cancer patients of lower socioeconomic status. *J. Clin. Oncol.* **20**, 3599–3604.
170. Powell, I. J., Land, S. J., Dey, J., et al. (2005). The impact of CAG repeats in exon 1 of the androgen receptor on disease progression after prostatectomy. *Cancer* **103**, 528–537.
171. Nam, R. K., Elhaji, Y., Krahn, M. D., et al. (2000). Significance of the CAG repeat polymorphism of the androgen receptor gene in prostate cancer progression. *J. Urol.* 164, 567–572.
172. Ding, D., Xu, L., Menon, M., Reddy, G. P., and Barrack, E. R. (2005). Effect of GGC (glycine) repeat length polymorphism in the human androgen receptor on androgen action. *Prostate* **62**, 133–139.
173. Salinas, C. A., Austin, M. A., Ostrander, E. O., and Stanford, J. L. (2005). Polymorphisms in the androgen receptor and the prostate-specific antigen genes and prostate cancer risk. *Prostate*. **65**, 58–65.
174. Hsing, A. W., Gao, Y. T., Wu, G., et al. (2000). Polymorphic CAG and GGN repeat lengths in the androgen receptor gene and prostate cancer risk: a population-based case–control study in China. *Cancer Res.* **60**, 5111–5116.
175. Makridakis, N. M., Ross, R. K., Pike, M. C., et al. (1999). Association of mis-sense substitution in SRD5A2 gene with prostate cancer in African-American and Hispanic men in Los Angeles, USA. *Lancet* **354**, 975–978.
176. Lunn, R. M., Bell, D. A., Mohler, J. L., and Taylor, J. A. (1999). Prostate cancer risk and polymorphism in 17 hydroxylase (CYP17) and steroid reductase (SRD5A2). *Carcinogenesis* **20**, 1727–1731.

177. Devgan, S. A., Henderson, B. E., Yu, M. C., et al. (1997). Genetic variation of 3 beta-hydroxysteroid dehydrogenase type II in three racial/ethnic groups: implications for prostate cancer risk. *Prostate* **33**, 9–12.
178. Stephens, E. A., Taylor, J. A., Kaplan, N., et al. (1994). Ethnic variation in the CYP2E1 gene: polymorphism analysis of 695 African-Americans, European-Americans and Taiwanese. *Pharmacogenetics* **4**, 185–192.
179. Hulla, J. E., Miller, M. S., Taylor, J. A., et al. (1999). Symposium overview: the role of genetic polymorphism and repair deficiencies in environmental disease. *Toxicol. Sci.* **47**, 135–143.
180. van Gils, C. H., Bostick, R. M., Stern, M. C., and Taylor, J. A. (2002). Differences in base excision repair capacity may modulate the effect of dietary antioxidant intake on prostate cancer risk: an example of polymorphisms in the XRCC1 gene. *Cancer Epidemiol. Biomarkers Prev.* **11**, 1279–1284.
181. Reichardt, J. K., Makridakis, N., Henderson, B. E., Yu, M. C., Pike, M. C., and Ross, R. K. (1995). Genetic variability of the human SRD5A2 gene: implications for prostate cancer risk. *Cancer Res.* **55**, 3973–3975.
182. Soderstrom, T., Wadelius, M., Andersson, S. O., et al. (2002). 5alpha-reductase 2 polymorphisms as risk factors in prostate cancer. *Pharmacogenetics* **12**, 307–312.
183. Zeigler-Johnson, C. M., Walker, A. H., Mancke, B., et al. (2002). Ethnic differences in the frequency of prostate cancer susceptibility alleles at SRD5A2 and CYP3A4. *Hum. Hered.* **54**, 13–21.
184. Shibata, A., Garcia, M. I., Cheng, I., et al. (2002). Polymorphisms in the androgen receptor and type II 5 alpha-reductase genes and prostate cancer prognosis. *Prostate* **52**, 269–278.
185. Oakley-Girvan, I., Feldman, D., Eccleshall, T. R., et al. (2004). Risk of early-onset prostate cancer in relation to germ line polymorphisms of the vitamin D receptor. *Cancer Epidemiol. Biomarkers Prev.* **13**, 1325–1330.
186. Williams, H., Powell, I. J., Land, S. J., et al. (2004). Vitamin D receptor gene polymorphisms and disease free survival after radical prostatectomy. *Prostate* **61**, 267–275.
187. Hawkins, G. A., Cramer, S. D., Zheng, S. L., et al. (2002). Sequence variants in the human 25-hydroxyvitamin D3 1-alpha-hydroxylase (CYP27B1) gene are not associated with prostate cancer risk. *Prostate* **53**, 175–178.
188. Ning, B., Wang, C., Morel, F., et al. (2004). Human glutathione S-transferase A2 polymorphisms: variant expression, distribution in prostate cancer cases/controls and a novel form. *Pharmacogenetics* **14**, 35–44.
189. Panguluri, R. C., Long, L. O., Chen, W., et al. (2004). COX-2 gene promoter haplotypes and prostate cancer risk. *Carcinogenesis* **25**, 961–966.
190. Lamba, J. K., Lin, Y. S., Schuetz, E. G., and Thummel, K. E. (2002). Genetic contribution to variable human CYP3A-mediated metabolism. *Adv. Drug Deliv. Rev.* **54**, 1271–1294.
191. Shibata, A. and Whittemore, A. S. (1997). Genetic predisposition to prostate cancer: possible explanations for ethnic differences in risk. *Prostate* **32**, 65–72.
192. Isaacs, W. B. and Bova, G. S. (2001). Prostate Cancer. In: Metabolic Basis of Inherited Disease, 8th Ed. McGraw-Hill, Inc., New York, NY.
193. Smith, J. R., Freije, D., Carpten, J. D., et al. (1996). Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search. *Science* **274**, 1371–1374.
194. Xu, J., Meyers, D., Freije, D., et al. (1998). Evidence for a prostate cancer susceptibility locus on the X chromosome. *Nat. Genet.* **20**, 175–179.
195. Xu, J., Zheng, S. L., Hawkins, G. A., et al. (2001). Linkage and association studies of prostate cancer susceptibility: evidence for linkage at 8p22-23. *Am. J. Hum. Genet.* **69**, 341–350.
196. Xu, J., Zheng, S. L., Chang, B., et al. (2001). Linkage of prostate cancer susceptibility loci to chromosome 1. *Hum. Genet.* **108**, 335–345.
197. Zheng, S. L., Xu, J., Chang, B., et al. (2001). Evaluation of linkage of HPC20 in 159 hereditary prostate cancer pedigrees. *Hum. Genet.* **1108**, 430–435.
198. Xu, J., Zheng, S. L., Carpten, J. D., et al. (2001). Evaluation of linkage and association of HPC2/ELAC2 in patients with familial or sporadic prostate cancer. *Am. J. Hum. Genet.* **68**, 901–911.
199. Cunningham, G. R., Ashton, C. M., Annegers, J. F., Souchek, J., Klima, M., and Miles, B. (2003). Familial aggregation of prostate cancer in African-Americans and white Americans. *Prostate* **56**, 256–262.
200. Gong, G., Oakley-Girvan, I., Wu, A. H., et al. (2002). Segregation analysis of prostate cancer in 1,719 white, African-American and Asian-American families in the United States and Canada. *Cancer Causes Control* **13**, 471–482.
201. Ahaghotu, C., Baffoe-Bonnie, A., Kittles, R., et al. (2004). Clinical characteristics of African-American men with hereditary prostate cancer: the AAHPC Study. *Prostate Cancer Prostatic Dis.* **7**, 165–169.
202. Brown, W. M., Lange, E. M., Chen, H., et al. (2004). Hereditary prostate cancer in African American families: linkage analysis using markers that map to five candidate susceptibility loci. *Br. J. Cancer* **90**, 510–514.
203. Parra, E. J., Marcini, A., Akey, J., et al. (1998). Estimating African American admixture proportions by use of population-specific alleles. *Am. J. Hum. Genet.* **63**, 1839–1851.
204. Parra, E. J., Kittles, R. A., Argyropoulos, G., et al. (2001). Ancestral proportions and admixture dynamics in geographically defined African Americans living in South Carolina. *Am. J. Phys. Anthropol.* **114**, 18–29.
205. Lautenberger, J. A., Stephens, J. C., O'Brien, S. J., and Smith, M. W. (2000). Significant admixture linkage disequilibrium across 30 cM around the FY locus in African Americans. *Am. J. Hum. Genet.* **66**, 969–978.

206.Wang, Y., Localio, R., and Rebbeck, T. R. (2004). Evaluating bias due to population stratification in case–control association studies of admixed populations. *Genet. Epidemiol.* **27**, 14–20.
207. Kittles, R. A., Chen, W., Panguluri, R. K., et al. (2002). CYP3A4-V and prostate cancer in African Americans: causal or confounding association because of population stratification? *Hum. Genet.* **110**, 553–560.
208. Clark, J. Y. and Thompson, I. M. (1994). Military rank as a measure of socioeconomic status and survival from prostate cancer. *South Med. J.* **87**, 1141–1144.
209. Pienta, K. J., Demers, R., Hoff, M., Kau, T. Y., Montie, J. E., and Severson, R. K. (1995). Effect of age and race on the survival of men with prostate cancer in the Metropolitan Detroit tricounty area, 1973 to 1987. *Urology* **45**, 93–101; discussion 101–102.
210. Powell, I. J., Schwartz, K., and Hussain, M. (1995). Removal of the financial barrier to health care: does it impact on prostate cancer at presentation and survival? A comparative study between black and white men in a Veterans Affairs system. *Urology* **46**, 825–830.
211. Park, J., Sokoll, L. J., Bruzek, D. J., et al. (2003). Comparison of total prostate-specific antigen and derivative levels in a screening population of black, white, and Korean-American men. *Clin. Prostate Cancer* **2**, 173–176.
211a. Scorilas, A., Plebani, M., Mazza, S., Basso, D., Soosaipillai, A. R., Katsaros, N., Pagano, F., and Diamandis, E. P. (2003). Serum human glandular kallikrein (hK2) and insulin-like growth factor 1 (IGF-1) improve te discrimination between prostate cancer and benign prostatic hyperplasia in combination with total and % free PSA. *Prostate* **54,** 220–229.
211b. Martin, B. J., Finlay, J. A., Sterling, ., Ward, M., Lifsey, D., Mercante, D., Jainto, J. M., Martin, L., and Rayford, W. (20040. Early detection of prostate cancer in African-American men through use of multiple biomarkers: human kallikrein 2 (hK2), prostate-specific antigen (PSA), and fre PSA (fPSA). *Prostate Cancer Prostatic Dis.* **&,** 132_137.
212. Martin, B. J., Finlay, J. A., Sterling, K., et al. (2004). Early detection of prostate cancer in African-American men through use of multiple biomarkers: human kallikrein 2 (hK2), prostate-specific antigen (PSA), and free PSA (fPSA). *Prostate Cancer Prostatic Dis.* **7**, 132–137.
213. Martin, B., Cheli, C., Pollard, S., et al. (2003). Similar age-specific PSA, complexed PSA, and percent cPSA levels among African-American and white men of southern Louisiana. Prostate-specific antigen. *Urology* **61**, 375–379.
214. Wu, A. H., Whittemore, A. S., Kolonel, L. N., et al. (1995). Serum androgens and sex hormone-binding globulins in relation to lifestyle factors in older African-American, white, and Asian men in the United States and Canada. *Cancer Epidemiol. Biomarkers Prev.* **4**, 735–741.
215. Gann, P. H., Hennekens, C. H., Ma, J., Longcope, C., and Stampfer, M. J. (1996). Prospective study of sex hormone levels and risk of prostate cancer. *J. Natl. Cancer Inst.* **88**, 1118–1126.
216. Heikkila, R., Aho, K., Heliovaara, M., et al. (1999). Serum testosterone and sex hormone-binding globulin concentrations and the risk of prostate carcinoma: a longitudinal study. *Cancer* **86**, 312–315.
217. Hryb, D. J., Khan, M. S., Romas, N. A., and Rosner, W. (1989). Solubilization and partial characterization of the sex hormone-binding globulin receptor from human prostate. *J. Biol. Chem.* **264**, 5378–5383.
218. Hryb, D. J., Nakhla, A. M., Kahn, S. M., et al. (2002). Sex hormone-binding globulin in the human prostate is locally synthesized and may act as an autocrine/paracrine effector. *J. Biol. Chem.* **277**, 26,618–26,622.
219. Ding, V. D., Moller, D. E., Feeney, W. P., et al. (1998). Sex hormone-binding globulin mediates prostate androgen receptor action via a novel signaling pathway. *Endocrinology* **139**, 213–218.
220. Hryb, D. J., Khan, M. S., Romas, N. A., and Rosner, W. (1990). The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. *J. Biol. Chem.* **265**, 6048–6054.
221. Usui, T., Ishibe, T., Nakatsu, H., Nakahara, M., Nihira, H., and Miyachi, Y. (1983). Immunohistochemical localization of testosterone-estradiol-binding globulin in a clinically hormone-independent prostatic carcinoma. *Urol. Int.* **38**, 16–18.
222. Kahn, S. M., Hryb, D. J., Nakhla, A. M., Romas, N. A., and Rosner, W. (2002). Sex hormone-binding globulin is synthesized in target cells. *J. Endocrinol.* **175**, 113–120.
223. Gregory, C. W., Johnson, R. T., Jr., Presnell, S. C., Mohler, J. L., and French, F. S. (2001). Androgen receptor regulation of G1 cyclin and cyclin-dependent kinase function in the CWR22 human prostate cancer xenograft. *J. Androl.* **22**, 537–548.
224. Gregory, C. W., Kim, D., Ye, P., et al. (1999). Androgen receptor up-regulates insulin-like growth factor binding protein-5 (IGFBP-5) expression in a human prostate cancer xenograft. *Endocrinology* **140**, 2372–2381.
225. Gregory, C. W., Hamil, K. G., Kim, D., et al. (1998). Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res.* **58**, 5718–5724.
226. Mohler, J. L., Morris, T. L., Ford, O. H., 3rd, Alvey, R. F., Sakamoto, C., and Gregory, C. W. (2002). Identification of differentially expressed genes associated with androgen-independent growth of prostate cancer. *Prostate* **51**, 247–255.
227. Kononen, J., Bubendorf, L., Kallioniemi, A., et al. (1998). Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med.* **4**, 844–847.
228. Kim, D. (1993). Multiscale image analysis of prostatic carcinoma and benign hyperplasia using confocal laser scanning microscope. *University of North Carolina*.
229. Kim, D., Gregory, C. W., Smith, G. J., and Mohler, J. L. (1999). Immunohistochemical quantitation of androgen receptor expression using color video image analysis. *Cytometry* **35**, 2–10.

230. Allen, M. V., Smith, G. J., Juliano, R., Maygarden, S. J., and Mohler, J. L. (1998). Downregulation of the beta4 integrin subunit in prostatic carcinoma and prostatic intraepithelial neoplasia. *Hum. Pathol.* **29**, 311–318.
231. Koochekpour, S., Maresh, G. A., Katner, A., et al. (2004). Establishment and characterization of a primary androgen-responsive African-American prostate cancer cell line, E006AA. *Prostate* **60**, 141–152.
232. Gregory, C. W., Johnson, R. T., Jr., Mohler, J. L., French, F. S., and Wilson, E. M. (2001). Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res.* **61**, 2892–2898.
233. Gregory, C. W., He, B., Johnson, R. T., et al. (2001). A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res.* **61**, 4315–4319.
234. Yang, G., Addai, J., Ittmann, M., Wheeler, T. M., and Thompson, T. C. (2000). Elevated caveolin-1 levels in African-American versus white-American prostate cancer. *Clin. Cancer Res.* **6**, 3430–3433.
235. Mouraviev, V., Li, L., Tahir, S. A., et al. (2002). The role of caveolin-1 in androgen insensitive prostate cancer. *J. Urol.* **168**, 1589–1596.
236. Grignon, D. J., Caplan, R., Sarkar, F. H., et al. (1997). p53 status and prognosis of locally advanced prostatic adenocarcinoma: a study based on RTOG 8610. *J. Natl. Cancer Inst.* **89**, 158–165.
237. Leibovich, B. C., Cheng, L., Weaver, A. L., Myers, R. P., and Bostwick, D. G. (2000). Outcome prediction with p53 immunostaining after radical prostatectomy in patients with locally advanced prostate cancer. *J. Urol.* **163**, 1756–1760.
238. Quinn, D. I., Henshall, S. M., Head, D. R., et al. (2000). Prognostic significance of p53 nuclear accumulation in localized prostate cancer treated with radical prostatectomy. *Cancer Res.* **60**, 1585–1594.
239. McDonnell, T. J., Troncoso, P., Brisbay, S. M., et al. (1992). Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res.* **52**, 6940–6944.
240. Scherr, D. S., Vaughan, E. D., Jr., Wei, J., et al. (1999). BCL-2 and p53 expression in clinically localized prostate cancer predicts response to external beam radiotherapy. *J. Urol.* **162**, 12–16; discussion 16–7.
241. Stackhouse, G. B., Sesterhenn, I. A., Bauer, J. J., et al. (1999). p53 and bcl-2 immunohistochemistry in pretreatment prostate needle biopsies to predict recurrence of prostate cancer after radical prostatectomy. *J. Urol.* **162**, 2040–2045.
242. Kuczyk, M., Machtens, S., Hradil, K., et al. (1999). Predictive value of decreased p27Kip1 protein expression for the recurrence-free and long-term survival of prostate cancer patients. *Br. J. Cancer* **81**, 1052–1058.
243. Yang, R. M., Naitoh, J., Murphy, M., et al. (1998). Low p27 expression predicts poor disease-free survival in patients with prostate cancer. *J. Urol.* **159**, 941–945.
244. Cairns, P., Okami, K., Halachmi, S., et al. (1997). Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res.* **57**, 4997–5000.
245. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. R. (1999). Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res.* **59**, 4291–4296.
246. Suzuki, H., Freije, D., Nusskern, D. R., et al. (1998). Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res.* **58**, 204–209.
247. Whang, Y. E., Wu, X., Suzuki, H., et al. (1998). Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc. Natl. Acad. Sci. USA* **95**, 5246–5250.
248. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* **274**, 784–787.
249. Palayoor, S. T., Youmell, M. Y., Calderwood, S. K., Coleman, C. N., and Price, B. D. (1999). Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. *Oncogene* **18**, 7389–7394.
250. Paweletz, C. P., Charboneau, L., Bichsel, V. E., et al. (2001). Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* **20**, 1981–1989.
251. Gioeli, D., Mandell, J. W., Petroni, G. R., Frierson, H. F., Jr., and Weber, M. J. (1999). Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res.* **59**, 279–284.
252. Shuch, B., Mikhail, M., Satagopan, J., et al. (2004). Racial disparity of epidermal growth factor receptor expression in prostate cancer. *J. Clin. Oncol.* **22**, 4725–4729.
253. Drobnjak, M., Melamed, J., Taneja, S., et al. (2003). Altered expression of p27 and Skp2 proteins in prostate cancer of African-American patients. *Clin. Cancer Res.* **9**, 2613–2619.
254. Caruso, R. P., Levinson, B., Melamed, J., et al. (2004). Altered N-myc downstream-regulated gene 1 protein expression in African-American compared with caucasian prostate cancer patients. *Clin. Cancer Res.* **10**, 222–227.
255. Bonkhoff, H. and Remberger, K. (1996). Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model. *Prostate* **28**, 98–106.
256. Kinbara, H., Cunha, G. R., Boutin, E., Hayashi, N., and Kawamura, J. (1996). Evidence of stem cells in the adult prostatic epithelium based upon responsiveness to mesenchymal inductors. *Prostate* **29**, 107–116.
257. Presnell, S. C., Werdin, E. S., Maygarden, S. J., Mohler, J. L., and Smith, G. J. (2001). Establishment of short-term primary human prostate xenografts for the study of prostate biology and cancer. *Am. J. Pathol.* **159**, 855–860.

20

Radiotherapy for the Treatment of Locally Advanced Prostate Cancer

Danny Y. Song and Theodore L. DeWeese

Summary

Radiation therapy (RT) is the primary modality of treatment for locally advanced prostate cancer, as well as patients with a high risk of disease recurrence, regardless of clinical stage. Advances in external beam radiation delivery have provided the ability to safely apply higher doses of radiation than previously possible, leading to improvements in biochemical control rates for certain subsets of patients. Brachytherapy using either high-dose rate or low-dose rate sources is an alternative means of performing dose escalation, most commonly used as a boost treatment in conjunction with external beam radiation. Androgen deprivation in conjunction with definitive radiation has been shown to improve both disease control and overall survival when compared with radiation alone in randomized studies, although toxicity may also be increased. The optimal duration and sequencing of androgen deprivation is still under investigation. For patients at significant risk of nodal metasatasis, preliminary results suggest that the addition of whole pelvic radiation improves biochemical control.

Key Words: Androgen; brachytherapy; conformal; dose escalation; intensity modulated; locally advanced; radiation; radiotherapy; whole pelvic.

1. INTRODUCTION

Radiotherapy has been used to treat prostate cancer for nearly a century. The use of interstitial radium was first described in 1911 by Pasteau and Degrais *(1)*. Smith and Pierson described the usefulness of high-voltage X-ray therapy in the treatment of prostate cancer in 1930, and, in the same year, Widmann subsequently noted an improvement in survival for patients with advanced prostate cancer treated with kilovoltage X-rays *(2,3)*. Before the advent of prostate-specific antigen (PSA), most patients were diagnosed based on clinically evident disease, which was often more advanced than the presentations commonly seen today. The activity of radiation in prostate cancer was evident by its effect on these large palpable tumors, which often resolved after treatment. The historical definition of locally advanced prostate cancer, thus, refers to patients with advanced clinical stage based on direct extension of disease beyond the prostatic capsule, either felt on digital rectal examination or seen on imaging, that is, stage T3 or T4. Such patients are at high risk for disease recurrence after surgical or nonsurgical treatment.

Widespread screening for prostate cancer led to an increasing frequency of the disease being detected in its early stages, however, a significant proportion of patients are still diagnosed with locally advanced disease. However, the change in disease presentation has led to modifications in what is meant by the term "locally advanced disease." In addition to tumor stage, a number of studies have correlated histological tumor grade and pretreatment PSA with not only extracapsular extension

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

of disease found at radical prostatectomy, but risk of disease recurrence after any form of therapy *(4–7)*. A more contemporary and commonly used method of defining locally advanced disease includes patients who have biologically more aggressive and, therefore, higher-risk disease, as predicted by prognostic factors such as PSA level and tumor grade. High-risk patients are generally considered to be those who have advanced clinical stage disease, high presenting PSA levels, and/or poorly differentiated histology (Gleason score 8–10) *(8)*. In addition, patients with involvement of regional pelvic lymph nodes are also frequently included in the category of locally advanced disease. As a result, patients seen in modern clinical practice who are classified as having locally advanced disease differ significantly from those termed as having locally advanced disease in previous eras, and this should be kept in mind when reviewing older data. Although the optimal treatment of this group of patients remains undefined, radiation is a commonly used primary modality. Historical results with either radical prostatectomy or RT alone are suboptimal, with biochemical control rates of less than 40% at 5 years *(9–12)*. We describe the innovations in radiation oncology that have been developed in an attempt to improve on these results by the use of dose escalation (using either external beam or brachytherapy as a boost), whole pelvic RT (WPRT), and androgen suppression (AS).

2. RATIONALE FOR DOSE-ESCALATED RADIOTHERAPY

Although many patients with locally advanced prostate cancer develop metastatic disease as the first sign of failure, local control with standard radiotherapy is poor and the intraprostatic tumor may serve as a reservoir for metastatic dissemination *(11,13)*. Coen and coworkers analyzed 1469 patients treated with RT who had greater than 2 years of follow-up data, and found Gleason score of at least 7, PSA level greater than 15, and T3 to T4 stage to be predictive of a higher incidence of distant failure. On multivariate analysis, local failure was the strongest predictor for distant metastasis, with 77% vs 61% distant metastasis-free survival at 10 years for patients with locally controlled vs uncontrolled disease *(14)*. Several long-term retrospective studies of patients treated with conventional techniques in the 1970s and 1980s describe a direct relationship between radiation dose and local control in men with locally advanced prostate cancer. Valicenti et al. reviewed the data from Radiation Therapy Oncology Group (RTOG) Phase III trials of external beam radiation, and, on multivariate analysis, found that dose greater than 66 Gy significantly reduced the relative risk of prostate cancer-related death in men with Gleason scores of 8 to 10 *(15)*. Hanks et al. reviewed data from the American College of Radiology Patterns of Care study on 624 patients with stage T3 disease. The actuarial 7-year local recurrence rate was 36% with doses of 60-64.9 Gy, 32% for 65 to 69.9 Gy, and 24% for at least 70 Gy *(16)*. Multivariate analysis of the MD Anderson experience in 1127 patients with stage T1 to T4 disease treated with radiotherapy from 1987 to 1997 found radiation dose to be an independent predictor of biochemical control in addition to pretreatment PSA level, Gleason score, and palpable stage. The patients who benefited most from higher doses were those with pretreatment PSA levels greater than 10 ng/mL *(17)*.

The radiotherapy technique used in the majority of patients in these studies relied on imprecise estimations of prostate boundaries based on surrogate landmarks, such as the pubic bones and rectal and bladder contrast media. The ability to escalate radiation dose to greater than 70 Gy with such conventional radiotherapy techniques was limited by rectal and bladder complications. In the Patterns of Care study, the rate of grade 3 or 4 complications increased from 3.5% to 6.9% when doses exceeding 70 Gy were delivered *(18)*. Smit et al. reported up to 30% incidence of late proctitis after conventional prostate RT to 70 Gy, with the incidence of proctitis correlating with the maximum rectal dose *(19)*. Schultheiss et al. attempted to identify factors predictive of late genitourinary (GU) and gastrointestinal (GI) morbidity in patients treated with doses higher than 65 Gy. Central axis dose was the only independent variable significantly related to the incidence of late GI morbidity *(20)*.

The past two decades have witnessed the development of innovative radiotherapy techniques including 3D conformal RT (3DCRT), which uses CT-based targeting as well as more sophisticated

beam delivery and treatment plan assessment techniques. In conjunction with the development of these 3DCRT, improvements in patient positioning accuracy and quantitation of prostate motion have created the ability to more accurately as well as conformally target the shape and location of the prostate, while minimizing treatment of surrounding tissues *(21)*. These benefits have resulted in the ability to decrease toxicity of treatment at isoeffective dose levels. A randomized trial of 64 Gy conventional radiotherapy vs similar doses delivered with 3DCRT demonstrated at least grade 1 proctitis occurring in 37% of the 3DCRT group vs 56% of the conventional group ($p = 0.004$). Grade 2 proctitis was also significantly lower, at 5% in the 3DCRT group vs 15% in the conventional group. There were no significant differences in bladder function after treatment between the groups *(22)*. A similar study by Koper et al. using doses of 66 Gy found a significant reduction in grade 2 GI toxicity with the use of 3DCRT compared with conventional techniques (19 vs 32%; $p = 0.02$). Again, there was similar at least grade 2, GU toxicity between the arms, despite a significant reduction in bladder volumes treated in patients receiving 3DCRT *(23)*.

3. TRIALS OF DOSE-ESCALATED RADIOTHERAPY

In an attempt to improve on the results observed with conventional treatment, clinical trials of dose escalated radiotherapy have been performed using the enhanced therapeutic ratio of 3DCRT. Long-term, 12-year follow-up of the initial Fox Chase dose escalation study cohort of 232 patients has been reported by Hanks. Doses ranged from 67 to 81 Gy, with median total dose (at the center of the prostate) being 74 Gy. The American Society for Therapeutic Radiology and Oncology (ASTRO) consensus definition of three consecutive rises in PSA was used to determine biological no evidence of disease (bNED) status *(24)*. For purposes of analysis, patients were divided into six prognostic subgroups by pretreatment PSA level, clinical stage, Gleason score, and presence of perineural invasion. Patients with pretreatment PSA levels of 10 to 20 ng/mL had statistically significant differences in bNED control when stratified by dose on univariate analysis. On multivariate analysis, radiation dose was a statistically significant predictor of bNED control for all patients and for unfavorable patients with a pretreatment PSA less than 10 ng/mL. No radiation dose response was observed for patients with pretreatment PSA greater than 20 ng/mL, but only 44 patients were in this group. No difference was noted in the frequency of grade 2 and 3 GU and grade 3 GI morbidity when patients were stratified by radiation dose, although a significant increase in grade 2 GI complications occurred as the dose increased *(25)*.

Pollack recently updated the Fox Chase dose escalation experience, reporting on 839 patients treated to doses from 63 to 84 Gy (median 74 Gy) with a median follow-up of 63 months. Multivariate analysis found radiotherapy dose and pretreatment PSA level to be the most significant factors prognostic for bNED survival, followed by Gleason score and T stage. Within six prognostic groups, higher dose was significantly associated with outcome in all but the most favorable (PSA < 10 ng/mL and Gleason score 2–6) and least favorable (PSA > 20 ng/mL and T3–T4) groups. However, there were only 22 patients available for analysis within the latter group. For patients with PSA level greater than 20 ng/mL and T1 to T2 clinical stage, dose was significant for bNED survival when analyzed by the Wilcoxon test *(26)*.

Bey et al. have reported preliminary results of a multi-institutional, randomized dose escalation study performed by a French collaborative group. Eligible patients had T1b to T3 disease; Gleason score and PSA level were not criteria for study entry. Thirty-one percent of patients had stage T3 disease, and 19% had pretreatment PSA level greater than 20 ng/mL. The control group received 66 to 70 Gy prescribed to the center of the prostate, whereas the experimental group was treated to doses ranging from 74 to 80 Gy. With mean follow-up time of 32 months, no statistical difference was observed between the groups with respect to grade at least 2 GI or GU late toxicity. The probability of achieving a posttreatment PSA nadir of at most 1 ng/mL was significantly higher in the dose-escalation group, and directly related to the dose of radiation given *(27)*.

Investigators at Memorial Sloan-Kettering also performed a dose escalation trial using 3DCRT in patients with stage T1 to T3 disease. Doses were 64.8 to 75.6 Gy prescribed at the prostate periphery, with isocenter doses approx 5 to 7% higher than the stated dose. Patients were stratified into three risk groups based on PSA level, Gleason score, and clinical stage. At 10 years, bNED survival (ASTRO definition) was significantly better with higher doses in all three risk groups. For intermediate risk patients, 10-year bNED survival was 50% in patients receiving 75.6 Gy, vs 42% in patients receiving 70.2 Gy ($p = 0.05$); for unfavorable risk patients, the corresponding rates were 42% vs 24% ($p = 0.04$). Grade 2 and grade at least 3 rectal toxicity was 17% and 1.5%, respectively, whereas grade 2 and grade at least 3 urinary toxicity were 15% and 2.5% *(28)*.

4. COOPERATIVE GROUP TRIAL OF DOSE ESCALATION

RTOG 94-06 was a phase I/II 3DCRT dose escalation study that enrolled patients with T1 to T3 tumors to sequentially escalated dose levels beginning at 68.4 Gy at 1.8 Gy per fraction, to 78 Gy at 2 Gy per fraction daily. Patients were grouped into three categories based on risk of seminal vesicle invasion greater than or less than 15% and presence of T3 disease. Patients with greater than 15% risk of seminal vesicle involvement were treated to both prostate and seminal vesicles to 55.8 Gy, followed by a reduction in target volume to the prostate alone. Patients with T3 disease received the full dose to both prostate and seminal vesicles. Doses were prescribed as a minimum dose to a target volume. Preliminary toxicity results from this trial have been reported. Michalski et al. evaluated late effects in 424 patients treated on the first two dose levels of 68.4 Gy (level I) and 73.8 Gy (level II). Average months at risk after completion of therapy ranged from 21.4 to 40.1 months for patients on dose level I, and 10.0 to 34.2 months for patients on dose level II. The incidence of RTOG grade at least 2 GU toxicity was 10 to 13%, and RTOG grade at least 2 GI toxicity was 7 to 8%, with most toxicity occurring within the first 2 years after therapy. When compared with historical RTOG data derived from previous studies using pre-3DCRT techniques, the incidence of grade at least 3 toxicity was reduced for all groups (despite the administration of higher doses), but this was associated with an increase in the rate of grade 1 complications. This resulted in an overall statistically significant increase in the number of patients with any late effects when compared with expected complications derived from the historical data. These data implied a reduction of higher-grade toxicity occurred, with the toxicity being shifted to lower grades, and an overall increase in low-grade toxicity, presumably caused by dose escalation *(29)*. Results in 173 patients with stage T1 and T2 disease treated on dose level III (79.2 Gy) have also been reported (T3 patients were excluded because of poor accrual). No patients experienced grade at least 3 acute toxicity. The incidences of late grade 1 and 2 GU toxicity were 31% and 11%, respectively, whereas the rates of grade 1 and 2 GI toxicity were 24% and 7%. The overall rate of toxicity was comparable to previous dose levels. With a median follow-up of 3.3 years, four patients (2.4%) experienced grade 3 late toxicity, with three GU events and one related to the rectum. There were no grade 4 or 5 late complications. Again, comparison with historical controls revealed that the observed rate of grade at least 3 late effects was significantly lower than expected *(30)*.

5. RANDOMIZED TRIALS OF DOSE ESCALATION

To test the hypothesis that dose escalation results in improved outcomes, Shipley and coworkers at the Massachusetts General Hospital carried out a phase III randomized study of dose escalation using proton beam irradiation as method of delivering higher doses to the prostate. Unlike the more commonly used photon beams, proton beams penetrate surrounding tissues, depositing only a small amount of energy until they reach a specified distance, at which point the dose is delivered. This lends the ability to minimize doses to the tissues around the target. Currently, proton beam for routine clinical use is available at only two centers within the United States, although construction is underway at additional facilities. The study enrolled only patients with locally advanced (stages T3–T4)

disease, randomizing them to either 67.2 Gy with conventional radiotherapy or 75.6 Gy equivalent with the last 25.2 Gy delivered by proton beam. The trial was performed from 1982 to 1992, and, therefore, used older definitions of treatment effectiveness, namely disease-specific survival was defined as PSA level less than 4 ng/mL, negative digital rectal exam, and negative rebiopsy (if performed). With a median follow-up of 61 months, there were no significantly differences in disease-specific survival, overall survival, or local control between the two arms. The only statistically significant benefit observed was in the planned subgroup analysis of patients with poorly differentiated tumors (Gleason score 4 or 5 of 5), in which the rates of local control were 19% vs 84% at 8 years ($p = 0.0014$). For the entire group, the incidence of grades 1 and 2 rectal bleeding was greater in the high-dose arm (32 vs 12%; $p = 0.002$) *(31)*. It must be noted that because of stage migration from the widespread adoption of PSA screening, the patients treated in the era when this study was performed are significantly different from the majority of patients seen today, even within the same category of T3 to T4 disease.

A more contemporary dose escalation study using proton beam irradiation has been reported by researchers at Massachusetts General Hospital and Loma Linda University. This study randomized 393 patients with stages T1b to T2b prostate cancer and PSA level less than 15 ng/mL to either 19.8 GyE (photon Gy equivalents) or 28.8 GyE, followed by a 3DCRT dose of 50.4 Gy (total doses 70.2 GyE vs 79.2 GyE, respectively). Only 2% of patients receiving 70.2 GyE and 1.5% of patients receiving 79.2 GyE had acute RTOG morbidity greater than grade 2. At a median follow-up of 4 years, the 5-year biochemical failure rate was significantly lower in the patients receiving higher-dose radiation (19.1% vs 37.3%; $p = 0.00001$). This difference also persisted when patients with intermediate risk disease were analyzed. The majority of the patients treated on this study had low- to intermediate-risk disease (only 8.4% had Gleason score $\geq$ 8), but this study deserves mention because it strongly supports the concept of dose escalation in prostate cancer *(32)*.

Pollack and colleagues have also performed a phase III randomized trial at the MD Anderson Cancer Center comparing 70 Gy vs 78 Gy radiotherapy using 3DCRT. A total of 305 patients with stage T1 through T3 tumors were treated using 2-Gy daily fractions prescribed to the isocenter; androgen ablation was not given as part of initial therapy. Treatment failure was defined by the ASTRO consensus definition or the initiation of salvage treatment. For the entire cohort, the freedom-from-failure (FFF) rates at 5 years were 64% and 70% for the 70 Gy and 78 Gy arms, respectively ($p = 0.03$). The patients who benefited most from the dose escalation were the subgroup with PSA levels greater than 10 ng/mL, in which the FFF rates were 43% and 62% ($p = 0.01$). Within this subgroup, there was also a significant difference in the rates of metastasis-free survival (98% vs 88%; $p = 0.056$), although no differences in overall survival were observed during the follow-up period to date. Within patients with stage T3 disease, there were also statistically significant differences in FFF (36% vs 61%; $p = 0.047$). There were no differences in FFF for the patients with Gleason scores of 7 to 10, in which groups, FFF rates were 64% and 68%, respectively ($p = 0.39$). There was an increase in the rate of grade at least 2 late rectal toxicity, with a significant direct correlation between the extent of treated rectal volume and grade at least 2 rectal toxicity; there was no difference in bladder toxicity. Among patients with detailed dose–volume histogram information available, eight of nine patients with grade at least 3 rectal toxicity had greater than 25% of the rectum receiving at least 70 Gy, and the majority of grade at least 2 rectal toxicity was also in patients with greater than 25% of the rectum receiving at least 70 Gy *(33,34)*.

6. NONRANDOMIZED DATA ON DOSE ESCALATION FOR HIGH-GRADE TUMORS

A retrospective analysis of a larger number of patients with high-grade (Gleason score 8–10) tumors is available from Roach et al. at the University of California, San Francisco. Fifty patients with T1 to T4 clinical stage and a median PSA level of 22.7 ng/mL were treated with a variety of

doses of external beam radiation. Biochemical failure was defined as increase in PSA level of 0.5 ng/mL per year, PSA level greater than 1.0 ng/mL, or positive posttreatment biopsy. The overall actuarial probability of freedom from biochemical failure at 4 years was 23%. Multivariate analysis of all patients revealed pretreatment PSA level to be the only predictor of PSA failure. In a multivariate analysis restricted to patients with PSA level less than 20 ng/mL, 83% of those treated to more than 71 Gy were free of progression compared with none of those treated to lower doses ($p = 0.03$) *(35)*. Fiveash and colleagues reported on the combined results of a group of 180 patients treated at the University of Michigan; University of California, San Francisco; and Fox Chase Cancer Center. Patients had T1 to T4 N0 to Nx adenocarcinoma with a pretreatment PSA level; 27% received adjuvant or neoadjuvant hormonal therapy. The total dose received was less than 70 Gy in 30%, 70 to 75 Gy in 37%, and greater than 75 Gy in 33%. At a median follow-up of 3.0 years, the 5-year freedom from PSA failure was 62.5% for all patients and 79.3% in T1 to T2 patients. Univariate analysis revealed T-stage, pretreatment PSA level, and RT dose predictive of freedom from PSA failure. Univariate analysis of likelihood of 5-year overall survival revealed RT dose to be the only significant predictive factor. When Cox proportional hazards model was performed separate for T1 to T2 and T3 to T4 tumors, none of the prognostic factors reached statistical significance for overall survival or freedom from biochemical progression in the T3 to T4 group, but lower RT dose and higher pretreatment PSA level did predict for PSA failure within the T1 to T2 patients *(36)*.

In summary, the available randomized evidence suggests that dose escalation with 3DCRT results in significant improvements in PSA-defined disease control in patients with "intermediate-risk" prostate cancer. The impact on disease control in patients with higher risk features, such as stage T3 to T4, Gleason score 8 to 10, and PSA level greater than 20 ng/mL in these studies is unclear, although most suffer from low patient numbers within this subgroup. Retrospective data suggest that patients with high-grade, low-stage tumors benefit from dose escalation. It remains to be seen whether these improvements will result in differences in overall survival, which would only become evident with very long-term follow-up. Although the use of 3DCRT results in less toxicity compared with conventional radiotherapy given at similar doses, escalated doses with these techniques seem to result in increased rectal toxicity, mostly limited to grade 2 or less. Further study will allow for development of better estimates of late toxicity associated with higher doses, as well as agreement on what constitutes an acceptable level of toxicity.

A more recent technological improvement in radiotherapy delivery is intensity-modulated radiotherapy (IMRT). When compared with 3DCRT, IMRT imparts the ability to create steeper dose gradients around the target, thereby reducing the volume of surrounding tissues receiving high doses of radiation. It also allows for even greater conformality of the treatment volume to the target *(37)*. Investigators at Memorial Sloan-Kettering have reported 6-year results in a cohort of 171 patients with T1c to T3 prostate cancer treated to 81 Gy using IMRT. Doses were prescribed to the isodose line encompassing the target volume. Fifty-four percent of patients received neoadjuvant androgen deprivation therapy before radiation. The 6-year PSA relapse-free survival rates for favorable, intermediate, and unfavorable risk disease were 91%, 73%, and 64% using the ASTRO consensus definition. Notably, the 6-year likelihood of grade 2 rectal bleeding was 4%, and 1.1% of patients developed grade 3 rectal toxicity. The 6-year risk of grade 2 GU toxicity was 10%. There were no grade 4 toxicities. These results appear favorable when compared with the toxicity profiles described previously for 3DCRT treatment *(38)*.

7. DOSE ESCALATION VIA HIGH-DOSE RATE BRACHYTHERAPY

Brachytherapy is another way by which it is possible to increase local dose to the prostate. Conformal high-dose rate brachytherapy (HDR) is an alternative means of dose escalation that offers a potential advantage over external beam radiation in that a steep dose gradient between the prostate and adjacent normal tissues can be generated. Other theoretical advantages are the elimination of

interfraction and intrafraction motion of the prostate gland, random and systemic treatment setup errors, gland edema, and imprecise target localization *(39,40)*. Briefly, the technique uses catheters placed into the prostate under intraoperative transrectal ultrasound guidance. The catheters are subsequently attached to an afterloading unit that sequentially feeds a single high activity (iridum-192) source into predetermined positions within the catheters. The dwell times within each position can be adjusted, thus, allowing for development of a treatment plan that optimally conforms to the target volume. Typically, the patient receives HDR in one to three fractions (separated by 4 to 6 hours) after each intraoperative catheter placement session, with the catheters remaining in place between fractions. A total of one to three sessions may take place during a treatment course, either following or interdigitated with conformal external beam radiation.

There may be radiobiological advantages to the use of large doses per HDR fraction. Traditionally, tumors have been considered to be less sensitive to large doses per fraction than normal tissues, and, therefore, delivering an increased number of fractions to the same total dose is associated with decreased normal tissue late effects. More recent analyses of clinical and laboratory data suggest that prostate cancer is more sensitive to large doses per fraction relative to normal tissues *(41,42)*. If true, this would also mean that isoeffective doses of hypofractionated HDR could be delivered with less acute toxicity than possible with smaller fractions.

One of the largest published experiences to date comes from Deger et al., who analyzed 230 patients with T2 (35%) and T3 (58%) tumors. The median PSA level was 12.8 ng/mL; 60% and 16% of patients had grade 2 and grade 3 histological grades, respectively. The mean time to PSA nadir was 15 months, and 47% of patients reached a nadir less than 0.5 ng/mL. The 5-year biochemical progression-free survival rate was 75% for T2 and 60% for T3 tumors. After modifications to technique and a decrease in the HDR fractional dose to 9 Gy, complications were reported to decrease from 16.4% to a rate of 6.9% *(43)*.

Investigators at William Beaumont Hospital have described their experience in a dose escalation trial of HDR with external beam radiotherapy (EBRT) in 207 patients with poor prognostic factors of PSA level of at least 10 ng/mL, Gleason score of at least 7, or clinical stage of at least T2b. No patient received hormonal therapy, and the ASTRO consensus definition was used for determining biochemical failure. Treatment consisted of EBRT 46 Gy to the prostate, with a total of 2 HDR treatments given during the 1st and 3rd weeks of treatment, respectively. The majority of patients (57%) had T2b to T2c disease; 19.8% had Gleason score 8 to 10 tumors, and most patients had pretreatment PSA levels between 4 and 10 ng/mL. At a mean follow-up of 4.7 years, the 5-year actuarial biochemical control rate was 74%. The 5-year biochemical control rate was 85% for one poor prognostic factor, 75% for two and 50% for all three. On Cox regression analysis, lower HDR dose and higher Gleason score were associated with biochemical failure *(44)*. Table 1 summarizes several institutional reports on HDR.

Because of the heterogeneity of patient groups treated in many of the reported experiences, it is difficult to draw conclusions regarding the efficacy of HDR relative to other treatments. However, the biochemical control rates seem favorable compared with historical results *(9–12)*. Given the comparably short median follow-up times, longer observation will be needed to better determine the incidence of late complications.

8. DOSE ESCALATION VIA PERMANENT INTERSTITIAL SEED IMPLANTATION

A similar means of performing dose escalation is by performing permanent interstitial seed zimplantation (low-dose rate brachytherapy) in addition to EBRT. The vast majority of the low-dose rate brachytherapy experiences reported have not included patients with high-risk disease because of concerns regarding extraprostatic involvement or microscopic systemic disease minimizing the impact of increased local dose. D'Amico et al. retrospectively analyzed outcomes with radical pros-

Table 1
Studies of High-Dose Rate Brachy-Therapy[a]

Group	No. of patients	T–stage (%)	Mean PSA	Median F/U (mo)	Biochemical control	HDR fractional dose	No. of fractions	GU toxicity	GI toxicity
William Beaumont	207	T1c–T3c	11.5	52.8	74% @ 5 yr	5.5–11.5	2–3	8.0% late grade 3	1.0% late grade 3–4
Seattle	104	T1b–T3c	8.1 (median)	45	84% @ 5 yr	3–4	4	6.7% stricture 1.9% marked uropathy	2.0% spotty bleeding
Kiel	144	T1b–T3	25.6	96	69% @ 8 yr	9	2	2.1% late grade 3	4.1% late grade 3
Goteburg	50	T1b–T3b	not stated	45	78% @ 5 yr	10	2	8.0% acute dysuria 4.0% chronic dysuria 2.0% chronic hematuria	26.0% mild diarrhea 8.0% moderate diarrhea 2.0% rectal bleeding
Berlin	230	T2–3	12.8 (median)	40	T2: 75% T3: 60%	9–10	2	10.0% frequency/dysuria @ 3 mo 7.4% urethral stricture 3.0% incontinence	1.7% rectourethral fistula
Munich	40	T2–3	40.7	74	79.5	9	2	5.0% prostate necrosis 10.0% urethritis 80.0% acute mild hematuria	2.5% rectovesical fistula 10.0% proctitis
Lahey Clinic	52	T1–2	10.4	11.8	92.2	6	3	11.0% grade 2	18.0% grade 2 2.0% rectal wall necrosis 2.0% rectal bleeding
Long Beach	200	T1c–T3b	10	30	93% @ 25 mo	5–6.5	3–4	10.0% acute grade 3–4 1.5% urethral stricture 0.5% incontinence	20% acute grade 3–4 1.5% late grade 3
Offenbach	102	T1–3	15.3 (median)	31	82%@ 3 yr	5–7 Gy	4	27.0% acute grade 2–3 12.0% late grade 2–3	8.0% acute grade 2 6.0% late grade 2 1 patient with prostato-urethral fistula
Gothenburg	214	T1–3	9.6 (median)	48	Low risk, 92% @ 5 yr Intermediate risk, 87%@ 5 yr High risk, 56% @ 5 yr	10	2	6.0% urethral stricture 1.5% recurrent retention requiring TURP 1.0% recurrent hematuria requiring TURP	
Kawasaki, Japan	98	T1c–T3b	11.7 (median)	43	T1–2 95.9% @ 5 yr T3 84% @ 5 yr	6	4	29.6% cystourethritis or proctitis 7.0% urethral stricture	29.6% cystourethritis or proctitis 10.2% late grade 1–2 proctitis
California Endocurietherapy	491	T1c–T3b	not stated	not stated	not stated	6	4	0.6% incontinence	0.2% rectal bleeding

[a] PSA, prostate-specific antigen; HDR, high-dose rate brachytherapy; GU, genitourinary; GI, gastrointestinal; mo, month; TURP, transurethral resection of the prostate.

tatectomy, permanent interstitial seed implantation, or EBRT in 1872 men treated at two institutions. High-risk patients were defined as those with stage T2c, PSA level greater than 20 ng/mL, or Gleason score at least 8. Within the 590 patients in this category, those treated with seed implantation or seed implantation combined with AS had significantly inferior PSA outcomes compared with patients receiving radical prostatectomy or external beam radiation (relative risk of failure 3.0 and 3.1, respectively) *(45)*. Potential criticisms of this study include the relatively small cohort of patients in the implant group and the fact that no external beam radiation was used, counter to common practice for such patients. However, if improved disease control in locally advanced disease via dose escalation is a valid concept, then permanent interstitial implantation may offer a method of achieving dose escalation when used in addition to external beam radiation. Stock et al. treated 139 patients with high-risk features (defined as Gleason score 8 to 10, PSA level greater than 20 ng/mL, stage T2b, or positive seminal vesicle biopsy) with 9 months of AS, seed implantation, and 45 Gy EBRT. Negative laparoscopic pelvic lymph node dissections were performed in 44% of patients. At 5 years, the actuarial overall freedom from PSA failure rate was 86%. Posttreatment prostate biopsies performed in 47 patients were negative in 100% of patients at last biopsy *(46)*. Sylvester and colleagues reported results of combined EBRT and seed implantation in 232 patients and evaluated the subset of high-risk patients as defined by the D'Amico risk criteria (Gleason score 8–10, PSA > 20 ng/mL, or stage T2c) *(8)*. The 10-year biochemical relapse-free survival in these patients was 48% *(47)*. Dattoli et al. published results in patients treated with 41 Gy EBRT followed by palladium-103 seed implantation for comparably more favorable disease (Gleason score $\geq$ 7 and/or PSA > 10 ng/mL). The 10-year freedom from biochemical progression rate was 79% using a strict PSA failure criteria of a nadir greater than 0.2 ng/mL *(48)*. These results are achieved at a cost of increased morbidity as compared with brachytherapy alone. Brandeis et al. prospectively evaluated quality of life after combined modality therapy or seed implantation alone, and found urinary, bowel, and sexual function as well as American Urological Association symptom score to be all statistically worse in patients receiving combined treatment *(49)*. Others have not found combined modality treatment to be more toxic than seed implantation alone *(50)*.

9. ROLE OF ANDROGEN DEPRIVATION THERAPY IN CONJUNCTION WITH RADIATION IN LOCALLY ADVANCED PROSTATE CANCER

9.1. Biological Considerations

Human prostate cancer cells vary in their in vitro sensitivity to both acute radiation (such as that delivered as external beam radiation) and low-dose rate radiation (such as that used in prostate brachytherapy). The radiosensitivity does not seem to be dependent on p53 status or the ability of the cell to initiate G_1 cell cycle arrest—both previously thought to be important to radiation response. These studies also do not reveal dependence on androgen responsiveness *(51)*. Androgen-responsive prostate cells undergo programmed cell death (apoptosis) when deprived of androgens *(52,53)*. Similar to radiation, this death does not seem to be p53 dependent *(52)*. Several investigators have performed in vivo analyses of radiation combined with androgen deprivation on androgen responsive tumors of both prostate and nonprostate origin *(54,55)*. None of these studies have conclusively demonstrated synergy between radiation and androgen deprivation, but they do suggest that timing of the androgen deprivation may be critical. In one study of androgen withdrawal in the Shionogi breast tumor model, Zietman et al. found that a lower dose of radiation was required to control 50% of the tumors if androgens were used in combination with radiation. Specifically, animals treated with radiation after maximal androgen withdrawal-mediated tumor regression required 42.1 Gy of radiation to control 50% of the tumors as compared with 89.0 Gy in animals treated with radiation only. The most direct interpretation of these data is that, after androgen withdrawal, there are a smaller number of tumor clonogens for the radiation to eradicate.

Historically, hormonal therapy was added to RT in an attempt to modify the outcome of patients with stage C (T3) prostate cancer *(56)*. This was based on the knowledge that these patients had an inferior outcome compared with patients with earlier stage prostate cancer treated with RT. These T3 tumors were often very large and it was thought that a course of cytoreductive therapy might provide a more favorable geometry for external irradiation as well as reduction of tumor burden *(57)*.

In the early 1980s, at least two institutional series reported encouraging results with the use of AS and EBRT in this group of patients *(57,58)*. Pilepich et al. also found that patients with histologically unfavorable lesions who had been treated on the RTOG 75-06 trial receiving AS and EBRT had a similar disease-free survival (DFS) and overall survival as patients with more favorable tumors who did not receive the AS *(59)*. In part, these studies provided the basis for the next series of RTOG phase II studies designed to test the efficacy of combined EBRT and AS, with the hypothesis that preradiation cytoreduction as well as concomitant radiation- and AS-induced cell death would improve local control in patients with locally advanced prostate tumors *(60,61)*. Based on this experience, RTOG study 86-10 was initiated *(62)*. This was a randomized, phase III trial of EBRT alone (standard treatment arm) vs neoadjuvant and concomitant total AS (TAS) and EBRT (experimental treatment arm). Eligible patients were those with bulky, locally advanced tumors (>25 cm2), stage T2b to T4, N0 to N1, and M0. Those patients randomized to receive TAS were treated with goserelin 3.6 mg acetate every 28 days and flutamide 250 mg three times a day for 2 months before the start of radiation and during RT. A total of 471 patients were enrolled and randomized to one of the two treatment arms. Analysis of this series revealed that those patients treated with TAS and radiation had a statistically significant improvement in local control at 5 years compared with those patients treated with radiation only ($p < 0.001$). An update of this study at a median follow-up of 6.7 years for all patients and 8.6 years for alive patients continued to show statistically significant differences in local control (42% vs 30%), biochemical DFS (24% vs 10%, and cause-specific mortality (23% vs 31%). Subset analysis indicated a preferential effect in patients with Gleason score 2 to 6, in whom there was a significant difference in overall survival between the arms (70% vs 52%) *(63)*. To date, there is no difference in the overall survival of the two groups of patients. This may have several possible explanations. It is possible that there may not be an overall survival benefit to patients when TAS is added to EBRT in this fashion and/or this patient population. However, it is important to note that this study was limited in that it did not routinely collect serum PSA on all patients before entry, a parameter that is now recognized to be an extremely important prognostic factor and indicator of disease extension. Therefore, there likely were a large number of patients with elevated serum PSA levels in the range frequently associated with a high risk of micrometastatic disease. The study also included patients with node-positive disease, also recognized as a poor risk factor and one in which the value of any treatment modality to overall survival can be debated. Nonetheless, this is an important study, performed in a rigorous fashion, revealing important and measurable benefit with the addition of AS to RT for this high-risk group of patients.

A concurrent study, the RTOG 85-31 trial, compared EBRT alone with EBRT followed by adjuvant goserelin indefinitely for patients with clinical or pathological stage T3 or node-positive prostate cancer. At a median follow-up of 6 years, investigators reported statistically significant differences in rates of local failure (23% vs 37%), distant metastasis (27% vs 37%), and DFS (36% vs 25%), but overall survival differences were only detected in the subset of patients with Gleason score 8 to 10 disease *(64)*. In an updated report with median follow-up of 7.6 years (11 years for surviving patients), these differences have translated into improved overall survival for all patients, with 10-year absolute survival rates of 49% vs 39% ($p = 0.002$) *(65)*.

Bolla et al. also published a long-term analysis of the European Organization for Research and Therapy of Cancer (EORTC) 22863 trial *(66)*. This phase III trial enrolled 415 patients with stage T3 to T4 any grade or stage T1 to T2 World Health Organization (WHO) grade 3 prostate cancer with no evidence of nodal or metastatic disease. Patients were randomized to receive either EBRT alone

(control arm) or AS plus EBRT (experimental arm). AS consisted of oral cyproterone acetate (50 mg thrice daily) for 4 weeks before radiation and an luteinizing hormone-releasing hormone (LHRH)-agonist started on the first day of radiation and continued every month for 3 years. A total of 401 patients were analyzed with a median follow-up of 66 months. The 5-year locoregional recurrence-free survival was 98% in the experimental arm vs 84% in the control arm ($p < 0.001$), and clinical relapse-free survival 74% in the experimental arm vs 40% in the control arm ($p < 0.001$). There was also a difference in favor of AS in the 5-year incidence of distant metastases (29% vs 9.8%; $p < 0.0001$). There was an overall survival advantage with the addition of AS to radiation with an estimated 5-year survival of 78% in the experimental arm vs 62% in the control arm ($p = 0.001$).

A more recent study from Harvard also demonstrated an overall survival advantage with the addition of AS to EBRT. This was a phase III randomized study comparing 70 Gy of 3DCRT alone vs 3DCRT and 6 months of LHRH agonist and flutamide. Eligible patients had PSA level of at least 10 ng/mL, Gleason score of at least 7, or radiographic evidence of extraprostatic disease. Patients also underwent cardiac stress testing before enrollment to exclude competing causes of mortality. Although the majority of these patients had "intermediate-risk" disease, 13% had PSA values of 20 to 40 ng/mL, and 64% had a Gleason score of at least 7. Patients who developed biochemical failure (PSA > 1.0 ng/mL with two consecutive PSA measurements) received salvage AS when the PSA level reached approx 10 ng/mL. The trial was stopped after interim analysis at median follow-up of 4.5 years revealed an overall survival benefit in favor of the AS arm (5-year survival 88% vs 78%; $p = 0.04$) *(67)*. Table 2 summarizes results of the randomized trials evaluating the benefit of adding AS to EBRT.

10. DURATION OF ANDROGEN-DEPRIVATION THERAPY

The EORTC series and others raise several significant issues, including the optimum duration of androgen-deprivation administration. Although results of the EORTC study and RTOG 85-31 suggest that long-term AS may be beneficial for disease control, prolonged AS has been associated with other causes of patient morbidity, including osteopenia, anemia, muscle wasting, and impotence *(68)*. These toxicities are especially worthy of consideration because patients treated for prostate cancer tend to be older.

The study by Laverdiere et al. provided important information in this regard. In this study, patients with stage T2a to T4 disease were randomized to receive either EBRT alone, 3 months of neoadjuvant AS (LHRH-agonist plus flutamide) followed by EBRT, or 3 months of TAS then AS plus EBRT followed by another 6 months of adjuvant AS. Interestingly, the addition of 3 months of TAS before radiation reduced the 2-year positive prostate biopsy rate from 65% (radiation only) to 28%. Those patients receiving neoadjuvant, concomitant, and adjuvant TAS and radiation had only a 5% positive prostate biopsy rate. These data would seem to confirm the idea that a more protracted course of AS is important, at least in terms of local control. Although impressive, the length of follow-up is too short to determine whether the addition of AS to radiation has made any significant impact on other meaningful end points, such as DFS or overall survival *(69)*. In contrast to these results, Laverdiere et al. have also reported an analysis based on patients with T2 to T3 disease enrolled in two studies of AS with EBRT, including the study described above. The second study randomized patients to neoadjuvant and concomitant AS (total 5 months) vs neoadjuvant, concomitant, and short course adjuvant (total 10 months) AS; both groups received EBRT. At a median follow-up of 5 years, there was a significant advantage in bNED survival rate with the addition of neoadjuvant and concomitant AS, but no advantage with the addition of short course adjuvant AS after EBRT *(70)*.

Another phase III randomized study (RTOG 92-02) sought to ascertain the benefit of prolonged AS after neoadjuvant and concomitant AS with EBRT. Patients with locally advanced (T2c–T4) prostate cancers with PSA levels of less than 150 ng/mL received 4 months of goserelin and flutamide,

Table 2
Randomized Studies of Radiotherapy With or Without Androgen Suppression[a]

Study	Eligibility criteria	No. of patients	Randomization	Interval measured	Disease-free survival	*p* value	Overall survival	*p* value
RTOG 86–10	Bulky T2b–T4, N0–1	471	65–70 Gy	@ 8 yr	10%	<0.0001	44%	0.1
			65–70 Gy + neoadjuvant and concomitant AS (4 mo)		24%		53%	
EORTC 22863	T3–T4, N0–1, or WHO grade 3	415	70 Gy	@ 5 yr	40%	0.0001	62%	0.0002
			70 Gy + concomitant and adjuvant AS (3 yr)		74%		78%	
RTOG 85-31	T3 or N1	977	65–70 Gy (60–65 Gy if surgical adjuvant)	@ 10 yr	9%	0.0001	39%	0.002
			65–70 Gy (60–65 Gy if surgical adjuvant) + adjuvant AS indefinitely		31%		49%	
Harvard	PSA ≥ 10 ng/mL, Gleason ≥7, or radiographic T3	206	70 Gy 3DCRT	median 54 mo	56%	<0.001	79%	0.04
			70 Gy + AS (6 mo)		79%		88%	

[a] RTOG, Radiation Therapy Oncology Group; AS, androgen suppression; EORTC, European Organization for Research and Therapy of Cancer; WHO, World Health Organization; PSA, prostate-specific antigen; 3DCRT, 3D conformal radiation therapy.

2 months before and 2 months during RT. A dose of 65 to 70 Gy was given to the prostate, and a dose of 44 to 50 Gy to the pelvic lymph nodes. Randomization was between no additional AS therapy after completion of RT, vs 24 months of additional goserelin; 1554 patients entered the study. The 5-year rate of biochemical DFS was 46.4% vs 28.1% in the long-term vs short-term AS arms, respectively ($p < 0.0001$). The long-term AS arm showed significant improvement in all end points except overall survival (80% vs 78.5% at 5 years; $p = 0.73$), although an unplanned subset analysis revealed a significant survival benefit for patients with Gleason score 8 to 10 tumors as determined by the institution (but not on central pathology review). Despite a statistically significant reduction in the rate of prostate cancer-related deaths in the long-term arm (38% vs 24%; $p = 0.001$), there was an absolute increase in the number of deaths as a result of other causes, resulting in a similar rate of overall survival. Cardiovascular disease at the time of enrollment was more prevalent in the patients treated on the long-term arm (55% vs 44%, respectively), but a detrimental effect of long-term AS therapy on other causes of death cannot be excluded *(71)*.

Other data also suggest that the benefit of long-term AS may be greater in patients with high Gleason scores. Roach et al. analyzed patients enrolled in five randomized RTOG studies that treated patients with radiotherapy and AS. Patients with stage T3 disease, nodal involvement, or Gleason score 7 had a disease-specific survival benefit with the addition of 4 months of goserelin and flutamide, whereas patients with Gleason score 8 to 10 disease or multiple high-risk factors had an approx 20% higher survival at 8 years with the use of up-front, long-term hormonal therapy *(72)*. However, it is unclear how much of this conclusion overlaps with that from RTOG 85-31, because it was included in the analysis. Another re-analysis of RTOG data comes from Horwitz et al., who assessed the benefit of long-term vs short-term androgen deprivation in studies 85-31 and 86-10. The benefit in bNED control, distant metastasis-free survival, and cause-specific survival from long-term AS was limited to those patients with centrally reviewed Gleason score of 7 to 10 tumors; multivariate analysis demonstrated Gleason score and the use of long-term AS to be independent predictors for all three outcome measures *(73)*.

Related data comes from a number of surgical series investigating the use of androgen suppressive therapy before radical prostatectomy. Several of these series have shown reduction in the positive margin rate, inferring AS-induced cell death. Relevant to this discussion is that a longer course of AS (i.e., 8 months) resulted in the greatest reduction in serum PSA, and only a 12% incidence of positive surgical margins compared with 23% in the 3-month AS arm *(74)*.

Although these data are not conclusive and the studies assess differing end points, they provide some useful information. Taken together, they would seem to support the theory that a longer course of AS may very well lead to improved local/regional control of large tumors or high-risk tumors that possess an elevated risk of extraprostatic extension. However, although differences have been shown in biochemical and clinical disease control, thus far, there is no demonstrated overall survival advantage of prolonged vs short-course AS.

11. SEQUENCING OF ANDROGEN DEPRIVATION AND RT

The question of optimal sequencing of AS and RT is similarly unanswered. It is not clear whether the effects of AS on tumor control are merely additive to the tumoricidal effects of radiation or whether they are synergistic, providing an enhancement of tumor killing that cannot be simply explained by the killing of each modality individually.

To assess the importance of AS timing in relation to EBRT, the RTOG performed a phase III trial (RTOG 94-13) in which patients were randomized to either 2 months of neoadjuvant AS followed by AS and EBRT (control arm) or EBRT followed by 4 months of adjuvant AS *(75)*. Patients were also randomized in a 2 2 factorial design to receive WPRT vs prostate-only radiation. Eligibility included localized prostate cancer with risk of lymph node involvement of at least 15% (based on an equation using pretreatment PSA level, Gleason score, and stage) *(76)*. At a median follow-up of 59

months, patients treated with neoadjuvant and concomitant AS experienced a similar 4-year progression-free survival compared with those receiving adjuvant AS (52% vs 49%; $p = 0.56$). However, the group that received WPRT and neoadjuvant and concomitant AS had a significantly improved progression-free survival rate compared with the other 3 arms (60% vs 44–50%; $p = 0.008$). Further information regarding the most effective duration of neoadjuvant treatment will be provided by RTOG 99-10, which randomized patients to either 6 months or 2 months of neoadjuvant AS followed by concurrent AS and radiation.

12. ALTERATION OF TOXICITY

In addition to improved tumor control, androgen-deprivation therapy adds its own side effect profile but may also alter possible radiation-induced side effects. In the randomized study by D'Amico et al., patients receiving AS with 3DCRT had statistically significant increases in grade 3 impotence (26% vs 21%) as well as gynecomastia, but no other significant differences in late toxicity were noted in this trial *(67)*. In contrast, Chen et al. found no significant differences in 1-year potency rates between men receiving 3DCRT with AS vs 3DCRT alone, and patients treated in RTOG 86-10 had no differences in frequency or time to return of sexual potency *(63,77)*.

Reductions in prostatic volume after neoadjuvant AS can result in smaller treatment fields and consequent reductions in the amount of surrounding normal tissue that is secondarily irradiated. For example, reduction in the volume of rectum irradiated is associated with a significant decrease in long-term rectal injury *(78)*. Comparisons of dose-volume histograms from 3DCRT plans generated on patients before and after neoadjuvant AS reveal a median reduction in prostate volume of 27 to 42%. This was also correlated with a decrease in the amount of irradiated rectum, bladder, and bowel *(79–81)*. Despite this expected benefit of AS, most studies do not show these effects translating into clinical benefit. Late grade 1 to 3 incontinence was increased within the combined therapy group in the Bolla study (29% vs 16%; $p = 0.002$) *(82)*. In a retrospective analysis by Sanguineti et al., rectal dose and the use of adjuvant AS were both significantly associated with higher risk of late rectal toxicity. The 2-year estimates of grade 2 to 4 late rectal toxicity were 30.3% vs 14.1% in patients receiving or not receiving AS, respectively *(83)*. The RTOG 94-13 study found the acute and 2-year rates of late grade 3 or higher GU/GI toxicity to be higher on the arm receiving whole pelvic EBRT with neoadjuvant and concomitant AS, but these differences did not reach statistical significance *(75)*. Valicenti et al. compared toxicity rates in patients enrolled in RTOG 94-06, in which approximately half of the men received neoadjuvant AS (with variable dose levels of radiation). On univariate analysis, AS significantly increased the incidence of grade 2 acute GU complications, but was not significant in the multivariate analysis when volume of bladder treated was taken into account. The use of AS did increase risk of acute GU effects in men with preexisting obstructive symptoms *(84)*. Data from RTOG 92-02 may be interpreted to suggest that long-term AS increases toxicity in and of itself. In that study, there was a small but statistically significant increase in the frequency of late grade 3 to 5 GI toxicity in the long-term compared with the short-term AS arm (2.6% vs 1.2% at 5 years; $p = 0.037$), although there is no obvious explanation for this difference *(71)*.

In summary, the addition of AS to EBRT for the treatment of locally advanced prostate cancer results in an apparent increase in local control and DFS as supported by several prospective, randomized trials. Three randomized studies to date (EORTC, Harvard, and RTOG 85-31) have also shown overall survival improvements with the addition of AS to EBRT, whereas another did not (RTOG 86-10). The most favorable duration as well as timing of AS in relation to radiation is not yet known. Because of the potential added toxicity of AS, especially when administered long term, the degree of added benefit vs toxicity should be weighed in each individual patient. Besides the well-recognized side effects of AS, such as hot flashes and decreased libido, other important physiological changes have been shown to occur with long-term use, including anemia, loss of muscle mass, decreased bone density, and depression *(68)*. Consideration of these potentially limiting side effects is required before instituting therapy.

13. WPRT FOR OCCULT NODAL METASTASIS

A significant proportion of patients with locally advanced prostate cancer will have clinically occult pelvic lymph node spread. The primary nodes at risk for involvement include the external iliac, hypogastric, presacral, and obturator nodes *(85)*. Theoretically, treatment of these nodal areas in patients with meaningful risk of nodal involvement might be of therapeutic benefit if sufficient doses of radiation are given to eradicate microscopic tumor.

Partin et al. developed predictive nomograms for lymph node involvement as well as other surgical findings based on multi-institutional data from radical prostatectomy specimens. Using these tables, it is possible to estimate the risk of lymph node involvement based on a patient's preoperative Gleason score, clinical stage, and PSA level *(86)*. Roach et al. developed an equation that estimates the risk of lymph node involvement based on the Partin tables. The equation is: lymph node risk = (2/3) PSA + ([Gleason score – 6] × 10) *(87)*. The true incidence of nodal involvement is most likely underestimated in data based on surgical series, because of the inherent false-negative rate of lymph node dissections as typically performed in modern surgical practice. Heidenreich et al. compared results in patients with similar risk characteristics undergoing extended pelvic lymphadenectomy vs standard pelvic lymphadenectomy. The incidence of lymph node detection was significantly higher in the extended lymphadenectomy group (26% vs 12%), and 42% of nodal metastases in the extended lymphadenectomy group were outside of areas dissected in a standard pelvic lymphadenectomy, including external iliac and obturator nodes. All except one patient with lymph node involvement had a PSA level greater than 10.5 ng/mL and a Gleason score of at least 7 *(88)*. Therefore, a larger proportion of patients may benefit from pelvic nodal irradiation than might be estimated by using the Partin nomograms.

Several retrospective, single institutional analyses have shown conflicting results regarding the benefit of WPRT. Some of these studies are flawed by the fact that they are from the pre-PSA era, making the determination of patients at risk of nodal involvement as well as evaluation of treatment efficacy more difficult. They also suffer from low patient numbers and use of outdated radiotherapy techniques *(89–91)*. However, some studies did find a benefit to the use of larger treatment fields that encompassed pelvic nodal areas *(92)*. Ploysongsang et al. reported statistically significant improvements in 5-year survival rates among stage B and stage C patients receiving WPRT in comparison with stage-matched controls treated to prostate only *(93)*. Seaward et al. analyzed a group of 506 patients treated at the University of California, San Francisco who had at least 15% risk of nodal involvement (based on the Roach equation). The median biochemical progression-free survival was significantly higher (34 months vs 21 months; $p = 0.0001$) in patients who received initial WPRT followed by prostate boost as opposed to prostate-only radiation *(94)*.

Three randomized trials have been performed to evaluate the benefit of WPRT. Bagshaw reported a series of 57 patients with surgically staged, node-negative prostate cancer who were randomized to either WPRT and prostate boost vs prostate-only radiotherapy. There was no statistically significant improvement in DFS with the use of WPRT, however, the sample size was small *(95)*. The RTOG has reported results of two randomized studies performed to date on the value of WPRT in prostate cancer. RTOG 77-06 evaluated WPRT in patients with stage A2 and stage B prostate cancer with no evidence of lymph node involvement on lymphangiogram or biopsy. Randomization was to either 6500 cGy to the prostate alone or to 4500 cGy WPRT with 2000 cGy prostatic boost. At a median follow-up of 7 years, there was no beneficial effect of WPRT on local control, distant metastases, DFS, or overall survival *(96)*. Because the patients in this study were at low risk of lymph node involvement, it is inconclusive in determining whether patients at moderate to high risk of nodal metastasis benefit from WPRT.

As discussed in the section on AS, RTOG 94-13 also looked at the role of WPRT in patients with either risk of nodal involvement at least 15% (based on the Roach equation) or with T2c to T4 disease; the maximum allowable pretreatment PSA level was 100 ng/mL. WPRT was associated with a 4-year progression-free survival of 54%, as compared with 47% in the arms treated with prostate-

only radiation. Interestingly, when comparing all four treatment arms, there was a progression-free survival advantage for WPRT plus neoadjuvant/concurrent AS compared with the other arms (60% vs 44–50%; $p = 0.008$). No overall survival differences were observed *(75)*.

The positive results in this study for WPRT may be attributed to the use of AS in addition to WPRT, which has been shown to be synergistic with radiation *(54)*. The optimal dose of WPRT associated with durable control of microscopic nodal disease has not been determined, but doses beyond 50 to 55 Gy have not been used because of the limited radiation tolerance of small bowel, which is often included in WPRT fields. In a retrospective analysis on 963 patients performed by Perez and colleagues, patients receiving WPRT doses of 50 to 55 Gy had fewer pelvic failures when compared with those receiving 40 to 45 Gy ($p = 0.07$). A statistically significant reduction in pelvic failures was noted in stage C poorly differentiated tumors when the pelvic nodes received doses greater than 50 Gy compared with lower doses (23% vs 46%; *p* ≤ 0.01) *(97)*. In RTOG 94-13, the addition of AS to an otherwise modest dose of radiation may have allowed the WPRT to cross a threshold of efficacy.

The technique of WPRT in the modern era is substantially enhanced by the use of CT-based treatment planning, which allows for better targeting of lymphatic regions at risk. Work by Forman et al. showed that the "standard" four-field pelvic radiation portal as practiced in the conventional radiotherapy era (including RTOG 94-13) often underdosed or missed portions of at risk nodal chains entirely. With CT, the location of the pelvic vasculature can be used as approximations of the lymph node locations, with margin added to cover the associated lymph nodes *(98,99)*.

14. CONCLUSIONS

RT is the current standard of care for patients with locally advanced prostate cancer. The addition of AS to EBRT results in increased local control and DFS (as shown in several prospective, randomized studies), and data are emerging to show an improvement in overall survival as well. The optimal sequencing and duration of AS when combined with radiotherapy remains to be determined, and considerations must be made for the impact of prolonged AS on overall health and quality of life. The use of dose-escalated radiotherapy using modern treatment planning and delivery techniques results in increased biochemical control rates in patients with "intermediate-risk" disease, but further study is needed regarding its impact on patients with "high-risk" prognostic features. The use of WPRT combined with AS in patients at meaningful risk of lymph node involvement is now supported by prospective randomized data.

REFERENCES

1. Pasteau, O., Degrais, J. (1914). The radium treatment of cancer of the prostate. *Arch. Roentgen Ray* **18**, 396.
2. Smith, G. S. and Pierson, E. L. (1930). The value of high voltage x-ray therapy in carcinoma of the prostate. *J. Urol.* **23**, 331.
3. Widmann, B. P. (1934). Cancer of the prostate. The result of radium and roentgen ray treatment. *Radiology* **22**, 153.
4. Kupelian, P., Katcher, J., Levin, H., and Klein, E. (1997). Stage T1-2 prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **37**, 1043–1052.
5. Partin, A., Mangold, L., Lamm, D., Walsh, P. C., Epstein, J. I., and Pearson, J. D. (2001). Contemporary update of prostate cancer staging nomograms (Partin tables) for the new millennium. *Urology* **58**, 843–848.
6. Roach, M., Lu, J., Pilepich, M., et al. (2000). Four prognostic groups predict long-term survival from prostate cancer following radiotherapy alone on Radiation Therapy Oncology Group clinical trials. *Int. J. Radiat. Oncol. Biol. Phys.* **47**, 609–615.
7. D'Amico, A. V., Whittington, R., and Malkowicz, S. B. (1998). Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA* **16**, 969–974.
8. D'Amico, A. V. (2001). Combined-modality staging for localized adenocarcinoma of the prostate. *Oncology (Huntington)* **15**, 49–59.
9. Zietman, A. L., Coen, J. J., Shipley, W. U., Willett, C. G., and Efird, J. T. (1994). Radical radiation therapy in the management of prostatic adenocarcinoma: the initial prostate specific antigen value as a predictor of treatment outcome. *J. Urol.* **151**, 640–645.

10. Kuban, D. A., el-Mahdi, A. M., and Schellhammer, P. F. (1995). Prostate-specific antigen for pretreatment prediction and posttreatment evaluation of outcome after definitive irradiation for prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **32**, 307–316.
11. Hanks, G. E., Hanlon, A. L., Hudes, G., Lee, W. R., Suasin, W., and Schultheiss, T. E. (1996). Patterns-of-failure analysis of patients with high pretreatment prostate-specific antigen levels treated by radiation therapy: the need for improved systemic and locoregional treatment. *J. Clin. Oncol.* **14**, 1093–1097.
12. Shipley, W. U., Thames, H. D., and Sandler, H. M. (1999). Radiation therapy for clinically localized prostate cancer. *JAMA* **281**, 1598–1604.
13. Coen, J. J., Zietman, A. L., Thakral, H., and Shipley, W. U. (2002). Radical radiation for localized prostate cancer: local persistence of disease results in a late wave of metastases. *J. Clin. Oncol.* **20**, 3199–3205.
14. Kuban, D. A., el-Mahdi, A. M., and Schellhammer, P. F. (1995). Potential benefit of improved local tumor control in patients with prostate carcinoma. *Cancer* **75**, 2373–2382.
15. Valicenti, R., Lu, J., and Pilepich M. (2000). Survival advantage from higher-dose radiation therapy for clinically localized prostate cancer treated on the radiation therapy oncology group trials. *J. Clin. Oncol.* **18**, 2740–2746.
16. Hanks, G. E., Martz, K. L., and Diamond, J. J. (1988). The effect of dose on local control of prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **15**, 1299–1305.
17. Pollack, A., Smith, L. G., and von Eschenbach, A. G. (2000). External beam radiotherapy dose response characteristics of 1127 men with prostate cancer treated in the PSA era. *Int. J. Radiation. Oncol. Biol. Phys.* **48**, 507–512.
18. Leibel, S. A., Hanks, G. E., and Kramer, S. (1984). Patterns of care outcome studies: results of the national practice in adenocarcinoma of the prostate. *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 401–409.
19. Smit, W. G., Helle, P. A., and Van Putten, W. L. (1990). Late radiation damage in prostate cancer patients treated by high dose external radiotherapy in relation to rectal dose. *Int. J. Radiat. Oncol. Biol. Phys.* **18**, 23–29.
20. Schultheiss, T. E., Lee, W. R., Hunt, M. A., et al. (1997). Late GI and GU complications in the treatment of prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **37**, 3–11.
21. Roach, M. (2004). Reducing the toxicity associated with the use of radiotherapy in men with localized prostate cancer. *Urol. Clin. North. Am.* **31**, 353–366.
22. Dearnaley, D. P., Khoo, V. S., Norman, A. R., et al. (1999). Comparison of radiation side-effects of conformal and conventional radiotherapy in prostate cancer: a randomised trial. *Lancet* **23**, 267–272.
23. Koper, P. C., Stroom, J. C., van Putten, W. L., et al. (1999). Acute morbidity reduction using 3DCRT for prostate carcinoma: a randomized study. *Int. J. Radiat. Oncol. Biol. Phys.* **43**, 727–734.
24. American Society for Therapeutic Radiology and Oncology Consensus Panel. (1997). Consensus statement: Guidelines for PSA following radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys.* **37**, 1035–1041.
25. Hanks, G. E., Hanlon, A. L., Epstein, B., and Horwitz, E. M. (2002). Dose response in prostate cancer with 8-12 years' follow-up. *Int. J. Radiat. Oncol. Biol. Phys.* **54**, 427–435.
26. Pollack, A., Hanlon, A. L., Horwitz, E. M., Feigenberg, S. J., Uzzo, R. G., and Hanks, G. E. (2004). Prostate cancer radiotherapy dose response: an update of the Fox Chase experience. *J. Urol.* **171**, 1132–1136.
27. Bey, P., Carrie, C., Beckendorf, V., et al. (2000). Dose escalation with 3D-CRT in prostate cancer: French study of dose escalation with conformal 3D radiotherapy in prostate cancer-preliminary results. *Int. J. Radiat. Oncol. Biol. Phys.* **48**, 513–517.
28. Zelefsky, M., Fuks, Z., Chan, H., Ling, C., Hunt, M., and Leibel, S. (2003). Ten-year results of dose escalation with 3-dimensional conformal radiotherapy for patients with clinically localized prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **57S**, 149–150.
29. Michalski J. M., Winter K., Purdy J. A., et al. (2003). Preliminary evaluation of low-grade toxicity with conformal radiation therapy for prostate cancer on RTOG 9406 dose levels I and II. *Int. J. Radiat. Oncol. Biol. Phys.* **56**, 192–198.
30. Ryu, J. K., Winter, K., Michalski, J. M., et al. (2002). Interim report of toxicity from 3D conformal radiation therapy for prostate cancer on 3DOG/RTOG 9406, level III (79.2 Gy). *Int. J. Radiat. Oncol. Biol. Phys.* **54**, 1036–1046.
31. Shipley, W. U., Verhey, L. J., Munzenrider, J. E., Suit, H. D., Urie, M. M., McManus, P. L. (1995). Advanced prostate cancer: the results of a randomized comparative trial of high dose irradiation boosting with conformal protons compared with conventional dose irradiation using photons alone. *Int. J. Radiat. Oncol. Biol. Phys.* **32**, 3–12.
32. Zietman, A. L., DeSilvio, M., Slater, J. D., et al. (2004). A randomized trial comparing conventional dose (70.2GyE) and high-dose (79.2GyE) conformal radiation in early stage adenocarcinoma of the prostate: results of an interim analysis of PROG 95-09. *Int. J. Radiat. Oncol. Biol. Phys.* **60**,–S131.
33. Pollack, A., Zagars, G. K., Smith, L. G., Lee, J., von Eschenbach, A. C., and Antolak, J. A. (2000). Preliminary results of a randomized radiotherapy dose-escalation study comparing 70 Gy with 78 Gy for prostate cancer. *J. Clin. Oncol.* **18**, 3904–3911.
34. Pollack, A., Zagars, G. K., Starkschall, G., Antolak, J. A., Lee, J. J., Huang, E. (2002). Prostate cancer radiation dose response: results of the M. D. Anderson phase III randomized trial. *Int. J. Radiat. Oncol. Biol. Phys.* **53**, 1097–1105.
35. Roach, M., 3rd, Meehan, S., Kroll, S., et al. (1996). Radiotherapy for high grade clinically localized adenocarcinoma of the prostate. *J. Urol.* **156**, 1719–1723.

36. Fiveash, J. B., Hanks, G., Roach, M. 3rd, et al. (2000). 3D conformal radiation therapy (3DCRT) for high grade prostate cancer: a multi-institutional review. *Int. J. Radiat. Oncol. Biol. Phys.* **47**, 335–342.
37. Luxton, G., Hancock, S. L., Boyer, A. L. (2004). Dosimetry and radiobiologic model comparison of IMRT and 3D conformal radiotherapy in treatment of carcinoma of the prostate. Int. J. Radiat. Oncol. Biol. Phys. **59**, 267–284.
38. Chan, H. M., Zelefsky, M. J., Fuks, Z., Hunt, M., Ling, C., Leibel, S. A. (2004). Long-term outcome of high dose intensity modulated radiotherapy for clinically localized prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **60**, S169–S170.
39. Ghilezan, M., Yan, D., Liang, J., Jaffray, D., Wong, J., Martinez, A. (2004). Online image-guided intensity-modulated radiotherapy for prostate cancer: how much improvement can we expect? A theoretical assessment of clinical benefits and potential dose escalation by improving precision and accuracy of radiation delivery. *Int. J. Radiat. Oncol. Biol. Phys.* **60**, 1602–1610.
40. Yan, D., Xu, B., and Lockman, D. (2001). The influence of interpatient and intrapatient rectum variation on external beam treatment of prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **51**, 1111.
41. Fowler, J., Chappell, R., and Ritter, M. (2001). Is alpha/beta for prostate tumors really low? *Int. J. Radiat. Oncol. Biol. Phys.* **50**, 1021–1031.
42. Duchesne, G. M. and Peters, L. J. (1999). What is the alpha/beta ratio for prostate cancer? Rationale for hypofractionated high-dose-rate brachytherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **44**, 747–748.
43. Deger, S., Boehmer, D., Turk, I., et al. (2002). High dose rate brachytherapy of localized prostate cancer. *European Urology* **41**, 420–426.
44. Martinez, A., Gonzalez, J., Spencer, W., et al. (2003). Conformal high dose rate brachytherapy improves biochemical control and cause specific survival in patients with prostate cancer and poor prognostic factors. *J. Urol.* **169**, 974–980.
45. D'Amico, A. V., Whittington, R., Malkowicz, B., et al. (1998). Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA* **280**, 969–974.
46. Stock, R. G., Cahlon, O., Cesaretti, J. A., Kollmeier, M. A., and Stone, N. N. (2004). Combined modality treatment in the management of high-risk prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* 1352–1359.
47. Sylvester, J. E., Blasko, J. C., Grimm, P. D., Meier, R., and Malmgren, J. A. (2003). Ten-year biochemical relapse-free survival after external beam radiation and brachytherapy for localized prostate cancer: the Seattle experience. *Int. J. Radiat. Oncol. Biol. Phys.* **57**, 944–952.
48. Dattoli, M., Wallner, K., True, L., Cash, J., and Sorace, R. (2003). Long-term outcomes after treatment with external beam radiation therapy and palladium 103 for patients with higher risk prostate carcinoma. *Cancer* **97**, 979–983.
49. Brandeis, J. M., Litwin, M. S., Burnison, C. M., and Reiter, R. E. (2000). Quality of life outcomes after brachytherapy for early stage prostate cancer. *J. Urol.* **163**, 851–857.
50. Gelblum, D. Y. and Potters, L. (2000). Rectal complications associated with transperineal interstitial brachytherapy for prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **48**, 119–124.
51. DeWeese, T. L., Shipman, J. M., Dillehay, L. E., and Nelson, W. G. (1998). Sensitivity of human prostatic carcinoma cell lines to low dose rate radiation exposure. *J. Urol.* **159**, 591–598.
52. Furuya, Y., Lin, X.S., Walsh, J. C., Nelson, W. G., and Isaacs, J. T. (1995). Androgen ablation-induced programmed death of prostatic glandular cells does not involve recruitment into a defective cell cycle or p53 induction. *Endocrinol.* **136**, 1898–1906.
53. Denmeade, S. R., Lin, X. S., and Isaacs, J. T. (1996). Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* **28**, 251–265.
54. Zietman, A. L., Nakfoor, B. M., Prince, E. A., and Gerweck, L. E. (1997). The effect of androgen deprivation and radiation therapy on an androgen-sensitive murine tumor: an in vitro and in vivo study. *Cancer J. Sci. Am.* **3**, 31–36.
55. Joon, D. L., Hasegawa, M., Sikes, C., et al. (1997). Supraadditive apoptotic response of R3327-G rat prostate tumors to androgen ablation and radiation. *Int. J. Radiat. Oncol. Biol. Phys.* **38**, 1071–1077.
56. Del Regato, J. (1968). Radiotherapy for carcinoma of the prostate. A report from the Committee for the Cooperative Study of Radiotherapy for Carcinoma of the Prostate, Colorado Springs, CO, Penrose Cancer Hospital.
57. Green, N., Bodner, H., Broth, E., et al. (1984). Improved control of bulky prostate carcinoma with sequential estrogen and radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 971–976.
58. Mukamel, E., Servadio, C., and Lurie, H. (1983). Combined external radiotherapy and hormonal therapy for localized carcinoma of the prostate. *Prostate* **4**, 283–287.
59. Pilepich, M. V., Krall, J. M., Johnson, R. J., et al. (1987). Prognostic factors in carcinoma of the prostate-analysis of the RTOG 75-06. *Int. J. Radiat. Oncol. Biol. Phys.* **13**, 339–349.
60. Pilepich, M. V., Krall, J. M., John, M. J., et al. (1989). Hormonal cytoreduction in locally advanced carcinoma of the prostate treated with definitive radiotherapy: Preliminary results of RTOG 83-07. *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 813–817.
61. Pilepich, M. V., John, M. J., Krall, J. M., McGowan, D., Hwang, Y. S., and Perez, C. A. (1990). Phase II Radiation Therapy Oncology Group study of hormonal cytoreduction with flutamide and zoladex in locally advanced carcinoma of the prostate treated with definitive radiotherapy. *Am. J. Clin. Oncol.* **13**, 461–464.

62. Pilepich, M. V., Krall, J. M., al-Sarraf, M., et al. (1995). Androgen deprivation with radiation therapy alone for locally advanced prostatic carcinoma: a randomized comparative trial of the Radiation Therapy Oncology Group. *Urology* **45**, 616–623.
63. Pilepich, M. V., Winter, K., John, M. J., et al. (2001). Phase III radiation therapy oncology group (RTOG) trial 86-10 of androgen deprivation adjuvant to definitive radiotherapy in locally advanced carcinoma of the prostate. *Int. J. Radiat. Oncol. Biol. Phys.* **50**, 1243–1252.
64. Lawton, C. A., Winter, K., Murray, K., et al. (2001). Updated results of the phase III radiation therapy oncology group (RTOG) trial 85-31 evaluating the potential benefit of androgen suppression following standard radiation therapy for unfavorable prognosis carcinoma of the prostate. *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 937–946.
65. Pilepich, M. V., Winter, K., Lawton, C. A., et al. (2005). Androgen suppression adjuvant to definitive radiotherapy in prostate carcinoma—Long-term results of Phase III RTOG 85-31. *Int. J. Radiat. Oncol. Biol. Phys.* **61**, 1285–1290.
66. Bolla, M., Collette, L., Blank, L., et al. (2002). Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): a phase III randomized trial. Lancet **360**, 103–108.
67. D'Amico, A. V., Manola, J., Loffredo, M., Renshaw, A. A., DellaCroce, A., and Kantoff, P. W. (2004). 6-month androgen suppression plus radiation therapy vs radiation therapy alone for patients with clinically localized prostate cancer. *JAMA* **292**, 821–827.
68. Holzbeierlein, J. M., Castle, E., and Thrasher, J. B. (2004). Complications of androgen deprivation therapy: prevention and treatment. *Oncology* **18**, 303–309.
69. Laverdiere, J., Gomez, J. L., Cusan, L., et al. (1997). Beneficial effect of combination hormonal therapy administered prior and following external beam radiation therapy in localized prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **37**, 247–252.
70. Laverdiere, J., Nabid, A., de Bedoya, L. D., et al. (2004). The efficacy and sequencing of a short course of androgen suppression on freedom from biochemical failure when administered with radiation therapy for T2-T3 prostate cancer. *J. Urol.* **171**, 1137–1140.
71. Hanks, G. E., Pajak, T. F., Porter, A., et al. (2003). Phase III trial of long-term adjuvant androgen deprivation after neoadjuvant hormonal cytoreduction and radiotherapy in locally advanced carcinoma of the prostate: the Radiation Therapy Oncology Group protocol 92-02. *J. Clin. Oncol.* **21**, 3972–3978.
72. Roach, M., Lu, J., Pilepich, M. V., et al. (2000). Predicting long-term survival and the need for hormonal therapy: a meta-analysis of RTOG prostate cancer trials. *Int. J. Radiat. Oncol. Biol. Phys.* **47**, 617–627.
73. Horwitz, E. M., Winter, K., Hanks, G. E., Lawton, C. A., Russell, A. H., and Machtay, M. (2001). Subset analysis of RTOG 85-31 and 86-10 indicates an advantage for long-term vs. short-term adjuvant hormones for patients with locally advanced nonmetastatic prostate cancer treated with radiation therapy. *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 947–956.
74. Gleave, M. E., Goldenberg, S. L., Chin, J. L., et al. (2001). Randomized comparative study of 3 versus 8-month neoadjuvant hormonal therapy before radical prostatectomy: biochemical and pathological effects. *J. Urol.* **166**, 500–507.
75. Roach, M. 3rd, DeSilvio, M., Lawton, C., et al. (2003). Phase III trial comparing whole-pelvic versus prostate-only radiotherapy and neoadjuvant versus adjuvant combined androgen suppression: radiation therapy oncology group 9413. *J. Clin. Oncol.* **21**, 1904–1911.
76. Roach, M. 3rd, Marquez, C., You, H. S., et al. (1994). Predicting the risk of lymph node involvement using the pre-treatment prostate specific antigen and Gleason score in men with clinically localized prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **28**, 33–37.
77. Chen, C. T., Valicenti, R. K., Lu, J., et al. (2001). Does hormonal therapy influence sexual function in men receiving 3D conformal radiation therapy for prostate cancer?. *Int. J. Radiat. Oncol. Biol. Phys.* **50**, 591–595.
78. Dearnaley, D. P., Khoo, V. S., Norman, A. R., et al. (1999). Comparison of radiation side-effects of conformal and conventional radiotherapy in prostate cancer: a randomised trial. *Lancet* **353**, 267–272.
79. Zelefsky, M. J. and Harrison, A. (1997). Neoadjuvant androgen ablation prior to radiotherapy for prostate cancer: Reducing the potential morbidity of therapy. *Urology* **49**, 38–45.
80. Forman J. D., Kumar R., Haas G., Montie J., Porter A. T., and Mesina C. F. (1995). Neoadjuvant hormonal downsizing of localized carcinoma of the prostate: Effects on the volume of normal tissue irradiation. *Cancer Invest.* **13**, 8–15.
81. Yang F. E., Chen G. T. Y., Ray P., et al. (1995). The potential for normal tissue dose reduction with neoadjuvant hormonal therapy in conformal treatment planning for stage C prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **33**, 1009–1017.
82. Bolla M., Gonzalez D., Warde P., et al. (1997). Improved survival in patients with locally advanced prostate cancer treated with radiotherapy and goserelin. *N. Eng. J. Med.* **337**, 295–300.
83. Sanguineti, G., Agostinelli, S., Foppiano, F., et al. (2002). Adjuvant androgen deprivation impacts late rectal toxicity after conformal radiotherapy of prostate carcinoma. *Br. J. Cancer* **86**, 1843–1847.
84. Valicenti, R. K., Winter, K., Cox, J. D., et al. (2003). RTOG 94-06: is the addition of neoadjuvant hormonal therapy to dose-escalated 3D conformal radiation therapy for prostate cancer associated with treatment toxicity? *Int. J. Radiat. Oncol. Biol. Phys.* **57**, 614–620.
85. Gray, H. (1985). Anatomy of the Human Body. Lea & Febiger, Philadelphia, PA.

86. Partin, A. W., Mangold, L. A., Lamm, D.M., Walsh, P. C., Epstein, J. I., Pearson, J. D. (2001). Contemporary update of prostate cancer staging nomograms (Partin Tables) for the new millennium. *Urology* **58**, 843–848.
87. Roach, M., 3rd, Marquez, C., Yho, H. S., et al. (1994). Predicting the risk of lymph node involvement using the pre-treatment prostate specific antigen and Gleason score in men with clinically localized prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **28**, 33–37.
88. Heidenreich, A., Varga, Z., and Von Knobloch, R. (2002). Extended pelvic lymphadenectomy in patients undergoing radical prostatectomy: high incidence of lymph node metastasis. *J. Urol.* **167**, 1681–1686.
89. Rosen, E., Cassady, J. R., Connolly, J., and Chaffey, J. T. (1985). Radiotherapy for prostate carcinoma: the JCRT experience (1968-1978). II. Factors related to tumor control and complications. *Int. J. Radiat. Oncol. Biol. Phys.* **11**, 723–730.
90. Aristizabal, S. A., Steinbronn, D., and Heusinkveld, R. S. (1984). External beam radiotherapy in cancer of the prostate. The University of Arizona experience. *Radiother. Oncol.* **1**, 309–315.
91. Zagars, G. K., von Eschenbach, A. C., Johnson, D. E., and Oswald, M. J. (1987). Stage C adenocarcinoma of the prostate. An analysis of 551 patients treated with external beam radiation. *Cancer* **60**, 1489–1499.
92. McGowan, D. G. (1981). The value of extended field radiation therapy in carcinoma of the prostate. *Int. J. Radiat. Oncol. Biol. Phys.* **7**, 1333–1339.
93. Ploysongsang, S. S., Aron, B. S., and Shehata, W. M. (2002). Radiation therapy in prostate cancer: whole pelvis with prostate boost or small field to prostate? *Urology* **40**, 18–26.
94. Seaward, S. A., Weinberg, V., Lewis, P., Leigh, B., Phillips, T. L., and Roach, M., 3rd. (1998). Improved freedom from PSA failure with whole pelvic irradiation for high-risk prostate cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **42**, 1055–1062.
95. Bagshaw, M. (1984). Radiotherapeutic treatment of prostatic carcinoma with pelvic node involvement. *Urol. Clin. N. Am.* **11**, 297–304.
96. Asbell, S. O., Krall, J. M., Pilepich, M. V., et al. (1988). Elective pelvic irradiation in Stage A2, B carcinoma of the prostate: analysis of RTOG 77-06. *Int. J. Radiat. Oncol. Biol. Phys.* **15**, 1307–1316.
97. Perez, C. A., Michalski, J., Brown, K. C., and Lockett, M. A. (1996). Nonrandomized evaluation of pelvic lymph node irradiation in localized carcinoma of the prostate. *Int. J. Radiat. Oncol. Biol. Phys.* **36**, 573–584.
98. Forman, J. D., Lee, Y., Robertson, P., Montie, J. E., Zingas, A. P., and Porter, A. T. (1993). Advantages of CT and beam's eye view display to confirm the accuracy of pelvic lymph node irradiation in carcinoma of the prostate, *Radiology* **186**, 889–892.
99. Taylor, A., Rockall, A. G., Usher, C., and Powell, M. E. (2004). Delineation of a reference target volume for pelvic lymph node irradiation using MR imaging with ultrasmall superparamagnetic iron oxide particles. *Int. J. Radiat. Oncol. Biol. Phys.* **60**, S225.

21

Recombinant Antibody Candidates for Treatment of Prostate Cancer

Anoop M. Meraney and Warren W. D. Heston

Summary

Understanding of the molecular basis of prostate cancer has led to development of newer treatment modalities. Identification of specific target molecules has led to the development of immune based therapeutic strategies for prostate cancer, such as therapy with vaccines and monoclonal antibody treatment. Chimeric and humanized antibodies directed against prostate cancer targets, such as prostate-specific membrane antigen, epidermal growth factor, and vascular endothelial growth factor have been developed. This chapter provides an overview of existing monoclonal antibody technology, clinical application, and future direction.

Key Words: Immune therapy; monoclonal antibody; prostate cancer.

1. INTRODUCTION

Despite continued advances in diagnosis and treatment, prostate cancer remains the second leading cause of cancer related death in the United States. Treatment of patients with recurrent, metastatic, or hormone-refractory prostate cancer continues to lack curative potential. Hormone deprivation remains the most powerful tool in our armamentarium for patients with advanced prostate cancer, and ultimately, patients will fail this treatment and progress to a hormone-refractory state.

Improved understanding of the molecular basis of cancer has led to development of newer treatment modalities. These include gene and immune therapy. Unlike renal cell cancer, prostate cancer has not been considered sensitive to immune therapy. Notwithstanding, identification of specific target molecules and advances in immune therapy have led to the development of immune-based therapeutic strategies for prostate cancer, such as therapy with vaccines and monoclonal antibody treatment. Monoclonal antibody technology burgeoned from the development of hybridoma technology, described initially by Kohler and Milstein in 1975 *(1)*. Several murine monoclonal antibodies, such as CYT-356 *(2)*, CC49 *(3)*, KC4 *(4)*, and L6 *(5)*, were evaluated for treatment of prostate cancer. However, a major limitation of these antibodies, which are essentially foreign to the human immune system, is the development of human-against-mouse antibodies (HAMA). More recently, advances in genetic engineering have enabled production of antibodies with human genetic constituents, including several recombinant monoclonal antibodies that are being evaluated in the treatment of prostate cancer.

This chapter provides an overview of recombinant monoclonal antibody therapy for prostate cancer.

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

2. DEVELOPMENT OF RECOMBINANT MONOCLONAL ANTIBODIES

Production of murine monoclonal antibodies involves challenging a rodent's immune system with an antigen and harvesting rodent splenocytes for antibody retrieval. Rodent splenocytes are then immortalized by fusing them with myeloma cells deficient in hypoxanthine-guanine phosphoribosyl transferase. Deficiency of the enzyme hypoxanthine-guanine phosphoribosyl transferase renders myeloma cells incapable of self-propelled growth or antibody production. This ensures that antibodies are produced exclusively from rodent splenocytes. Although murine monoclonal antibodies produced in this fashion result in robust and specific antibody-mediated responses, a major shortcoming is the development of HAMA, which precludes repeat dosing *(1)*.

To date, recombinant monoclonal antibody production for the most part still relies on this basic technical design. Advances have enabled production of antibodies that are more humanlike *(6,7)*. Recombinant monoclonal antibody technology involves modification of murine antibodies to prevent development of HAMA, which includes production of chimeric or humanized antibodies or *de novo* production of "non-immunogenic" antibodies by methods such as phage display technology or by transgenic mice. Chimeric antibodies are two-thirds human, and are produced by combining the variable region of the murine antibody with the constant region of a human antibody. DNA sequences that code for the variable region of the murine antibody are spliced to sequences that encode the human constant region of an antibody. In comparison, humanized antibodies are 95% human and are produced by grafting complement-determining regions, wherein actual antigen binding sites on the hypervariable region of the murine antibody are identified and the responsible murine genes are spliced into the DNA sequence of a human antibody. Both of these technologies have limitations, and despite containing less than 5 to 33% murine elements, their use may result in formation of human-against-human antibodies (HAHA) or HAMA. Moreover, humanization is a complex process, and its high selectivity may compromise the potency of the antibodies produced. In this regard, a process called deimmunization (Biovation, Aberdeen, United Kingdom) involves precise identification and replacement of antibody peptides using computer-generated technology to minimize the occurrence of HAMA responses and maintaining antibody potency *(8,9)*. The antibodies are genetically engineered by removal of helper T-cell epitopes from antibodies. Murine antibodies are assessed, and peptides on the antibody that might bind to major histocompatibility complex (MHC) II molecules are identified. Peptides on variable heavy and variable light regions are identified in this fashion and substituted with alternative peptides. Various substitutions are evaluated to prevent any alteration that might render the antibody ineffective.

2.1. *Transgenic Mice (Xenomouse, Abgenix, Fremont, CA)*

Strategic gene silencing of heavy chain and κ-light chain genes in mice blocks murine antibody production while preserving the apparatus necessary for immunoglobulin production *(6)*. These knockout mice are essentially rendered incapable of any murine antibody production. After transfer of human heavy chain and κ-light chain genes into the mouse genome, these mice can produce human antibodies. Human genes that are several megabases long are inserted into the mouse genome by yeast artificial chromosomes. Using this technology, large portions of human immunoglobulin loci can be introduced into the mouse genome, and this has improved the stability and diversity of human antibody production by transgenic mice *(10)*. Because these antibodies are essentially all human, they are not associated with HAMA responses. However, transgenic mice lack the complete set of human antibody-producing loci and, hence, only a limited antibody repertoire can be produced in this fashion *(11)*. However, proposed efforts directed toward overcoming these shortcomings, such as the introduction of human Igλ into the murine genome, which represents 40% of the human antibody repertoire, or use of antibody display libraries from hyperimmunized animals to recover monoclonal antibodies in vitro, may enhance the effectiveness of this technology. Another potential disadvantage of this technology is that because antibodies produced in this fashion are completely foreign to the

host animal, targeting of host tissues might occur after antibody production. It remains to be seen whether this might influence the quality of antibodies produced.

2.2. Phage Display

Antibody fragments may be produced by phage display technology. Antibody V genes are displayed on the surface of flagellated bacteriophages, which then produce antibody fragments that are transfected into bacteria, such as *Escherichia coli*. Single-chain Fv (scFv) fragments are fused to the PIII phage coat protein. Typically, antibodies consist of heavy and light proteins, each of which are synthesized by separate genes. In single-chain antibodies, the light and heavy proteins are fused into a single fused domain, and are encoded by a single gene. As such, single genes are more easily transferred and are, therefore, used in recombinant technology. The PIII coat protein of the bacteriophage consists of N- and C-terminal domains. The N-terminal domains (N1, N2) are involved with phage infectivity, whereas the C terminal is essential for phage assembly. The scFv fragments are bound to the N1 terminal of the PIII coat protein. ScFv–PIII fusions are then encoded by phagemids. Fusion of scFv to the N-terminal domain renders the phage noninfective and, therefore, bacteria are infected using helper phage units. The antibody fragments are then selected by antigen binding. Repeat antigen exposure and mutations result in affinity maturation of the fragments, which increases their specificity *(12)*. A limitation of this technique is that only antibody fragments and not whole antibodies are produced. Compared with whole antibodies, the fragments lack Fc portions essential in recruiting natural effector functions *(7)*. However, a clear benefit of this technology is that antibody production occurs in vitro. Moreover, antibody fragments can be produced rapidly by this technique, and affinity maturation and the potential for property selection results in production of fragments with increased specificity.

3. MONOCLONAL ANTIBODIES: TARGET MOLECULES AND CLINICAL TRIALS

Monoclonal antibodies for cancer therapy are designed to target antigens expressed by tumor cells. Therapy with monoclonal antibodies is capable of eliciting host antitumor responses, which include antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, production of interleukin 6, and increased vascular permeability *(13)*. Antitumor responses by monoclonal antibodies may be further bolstered by tagging the antibodies with cytotoxins, such as radioactive compounds or chemotherapeutic agents. Radiometals used for this purpose include radioactive iodine, yttrium, and lutetium (Table 1) *(8)*. O'Donnell and colleagues compared response rates achieved after treatment of nude mice with HBT-3477 tumors with labeled and unlabeled ChL6 antibodies. A response rate of 79% was achieved in mice treated with yttrium-labeled antibodies compared with rates of only 13% and 18% in unlabeled and untreated mice, respectively *(14)*.

Ideally, target antigens should be predominantly, if not exclusively, expressed by tumor cells. For prostate cancer, the antigen should also be expressed by metastatic and androgen-independent cells, and expression of the antigen should not diminish with increased cancer aggressiveness. Additionally, antigens should be stably expressed as cell surface proteins, making them susceptible to antibody targeting. Also, binding with an antibody should induce antitumor responses. In this regard, molecules that are secreted and not expressed by prostate cancer tissues are not considered valuable targets for monoclonal antibody therapy. These include secreted proteins, such as prostate-specific antigen (PSA) and prostate acid phosphatase, which are useful as screening tools for prostate cancer. In this section, we review recombinant monoclonal antibody targets for prostate cancer therapy, and describe clinical trials evaluating the efficacy of these antibodies for treatment of prostate cancer.

3.1. Antibodies to Prostate-Specific Membrane Antigen

Prostate-specific membrane antigen (PSMA) is a 100-kDa, 750-amino acid type II integral transmembrane glycoprotein (Fig. 1). It has a short cytoplasmic N-terminal amino acid domain contain-

Table 1
Properties of Radioactive Metals Used for Labeling of Monoclonal Antibodies [a]

Radioactive metal	Type of radiation	Depth of penetration (mm)	Maximum energy (MeV)	Half-life (days)	Comments
^{131}I	βγ	0.4	0.2	8	• Intracellular dehalogenation results in removal from cells • Associated with thyroid toxicity
^{90}Y	β	12	2.2	2.7	• Pure β emitter. Requires a surrogate agent such as indium for imaging • Irreversibly trapped within cells
^{177}Lu	βγ	2.2	0.5	6.7	• Irreversibly trapped within cells

[a] Adapted from references *31* and *32*.

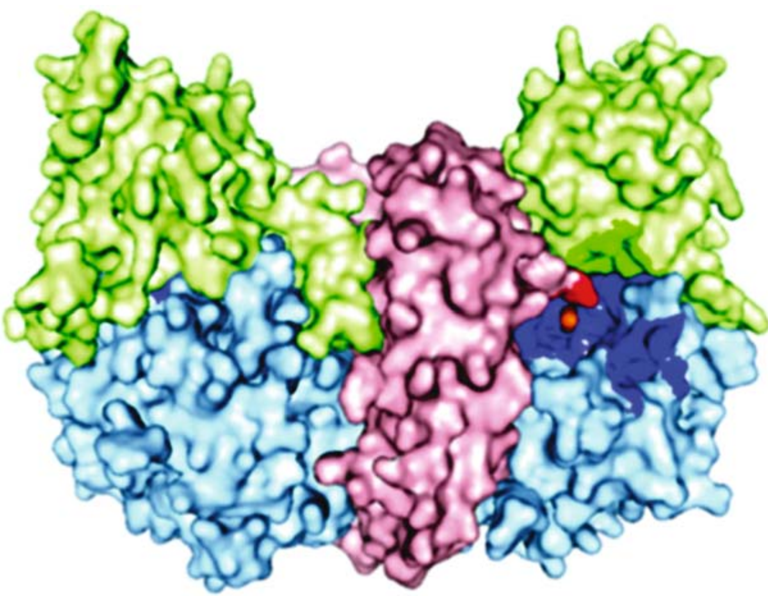


Fig. 1. Crystallographic structure of prostate-specific membrane antigen (reprinted with permission from ref. *56*).

ing 19 amino acids, and a larger extracellular tail with a carboxyl-terminal containing 707 amino acids. The transmembrane domain is comprised of 24 amino acids *(15)*. Although the precise function of this protein is yet to be determined, it has been demonstrated that PSMA is homologous to glutamate carboxypeptidase II. It has folate hydrolase and N-acetylated α-linked acidic dipeptidase activity *(16–18)*. The *PSMA* gene has been mapped to chromosome 11q11.12 *(19)*. PSMA was initially described in 1987 after isolation of monoclonal antibodies directed toward cell membrane preparations of LNCaP cells *(20)*. Clinically, PSMA is expressed by prostate cancer, and increased expression of PSMA has been identified in patients with advanced, metastatic, and hormone-refractory prostate cancer *(21,22)*. PSMA expression has also been demonstrated in small intestinal brush

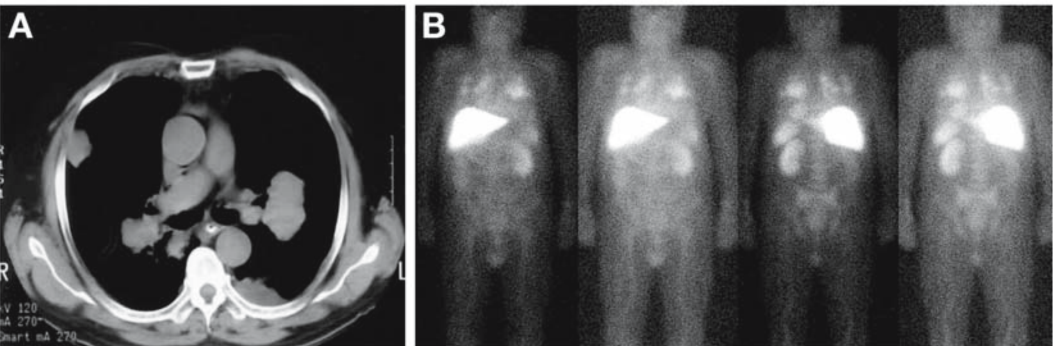


Fig. 2. Prostate-specific membrane antigen (PSMA) expression by neovascular endothelium has enabled targeting of nonprostate solid organ tumors with antibodies to PSMA (reprinted with permission from ref. *57*).

border cells, renal proximal collecting tubular cells, cells within salivary glands, and endothelium of tumor neovasculature *(23)*. Of note, expression of PSMA by nonprostate tissues is typically 100- to 1000-fold less than that expressed by the prostate, which permits safe and efficacious monoclonal antibody directed targeting of the prostate. Vascular PSMA expression is observed only in the tumor neovasculature of nonprostate-related cancers, including solid organ carcinomas and sarcomas, and not in normal vascular endothelium *(8)*. This feature has enabled targeting nonprostate solid organ cancers expressing PSMA by monoclonal antibodies to PSMA (Fig. 2).

The monoclonal antibody 7E11 recognizes the intracellular epitope of the PSMA receptor. Hence, it is thought that the antibody selectively binds to dead cells. Murine 7E11 monoclonal antibodies conjugated with ^{111}indium are used for tumor imaging using the ProstaScint scan (Cytogen Corp., Princeton, NJ). The ProstaScint scan typically detects soft tissue involvement with more precision than osseous involvement *(8)*. This might be secondary to increased numbers of apoptotic cells within soft tissue metastases.

Several antibodies directed against the extracellular epitope of PSMA have since been isolated. Initially, Liu and colleagues, from their work with LNCaP cells, reported the isolation of four monoclonal antibodies, namely, J591, J415, J533, and E99 *(24)*. In this study, the antibodies were demonstrated to bind to two extracellular PSMA epitopes, more accessible than the internal epitope targeted by 7E11. Using immunohistochemistry, the authors demonstrated that staining of human prostate cancer tissue was more homogenous and consistent with the newer antibodies compared with 7E11. Additionally, prominent staining of tumor vascular endothelium was also noted.

Smith-Jones and colleagues compared the pharmacokinetics, biodistribution, and tumor uptake of antibodies directed against internal and external domains of PSMA in nude mice bearing LNCaP tumors *(25)*. Using antibodies labeled with indium, the authors reported that antibodies directed against the external domain were localized to areas of viable tumors, whereas antibodies to the internal domain were predominantly localized to necrotic regions within the tumor. Also, unlike the ProstaScint scan, imaging with labeled antibodies to the external domain of PSMA can be used to identify bone metastases (Fig. 3). Other antibodies to PSMA targeting the external domain of PSMA that have been isolated include 3E11, EC2, 4E10-1.14, 3C9, 1G3, and PEQ226.5 *(26,27)*.

3.1.1. Human J591

Human J591 (huJ591) antibodies to PSMA are derived from murine J591 antibodies to PSMA by the process of deimmunization (Biovation, Aberdeen, United Kingdom). The F(ab) region of the

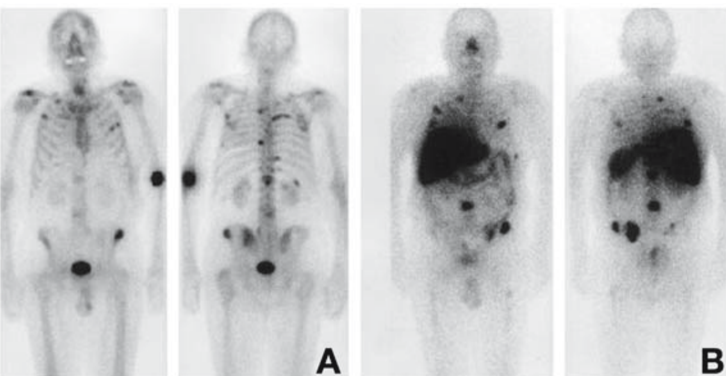


Fig. 3. Bone imaging with labeled antibodies to prostate-specific membrane antigen (PSMA) antibodies to the external domain of PSMA (reprinted with permission from ref. *8*).

antibody is modified by replacing potentially immunogenic murine sequences with sequences that would be nonimmunogenic to humans, while maintaining the effectiveness of the antibody. A human IgG1Fc portion is then attached.

Radioactive iodine- and yttrium-labeled huJ591 antibodies were initially evaluated in nude mice implanted with LNCaP tumors *(8,28)*. The study assessed the effectiveness of yttrium- and radioidine-labeled antibodies in the treatment of large tumors (300–400 mg) and small tumors (100–200 mg). Various doses were administered to evaluate the maximum tolerated dose, and to evaluate antitumor response to re-dosing schedules. Intracellular retention of the radiopharmaceutical compound was shorter with radioactive iodine compared with yttrium. This is secondary to rapid dehalogenation of the antibody and clearance of iodine from the cells. In contrast to this, yttrium, which is a radiometal, is irreversibly trapped within cells, resulting in a longer half-life and increased effectiveness. As a result, the radiation dose delivered by yttrium-labeled antibodies was greater compared with radioiodine-labeled antibodies *(29)*. Indium-labeled antibodies were used for surrogate imaging in animals within the yttrium group. Yttrium is a pure β-emitter and cannot be visualized with a γ-camera. Biodistribution of the yttrium- and iodine-labeled antibodies was assessed using indium- and iodine-labeled antibodies, respectively. Indium-labeled huJ591 was found to bind to viable regions within the LNCaP xenografts (Fig. 4). It was demonstrated that distribution of indium-labeled antibodies was higher in the tumor tissue, and in the liver, kidney, and spleen compared with radioiodine-labeled antibodies. In addition, this study demonstrated the effectiveness of multiple fractionated doses in the animal model. In this study, for mice with smaller tumors, survival was 80 to 100 days after administration of a radiation dose of 3.7 to 5.55 MBq. Using a fractionated dose regimen, which delivered a cumulative dose of 3.33 to 6.7 MBq, survival was prolonged to 120 days.

Bander and colleagues, in a phase I clinical trial, assessed the biodistribution, pharmacokinetics, and toxicity of huJ591 *(30)*. The antibody was labeled with indium using a DOTA chelate. Patients received a 4-week course comprising an initial dose followed by three maintenance doses, which were 50% of the initial dose. Initial doses administered were 25, 50, 100, or 200 mg/m^2. Within an hour after administration of the initial dose, γ-camera total body images were obtained. Subsequently, additional total body images were also obtained on three separate occasions during the following week. Patients with progressive prostate cancer were enrolled in the trial. The antibody demonstrated good tumor localization. Prominent liver uptake and excretion was also observed. None of the patients developed human antihuman responses, and no dose-limiting toxicity was observed. One patient developed hypotension, secondary to rapid infusion.

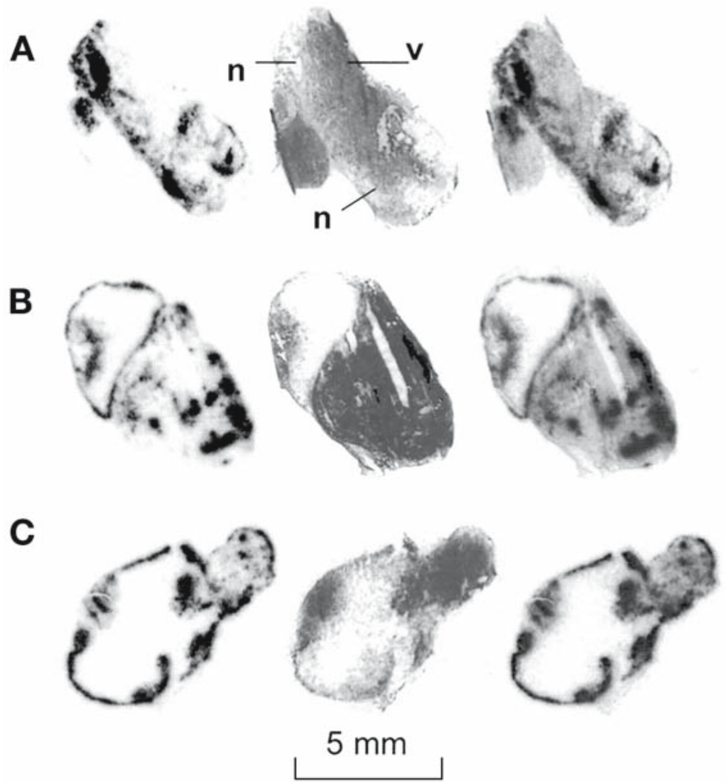


Fig. 4. Uptake of 7E11 to areas of necrosis (n) and uptake of J415 and J591 to viable tumor regions (v). Autoradiographs and H&E-stained sections of LNCaP xenografts harvested 4–6 days after intravenous injection of ^{131}I-labeled 7E11 **(A)**, J591 **(B)**, and J415 **(C)**. Column 1, autoradiograph; column 2, stained section; column 3, composite image of autoradiograph and stained section (reprinted with permission from ref. *25*).

Subsequently, phase I trials were designed to assess the safety, dosimetry, and pharmacokinetics of radiolabeled huJ591 monoclonal antibodies. Yttrium- and lutetium-labeled antibodies were evaluated in two separate trials *(8,31,32)*. Radiolabeled antibodies were created by linking the radioisotope to huJ591 via a DOTA chelate. Patients enrolled into the trial were those with hormone-refractory prostate cancer with evidence of biochemical progression or metastatic progression based on radiographic evidence. The phase I trial involving yttrium-labeled antibodies enrolled 29 patients. Initially, patients received indium-labeled antibodies to determine pharmacokinetics, biodistribution, and dosimetry. Subsequently, total body images were obtained with a γ-camera 1 hour after administration of the antibody, followed by subsequent imaging on day 1, 2, 3, and either on day 6 or 7. Imaging of indium-labeled antibodies demonstrated significant activity in the liver followed by the spleen and kidneys. The half-life for indium-labeled huJ591 was 32 ± 8 hours. ^{90}Y-labeled antibodies with a specific activity of 3 to 5 mCi/mg were administered 1 week later. Radiation dosage of yttrium was based upon the information obtained after administration of indium-labeled antibodies. Yttrium-labeled antibody was administered at one of five initial dose levels: 5, 10, 15, 17.5, or 20 mCi/m^2. Four patients received an initial dose of 5 mCi/m^2, seven patients received 10 mCi/m^2, eight patients received 15 mCi/m^2, six patients received 17.5 mCi/m^2, and four patients received 20 mCi/m^2.

Re-dosing was determined by recovery of platelet and neutrophil counts. One patient in the 15 mCi/m^2 group died of an unrelated pulmonary embolism, and two patients who received an initial dose of 20 mCi/m^2 developed grade 3 thrombocytopenia with bleeding episodes. Toxicities in all three patients were considered to be dose limiting. Two patients developed grade 3 anemia, one who received 10 mCi/m^2 and another who received 15 mCi/m^2. Three patients were re-dosed with the yttrium-labeled antibody. Two patients received 17.5 mCi/m^2, and one patient received 20 mCi/m^2. Of these three patients, one patient who received 17.5 mCi/m^2 and one patient who received 20 mCi/m^2 developed grade 3 thrombocytopenia and neutropenia. Other side effects included fatigue, anorexia, nausea, and elevated levels of transaminases (n = 11). One patient developed an upper extremity deep vein thrombosis secondary to a central venous catheter. The maximum tolerated dose was determined to be 17.5 mCi/m^2. No human antihuman antibody responses occurred. Although the study was not designed to assess effectiveness of treatment, PSA stabilization was noted in six patients. In addition, a decline in serum PSA was observed in two patients. In these two patients, PSA levels declined by 85% and 70%, respectively. PSA returned to baseline after 8 and 8.6 months, respectively.

Another phase I trial that assessed the safety, dosimetry, and pharmacokinetics of lutetium was recently completed. The study accrued 35 patients. Eligibility criteria used for the study were similar to those used for the phase I trial with yttrium-labeled antibodies. Total body images were obtained with a γ-camera within 1 hour after administration of the radiolabeled antibody, and then at four additional time points on day 2, day 4 or 5, day 6 or 7, and day 12 or 14. Plasma half-life was 39 ± 13 hours. Various initial dose levels were investigated, including 10, 15, 30, 45, 60, 70, and 75 mCi/m^2. Retreatment doses were also evaluated. Of patients receiving 75 mCi/m^2 (n = 3), one patient developed grade 4 thrombocytopenia and two patients developed grade 3 thrombocytopenia. In addition, grade 4 neutropenia occurred in all three patients. Of patients receiving 70 mCi/m^2, two patients developed grade 4 neutropenia, and one patient developed a dose-limiting toxicity of grade 4 thrombocytopenia. Subsequently, 70 mCi/m^2 was determined to be the maximum tolerated dose. Significant hematological toxicity was noted in patients re-dosed with 45 mCi/m^2. However, patients re-dosed with 30 mCi/m^2 tolerated multiple doses better. Within this trial, a PSA decline of at least 50% was observed in 4 patients, and PSA stabilization was noted in 16 patients. Of note, responders were patients with elevated PSA levels and without measurable disease at the outset of the study. In contrast to this, responders within the yttrium-labeled antibody phase I trial were those with measurable disease. Based on this data, the authors suggest that yttrium may be a more suitable agent for patients with measurable disease, whereas Lu may be indicated in those with elevated PSA levels without any evidence of measurable disease. Although it is encouraging that initial results indicated that select patients did respond to the treatment, further testing is required.

The phase I trials with yttrium- and lutetium-labeled huJ591 demonstrated that myelosuppression is the most common and most severe side effect with this treatment. Vallabhajosula and colleagues evaluated the results of the phase I trials with yttrium- and lutetium-labeled huJ591 to determine the possibility of predicting bone marrow toxicity based on the dose of radioisotope administered. For patients receiving lutetium-labeled antibodies, bone marrow radiation absorbed dose and fractional decrease in platelets correlated well with the radiation dose administered. However, by comparison, the radioactive dose of yttrium-labeled antibodies did not correlate with bone marrow radiation dose *(33)*.

The monoclonal antibody J591 has also been conjugated with DM1, which is a cytotoxin derived from maytansine *(8)*. Conjugates are not active until the cytotoxin is internalized into the cell. This feature should minimize gastrointestinal and neurologic toxicity that might otherwise occur with use of DM1. Currently, DM1 conjugated with J591 is being evaluated in a phase I trial.

Although not a monoclonal antibody treatment *per se*, a novel treatment strategy is under investigation involving PSMA that deserves mention. It entails modification of T-cell receptors to recog-

nize PSMA molecules on prostate tissue. A unique genetically engineered receptor called pz1, designed to recognize PSMA molecules, has been developed. The pz1 receptor is a hybrid, with the extracellular portion being a single-chain antibody against PSMA, and the intracellular portion comprising the intracellular signaling region of the T-cell receptor. By this strategy, T lymphocytes with the modified pzl receptors are able to recognize, target, and lyse tumor cells expressing PSMA independent of MHC molecule expression. Thus far, Gong and colleagues have reported results obtained in vitro, which seem promising *(34)*.

3.1.2. ChL6

L6 is murine IgG2a monoclonal antibody obtained after immunization of mice with human non small-cell lung cancer cells *(5)*. The antibody binds to a 24-kDa integral membrane protein, which is expressed on tumor cells. In addition to lung cancer, L6 has been found to bind to tumor-associated antigens expressed on various other tumors, including tumors of the breast, colon, ovary, and prostate. The chimeric form of the antibody (ChL6) incorporates the murine L6 binding sites combined with human IgG1 and κ-constant regions. In vitro and animal experiments have demonstrated binding of L6/ChL6 to human prostate cancer cell lines, especially the PC3 and DU-145 cell lines. Nude mice implanted with PC3 tumors and treated with ^{90}Y–DOTA–peptide–ChL6 demonstrated durable response rates with high doses of radioimmunotherapy *(35)*. Mice that received 260 μCi demonstrated 20% complete response rates. However, this group was associated with 60% mortality secondary to severe myelosuppression. Good partial response rates of up to 100% were achieved in mice receiving lower doses of 112 μCi and 150 μCi. Human trials with this chimeric monoclonal antibody have not been reported in the literature. A limitation of ChL6 is that chimeric antibodies are likely to result in formation of HAHA responses. In addition, the target is a not specific for prostate cancer.

3.1.3. ABX-EGF (Abgenix, Fremont, CA)

ABX-EGF is a human monoclonal antibody against epidermal growth factor (EGF) receptor. Monoclonal antibodies against EGF are being investigated for treatment of colorectal, head, neck, and breast cancers, and also currently being evaluated for treatment of hormone refractory prostate cancer *(36,37)*. Animal studies in athymic mice implanted with DU-145 and PC3 xenografts demonstrated inhibition of growth after treatment with ABX-EGF. Combining monoclonal antibody treatment with doxorubicin chemotherapy resulted in a more profound effect. However, concerns with this treatment include the fact that EGF is a molecule expressed normally by cells, and not a tumor specific molecule. In addition, the level of expression of EGF receptor by prostate cancer cells is lower than that seen in some other tumors, such as squamous cell cancers, which express more than 1 million sites per cell. In contrast to this, DU-145 cells express 100,000 sites per cell, LNCaP cells express 80,000 sites per cell, and PC3 cells express only 30,000 sites per cell *(38)*. There is also concern that antibody response with antibodies produced using xenomouse technology may not be as robust, because transgenic mice may lack certain alleles of heavy and light chain regions that determine antibody specificity *(8)*.

3.1.4. Trastuzumab (Herceptin, Genentech, Cambridge, MA)

Trastuzumab is a monoclonal antibody directed against Her-2/neu, a member of the EGF receptor family. This receptor is not specific for prostate cancer tissue, and overexpression is commonly seen in patients with breast cancer. Trastuzumab is an IgG1 humanized monoclonal antibody directed against the extracellular component of Her-2 receptor. The *Her-2* gene is homologous to *neu*, which is a rat gene, and, hence, the gene is often referred to as *Her-2/neu*. Low levels of expression have been demonstrated in prostate cancer tissue by immunohistochemistry and fluorescence *in situ* hybridization. Expression of Her-2/Neu seems to be higher in hormone-refractory cases. Ziada and colleagues reported results after completion of a phase II trial in 18 patients with hormone refractory prostate cancer *(39)*. Expression of Her-2 was detected in only 2 of 18 cases. No objective responses

were attained based on PSA and radiographic criteria. Of note, this agent is associated with potential for cardiac complications, which was observed in 2 of the 18 patients within this trial. Combining Herceptin with paclitaxel seems to be associated with increased effectiveness *(40)*. It remains to be seen whether this form of treatment will benefit patients with prostate cancer, although this certainly seems less likely at this time, because of the low incidence of Her-2 expression in patients with prostate cancer.

3.1.5. Bevacizumab (Avastin, Genetec, Cambridge, MA)

Bevacizumab is a humanized murine monoclonal antibody to vascular endothelial growth factor (VEGF) *(36)*. VEGF is responsible for proliferation of neovasculature within tumors. Although associated with limited efficacy when used in isolation, combination treatment with 5-Fluorouracil and bevacizumab in patients with advanced colorectal cancer has been associated with improved survival *(41,42)*. VEGF receptors have also been linked to prostate cancer cells, and, currently, a phase II trial involving a combination of bevacizumab, docetaxel, thalidomide, and prednisone is recruiting patients with metastatic prostate cancer *(43)*. It remains to be seen whether this combined approach will result in treatment benefits.

3.1.6. MDX-010 (Medarex, Princeton, NJ)

MDX-010 is a humanized monoclonal antibody against cytotoxic T lymphocyte-associated antigen (CTLA)-4, manufactured using transgenic mouse technology *(44)*. CTLA-4 is a protein present on cytotoxic lymphocytes. Normally, CTLA-4 functions as an immune regulatory antigen. CTLA-4 binds B7 and this, in turn, attenuates antitumor responses. MDX-010 is being evaluated for treatment in malignant melanoma, and a trial assessing its efficacy for treatment of stage 4 renal cell cancer is underway. In addition, a phase I trial was conducted in 14 patients with hormone refractory prostate cancer, who were treated with a dose of 3 mg/kg of MDX-010. Of these patients, seven had undergone prior chemotherapy. A favorable PSA response was noted in two of seven patients who had not received previous chemotherapy. Side effects included pruritis and rash. MDX-010 was detectable in the plasma for up to 4 months. No HAHA responses were noted *(45)*. Currently, a phase I trial is underway to evaluate the combined use of MDX-010 and granulocyte colony-stimulating factor in the treatment of advanced prostate cancer *(46)*. Studies in transgenic adenocarcinoma of the mouse prostate have demonstrated the efficacy of combining CTLA-4 antibody treatment with granulocyte colony-stimulating factor vaccination of tumor cells *(47)*. The efficacy of concurrent use of the co-stimulatory pathways remains to be evaluated.

4. DISCUSSION

Several monoclonal antibody therapies are currently being evaluated (Table 2). The majority of the antibodies being evaluated are not prostate specific. PSMA is currently the only organ-specific antibody being evaluated. Because PSMA is expressed in both hormone-naïve and hormone-refractory cases, it forms an important target for immune-based treatment strategies. A feature of PSMA that makes it a unique target is internalization of receptor–ligand complexes. This allows for intracellular delivery of radiopharmaceutical or chemotherapeutic agents bound to monoclonal antibodies. Internalization occurs via clathrin-coated pits. The process is mediated by the MXXXL motif of the intracytoplasmic domain of the PSMA molecule *(48)*.

Results of clinical trials with administration of radiolabeled huJ591 antibodies have demonstrated favorable responses in select patients, although the data available are from phase I trials, which are not designed to evaluate treatment outcomes. However, within these studies, bone marrow toxicity has limited the use of more aggressive regimens. It remains to be seen whether utilization of allogenic bone marrow transplantation into future trials will enable incorporation of more aggressive treatment regimens while minimizing bone marrow toxicity and, thereby, achieving superior outcomes. Currently, this form of treatment is used predominantly for hematologic malignancies.

Table 2
Recombinant Monoclonal Antibodies Being Evaluated for Treatment of Prostate Cancer

Monoclonal antibody	Immunoreactivity	Target molecule
huJ591	Humanized	Prostate-specific membrane antigen (PSMA)
ChL6	Chimeric	Surface protein on adenocarcinoma cells
ABX-EGF	Humanized	Epidermal growth factor (EGF)
Herceptin	Humanized	Her-2/neu
Bavacizumab	Humanized	Vascular endothelial growth factor (VEGF)
MDX-010	Humanized	Cytotoxic T lymphocyte antigen-4 (CTLA-4)

Research involving chelate-linkers to bind radiometals to monoclonal antibodies has demonstrated reduced liver toxicity associated with the use of a novel chelate–peptide linker. As such, direct linkage of radiometals to monoclonal antibodies results in an unstable bond. DOTA is a macrocyclic chelator that stably binds radiometals such as indium, yttrium, and lutetium to the monoclonal antibody. As a result, radiolabeled monoclonal antibodies are stable until delivered into the tumor tissue. This also minimizes uptake of radiometal compounds by normal body tissues.

Recently, DeNardo and colleagues reported the use of peptide with a cathepsin B cleavage site for linkage of DOTA–radiometal conjugates to monoclonal antibodies *(49)*. The DOTA–peptide chelate-linker is cleaved by liver endopeptidases resulting in rapid urinary excretion of radiometal–DOTA, thereby reducing hepatic toxicity. The authors evaluated biodistribution of this new compound in eight patients. The monoclonal antibody used for this trial was m170, a murine IgG that binds to human adenocarcinoma cells. Of 25 patients enrolled in this trial, 17 patients received indium–DOTA-labeled m170 Abs, and 8 patients received indium–DOTA–peptide-labeled antibodies. Patients within the DOTA–peptide group demonstrated 30% less liver radiation doses. Radiation doses to bone marrow and to the tumors were comparable between the two groups. It remains to be seen whether the use of this peptide linkage will be associated with better tolerance to therapeutic approaches involving dose escalation.

Besides the use of labeled monoclonal antibodies, various other strategies to enhance the effectiveness of monoclonal antibody therapy are being evaluated. These include the use of combined modality radioimmunotherapy and concurrent use of immune modulators. Combined modality radioimmunotherapy is a combination of radioimmunotherapy and radiosensitizing chemotherapy. Taxanes, such as paclitaxel and docetaxel, induce G_2/M cell arrest. This phase of the cell cycle is particularly sensitive to radiation-induced apoptosis. An animal study evaluated combined modality radioimmunotherapy using radiolabeled ChL6 monoclonal antibodies and taxanes *(50)*. Uptake of monoclonal antibodies by tumor cells was complete within 24 to 48 hours of administration, and because nontargeted radiolabeled antibodies are eliminated by this time, the chemotherapeutic agents were administered intraperitoneally 24 hours after administration of the radiolabeled antibodies (^{90}Y–DOTA–peptide–ChL6). Mice that received combined therapy demonstrated 79% response rates, and an 84-day survival of 88%, whereas animals that received only radiolabeled antibody treatment or taxane treatment demonstrated a response rate of 19% and an 84-day survival of 19%. Although no treatment-related deaths occurred, bone marrow toxicity was greater in animals that received combined treatment. Human trials involving the use of ChL6 have not yet been reported. Because the antibody was derived from lung cancer cell antigens, its binding with human prostate cancer tissue needs to be evaluated further. Nonetheless, presensitization with chemotherapeutic drugs seems to be a promising treatment modality that can be combined with any radiolabeled monoclonal antibody to enhance the effectiveness of therapy. It remains to be seen whether responses demonstrated in the animal model can be duplicated in human trials.

After initial phase I and II trials to evaluate therapy with CC49, a murine monoclonal pan-adenocarcinoma antibody against TAG-72, two additional trials were reported involving pretreatment with interferon-γ and interferon-α 2a, followed by administration of radioiodine-labeled CC49 monoclonal antibodies *(51–53)*. These studies were based on observations that pretreatment with interferons might upregulate TAG-72 expression. In patients who received interferon-γ, increased TAG72 expression was noted in 3 of 16 patients. The dose of radioiodine was similar to that used in a previous phase II trial (75 mCi/m^2). However, similar to results of CC49 monoclonal antibody treatment without any pretreatment, PSA responses were not observed in any of the patients. However, pretreatment with interferon-α 2b followed by administration of radioiodine labeled antibody resulted in higher mean tumor doses of radiation. Six weeks after treatment, two patients were described as having minor radiographic responses and three patients demonstrated PSA responses (>50% reduction). PSA remained stable in another six patients. Pretreatment with interferon-α 2a seemed to be more effective than pretreatment with interferon-γ or radiolabeled antibody alone.

In patients with prostate cancer, definitive cure is only achieved in select patients with organ-confined disease. Patients with biochemical progression are treated with hormone manipulation. Progression to a hormone-refractory state and development of metastatic disease portend a poor prognosis. Although recent studies have demonstrated survival benefits in patients with metastatic disease treated with chemotherapy *(54)*, even patients in this category have a poor prognosis. Clearly, there is need for newer and more effective treatment strategies. Fortunately, our understanding of prostate cancer at the molecular level is advancing. Patients with prostate cancer may harbor clonally varied tumor cells. Autopsy studies have demonstrated that metastatic prostate cancer is a phenotypically diverse disease *(55)*. Phenotypic heterogeneity may be observed within different metastatic sites within the same patient. It is likely that, in the individual patient, tumor cells with phenotypic heterogeneity will likely respond in a varied manner to similar treatments, with some cells escaping from therapy. This demonstrates the need for a multifaceted approach to prostate cancer treatment, with tailored treatment plans for individual patients. This will include monoclonal antibody therapy. Monoclonal antibody therapy is a rapidly advancing and promising treatment for prostate cancer. However, this therapy is still evolving. Identification of new targets and technological innovations improving antibody specificity will likely shape the future of monoclonal antibody strategies.

REFERENCES

1. Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256(5517)**, 495–497.
2. Deb, N., Goris, M., Trisler, K., et al. (1996). Treatment of hormone-refractory prostate cancer with 90Y-CYT-356 monoclonal antibody. *Clin. Cancer Res.* **2(8)**, 1289–1297.
3. Mulligan, T., Carrasquillo, J. A., Chung, Y., et al. (1995). Phase I study of intravenous Lu-labeled CC49 murine monoclonal antibody in patients with advanced adenocarcinoma. *Clin. Cancer Res.* **1(12)**, 1447–1454.
4. Abdel-Nabi, H., Spaulding, F. F., Derby, L., Lamonica, D., and Glenn, S. (1995). Treatment of refractory prostate cancer with 90Y KC4. *J. Nuclear Med.* **36[Suppl]**, 213.
5. O'Donnell, R. T., DeNardo, S. J., Shi, X. B., et al. (1998). L6 monoclonal antibody binds prostate cancer. *Prostate* **37(2)**, 91–97.
6. Davis, C. G., Gallo, M. L., and Corvalan, J. R. (1999). Transgenic mice as a source of fully human antibodies for the treatment of cancer. *Cancer Metastasis Rev.* **18(4)**, 421–425.
7. Holliger, P. and Bohlen, H. (1999). Engineering antibodies for the clinic. *Cancer Metastasis Rev.* **18(4)**, 411–419.
8. Bander, N. H., Nanus, D. M., Milowsky, M. I., Kostakoglu, L., Vallabahajosula, S., and Goldsmith, S. J. (2003). Targeted systemic therapy of prostate cancer with a monoclonal antibody to prostate-specific membrane antigen. *Sem. Oncol.* **30(5)**, 667–676.
9. Hamilton, A., King, S., Liu, H. (1998). A novel humanized antibody against prostate specific membrane antigen (PSMA) for in vivo targeting and therapy. *Proc. Am. Assoc. Cancer Res.* **39**, 440 (abstr).
10. Kellermann, S. A. and Green, L. L. (2002). Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for therapeutics. *Curr. Opin. Biotechnol.* **13(6)**, 593–597.
11. Belldegrun, A., Bander, N. H., Lerner, S. P., Wood, D. P., and Pantuck, A. J. (2001). Society of Urologic Oncology Biotechnology Forum: new approaches and targets for advanced prostate cancer. *J. Urol.* **166(4)**, 1316–1321.

12. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Ann. Rev. Immunol.* **12**, 433–455.
13. DeNardo, S. J., Mirick, G. R., Kroger, L. A., et al. (1994). The biologic window for chimeric L6 radioimmunotherapy. *Cancer* **73(3 Suppl.)**, 1023–1032.
14. O'Donnell, R. T. DeNardo, S. J. Shi, X. B., et al. (1998). L6 monoclonal antibody binds prostate cancer. *Prostate* **37(2)**, 91–97.
15. Israeli, R. S., Powell, C. T., Fair, W. R., and Heston, W. D. (1993). Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.* **53(2)**, 227–230.
16. Pinto, J. T., Suffoletto, B. P., Berzin, T. M., et al. (1996). Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.* **2(9)**, 1445–1451.
17. Carter, R. E., Feldman, A. R., and Coyle, J. T. (1996). Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc. Natl. Acad. Sci. USA* **93(2)**, 749–753.
18. Luthi-Carter, R., Barczak, A. K., Speno, H., and Coyle, J. T. (1998). Molecular characterization of human brain N-acetylated alpha-linked acidic dipeptidase (NAALADase). *J. Pharmacol. Exp. Ther.* **286(2)**, 1020–1025.
19. O'Keefe, D. S., Su, S. L., Bacich, D. J., et al. (1998). Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim. Biophys. Acta.* **1443(1–2)**, 113–127.
20. Horoszewicz, J. S., Kawinski, E., and Murphy, G. P. (1987). Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res.* **7(5B)**, 927–935.
21. Sweat, S. D., Pacelli, A., Murphy, G. P., and Bostwick, D. G. (1998). Prostate-specific membrane antigen expression is greatest in prostate adenocarcinoma and lymph node metastases. *Urology* **52(4)**, 637–640.
22. Bostwick, D. G., Pacelli, A., Blute, M., Roche, P., and Murphy, G. P. (1998). Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. *Cancer* **82(11)**, 2256–2261.
23. Troyer, J. K., Beckett, M. L., and Wright, G. L., Jr. (1995). Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int. J. Cancer* **62(5)**, 552–558.
24. Liu, H., Moy, P., Kim, S., et al. (1997). Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. *Cancer Res.* **57(17)**, 3629–3634.
25. Smith-Jones, P. M., Vallabhajosula, S., Navarro, V., Bastidas, D., Goldsmith, S. J., and Bander, N. H. (2003). Radiolabeled monoclonal antibodies specific to the extracellular domain of prostate-specific membrane antigen: preclinical studies in nude mice bearing LNCaP human prostate tumor. *J. Nucl. Med.* **44(4)**, 610–617.
26. Grauer, L. S., Lawler, K. D., Marignac, J. L., Kumar, A., Goel, A. S., and Wolfert, R. L. (1998). Identification, purification, and subcellular localization of prostate-specific membrane antigen PSM' protein in the LNCaP prostatic carcinoma cell line. *Cancer Res.* **58(21)**, 4787–4789.
27. Murphy, G. P., Greene, T. G., Tino, W. T., Boynton, A. L., and Holmes, E. H. (1998). Isolation and characterization of monoclonal antibodies specific for the extracellular domain of prostate specific membrane antigen. *J. Urol.* **160(6 Pt 2)**, 2396–2401.
28. Smith-Jones, P. M., Vallabhajosula, S., Navarro, V., Bastidas, D., Goldsmith, S. J., and Bander, N. H. (2003). Radiolabeled monoclonal antibodies specific to the extracellular domain of prostate-specific membrane antigen: preclinical studies in nude mice bearing LNCaP human prostate tumor. *J. Nucl. Med.* **44(4)**, 610–617.
29. Vallabhajosula, S., Smith-Jones, P. M., Navarro, V., Goldsmith, S. J., and Bander, N. H. (2004). Radioimmunotherapy of prostate cancer in human xenografts using monoclonal antibodies specific to prostate specific membrane antigen (PSMA): studies in nude mice. *Prostate* **58(2)**, 145–145.
30. Bander, N. H., Nanus, D., Goldsmith, S., et al. (2000). Phase I trial of humanized monoclonal antibody (mAb) to prostate specific membrane antigen/extracellular domain (PSMAext). *Proc. Am. Soc. Clin. Oncol.* **19**, 477a (abstr 1872).
31. Milowsky, M. I., Nanus, D. M., Kostakoglu, L., Vallabhajosula, S., Goldsmith, S. J., and Bander, N. H. (2004). Phase I trial of yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer. *J. Clin. Oncol.* **22(13)**, 2522–2531.
32. Bander, N. H., Milowsky, M. I., Nanus, D., Kostakoglu, L., Vallabhajosula, S., Goldsmith, S. (2005). Phase 1 trial of 177Lutetium-labeled J591, a monoclonal antibody to prostate specific membrane antigen, in patients with androgen-independent prostate cancer. *J. Clin. Oncol.* **23(21)**, 4591–4601.
33. Vallabhajosula, S., Goldsmith, S. J., Hamacher, K. A., et al. (2005). Prediction of myelotoxicity based on bone marrow radiation-absorbed dose: radioimmunotherapy studies using 90Y- and 177Lu-labeled J591 antibodies specific for prostate-specific membrane antigen. *J. Nucl. Med.* **46(5)**, 850–858.
34. Gong, M. C., Latouche, J. B., Krause, A., Heston, W. D., Bander, N. H., and Sadelain, M. (1999). Cancer patient T cells genetically targeted to prostate-specific membrane antigen specifically lyse prostate cancer cells and release cytokines in response to prostate-specific membrane antigen. *Neoplasia (New York)* **1(2)**, 123–127.
35. O'Donnell, R. T., DeNardo, S. J., DeNardo, G. L., et al. (2000). Efficacy and toxicity of radioimmunotherapy with (90)Y-DOTA-peptide-ChL6 for PC3-tumored mice. *Prostate* **44(3)**, 187–192.
36. Ross, J. S., Schenkein, D. P., Pietrusko, R., et al. (2004). Targeted therapies for cancer 2004. *Am. J. Clin. Pathol.* **122(4)**, 598–609.

37. http://www.clinicaltrials.gov/show/NCT00061126.
38. http://www.abgenix.com/documents/6-AACR2002%20prostate.pdf.
39. Ziada, A., Barqawi, A., Glode, L. M., et al. (2004). The use of trastuzumab in the treatment of hormone refractory prostate cancer; phase II trial. *Prostate* **60(4)**, 332–337.
40. Small, E. J., Bok, R., Reese, D. M., Sudilovsky, D., and Frohlich, M. (2001). Docetaxel, estramustine, plus trastuzumab in patients with metastatic androgen-independent prostate cancer. *Sem. Oncol.* **28(4 Suppl. 15)**, 71–76.
41. McCarthy, M. (2003). Antiangiogenesis drug promising for metastatic colorectal cancer. *Lancet* **361(9373)**, 1959.
42. Sparano, J. A., Gray, R., Giantonio, B., O'Dwyer, P., and Comis, R. L. (2004). Eastern Cooperative Oncology Group Portfolio of Clinical Trials. Evaluating antiangiogenesis agents in the clinic: the Eastern Cooperative Oncology Group Portfolio of Clinical Trials. *Clin. Cancer Res.* **10(4)**, 1206–1211.
43. http://www.clinicaltrials.gov/ct/show/NCT00091364?order=1.
44. Phan, G. Q., Yang, J. C., Sherry, R. M., et al. (2003). Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. USA* **100(14)**, 8372–8377.
45. http://www.asco.org/ac/1,1003,_12-002511-00_18-0016-00_19-002905,00.asp.
46. http://www.clinicaltrials.gov/ct/show/NCT00064129?order=12.
47. Hurwitz, A. A., Foster, B. A., Kwon, E. D., et al. (2000). Combination immunotherapy of primary prostate cancer in a transgenic mouse model using CTLA-4 blockade. *Cancer Res.* **60(9)**, 2444–2448.
48. Rajasekaran, S. A., Anilkumar, G., Oshima, E., et al. (2003). A novel cytoplasmic tail MXXXL motif mediates the internalization of prostate-specific membrane antigen. *Mol. Biol. Cell.* **14(12)**, 4835–4845.
49. DeNardo, S. J., DeNardo, G. L., Yuan, A., et al. (2003). Enhanced therapeutic index of radioimmunotherapy (RIT) in prostate cancer patients: comparison of radiation dosimetry for 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA)-peptide versus 2IT-DOTA monoclonal antibody linkage for RIT. *Clin. Cancer Res.* **9(10 Pt 2)**, 3938S–3944S.
50. O'Donnell, R. T., DeNardo, S. J., Miers, L. A., et al. (2002). Combined modality radioimmunotherapy for human prostate cancer xenografts with taxanes and 90yttrium-DOTA-peptide-ChL6. *Prostate* **50(1)**, 27–37.
51. Meredith, R. F., Bueschen, A. J., Khazaeli, M. B., et al. (1994). Treatment of metastatic prostate carcinoma with radiolabeled antibody CC49. *J. Nucl. Med.* **35(6)**, 1017–1022.
52. Meredith, R. F., Khazaeli, M. B., Macey, D. J., et al. (1999). Phase II study of interferon-enhanced 131I-labeled high affinity CC49 monoclonal antibody therapy in patients with metastatic prostate cancer. *Clin. Cancer Res.* **5(10 Suppl.)**, 3254s–3258s.
53. Slovin, S. F., Scher, H. I., Divgi, C. R., et al. (1998). Interferon-gamma and monoclonal antibody 131I-labeled CC49: outcomes in patients with androgen-independent prostate cancer. *Clin. Cancer Res.* **4(3)**, 643–651.
54. Petrylak, D. P., Tangen, C. M., Hussain, M. H., et al. (2004). Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer.[see comment]. *New Engl. J. Med.* **351(15)**, 1513–1520.
55. Shah, R. B., Mehra, R., Chinnaiyan, A. M. et al. (2004). Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **64(24)**, 9209–9216.
56. Davis, M. I., Bennett, M. J., Thomas, L. M., and Bjorkman, P. J. (2005). Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase. *Proc. Natl. Acad. Sci USA* **102**, 5981–5986.
57. Nanus, D. M., Milowsky, M. I., Kostakoglu, L., et al. (2003). Clinical use of monoclonal antibody HuJ591 therapy: targeting prostate specific membrane antigen. *J. Urol.* **170(6 Pt 2)**, S84–88; discussion S88–S89.

22

Differentiation Agents and Epigenomic Therapies

An Overview and Potential Use for Prostate Cancer

Madeleine S. Q. Kortenhorst, Michael A. Carducci, and Shabana Shabbeer

Summary

The role of epigenetics in the development and treatment of cancer continues to gain interest. Although the field of epigenetics as a therapeutic target is in its infancy, discoveries and new agents targeting epigenetics are being translated into the clinic at remarkable speed. Histone deacetylase inhibitors (HDACIs), one class of agents targeting epigenetic changes, are in phase I trials and are moving to phase II trials for several types of cancer, including prostate cancer. DNA methyl transferase inhibitors, such as 5-azacytidine, have undergone limited testing in prostate cancer. In this chapter, a summary of epigenetic targets and novel agents in general and as it relates to prostate cancer is provided.

Subjects to be covered include chromatin remodeling, chromatin modification, histone code, and effects of HDACIs and DNA methyl transferase inhibitors in laboratory and in clinical studies, with a focus on prostate cancer models and patients.

Key Words: Acetylation; chromatin modification; chromatin remodeling; clinical trials; epigenetics; histone acetyl transferase; histone code; histone deacetylase; histone deacetylase inhibitor; methylation; methyl transferase inhibitor.

1. INTRODUCTION: EPIGENETICS MAKE THEIR MARK

The development of cancer by inappropriate cell proliferation and/or altered patterns of cell death has been associated with a myriad of genetic and nongenetic factors, such as age *(1,2)*; diet *(3)*; environmental factors, such as chemical *(4)* and sun exposure *(5)*; hereditary predisposition *(6)*; and familial susceptibility *(7,8)*. In recent years, the role of epigenetics in the development and treatment of cancer has gained interest and the effects of internal and external factors on the epigenetic profile are under investigation. The term "epigenetics" refers to modifications that influence phenotype without altering genotype. Epigenetic changes are potentially reversible, but generally stably maintained during the cell cycle. Since Feinberg *et al*. described differences in DNA methylation in human cancer in 1983 *(9)*, several mechanisms of epigenetic control have been identified, such as DNA methylation and histone modification, including acetylation, methylation, and phosphorylation. Epigenetic abnormalities can also be acquired during fetal development and during the course of life, contributing to common cancer risk in adults *(10)*.

Recently Fraga *et al*. reported that although monozygotic twins are epigenetically identical during the first years of life, the patterns of global and locus-specific epigenetic modifications and gene expression patterns in monozygotic twin pairs diverge as they become older *(11)*. These differences could be explained by the influence of external factors, such as smoking, physical activity, and diet

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

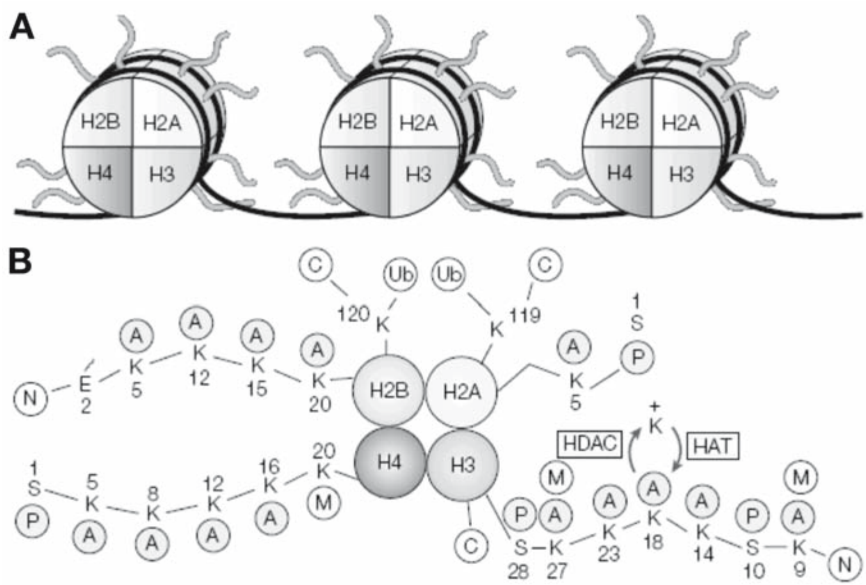


Fig. 1. Schematic structure of histones in nucleosomes. **(A)** The core proteins of nucleosomes are designated histone H-2A, H2B, H3, and H4. Each histone is present in two copies, so the DNA (*black*) wraps around an octamer of histones called the core nucleosome. **(B)** The amino-terminal tails of core histones, lysines (K) in the amino-terminal tails of H2A, H2B, H3, and H4 are potential acetylation/deacetylation sites for histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation neutralizes the charge on lysines. A, acetyl; C, carboxyl terminus; E, glutamic acid; M, methyl; N, amino terminus; P, phosphate; S, serine; Ub, ubiquitin. (Reprinted with permission from ref. *238*).

(12,13), as well as by accumulation of small defects in epigenetic information that could be considered an "epigenetic drift" associated with the aging process *(14)*.

Although the field of epigenetics is in its infancy, discoveries and translation to the clinic have moved at remarkable speed. Drugs targeting epigenetic changes are in phase I trials and moving to phase II trials for several types of histone deacetylase inhibitors (HDACIs). DNA methyl transferase (DNMT) inhibitors were recently approved by the US Food and Drug Administration (FDA) for their clinical benefit in patients with myelodysplastic syndrome. There is, however, only limited preclinical data available for prostate cancer. Nevertheless, the basic understanding of epigenetics and its clinical translation will gradually move forward to a therapeutic role in prostate cancer.

Epigenetic modifications take place posttranslationaly in the nucleosome. The nucleosome core consists of 147-base pairs *(15)* double-stranded DNA wrapped 1.65 times around an octamer of two copies each of histone H-2A, H2B, H3, and H4 protein (Fig. 1). The nucleosome core makes more than 120 direct atomic interactions with the DNA backbone at 14 super helix locations *(16)*. The repeating nucleosome core particles assemble into higher order helices that are stabilized by the linker histone H1 to further condense the chromatin and together make up the nucleosome. Approximately 25% of nucleosome core histones are comprised of amino-terminal tails that protrude into the enveloping DNA double helix *(17)*. Although they are unstructured on a single nucleosome level, these N-terminal tails are thought to mediate interactions with other nucleosomes and chromatin proteins affecting higher order chromatin structure. The highly efficient way of DNA packaging by nucleosome formation compresses the DNA approx 10,000 times, forcing chromatin into a repres-

sive state inaccessible for nuclear processes such as transcription. When Oudet *et al.* provided the first images of repeating uniformly sized particles in DNA, they hypothesized that their observations might have a function in genomic expression. Consistent with these predictions, Boeger *et al.* showed in 2003 that nucleosomes can unfold completely at transcriptionally active promoters *(18)*.

An overview of the dynamic structure and function of chromatin was published by Hansen in 2002 *(19)*.

2. NUCLEOSOME DYNAMICS AND EPIGENETIC MODIFICATIONS

Although DNA packaging is necessary to store the approx 2 m of DNA that make up the human genome in the nucleus, the complex structure of nucleosomes and many interactions with the DNA backbone impairs accessibility to chromatin. Access to chromatin is essential for proteins that regulate biological processes, such as transcription, DNA repair, and replication, to exert their function. To counterbalance the repressive nature of chromatin, a sophisticated mechanism has evolved that regulates chromatin accessibility.

2.1. Chromatin Remodeling and Modification

Unlike assumed previously, nucleosomes have emerged to be highly specialized, serving many functions in the regulation of individual genes and chromosome regions. Although all nucleosomes contain a histone octamer around which DNA is wrapped, chromatin remodeling and chromatin modifying complexes allow nucleosomes to have a specialized and dynamic composition *(20)*.

2.1.1. Chromatin Remodeling Complexes and Chromatin Modifying Complexes

N-terminal histone tails, which protrude from the surface of the chromatin polymer, possess many posttranslational modification sites. These modification sites can be recognized by ATP-dependent nucleosome-remodeling complexes that possess chromatin binding domains that recognize specific modification patterns. ATP-dependent chromatin-remodeling complexes regulate chromatin accessibility using ATP hydrolysis to weaken histone–DNA contacts. At least three ways of creating access to nucleosomal DNA by chromatin remodeling complexes have been identified *(21)*: octamer sliding, DNA looping, and histone substitution *(22–24)*. By weakening DNA contacts, these complexes are able to expose DNA to proteins that, for instance, regulate transcription to reach their target sites. An overview of recent literature on chromatin remodeling complexes is provided by Cosgrove *et al.* *(25)* and Cairns *(20)*.

The ε-amino groups on evolutionarily conserved lysine residues of histones tails can be posttranslationally modified by chromatin-modifying enzymes, by acetylation, methylation, and ubiquitination. Such seemingly small modifications determine not only the structural organization of chromatin, by virtue of their ionic charges, but also attract other proteins that possess modification-specific chromatin binding domains to sites in the chromatin where transcription may be regulated *(26)*.

2.1.2. Chromatin-Binding Domains

As described, both chromatin remodeling and chromatin modification make use of chromatin-binding domains, called protein motifs, which recognize specific modifications on histone tails and nonhistone proteins. Several chromatin-binding domains can be identified. Three domains important in the context of the histone code hypothesis will be discussed next: bromodomains, chromodomains, and SANT so called for the proteins that comprise this group: SWI3, ADA2, N-CoR and TF111B domains.

2.1.2.1. Bromodomains

Bromodomains form an extensive family of small protein domains that preferentially bind acetylated peptides irrespective of the protein to which they belong *(27)*. Bromodomains are widely dis-

tributed among different enzymes, such as chromatin remodeling enzymes that use ATP to modify chromatin structure, and also in subunits of the chromatin remodeling complexes that do not have a catalytic function. In that case, bromodomains mainly help to recognize previously modified chromatin and stabilize the complex *(28)*. Bromodomains have also been found in enzymes that cause methylation and acetylation. Interestingly, because histone acetyltransferases (HATs) acetylate a wide variety of target proteins the presence of a bromodomain on most HATs suggests self-perpetuation through a positive feedback loop *(27)*.

2.1.2.2. Chromodomains

As with bromodomains, chromodomains bind to their target protein, independent of the protein to which they belong. Although their distribution among chromatin modifying enzymes is more restricted, chromodomains have been found in ATP-dependent chromatin remodeling factors, HATs, and also methyl transferases. Chromodomains have been shown to recognize methyl-lysines *(29)*, DNA, and RNA, and point to an involvement in protein–protein interactions *(30)*. Their exact function in the context of gene expression is not yet fully understood.

2.1.2.3. SANT domains

Unlike bromodomains and chromodomains, SANT domains primarily mediate interactions between remodeling complexes and unmodified chromatin substrates through the recruitment of chromatin modifying enzymes and by mediating interactions between histones and enzymes. SANT domains have been shown to be present in several components of complexes containing histone deacetylase (HDAC) or HAT activity; although no HAT or HDAC enzyme itself has been found to possess a SANT domain to date *(27)*. In ATP-dependent chromatin remodeling complexes, SANT domains are broadly present. By direct binding, ATP-dependent chromatin remodeling complexes cause conformation of histone tails, thereby promoting binding of modifying enzymes and subsequent catalytic processes. As suggested by Yu *et al*., the interaction of SANT domains with unacetylated histone tails could block the binding of HATs, thereby maintaining a deacetylated state *(31)*. A review of the unique function of SANT domains was written by Boyer *et al*. in 2004 *(32)*. A more extensive review of the diversity of proteins containing protein motifs and their function was published by de la Cruz *et al*. in 2005 *(27)*.

3. THE HISTONE CODE

In 1993, Turner presented the first evidence that posttranslational modification of histone tails was functionally significant *(33)*. In the years that followed, increasing experimental data provided support for the hypothesis that distinct patterns of covalent histone marks make up a histone “language.” Encoded on histone tail domains and read by other proteins or protein modules, these modifications are thought to determine the transcriptional state of genes. Posttranslational modifications important in the development and progression of cancer include acetylation, methylation, phosphorylation, and ubiquitination. Figure 1 gives a schematic overview of the core nucleosome and important modification sites on amino terminal tails.

In 2000, Strahl and Allis referred for the first time to this language as the “histone code,” defining it as “multiple histone modifications acting in combinatorial or sequential fashion on one or multiple histone tails, specifying unique downstream functions.” *(34)*. An important concept in this hypothesis is that the histone code uses combinations of modifications on each histone and that modifications on different histone tails may be interdependent. It is, therefore, essential that histone modifications are site-specific and that these modifications cause site-specific chromatin modification *(34)*. Recently, two other hypotheses have been added to explain the important functions histone modifications serve. The binary switch model proposes that numerous residues in linear strings of densely modifiable sites can have a large array of different biological readouts by forming cassettes. (In the second model,) [neighboring modifications act together as “binary switches”] *(35)*.

Table 1
Classification of HDAC Subfamilies

Class I	Class II	Class III	Class IV
HDAC1	HDAC4	SIRT1	HDAC11
HDAC2	HDAC5	SIRT2	
HDAC3	HDAC6	SIRT3	
HDAC8	HDAC7	SIRT4	
	HDAC9	SIRT5	
	HDAC10	SIRT6	
		SIRT7	

[a] The classification is based on the homology of human HDACs to yeast HDACs, their subcellular expression, and enzymatic activity. HDAC, histone deacetylase; SIRT, Silent Information Regulator.

4. ACETYLATION

As briefly discussed in 2.1.1., modification of histone tails is an important feature in the regulation of gene expression. Limited by the 20 encoded amino acids available, posttranslational or cotranslational modifications of proteins extends the range of molecular structures and functions possible. Out of the more than 200 covalent modifications that have been reported, acetylation is the most common modification of eukaryotic proteins, affect ing many protein functions, such as transcription, nuclear import, microtubule function, hormone responses, peptide–receptor recognition, DNA binding, and protein–protein interactions. Modification of proteins not only changes the molecular structure of a certain protein, but also provides binding sites for modification-recognizing protein motifs, such as bromodomains. The acetylation status of histones is regulated by the opposing actions of HATs and HDACs. Both HATs and HDACs function as part of multiprotein complexes with other proteins *(36,37)*, or self-associate with each other *(38,39)*.

4.1. HDACs

HDACs may be classified into four subfamilies, (Table 1) *(40)*. The class I HDACs have a molecular weight of 22 to 55 kDa and share homologous catalytic sites. The class II HDACs range from 120 to 135 kDa and, unlike the class I HDACs, exhibit greater diversity within the class. For example, HDAC6 and HDAC10 are characterized by duplicated HDAC catalytic domains, whereas the other members of the same class display only one catalytic domain *(39)*. Class I HDACs are located exclusively in the nucleus, with the exception of HDAC8, which has recently been shown to be predominantly expressed in the cytosol *(41)*. Class II HDACs shuttle between the nucleus and cytoplasm, depending on perceived cellular signals. Class III HDACs, on the other hand, are known as the Sir2-like HDACs, named because of their homology to yeast Sir2 proteins. Although classes I and II are Zn-dependent HDACs, the class III HDACs are Zn-independent and NAD-dependent. The HDAC11-related enzymes, which share the features of class I and II HDACs but may have a distinct physiological role, could potentially constitute a fourth class of HDACs.

Despite the existence of several HDAC subfamilies, the different HDACs are by no means redundant. Robyr *et al.* generated "acetylation maps" by inactivating six different HDACs, demonstrating that only a small degree of functional overlap was present among the different HDACs *(42)*. For example, HDAC5 and HDAC9 are involved in stress response of the heart *(43)*, HDAC2 in apoptosis *(44)*, HDAC1 in modulating the cell cycle, and HDAC8 in smooth muscle contractility *(45)*. The

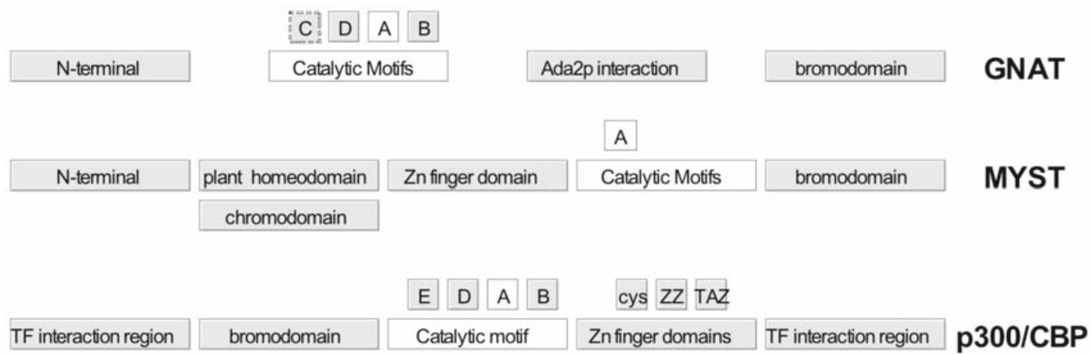


Fig. 2. Functional domains of histone acetyltransferases (HATs). For the function of the different domains, see text. (Modified from ref. *37*).

HDAC activity differs between different types of tissues *(46,47)*. For instance, screening of HDAC expression in human prostate cancer revealed distinct class I HDAC profiles between stromal and epithelial cells *(41)*, and *in vitro* experiments with prostate cancer cell lines showed a marked increase in HDAC level for most HDACs compared with normal prostate tissue *(48)*. Furthermore, different isoforms of HDACs may have their distinct localization and functions *(49)*. Even during embryonic development, the levels of HDACs are continuously changing *(50)*. The targeted disruption of both HDAC1 alleles resulted in severe proliferation defects and retardation in development, leading to embryonic lethality *(51)*. Certain disease states can be characterized by loss or gain of specific or generalized HDAC activity, for example, reduced protein expression of HDAC1 and HDAC2 proteins and decreased enzymatic HDAC activity is observed in asthma patients *(52)*, whereas class II HDACs suppress cardiac hypertrophy *(53)*, and higher expression of HDAC2 and HDAC9 has been reported in many colon cancer cell lines compared with the primary cell from corresponding normal tissue *(44,49)*.

Malignant diseases are exemplified by aberrant transcriptional regulation that may be triggered by increased recruitment of HDACs to the site of transcriptional initiation. Because malignant cells express increased HDAC activity, irrespective of the trigger of such activity, it was thought that inhibitors of HDACs would return this transient aberrant transcription in malignant cells to the transcription status of their normal cell counterpart, while leaving the surrounding normal cells unaltered. With this premise, HDAC inhibitors were investigated for activity and found to be relatively nontoxic to normal cells *in vitro* and *in vivo*, despite the accumulation of acetylation in normal cells as well as tumor cells *(54)*. Successful preclinical studies and development of animal models has resulted in a number of HDACIs being evaluated in human clinical trials (*see* below).

4.2. *Histone Acetyltransferases*

Acetylation of internal lysine residues is facilitated by the action of HATs. By definition, HATs acetylate histone tails by transferring acetyl groups from acetyl coenzyme A (CoA) onto the ε-amino group of conserved lysine residues. However, HATs are also able to acetylate nonhistone proteins and are, therefore, sometimes referred to as factor acetyltransferases. To date, posttranslational acetylation of lysine residues has been shown to occur in histones, high-mobility group proteins, transcription factors, nuclear receptors, and α-tubulin *(55)*. Important in the context of cancer is that posttranslational acetylation of the ε-amino group of lysine residues is known to be reversible, making it an attractive therapeutic target.

Fig. 3. Structural features of hydroxamic acid-based histone deacetylase inhibitors (HDACIs). (Reprinted with permission from ref. *239*).

HATs are composed of several domains, making them substrate- and site-specific under distinct physiological situations. Three superfamilies of HATs can be identified based on their composition: GNAT (Gcn5-related *N*-acetyl transferases) *(56)*, MYST (named after its founding members monocytic leukemia zinc finger [MOZ], Ybf2/Sas3, Sas2, and Tip60) *(57)* and p300/CREB-binding protein (CBP) *(58,59)*.

4.2.1. GNAT Superfamily

The GNAT superfamily acetylates a distinct subset of genes. All GNAT enzymes contain four functional domains (Fig. 2), an amino terminal, a catalytic motif domain, an Ada2p interaction domain, and a carboxy-terminal bromodomain. The amino terminal is variable in length and is thought to facilitate recognition of nucleosomal substrates *(60)*. The catalytic motif domain actually contains up to four conserved motifs, labeled A to D, of which motif A, the highly conserved acetyl-CoA binding site, is common to HATs from all superfamilies and essential for HAT activity *(61)* (Fig. 2). The Ada2p interaction domain enables GNATs to acetylate physiologically relevant nucleosomal substrates *in vivo*. Ada2 proteins interact with DNA-bound transcriptional activators *(61)*. The C-terminal bromodomain binds to acetylated lysine residues facilitating protein–protein interactions. Secondly, the bromodomain is able to affect HAT activity by autoacetylation, causing the HAT to fold into an inactive state *(62)*.

4.2.2. MYST Superfamily

Besides an amino-terminal tail, an acetyltransferase domain A, and a C-terminal domain, MYST superfamily HATs sometimes contain zinc finger domains and a chromodomain. The function of the chromodomain in the context of HATs is unclear. It may be likely that chromodomains serve as chromatin-targeting modules for the MYST family, similar to the bromodomains of the GNAT family (Fig. 2).

4.2.3. p300/CBP Superfamily

Contrary to the GNAT super family, the p300/CBP superfamily generally acts as a global transcriptional activator. p300 and CBP are two of the most widely studied HATs in the context of transcriptional regulation. They are largely interchangeable in function and are, therefore, often referred to as p300/CBP. As coactivators for transcription, p300/CBP is particularly recruited to promoters by DNA-bound transcription factors that need p300/CBP to function in transcriptional activation. p300/CBP contain at least two independent regions for interaction with multiple transcription factors, such as c-Jun *(63)* and nuclear hormone receptors *(64)*. Furthermore, p300/CBP contains a bromodomain, three zinc finger regions (cys, ZZ, and TAZ domains), and a HAT domain containing a p300/CBP-specific E motif (Fig. 2). The distribution of p300/CBP among the different target proteins might provide a mechanism for integrating several signaling and transcription response pathways because p300/CBP is present in limited amounts in the cell *(64)*. Research on HATs to date is basic and preclinical, with limited, if any, clinical candidates. HATS have been included in this chapter to

highlight their potential as a therapeutic target. A detailed review of HATs in 2001 was published by Roth *et al. (37)*.

5. EPIGENOMICS AND DIFFERENTIATION THERAPY

Differentiation therapy and especially epigenomic therapy are promising approaches in the treatment of cancer, including prostate cancer. Among the best studied differentiating agents in prostate cancer are retinoids, vitamin D, peroxisome proliferator-activated receptor-γ ligands, and, most recently, the epigenetic modifiers, HDACIs and DNA methyltransferase inhibitors. The first edition of this book focused on differentiating agents such as retinoids. This edition focuses on the newer differentiating agents, mainly HDACIs, and only briefly cover the DNMT Is because the latter are extensively discussed in Chapter 5.

5.1. DNMT Inhibitors in the Preclinical and Clinical Settings

It is important to emphasize that histone modifications are not the only determinants of transcriptional regulation. They are one layer of modification, mostly transient, which may be preceded or followed by changes that are more permanent on the chromatin, such as DNA methylation *(65)*. Methylated DNA in the promoter regions recruits HDACs in addition to other repressor proteins, which act in concert to inhibit transcription initiation *(66)*. Furthermore, DNA methylation may override any amount of deacetylation inhibition *(67)*. It has long been known that cancer cells are characterized by genome-wide hypomethylation and promoter-specific hypermethylation (reviewed in ref. 65). In malignant prostate cancer cells, the most widely reported methylated gene is the *GSTPi* gene *(68)*. Because of such a cancer promoter-specific methylation, DNA methylation apparently seems to be an attractive therapeutic target. However, known demethylating drugs cannot control the chromosomal site of demethylation. *In vitro* studies have demonstrated substantial genome-wide demethylation in mice carrying a hypomorphic *DNMT1* allele, which reduces DNMT1 expression to 10% of wild-type levels *(69)*. These mice developed aggressive T-cell lymphoma. Hence, caution must be exercised in using drugs that alter methylation status.

The most commonly used DNMT Is are 5-azacytidine (5AC), a cell-cycle specific ring analog of the pyrimidine nucleoside cytosine, and its deoxynucleotide analog, 5-aza-2-deoxycytidine (DAC), also known as decitabine. Besides 5AC and DAC, Zebularine *(70)*, Procainamide *(71)*, and EGCG *(72)* have been evaluated for their DNMT inhibitory potential in preclinical studies. Procainamide was used in a mouse xenograft model of prostate cancer and shown to inhibit growth of the xenograft with concomitant reexpression of the silenced *GSTPi* gene *(73)*. Treatment of human esophageal cancer KYSE 510 cells with 5 to 50 μ*M* of EGCG for 12 to 144 hours caused a concentration- and time-dependent reversal of hypermethylation of p16 (ink4a), *RAR*β, *MGMT*, and *hMLH1* genes, as determined by the appearance of the unmethylated specific bands in PCR. A combination of DNMTIs and HDAC Is caused a synergistic reduction in murine lung cancer *(74)*.

The first experimental evidence for a role for DNA modification in differentiation was shown by Jones and Taylor in 1980 *(75)*. Cytidine analogs containing an altered 5 position perturbed previously established methylation patterns to yield new cellular phenotypes in cultured mouse embryo cells. In contrast, nucleoside analogs without this modification either did not induce or were poor inducers of muscle cell differentiation and poor inhibitors of DNA methylation. Before that discovery, 5AC had been tested in clinical trials and demonstrated to have consistent antitumor activity in patients with acute myelogenous leukemia resistant to previous treatment by mechanisms other than its demethylating or differentiating properties *(76)* (summarized in Table 2). However, there were setbacks; an early European Organization for Research and Therapy of Cancer (EORTC) trial, for example, failed in causing any response in patients when using DNMT Is *(77)*. Because of the cytoxicity associated with high doses of this drug, interest in this therapy declined. It was only later, when its DNMT inhibitory potential was discovered, that renewed interest led to additional clinical

Table 2
Clinical Trials of DNA Methyl Transferase Inhibitors[a]

DNMTI (tumor Type)	Schedule	Outcome	Adverse events	Reference
5AC (RAEB, RAEB-T) Phase II	75 mg/m^2/d continuous iv, 7 d every 4 wk	21 of evaluable 43 patients (49%): 5 (12%) CR, 11 (25%) PR, 5 (12%) improved. A trilineage improvement (CR and PR) occurred in 37% of the patients. Median survival for all patients: 13.3 mo. Median duration of remission for those with CR and PR: 14.7 mo Mild to moderate nausea and/or vomiting was the most common side effect (63%)	Myelosuppression, either bone marrow hypoplasia or drug related cytopenias requiring a reduction in the dose of 5AC, occurred in 33% of the patients	*78*
5AC (MDS) Phase III	75 mg/m^2/d subcutaneously, 7 d every 4 wk	60% of patients on the 5AC arm (7% CR, 16% PR, 37% improved) compared with 5% (improved) receiving supportive care. Median time to leukemic transformation or death: 21 mo for 5AC vs 13 mo for supportive care	Not reported	*79*
DAC (hormone refractory CaP) Phase II	75 mg/m^2/8 h iv in 1 h infusion, three doses every 5–8 wk	2 African American patients of 14 included men with progressive, metastatic CaP (recurrent after total androgen blockage and flutamide with drawal) had stable disease for more than 10 wk	Toxicity was similar to previously reported experience	*154*

(Continued on next page)

Table 2 *(Continued)*
Clinical Trials of DNA Methyl Transferase Inhibitors[a]

DNMTI (tumor Type)	Schedule	Outcome	Adverse events	Reference
MG98 (solid tumors)	240 mg/m^2 iv in 2-h infusion, 2 d/wk, 3 of 4 wk	Tumor samples demonstrated general demethylation post-MG98 treatment, including genes implicated in head and neck cancer, such as *PTEN* and *CDH1*. In PBMC samples and adjacent normal tissues, no systematic methylation pattern changes were observed	Not reported	*155*
MG98 (advanced solid tumors) Phase II	100, 125, 160, 200, and 250 mg/m^2/d continuous iv, 7 d every other wk	Trial is ongoing (n = 23 to date). Median PS: treatment DNMT1 expression in PBL decreased by between 6 and 69% on cycle 1, and between 34 and 85% on cycle 2. SD: 2 patients (esophageal and GIST) > 4 mo. Recommended phase II dose: 200 mg/m^2/d	DLT: 200mg/m^2/d (n = 1, G3 transaminitis) MTD: <250 mg/m^2/d (2/4 patients: G3 transaminitis, G3 thrombocytopenia) Other AEs: fatigue, headache, nausea, anorexia, and constipation (all G2)	*156*
MG98 (advanced solid tumors) Phase I	40, 80, 160, and 240 mg/m^2/d continuous iv infusion for 21 d every 4 wk	Not available yet	DLT: G3 transminalase elevation (2/2 patients at 240 mg/m^2/d) Other AEs (G1–2): fever, sweats, fatigue, transient rise in PTT, thrombocytopenia	*157*

[a]DNMTI, DNA methyl transferase inhibitors; 5AC, 5-aza-cytidine; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation; iv, intravenous; CR, complete response; PR, partial response; MDS, myelodysplastic syndrome; DAC, 5-aza-2'-deoxycytidine; CaP, prostate cancer; PBMC, peripheral blood mononuclear cells; PS, performance status; PBL, peripheral blood lymphocytes; SD, stable disease; GIST, gastrointestinal stromal tumor; DLT, dose-limiting toxicity; MTD, maximum tolerated dose; AE, adverse event; PTT, protrombin time.

trials. Similar to HDACIs, DNMT Is have shown greater efficacy in treating blood cancers than solid cancers. In fact, 5AC was approved by the FDA in 2004 to treat myelodysplastic syndrome. Investigators in prostate cancer may learn from studies led by Silverman et al. *(78,79)* at Mt. Sinai and the Cancer and Leukemia Group B (CALGB) study using lower doses of 5AC, initially intravenously and subsequently subcutaneously, to arrive at the most effective schedule (summarized in Table 2). Our prostate cancer program has recently completed a Phase I study of 5AC in patients with refractory solid tumors across a range of doses. Although no tumor responses were seen in the 27 patients treated, inhibition of DNMT activity was noted in a subset of patients *(80)*.

6. HISTONE DEACETYLASE INHIBITORS

A number of HDACIs are now known to selectively block the catalytic site of class I and II HDACs, leading to maintenance of acetylation of target sites. Research on hydroxamic acid based HDACIs has identified structural features common to most HDACI that are responsible for their inhibitory function on HDACs *(81)* (Fig. 3). Treatment of cancer cells with HDACIs upregulates as well as downregulates a variety of genes, leading to growth arrest, differentiation, and apoptosis. Several mechanisms have been identified to explain these effects.

6.1. Classes of HDACIs

The microbial toxin Trichostatin A (TSA) was an early compound that was identified to possess HDAC inhibitory activity. After establishing its efficacy as an HDACI, TSA was used to model other HDACIs (Table 3), such as suberoylanilide hydroxamic acid (SAHA) and m-carboxycinnamic acid *bis*-hydroxamide (CBHA; Memorial Sloan-Kettering Cancer Center) *(82)*, which, in turn, served as a template for PXD-101 (Prolifix Ltd/CuraGen Corp;) and LAQ-824 (Novartis AG). Another natural compound to be discovered as a noncompetitive inhibitor of HDAC was sodium butyrate (SB) *(83)*. Similar to other HDACIs of the same structural type, the clinical relevance of SB was limited by its short half-life and low potency. However, several improved derivatives of SB were later characterized and screened, including the drugs phenyl butyrate (PB) (Table 3), phenyl acetate (which is the β-oxidation product of PB in vivo), valproic acid, and AN-9 (pivaloyloxymethyl butyrate, Titan Pharmaceuticals Inc.) (Table 3), the prodrug form of butanoic acid *(84)*.

The rate of development of newer HDACIs has greatly increased since the elucidation of the HDACI–HDAC interaction *(81)*. The ability of HDACIs to chelate zinc bound to the active site of the HDAC seemed to be crucial to maximum inhibitory activity, as exemplified by the general structure illustrated in Fig. 3, which is based on SAHA. This explains why HDACIs, such as the aliphatic acids, which lack a zinc-binding moiety, required higher concentrations (millimolar) of drug for activity compared with the nanomolar quantities of drugs of the hydroxamic acid type. Because of this research, a novel class of HDACIs with a Zn^{2+}-chelating motif tethered to short-chain fatty acids has been synthesized *(85)*.

Cyclic peptides, such as FK-228 (National Cancer Institute/Gloucester Pharmaceuticals Inc.) (Table 3), which do not possess the characteristic structure outlined in Fig. 3, also actively inhibit HDAC at nanomolar concentrations. Research conducted by Furumai *et al.* helped to decipher the mechanism of action of such compounds *(86)*. Cellular reducing activity converts FK-228 to its active, reduced form (redFK), which possesses a functional sulfhydryl group capable of interacting with the zinc in the active-site pocket of HDACs. Because FK-228 is more stable than redFK in medium and serum, FK-228 may be the natural prodrug activated after incorporation into the cells. Newer HDACIs that are being developed more recently belong to the class of electrophilic ketones and include various trifluoromethyl ketones and α-ketoamides and active at micromolar concentrations *(87,88)*. Special attention should also be given to the emergence of HDAC-specific HDACIs, such as Tubacin, an HDAC6-specific HDACI *(89)*, and MGCD0103, an HDAC1- to HDAC3-specific HDACI.

Table 3
Types of HDAC Inhibitors[a] With Proven Efficacy *In Vitro* and/or *In Vivo*

Class (concentration)	Compound	Structure	HDAC isoform selectivity	Reference
Hydroxamate (nM)	Trichostatin A (TSA)		Not selective for a particular HDAC *(158)*; Acetylates α-tubulin *(89)*	*159*
	Suberoylanilide hydroxamic acid (SAHA)		Inhibits HDAC1 *(160)*; Acetylates α-tubulin *(89)*	*82*
	Pyroxamide		Potent inhibitor of affinity-purified HDAC1 [ID(50) = 100 nM] *(132,161)*	*132*
	m-carboxycinnamic acid bishydroxamide (CBHA)		Inhibits HDAC1 and 3 in vitro *(161)*	*82*
	PXD-101		Unknown HDAC specificity	*131*
	LAQ-824		Unknown HDAC specificity	*162,163*
	Scriptaid		Unknown HDAC specificity	*164*
	Oxamflatin		Unknown HDAC specificity	*165*
Cyclic peptides (nM)	Depsipeptide (FK-228)		Preferentially inhibit HDAC1 and 2 over HDAC4 and 6 *(86,166,167)*; Acetylates chaperone protein Hsp90 *(123)*.	*168*

	Apicidin		Unknown HDAC specificity. Slow dissociation of Enzyme-inhibitor complex makes it an irreversible inhibitor *(169)*	*170*
	Trapoxin-hydroxamic acid (TPX-HA) analog; cyclic hydroxamic acid-containing peptide (CHAP)		Trapoxin is an irreversible inhibitor of HDAC *(171,172)*	*166*
Benzamides (µM)	MS-275		Preferentially inhibit HDAC1,2,4, and 6 over HDAC3 *(173)*	*174*
	CI-994 [N-acetylamide]		In assays of isolated enzymes, CI-994 inhibited HDAC-1 and HDAC-2 *(175)*	*176*
	MGCD0103		Inhibits HDAC1, HDAC2 and HDAC3	Unpublished data
Aliphatic acid (mM)	Sodium butyrate		Unknown HDAC specificity	*83*
	Phenyl butyrate		Unknown HDAC specificity	*99*
	Valproic acid		Unknown HDAC specificity	*177*

[a] Classified on the basis of their structural characteristics.

6.1.1. Mechanisms of Action

By changing the acetylation status of histone tails, HDACIs modify the histone code leading to changes in gene expression *(34)*. By accumulating acetylated histones, HDACIs relax chromatin structure enhancing the accessibility of the transcription machinery to DNA, and leading to increased transcription *(26)*. The accumulation of acetylated histones may also affect cell cycle progression. By inhibition of chromatin separation, the ability of tumor cells to undergo mitosis is altered *(90)*. Additionally, HDACIs specifically affect the cell cycle of tumor cells by targeting cells with deficient cell cycle checkpoint controls, causing apoptosis *(91)*. Generation of reactive oxygen species (ROS) also plays a role in HDACI-induced cell death. In a study published in 2005, Ruefli *et al.* showed that SAHA-induced Bid cleavage and disruption of the mitochondrial membrane lead to cell death in the absence of activation of key caspases *(92)*. Recent work by Xu *et al.* suggests that antitumor effects of SAHA in treated cancer cells is achieved in part by induction of polyploidy followed by senescence *(93)*.

6.2. The Cellular and Biological Effects of HDACIs

HDACIs demonstrate pleiotropic effects on cells in culture or *in vivo*. The reasons behind this are multifactorial and include cell type, dose, and duration of treatment with HDACIs, and whether used alone or in combination with other agents.

6.3. Activity In Vitro of HDACIs

TSA was evaluated for its biological activity in a variety of mammalian cell lines, and was demonstrated to prolong the half-life of acetylated histones in mouse mammary gland tumor cells *(94)*. Pulse–chase experiments revealed that histone hyperacetylation induced by TSA was not caused by increased acetylation but was a result of decreased deacetylation of histones. Many mammalian cell lines have since been investigated for their sensitivity to various HDACIs. Examples include differentiation by TSA in breast cancer cell lines *(95)*, apoptosis in endometrial cancer cell lines *(96)* and hepatoma cells *(97)*, and apoptosis of neuroblastoma cell lines by CBHA either administered alone or in combination with retinoic acid *(98)*. Without testing its ability to act as an HDACI, Carducci *et al.* demonstrated the pro-apoptotic effect of PB on prostate cancer cell lines *(99)*. Later, Butler *et al.* demonstrated the antiproliferative effect of SAHA on prostate cancer cell lines and simultaneously demonstrated the HDACI activity by detecting the presence of acetylated histones *(100)*.

6.3.1. Cell Cycle Effects

HDACIs arrest growth in different phases of the cell cycle in different cell lines *(101)*. All HDACIs, with the exception of tubacin, exert a dose-dependent effect on growth arrest in G_1 *(89)*. The arrest is mediated by the p53-independent induction of $p21^{CIP1/WAF1}$ *(102,103)*, loss of activity of Cdks *(104)* and transcriptional inactivation of the enzymes CTP synthase and thymidylate synthetase, which are responsible for DNA synthesis in the S phase *(45)*. G_2 arrest has been detected in comparatively fewer cell lines and requires a higher dose of HDACIs compared with G_1 arrest *(104,105)*. However, higher doses of HDACIs induce apoptosis of cancer cells. Research by Qiu *et al.* and Warrener *et al.* emphasized this dilemma by highlighting the role of checkpoints in cell cycle progression *(91,101)*. Cells exhibiting an intact G_2 checkpoint were growth-arrested on treatment with HDACIs, whereas cells possessing a defective G_2 checkpoint undergo apoptosis within hours of mitotic exit *(91)*.

6.3.2. Induction of Apoptosis

Treatment of cells with HDACIs can induce or repress genes to cause cell cycle arrest. HDACIs can also increase expression of pro-apoptotic genes, such as *Bak*, *Bax*, *CD95*, CD95 ligand, gel-

solin, *p53*, *GADD45*-β, *DRAK1*, *Apaf-1*, *DFF45*-α, caspase-9, caspase-8, caspase-3, *Bim*, *Bid*, and *Bad*, and decrease expression of anti-apoptotic genes, including *Bcl-2*, *Bcl-xL*, *c-FLIP*, survivin, *XIAP*, *Mcl-1*, and *NF*-κ*B* *(106–113)*. Apoptosis, mediated by varied pathways, was induced in many cell lines treated with HDACIs as a function of dose and duration of incubation. In U937 human leukemia cells, SAHA induced differentiation at low doses (less than micromolar), but, at higher doses, the drug caused apoptosis by triggering mitochondrial injury *(103)*. The apoptotic effect of HDACIs is also cell line specific, as demonstrated by the analysis of a large panel of HDACI-sensitive cell lines, in which selected cell lines required up to 10-fold lower concentrations of HDACIs to achieve apoptosis *(105)*. Cancer cells were revealed to be 10-fold more sensitive to hydroxamates than normal fibroblasts, possibly because of the loss of the G_2 checkpoint, a feature exploited by HDACIs to selectively kill proliferating and nonproliferating tumor cells but not normal cells *(114)*.

Recently, apoptosis has been reported to be induced by generation of ROS by various HDACIs (reviewed in ref. *115*). ROS production leads to activation of the caspase cascade and degradation of critical proteins such as $p21^{CIP1/WAF1}$, $p27^{KIP}$, Bcl-2, and pRb. Proof of this was established by administration of the free radical scavenger L-*N*-acetylcysteine, which blocked MS-275 (Schering AG)-mediated mitochondrial injury and apoptosis *(116)*. In acute T-cell leukemic cell lines, SAHA induced a cell-death pathway acting via cleavage of Bid and production of ROS *(92)*. Conversely, SB, which induces apoptosis independently of ROS generation or activation of the extrinsic pathway, prompted degradation of the anti-apoptotic proteins Bcl-2 and $p21^{CIP1/WAF1}$ in human leukemia cell lines *(117)*. Induction of $p21^{CIP1/WAF1}$ was also observed in breast cancer cell lines co-treated with SB and tumor necrosis factor-α, tumor necrosis factor-related apoptosis inducing ligand, or an antibody to FAS agonist *(118)*.

6.3.3. Gene Expression Affected by HDAC Inhibition

Gene expression profiles of T24 bladder and MDA breast carcinoma cells treated with TSA or other HDACIs were studied to define a common set of genes that are induced or repressed by HDAC inhibition *(119)*. Examples of genes induced and repressed are provided in Table 4. Data from our own laboratory confirm that prostate cancer cell lines treated with HDACIs show comparable gene expression patterns (data not published).

6.3.4. Acetylation of Nonhistone Proteins

Phylogenetic studies have proven that the evolution of HDACs predates the evolution of histones, indicating that there are nonhistone substrates of HDACs *(40)*. p53 is one example of a nonhistone protein that is maintained in the acetylated state by HDACIs *(120,121)*. Other examples include the transcriptional repressor Bcl-6 *(122)*, the 90-kDa heat shock protein (Hsp90) *(123)*, and tubulin *(89)*. Hyperacetylation of Ku70 protein on treatment with HDACIs results in release of Bax, which may provide one of the underlying mechanisms behind apoptosis *(124)*.

6.4. Activity In Vivo

The antitumor activity of HDACIs was demonstrated in several *in vivo* models of cancer, including human xenografts. Significant reduction in tumor volume of breast cancer xenografts and lung metastasis was observed in animals treated with valproic acid *(125)*. MS-275, pyroxamide (Aton Phama/National Cancer Institute), and SAHA have also exhibited antitumor activity in many cancer cell lines *in vivo (100,126)*. In addition to their role in direct growth arrest, death, and differentiation of tumor cells *in vivo*, HDACIs act as chemopreventive agents to inhibit tumor growth by preventing neovascularization of the tumor, thus, exerting an anti-angiogenesis function. TSA, SAHA, FK-228, and LAQ-824 have all demonstrated an anti-angiogenic effect *in vivo (127–129)*, and, recently, the chemopreventive sulforaphane has also indicated HDACI activity *(130)*.

Table 4
Genes Induced or Repressed by HDACIs [a]

Induced genes	Repressed genes
• *p21*$^{CIP1/WAF1}$ *(102)*, and *p27*^{KIP1} *(178)*	• Thymidylate synthetase *(119)*
• Retinoic acid receptor-β (*RAR*β) *(179)*	• CTP synthetase *(119)*
• Estrogen receptor *(180)*	• Genes controlled by signal transducer and activator of transcription-5 (*STAT5*) *(181)*
• TRAIL cell surface death receptors: *TRAIL-R1*/*DR4* and *TRAIL-R2*/*DR5 (111)*	• *Bcr–Abl* fusion gene *(182,183)*
• Semaphorin III, a vascular endothelial cell growth factor (VEGF) competitor *(127)*	• VEGF receptors *VEGFR-1* and *VEGFR-2*, and neuropilin-1 *(127,129)*
• α-Fucosidase *(119)*	• Inactivation of the heat shock protein-90 (*Hsp90*) molecular chaperone leading to mutant and wild-type androgen receptor depletion; also, other Hsp90 client proteins, HER-2 (ErbB2), Akt/PKB, and Raf-1 *(184)*
• Histone H2B *(119)*	• Lipopolysaccharide (LPS)-induced inflammatory cytokines *TNF*-α, interleukin (*IL*)-*1*β, *IL-6*, and interferon (*IFN*)-γ *(185)*
• α-Tubulin *(119)*	
• Glutaredoxin *(119)*	
• Metallothionein 1L *(119)*	

[a] HDACI, histone deacetylase inhibitors; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

6.4.1. Histone Acetylation

With the exception of the small molecule, tubacin, all known HDACIs maintain histones in the acetylated state *(89)*. For example, hyperacetylated histone H4 was detected in peripheral blood mononuclear cells at 1 hour and 2 hours after a single injection of PXD-101 in tumor-bearing mice *(131)*. In another example, xenografts from mice treated with pyroxamide displayed increased levels of histone acetylation and increased expression of the cell cycle regulator p21$^{CIP1/WAF1}$ compared with tumors from vehicle-treated control animals *(132)*.

7. HAT REGULATION: THE OTHER SIDE OF ACETYLATION

During the past years, substantial progress has been made in the field of HDACIs, but less research has been performed in the field of HAT inhibitors. Several papers, however, have established a direct relationship between HAT activity and the development or progression of disease *(133)*. As mentioned earlier, because CBP and p300 are available in limited concentrations in the cell, competition for them between different transcription factors can facilitate integration of several signaling and transcription response pathways.

7.1. Gain of p300/CBP and HAT Inhibitors

As a result of fusion to other proteins, HATs can become oncogenic. These gain-of-function mutations presumably increase proliferation by inappropriately enhancing activation of certain transcription pathways. MOZ, a putative HAT, has been described in acute myeloid leukemia (AML) to be fused with at least two different gene products: CREB-binding protein (CBP) *(57)* and TIF2, a nuclear receptor coactivator *(134)*, causing gain-of-function. Furthermore, the fusion of mixed-lineage leukemia (MLL), a homeotic regulator with CBP in patients with therapy-related AML, myelodysplastic syndrome, and chronic myelogenous leukemia (CML), and the fusion of MLL with p300 in patients with AML, suggests an important (oncogenic) role for these gain-of-function mutations. Initially, HAT inhibitors were synthesized as mechanistic tool for research focused on the identification of functional effects of protein acetylation in specific pathways *(135)*.

Lys-CoA, a conjugate of the amino acid lysine and Coenzyme A, specifically blocks the HAT activity of p300. Although Lys-CoA has been intensively used for transcription studies *in vitro* and *in vivo*, the use of microinjection or cell permeabilizing agents is necessary because Lys-CoA is not easily taken up by cells in cell culture conditions. The same holds true for H3-CoA-20, a p300/CBP associated factor (PCAF)-specific inhibitor of the same class *(136)*, and anacardic acid (AA), the first naturally occurring HAT inhibitor. AA was isolated from cashew nut shell liquid, which inhibits HAT activity from both p300 and PCAF very effectively *(137)*. Their inability to penetrate the cell membrane makes them unsuitable for future use in animal models and humans. Recognizing the importance of HAT inhibitors in a clinical setting, the first cell-permeable HAT inhibitor, garcinol, was reported by Balasubramanyam *et al.* in 2004. Garcinol is a polyisoprenylated benzophenone derived from *Garcinia indica* fruit rind and is a potent inhibitor of both p300 and PCAF HATs *in vitro* and *in vivo*. Treatment of HeLa cells with garcinol was shown to inhibit activated histone acetylation, induce apoptosis, and downregulate gene expression of protooncogenes *(138)*. However, the effect of garcinol in normal (untransformed) cells and other cancer types remains to be elucidated.

7.2. Loss of p300/CBP and HAT Activators

In addition to functioning as oncogenes, *p300*, as well as *CBP*, have been shown to be potent tumor suppressor genes. Mice heterozygous for loss of CBP have been shown to develop tumors. Consistent with these data, Rubenstein-Taybi syndrome patients lacking one functional allele of CBP show a predisposition to cancer *(139)*. Interestingly, mice heterozygous for loss of p300 have not been shown to develop tumors. Despite this lack of direct evidence for p300 acting as a tumor suppressor gene, heterozygosity studies show p300 involvement in a number of different cancer types in humans. Analysis of colorectal, gastric, and epithelial cancer samples, for instance, shows missense mutations as well as deletion mutations in the p300 gene *(140)*, and it has been found that colorectal tumors as well as 80% of glioblastomas are associated with a loss of heterozygosity of the p300 gene. In 10 of 193 tumor samples and cancer cell lines (breast, colorectal, ovarian, lung, pancreatic cancer, and glioma), Gayther *et al.* identified truncation mutations, insertions, and missense mutations of p300 with or without inactivation or deletion of the second allele. Although their study indicates that p300 mutations are relatively rare, they do support the idea that loss of p300 activity contributes to tumor development because the nature of the p300 mutations suggests that most of the mutations would clearly lead to a loss of function *(141)*. From that perspective, it would be interesting to use a HAT activator to study its effect on p300 function. Little has been published on HAT activators, especially in the context of cancer. By using the HAT inhibitor, AA, Balasubramanyam *et al.* synthesized the first small molecule HAT activator, CTPB. However, just like AA, cells are poorly permeable to CTPB *(138)*. It remains to be seen, *in vitro* and *in vivo*, whether HAT activators can serve as potential anticancer agents. The implications of small molecule activators and inhibitors of HATs in chromatin therapy was reviewed in 2004 by Varier *et al. (142)*.

8. HDACIS IN CLINICAL TRIALS

During the last 5 years, more than 20 HDACIs have been investigated in cancer clinical trials, either alone or in combination with other agents. Table 5 presents a brief summary of some selected clinical trials.

8.1. Clinical Toxicity and Antitumor Activity

Dose-limiting clinical toxicities and reported antitumor responses have been noted in Phase I and II clinical trials for the limited number of structurally varied HDACIs that have entered clinical testing. Short-chain fatty acids, such as phenylbutyrate, show a dose-limiting toxicity of somnolence and confusion when administered using prolonged intravenous infusion. This neurotoxicity has not

been reported for the benzamide or hydroxamate HDACIs or for the carboxylate prodrug AN-9. Despite thrombocytopenia being a dose-limiting toxicity for both CI-994 and depsipeptide, evidence for antitumor clinical activity on oral daily dosing of CI-994 has been noted in patients with several epithelial types of advanced solid malignancies (including non-small cell lung cancer, renal cell carcinoma, and bladder cancer). Likewise, two Phase I trials of depsipeptide have suggested that patients with T cell leukemia or lymphoma, as well as other occasional cases of refractory malignancies, may achieve clinical benefit from this HDACI. Unfortunately, depsipeptide has been reported to be associated with a significant incidence of cardiac dysrhythmias and nonspecific EKG abnormalities. In some patients, the hydroxamates LAQ824 and LBH-589 have also demonstrated some EKG changes. Fatigue was commonly observed with SAHA treatment but was not dose limiting, and was similar to that previously reported for depsipeptide. Importantly, many patients with solid cancers showed some degree of clinical improvement.

8.2. Pharmacokinetics and Pharmacodynamics

Because of the reversible nature of epigenetic modifications, and assuming these to be the key determinants of tumor growth, inhibition of intracellular HDAC activity or demethylation commonly will require continuous drug exposure to achieve maximal tumor cytostasis or apoptosis and clinical response. Rapid clearance, a high degree of protein binding, rapid metabolism, or rapid inactivation of reactive functional groups (i.e., epoxy groups) are factors that can adversely affect HDACI bioavailability and antitumor activity. Most HDACIs are rapidly metabolized in rodents and dogs. With a few exceptions, for example, LBH-589A (Novartis AG; $t_{1/2}$, 15 to 20 hours) *(143)* and MS-275 ($t_{1/2}$, 100 hours) *(144,145)*, reported half-lives for HDACIs are a maximum of 1 hour in humans. This short half-life poses a significant limitation to the design of both *in vivo* studies and clinical trials with HDACIs. In particular, butyrate and phenylbutyrate degraded rapidly after intravenous administration, requiring doses of at least 400 mg/kg/d to be administered as a continuous intravenous infusion for 120 hours (repeated every 21 days) in certain clinical trials *(146,147)*.

Most phase I trials continue to focus on the pharmacokinetics of different HDACIs. In case of drugs with a shorter half-life, continuous dosing may be required. However, continuous dosing is not always desirable. Therefore, drugs available as oral formulations, such as valproic acid and SAHA, are potentially more attractive candidates. In the case of valproic acid, the well-investigated neuropsychiatric drug with a pharmacokinetic profile including a $T_{1/2}$ of approx 14 hours *(148)* at therapeutically tolerated doses, effective plasma concentrations are achievable in the 0.5 to 0.75 m*M* range. The drug is conveniently bioavailable in an oral formulation. Trials on prostate cancer and other malignancies with this drug have been guided by this preexisting knowledge.

8.3. Biomarkers of Evaluation

Because of the differentiating properties of epigenetic modifiers, the conventional marker of evaluation, prostate-specific antigen, is often upregulated even when tumor burden is reduced *(146)*. Hence, prostate-specific antigen cannot be used as a marker of disease prognosis when using differentiating agents. To date, acetylation of histones in peripheral blood mononuclear cells is relied upon as a marker of exposure to HDACI in both blood and solid cancers. As markers of effect in blood cancers, acetylation and upregulation of p21 is a vital end point of drug activity but not of antitumor response. To avoid biopsies, and in the absence of validated biomarkers of effect in surrogate tissue, evaluating acetylated histones in solid tumors has led to difficulty in trial design. However, many novel designs for evaluating patient and tumor response to HDACI are currently underway. These designs include treating patient populations with HDACIs in the window period before surgery (for example, before prostatectomy) and evaluating biomarkers of drug activity in postsurgery samples. Similarly, in the case of DNMTIs, the frequency of methylation markers, such as *GSTPi*, *RASSF1A*, *CDH1*, and *EDNRB1*, which are frequently methylated in prostate cancer *(149,150)*, may be evaluated. However,

Table 5
Phase I/II Clinical Trials With Histone Deacetylase Inhibitors

HDACI (tumor type)	Schedule	Outcome	Adverse events	References
FK-228 (CTCL + PTCL) Phase II	4-h infusion d 1, 8, and 15 of a 28 d cycle	CR: 21% (3/14 CTCL) PR: 29% (4/14 CTCL) PR: 24% (4/17 PTCL)	• Fatigue, N/V, granulocytopenia, hypocalcemia, neutropenia, thrombocytopenia • Cardiac: nonspecific ST-T wave changes, but no change in cardiac function	*186*
AN-9 (NSCLC) Phase II	2.34 $g/m^2/d$ iv in 6-h infusion on 3 consecutive days every 3 wk	PR: 4.3% (2/47) SD > 12 wk: 36% (18/47)	• Not reported	*187*
Phenylacetate (recurrent malignant glioblastoma) Phase II	400 mg/kg/d continuous iv infusion 2 wk/4 wk	CR: none PR: 7.5% (3/40) SD: 17.5% (7/40) >50% reduction of tumor: 7.5% (3/40) Treatment failure < 2 months: 75% (30/40)	• Infection, malaise, fatigue, lethargy, reversible disorientation, somnolence, weakness, N/V, edema, granulocytopenia	*188*
Phenylacetate (solid tumors) Phase I	Single iv bolus followed by 14-d continuous iv infusion (maintaining blood concentration at 200–300 µg/mL)	SD > 9 mo: 17% (1/6 glioblastoma) SD > 2 mo: 33% (3/9 HRPC) Nonlinear PK, induction of drug clearance	• Confusion, lethargy, emesis • DLT: reversible CNS depression	*189*
Phenylbutyrate (solid tumors) Phase I	150–515 mg/kg/d 120-h iv infusion every 21 d	CR: none SD: 8% (2/24, 19 CaP) Significant bone pain reduction in HRPC Recommended phase II dose: 410 mg/kg/d for 120 h	• DLT: neurocortical (excessive somnolence, confusion), hypokalemia, hyponatremia, hyperuricemia (2 patients, 515 and 345 mg/kg/d) • MTD: 410 mg/kg/d for 5 d • Other toxicities: fatigue, nausea	*146*
Phenylbutyrate (solid tumors) Phase I	9–45 g/d po in 3 doses/d	PR, CR: none SD > 6 mo: 25% (7/28, 12 CaP) Recommended phase II dose: 27 g/d	• Dyspepsia (G1–2), edema (G1–4), fatigue, neurocortical toxicity, N/V • DLT: N/V, hypocalcemia at 36 g/d (2/7) • MTD: 27 g/d	*190*
Phenylbutyrate + 5AC (solid tumors + hematological malignancies)	25 $mg/m^2/d$ 5AC on d 1–14; 400 mg/kg/d phenylbutyrate on d 6 and 13 for 5 wk in continuous iv	No change in pre/post tumor specimens was observed for methyltransferase or GSTPi. 5AC: mean T_{max}: 0.47 h;$T_{1/2}$: 1.5 ± 2.3 h, inhibition of DNMT methyl transferase	• Not reported	*80* *191*

(Continued on next page)

Table 5 ***(Continued)***

HDACI (tumor type)	Schedule	Outcome	Adverse events	References
SAHA (solid tumor + hematological malignancies)	200 mg qd, 400 mg qd, 600 mg qd, or 400 mg bid po	CR: 1/73 PR: 4% (3/73 + 2 unconfirmed) Linear PK from 200 to 600 mg Mean $T_{1/2}$: 91–127 min Dose-dependent accumulation of acetylated histones from 200–600 mg	• MTD: 400 mg qd and 200 mg bid for continuous daily dosing and 300 mg bid for 3 consecutive days per week dosing. • DLT: anorexia, dehydration, diarrhea, and fatigue	*192*
SAHA (Solid tumors + hematological malignancies + refractory lymphomas) Phase I	75–900 mg/m^2/d in 2-h iv infusion 3–5 d/wk for 1–3 wk	Objective tumor regression with clinical improvement in tumor related symptoms: 4 patients Mean $T_{1/2}$: 21–58 min	• Fatigue (G1–3), anorexia (G1–2), vomiting (G1–2), diarrhea (G1–3), constipation (G1–4), hypokalemia, nonspecific EKG changes, increased creatinine, dysgeusia • MTD: 300 mg/m^2/d × 5 d for 3 wk (2/5 hematological), 900 mg/m^2/d × 5 d for 3 wk (1/6) • DLT: leucopenia (G3–4), thrombocytopenia (G3–4), acute respiratory distress, hypotension (G3)	*193*
Depsipeptide (hematological malignancies) Phase I	13 mg/m^2 iv on d 1, 8, and 15 every 4 wk	CR, PR: none Evidence of antitumor activity and histone acetylation increases of at least 100% Increase in p21 promoter H4 acetylation, p21 protein, and 1D10 antigen expression	• Progressive fatigue, nausea, and other constitutional symptoms prevented repeated dosing • Neither life-threatening toxicities nor cardiac toxicities were noted.	*194*
MS-275 (solid tumors + lymphomas) Phase I	>2 mg/m^2/d (dose escalation) po 4/10 wk or 4/6 wk	$T_{1/2}$: 39–80 h Linear PK suggested PBMC: increased histone H3 acetylation was apparent at all dose levels	• MTD: 10 mg/m^2 • DLT: N/V, anorexia, and fatigue	*130*
MS-275 (solid tumors + lymphomas) Phase I	2–6 mg/m^2/2 wk po or 2 mg/m^2 po twice weekly for 2/3 wk or 4 mg/m^2/wk po for 3 wk	$T_{1/2}$: 100 h T_{max}: 0.5–2.0 h PBL: increased histone H3 acetylation HDACI observed PR: 6% (1/17) SD: 17% (3/17)	• Schedule A: Hypophosphatemia, asthenia, nausea, anorexia. MS-275 po on the daily schedule was intolerable at a dose and schedule explored. • AE: hypophosphatemia, asthenia, nausea and anorexia (all G1–3)	*144* *145*
LAQ-824 (advanced solid tumors) Phase I	Dose-escalating iv infusion on d 1–3 for 21 d	Increase and maintenance of acetylation for 24 h, inhibition of Hsp90 activity with increased expression of Hsp70 and decreased downs tream target c-Raf	• Not reported	*195*

HDACI (tumor type)	Schedule	Outcome	Adverse events	References
LAQ-824 (advanced solid tumors + hematological malignancies) Phase I	6–200 mg/m^2/d as 3-h iv infusion on d 1–3 for 21 d	PK analysis showed dose proportionality, $T_{1/2}$: 6–26 h PBL: Sustained histone acetylation 24 h after dosing of ≥36 mg/m^2/d	• QTcF day 1: no change • QTcF day 3: dose-related increases of <20 ms • QTcF >60 ms: 10% (8/77) at 36–200 mg/m^2 • QTc > 500 ms: 1/77 (200mg/m^2) • Frequent nonspecific T-wave flattening • Small increase in troponin without increase in CK (2/77)	*196*
LBH-589 (solid tumors) Phase I	1.2–7.2 mg/m^2/d as 30 min iv infusion d 1–3 and 8–10 every 3 wk (arm 1) or d 1–3 and 15–17 every 4 wk (arm 2)	SD: 46% (6/13) Rapid onset (1 h) of prolonged acetylation (up to 7 d) in some patients $T_{1/2}$: 15–20 h	• Neutropenia (G3, 1/13), hypoglyce mia (G3, 1/13), thrombocytopenia (G2, 2/13),anemia (G2, 2/13) • DLT: prolonged thrombocytopenia (G2, 7.2 mg/m^2/d in arm 1)	*143*
Valproic acid (advanced cancer) Phase I	30–120 mg/kg/d as 2 × 1-h iv infusion for 5 d every 2 wk	PBL: hyperacetylation observed in majority of patients Recommended phase II dose: 60 mg/kg	• Neurological toxicity (G3–4, 9/26, dose 75-, 90- and 120-mg/kg) • No hemalogical toxicity >G3–4 • MTD	*197*
PXD101 (advanced solid tumors) Phase I	150, 300, 600, 900, and 1200 mg/m^2 as 30 min iv infusion 5 d every 3 wk Ongoing schedule: 1000 mg/m^2	SD >2 cycles: 7/21 linear kinetics displayed $T_{1/2}$: 0.5–1 h. PBL: dose-dependent histone H4 Hyperacetylation, increases in p21, p19, and Apaf-1 expression	• DLT: 14% (3/21, all 1200 mg/m^2), fatigue (G3), reversible atrial fibrillation (G3), diarrhea (G3), and lethargy • All other AE were ≤ G2 • No hematological toxicity was observed	*198*
MGCD0103 (advanced solid tumors) Phase I	12.5, 20, 27, and 36 mg/m^2/d 3 d/wk, 2 of 3 wk	Trial is ongoing (n = 14 to date). SD (>2 cycles) seen in renal cell cancer (2 patients, 4 and 6 cycles) and colorectal cancer (1 patient, 4 cycles) $T_{1/2}$: 9.47 ± 3.15 h Maximal HDACI at 24 h after dosing. Dose-dependent histone acetylation	• AEs: grade 1–3 fatigue (57%), grade 1–2 nausea and vomiting (50%), anorexia (21%), constipation and dehydration (14%). • MTD has not yet been reached	*199*
MGCD0103 (advanced solid tumors + NHL) Phase I	12.5–27 mg/m^2/d po	SD >6 cycles: 1/27 to date (thymic carcinoma) HDACI correlates positively with histone acetylation in PBL lasting >24 h after dosing $T_{1/2}$: 8.8 h	• Fatigue (20/27, 5/20 G3), nausea 13 pts (13/27, 1/13 G3), anorexia (8/27), vomiting (6/27) and diarrhea (5/27)	*200* *201*

[a] HDACI, histone deacetylase inhibitor; CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma; CR, complete response; PR, partial response; N/V, nausea/vomiting; NSCLC, non-small cell lung cancer; SD, stable disease; HRPC, hormone refractory prostate cancer; DLT, dose-limiting toxicity; CNS, central nervous system; MTD, maximum tolerated dose; po, per oral; 5AC, 5-aza-cytidine; GSTPi, glutathione-S-transferase-π; SAHA, suberoylanilide hydroxamic acid; PBMC, peripheral blood mononuclear cells; PK, pharmacokinetics; PBL, peripheral blood lymphocytes; QTcF, Fridericia correction to QT interval; CK, creatin kinase; AE, adverse event; NHL, non-Hodgkin's lymphoma.

it is still not known whether decreases in methylation of these genes or others have independent prognostic significance.

9. COMBINATION STUDIES WITH HDACIS

HDACIs have been combined with an array of chemically and structurally diverse compounds. In some cases, the combination was supported by a rationale, and, in other cases, the mechanism underlying the synergistic activity of the combination was analyzed after experimentation. A summary of selected preclinical combination studies is provided in Table 6. Many of these preclinical observations have now moved forward to phase I clinical trials.

10. CONCLUSION

Several small molecules are now being considered for phase I/II clinical trials in prostate cancer because *in vitro* studies have shown tumor sensitivity to induction of differentiation. Selective oxidation/reduction-dependent inhibitors of 5-lipoxygenase were shown to prevent PC3 prostate cancer cells from proliferating and induced limited differentiation followed by apoptosis *(151)*. AN-7, another prodrug of butyric acid, induced histone hyperacetylation and differentiation and inhibited proliferation of human prostate 22Rv1 cancer cells *in vitro* and *in vivo*. In nude mice implanted with these cells, 50 mg/kg AN-7 administered orally thrice a week led to inhibition of tumor growth and metastasis, tumor regression in greater than 25% of animals, and increased survival *(152)*. In another study, the *in vivo* efficacy of the orally available tributyrin against PC3 and TSU-Pr1 was compared with SB, and found to be equally efficacious *(153)*.

HDACIs demonstrate pleiotropic effects by altering transcriptional status and preventing/inhibiting proliferation of tumor cells. Studies have shown synergistic gene reexpression and antiproliferative effects if HDACIs are combined with other agents. The pleiotropy arises from the variety of HDAC substrates, many of which are still unknown. The name "histone" deacetylase inhibitors may be misleading, because many "nonhistone" substrates have been identified for HDACs. In the clinic, HDACIs are competing with conventional chemotherapeutic drugs, and are favorable because of their low toxicities. Further analysis into the common pathways of conventional drugs and HDACIs, the significance of individual HDACs and their isoforms, and other substrates of HDACs will reveal the mechanisms behind the success of HDACIs in the clinic. Currently, studies on the mechanisms of action of HDACIs are ongoing, and the next few years should see a continued increase in the number of HDACIs under investigation in clinical trials.

REFERENCES

1. Dix, D. (2003). On the role of genes relative to the environment in carcinogenesis. *Mech. Ageing Dev.* **124**, 323–332.
2. Nejako, A., Aranton, B., and Dix, D. (2005). Carcinogenesis: a cellular model for age-dependence. *Anticancer Res.* **25**, 1385–1389.
3. Chen, C. and Kong, A. N. (2005). Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. *Trends Pharmacol. Sci.* **26**, 318–326.
4. Luch, A. (2005). Nature and nurture—lessons from chemical carcinogenesis. *Nat. Rev. Cancer* **5**, 113–125.
5. Melnikova, V. O. and Ananthaswamy, H. N. (2005). Cellular and molecular events leading to the development of skin cancer. *Mutat Res.* **571**, 91–106.
6. Garber, J. E. and Offit, K. (2005). Hereditary cancer predisposition syndromes. *J. Clin. Oncol.* **23**, 276–292.
7. Hemminki, K., Li, X., and Czene, K. (2004). Familial risk of cancer: data for clinical counseling and cancer genetics. *Int. J. Cancer* **108**, 109–114.
8. Lichtenstein, P., Holm, N. V., Verkasalo, P. K., et al. (2000). Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.* **343**, 78–85.
9. Feinberg, A. P. and Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* **301**, 89–92.
10. Feinberg, A. P. and Tycko, B. (2004). The history of cancer epigenetics. *Nat. Rev. Cancer* **4**, 143–153.

Table 6
Preclinical Combination of HDACIs With Other Agents

HDACI	Combination agent	Rationale/mechanism underlying synergism	References
TSA FK-228 Phenylbutyrate SAHA CBHA	Drugs or hormones that act on retinoic acid, estrogen receptors or other nuclear receptors	Aberrant fusion proteins bind to RARE (or other nuclear receptors) where HDAC-containing repressor complexes are recruited to silence expression of genes from these promoters. Ligands and HDACIs reactivate such silenced and repressed chromatin to cause expression of hormone-inducible genes to overcome retinoid resistance	*98,179,202–206*
TSA SAHA FK-228 Phenylbutyrate FR901228	5-Aza-2'-deoxycytidine	Eliminates the dominant effect of hypermethylation of promoters	*74,207–214*
3*n*-Butyrate	5-Fluorouracil	Enhances apoptosis	*215*
FK-228 Phenylbutyrate SAHA	Flavopiridol (NCI)	Flavopiridol, a synthetic Cdk inhibitor, interfers with expression of the cellular Cdk inhibitor $p21^{CIP1/WAF1}$ to cause apoptosis as opposed to cell cycle arrest and differentiation induced by HDACIs	*216–218*
SAHA Phenylbutyrate Apicidin LAQ-824	STI-571	HDACIs cause apoptosis in Imantinib-resistant cells and enhance apoptosis in Bcr-Abl expressing cells	*182,183,219–221*
Sodium butyrate	Topoisomerase II inhibitors (e.g., etoposide)	HDACIs upregulate topoisomerase II expression, which, in turn, renders cells sensitive to topoisomerase II inhibitors	*222*
LAQ-824 LBH-589	17-AAG (Hsp90 antagonist)	Inhibition of chaperone association of Hsp90 with Flt-3 and Bcr-Abl, resulting in poly-ubiquitination and proteosomal degradation of Flt-3 and Bcr-Abl. Levels of Flt-3 and Bcr-Abl are greatly attenuated resulting in enhanced apoptosis	*223,224*
SAHA FK-228	Standard chemotherapy agents (e.g., VP-16, ellipticine, doxorubicin, and cisplatin)	Enhances apoptosis	*225*

(Continued on next page)

Table 6 *(Continued)*

HDACI	Combination agent	Rationale/mechanism underlying synergism	References
TSA SAHA MS-275 FK-228	γ-Irradiation	γ-H2AX foci expression is prolonged and histones inhibited from participating in DNA repair. SAHA also causes differential expression of several oncoproteins and DNA damage repair proteins (epidermal growth factor receptor, AKT, DNA-PK, and Rad51) that affect susceptibility of cells to radiation-induced damage response	*226–231*
SAHA Sodium butyrate	Bortezomib	Enhances apoptosis	*220*
Sodium butyrate TSA SAHA	Activators of extrinsic, receptor-mediated apoptotic pathway (TRAIL, TNF-α)	HDACIs sensitizes cells to TRAIL by decreasing FLIP protein expression to cause cell death	*232–234*
LAQ824	Apo-2L/TRAIL	Exposure to LAQ824 increases the mRNA and protein expressions of the death receptors DR5 and/or DR4, but reduces the mRNA and protein levels of cellular FLICE-inhibitory protein (c-FLIP). As compared with treatment with Apo-2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or LAQ824 alone, pretreatment with LAQ824 increases the assembly of Fas-associated death domain and caspase-8, but not of c-FLIP, into the Apo-2L/TRAIL-induced death-inducing signaling complex. This increases the processing of caspase-8 and Bcl-2 interacting domain (BID), augmenting cytosolic accumulation of the prodeath molecules cytochrome-c, Smac and Omi, as well as leading to increased activity of caspase-3 and apoptosis. Treatment with LAQ824 also downregulates the levels of Bcl-2, Bcl-x(L), XIAP, and survivin. Partial inhibition of apoptosis caused by LAQ824 or Apo-2L/TRAIL exerted by Bcl-2 overexpression is reversed by cotreatment with LAQ824 and Apo-2L/TRAIL	*235*
SAHA	Signal transduction modulators (e.g., STI-571)	Effectively induces apoptosis in Bcr/Abl^{+} cells that are STI-571-resistant and nonresistant	*182,183,219*
SAHA	ErbB signaling inhibitors (e.g., CI-1033 [Pfizer Inc.])	Dual targeted therapy abrogates EGFR and Akt signaling	*236*
LAQ-824	VEGFR tyrosine kinase inhibitor (PTK-787/ ZK-222584)	Increases anti-angiogenic factors in tumor and surrounding endothelial cells, and inhibits tumor growth	*129*
LAQ-824	Herceptin and/or Taxotere or Epothilone B against human breast cancer	Causes downregulation of the mRNA and protein expression of Her-2, which correlated with the attenuation of pAKT. Promotes the proteasomal degradation of Her-2 and sensitizes human breast cancer cells (with Her-2 amplification) to Herceptin and apoptosis caused by tubulin polymerizing agents, Taxotere and Epothilone B	*237*

[a] HDACI, histone deacetylase inhibitor; HDAC, histone deacetylase; TSA, Trichostatin A; SAHA, suberoylanilide hydroxamic acid; CBHA, m-carboxycinnamic acid *bis*-hydroxamide; RARE, Retinoic Acid Response Element; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; FLIP, Flice inhibitory protein; mRNA, messenger RNA.

11. Fraga, M. F., Ballestar, E., Paz, M. F., et al. (2005). Epigenetic differences arise during the lifetime of monozygotic twins. *Proc. Natl. Acad. Sci. USA* **102**, 10,604–10,609.
12. Eden, A., Gaudet, F., Waghmare, A., and Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* **300**, 455.
13. Bjornsson, H. T., Fallin, M. D., and Feinberg, A. P. (2004). An integrated epigenetic and genetic approach to common human disease. *Trends Genet.* **20**, 350–358.
14. Bennett-Baker, P. E., Wilkowski, J., and Burke, D. T. (2003). Age-associated activation of epigenetically repressed genes in the mouse. *Genetics* **165**, 2055–2062.
15. Davey, C. A., Sargent, D. F., Luger, K., Maeder, A. W., and Richmond, T. J. (2002). Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. *J. Mol. Biol.* **319**, 1097–1113.
16. Luger, K. (2003). Structure and dynamic behavior of nucleosomes. *Curr. Opin. Genet. Dev.* **13**, 127–135.
17. Wolffe, A. P. and Hayes, J. J. (1999). Chromatin disruption and modification. *Nucleic Acids Res.* **27**, 711–720.
18. Boeger, H., Griesenbeck, J., Strattan, J. S., and Kornberg, R. D. (2003). Nucleosomes unfold completely at a transcriptionally active promoter. *Mol. Cell* **11**, 1587–1598.
19. Hansen, J. C. (2002). Conformational dynamics of the chromatin fiber in solution: determinants, mechanisms, and functions. *Annu. Rev. Biophys. Biomol. Struct.* **31**, 361–392.
20. Cairns, B. R. (2005). Chromatin remodeling complexes: strength in diversity, precision through specialization. *Curr. Opin. Genet. Dev.* **15**, 185–190.
21. Khorasanizadeh, S. (2004). The nucleosome: from genomic organization to genomic regulation. *Cell* **116**, 259–272.
22. Kassabov, S. R., Zhang, B., Persinger, J., and Bartholomew, B. (2003). SWI/SNF unwraps, slides, and rewraps the nucleosome. *Mol. Cell* **11**, 391–403.
23. Fan, H. Y., He, X., Kingston, R. E., and Narlikar, G. J. (2003). Distinct strategies to make nucleosomal DNA accessible. *Mol. Cell* **11**, 1311–1322.
24. Mizuguchi, G., Shen, X., Landry, J., Wu, W. H., Sen, S., and Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science* **303**, 343–348.
25. Cosgrove, M. S., Boeke, J. D., and Wolberger, C. (2004). Regulated nucleosome mobility and the histone code. *Nat. Struct. Mol. Biol.* **11**, 1037–1043.
26. Waterborg, J. H. (2002). Dynamics of histone acetylation in vivo. A function for acetylation turnover? *Biochem. Cell. Biol.* **80**, 363–378.
27. de la Cruz, X., Lois, S., Sanchez-Molina, S., and Martinez-Balbas, M. A. (2005). Do protein motifs read the histone code? *Bioessays* **27**, 164–175.
28. Kasten, M., Szerlong, H., Erdjument-Bromage, H., Tempst, P., Werner, M., and Cairns, B. R. (2004). Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. *Embo J.* **23**, 1348–1359.
29. Bannister, A. J., Zegerman, P., Partridge, J. F., et al. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**, 120–124.
30. Bouazoune, K., Mitterweger, A., Langst, G., et al. (2002). The dMi-2 chromodomains are DNA binding modules important for ATP-dependent nucleosome mobilization. *Embo J.* **21**, 2430–2440.
31. Yu, J., Li, Y., Ishizuka, T., Guenther, M. G., and Lazar, M. A. (2003). A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. *Embo J.* **22**, 3403–3410.
32. Boyer, L. A., Latek, R. R., and Peterson, C. L. (2004). The SANT domain: a unique histone-tail-binding module? *Nat. Rev. Mol. Cell. Biol.* **5**, 158–163.
33. Turner, B. M. (1993). Decoding the nucleosome. *Cell* **75**, 5–8.
34. Strahl, B. D. and Allis, C. D. (2000). The language of covalent histone modifications. *Nature* **403**, 41–45.
35. Fischle, W., Wang, Y., and Allis, C. D. (2003). Binary switches and modification cassettes in histone biology and beyond. *Nature* **425**, 475–479.
36. Gray, S. G. and Ekstrom, T. J. (2001). The human histone deacetylase family. *Exp. Cell Res.* **262**, 75–83.
37. Roth, S. Y., Denu, J. M., and Allis, C. D. (2001). Histone acetyltransferases. *Annu. Rev. Biochem.* **70**, 81–120.
38. Grozinger, C. M., Hassig, C. A., and Schreiber, S. L. (1999). Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc. Natl. Acad. Sci. USA* **96**, 4868–4873.
39. Verdin, E., Dequiedt, F., and Kasler, H. G. (2003). Class II histone deacetylases: versatile regulators. *Trends Genet.* **19**, 286–293.
40. Gregoretti, I. V., Lee, Y. M., and Goodson, H. V. (2004). Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J. Mol. Biol.* **338**, 17–31.
41. Waltregny, D., North, B., Van Mellaert, F., de Leval, J., Verdin, E., and Castronovo, V. (2004). Screening of histone deacetylases (HDAC) expression in human prostate cancer reveals distinct class I HDAC profiles between epithelial and stromal cells. *Eur. J. Histochem.* **48**, 273–290.
42. Robyr, D., Suka, Y., Xenarios, I., et al. (2002). Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell* **109**, 437–446.
43. Chang, S., McKinsey, T. A., Zhang, C. L., Richardson, J. A., Hill, J. A., and Olson, E. N. (2004). Histone deacetylases

5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. *Mol. Cell Biol.* **24**, 8467–8476.
44. Zhu, P., Martin, E., Mengwasser, J., Schlag, P., Janssen, K. P., and Gottlicher, M. (2004). Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. *Cancer Cell* **5**, 455–463.
45. Waltregny, D., Glenisson, W., Tran, S. L., et al. (2005). Histone deacetylase HDAC8 associates with smooth muscle alpha-actin and is essential for smooth muscle cell contractility. *Faseb J.* **19**, 966–968.
46. Choi, J. H., Kwon, H. J., Yoon, B. I., et al. (2001). Expression profile of histone deacetylase 1 in gastric cancer tissues. *Jpn. J. Cancer Res.* **92**, 1300–1304.
47. Khochbin, S., Verdel, A., Lemercier, C., and Seigneurin-Berny, D. (2001). Functional significance of histone deacetylase diversity. *Curr. Opin. Genet. Dev.* **11**, 162–166.
48. Kachhap, S. K., Kortenhorst, M. S. Q., Shabbeer, S., Washington, E., and Carducci, M. A. (2005). Comparison of expression of Class I and Class II histone deacetylase in prostate cancer cell lines and normal immortalized prostate epithelial cells. In: Proceedings of AARC Annual Meeting. 2005, Washington, DC.
49. Petrie, K., Guidez, F., Howell, L., et al. (2003). The histone deacetylase 9 gene encodes multiple protein isoforms. *J. Biol. Chem.* **278**, 16,059–16,072.
50. McGraw, S., Robert, C., Massicotte, L., and Sirard, M. A. (2003). Quantification of histone acetyltransferase and histone deacetylase transcripts during early bovine embryo development. *Biol. Reprod.* **68**, 383–389.
51. Lagger, G., O'Carroll, D., Rembold, M., et al. (2002). Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. *Embo J.* **21**, 2672–2681.
52. Ito, K., Caramori, G., Lim, S., et al. (2002). Expression and activity of histone deacetylases in human asthmatic airways. *Am. J. Respir. Crit. Care Med.* **166**, 392–396.
53. McKinsey, T. A. and Olson, E. N. (2004). Dual roles of histone deacetylases in the control of cardiac growth. *Novartis Found Symp.* **259**, 132–141; discussion 141–135, 163–139.
54. Insinga, A., Minucci, S., and Pelicci, P. G. (2005). Mechanisms of selective anticancer action of histone deacetylase inhibitors. *Cell Cycle* **4**, 741–743.
55. Polevoda, B. and Sherman, F. (2002). The diversity of acetylated proteins. *Genome Biol.* **3**, reviews Epub.
56. Neuwald, A. F. and Landsman, D. (1997). GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem. Sci.* **22**, 154–155.
57. Borrow, J., Stanton, V. P., Jr., Andresen, J. M., et al. (1996). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat. Genet.* **14**, 33–41.
58. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953–959.
59. Bannister, A. J. and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. *Nature* **384**, 641–643.
60. Xu, W., Edmondson, D. G., and Roth, S. Y. (1998). Mammalian GCN5 and P/CAF acetyltransferases have homologous amino-terminal domains important for recognition of nucleosomal substrates. *Mol. Cell. Biol.* **18**, 5659–5669.
61. Candau, R., Zhou, J. X., Allis, C. D., and Berger, S. L. (1997). Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function in vivo. *Embo J.* **16**, 555–565.
62. Santos-Rosa, H., Valls, E., Kouzarides, T., and Martinez-Balbas, M. (2003). Mechanisms of P/CAF auto-acetylation. *Nucleic Acids Res.* **31**, 4285–4292.
63. Bannister, A. J., Oehler, T., Wilhelm, D., Angel, P., and Kouzarides, T. (1995). Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro. *Oncogene* **11**, 2509–2514.
64. Kamei, Y., Xu, L., Heinzel, T., et al. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403–414.
65. Herman, J. G. and Baylin, S. B. (2003). Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* **349**, 2042–2054.
66. Jones, P. A. and Baylin, S. B. (2002). The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* **3**, 415–428.
67. El-Osta, A., Kantharidis, P., Zalcberg, J. R., and Wolffe, A. P. (2002). Precipitous release of methyl-CpG binding protein 2 and histone deacetylase 1 from the methylated human multidrug resistance gene (MDR1) on activation. *Mol. Cell Biol.* **22**, 1844–1857.
68. Lee, W. H., Morton, R. A., Epstein, J. I., et al. (1994). Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc. Natl. Acad. Sci. USA* **91**, 11,733–11,737.
69. Gaudet, F., Hodgson, J. G., Eden, A., et al. (2003). Induction of tumors in mice by genomic hypomethylation. *Science* **300**, 489–492.
70. Cheng, J. C., Yoo, C. B., Weisenberger, D. J., et al. (2004). Preferential response of cancer cells to zebularine. *Cancer Cell* **6**, 151–158.
71. Cornacchia, E., Golbus, J., Maybaum, J., Strahler, J., Hanash, S., and Richardson, B. (1988). Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *J. Immunol.* **140**, 2197–2200.

72. Fang, M. Z., Wang, Y., Ai, N., et al. (2003). Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.* **63**, 7563–7570.
73. Lin, X., Asgari, K., Putzi, M. J., et al. (2001). Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. *Cancer Res.* **61**, 8611–8616.
74. Belinsky, S. A., Klinge, D. M., Stidley, C. A., et al. (2003). Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res.* **63**, 7089–7093.
75. Jones, P. A. and Taylor, S. M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**, 85–93.
76. Von Hoff, D. D., Slavik, M., and Muggia, F. M. (1976). 5-Azacytidine. A new anticancer drug with effectiveness in acute myelogenous leukemia. *Ann. Intern. Med.* **85**, 237–245.
77. Abele, R., Clavel, M., Dodion, P., et al. (1987). The EORTC Early Clinical Trials Cooperative Group experience with 5-aza-2'-deoxycytidine (NSC 127716) in patients with colo-rectal, head and neck, renal carcinomas and malignant melanomas. *Eur. J. Cancer Clin. Oncol.* **23**, 1921–1924.
78. Silverman, L. R., Holland, J. F., Weinberg, R. S., et al. (1993). Effects of treatment with 5-azacytidine on the in vivo and in vitro hematopoiesis in patients with myelodysplastic syndromes. *Leukemia* **7(Suppl. 1)**, 21–29.
79. Silverman, L. R., Demakos, E. P., Peterson, B. L., et al. (2002). Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J. Clin. Oncol.* **20**, 2429–2440.
80. Gilbert, J., Baker, S. D., Donehower, R. C., Herman, J. G., DeMarzo, A., and Carducci, M. (2001). Methyltransferase (MT) activity and gene expression in tumor biopsies from patients enrolled in a phase I study of the MT inhibitor, 5-azacytidine (5AC), and the histone deacetylase inhibitor, phenylbutyrate (PB), in refractory solid tumors. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology, USA, San Francisco, California, USA.
81. Finnin, M. S., Donigian, J. R., Cohen, A., et al. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* **401**, 188–193.
82. Richon, V. M., Webb, Y., Merger, R., et al. (1996). Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. *Proc. Natl. Acad. Sci. USA* **93**, 5705–5708.
83. Candido, E. P., Reeves, R., and Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell* **14**, 105–113.
84. Nudelman, A., Gnizi, E., Katz, Y., et al. (2001). Prodrugs of butyric acid. Novel derivatives possessing increased aqueous solubility and potential for treating cancer and blood diseases. *Eur. J. Med. Chem.* **36**, 63–74.
85. Lu, Q., Yang, Y. T., Chen, C. S., et al. (2004). Zn2+-chelating motif-tethered short-chain fatty acids as a novel class of histone deacetylase inhibitors. *J. Med. Chem.* **47**, 467–474.
86. Furumai, R., Matsuyama, A., Kobashi, N., et al. (2002). FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. *Cancer Res.* **62**, 4916–4921.
87. Frey, R. R., Wada, C. K., Garland, R. B., et al. (2002). Trifluoromethyl ketones as inhibitors of histone deacetylase. *Bioorg. Med. Chem. Lett.* **12**, 3443–3447.
88. Vasudevan, A., Ji, Z., Frey, R. R., et al. (2003). Heterocyclic ketones as inhibitors of histone deacetylase. *Bioorg. Med. Chem. Lett.* **13**, 3909–3913.
89. Haggarty, S. J., Koeller, K. M., Wong, J. C., Grozinger, C. M., and Schreiber, S. L. (2003). Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. *Proc. Natl. Acad. Sci. USA* **100**, 4389–4394.
90. Cimini, D., Mattiuzzo, M., Torosantucci, L., and Degrassi, F. (2003). Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects. *Mol. Biol. Cell.* **14**, 3821–3833.
91. Warrener, R., Beamish, H., Burgess, A., et al. (2003). Tumor cell-selective cytotoxicity by targeting cell cycle checkpoints. *Faseb J.* **17**, 1550–1552.
92. Ruefli, A. A., Ausserlechner, M. J., Bernhard, D., et al. (2001). The histone deacetylase inhibitor and chemotherapeutic agent suberoylanilide hydroxamic acid (SAHA) induces a cell-death pathway characterized by cleavage of Bid and production of reactive oxygen species. *Proc. Natl. Acad. Sci. USA* **98**, 10,833–10,838.
93. Xu, W. S., Perez, G., Ngo, L., Gui, C. Y., and Marks, P. A. (2005). Induction of polyploidy by histone deacetylase inhibitor: a pathway for antitumor effects. *Cancer Res.* **65**, 7832–7839.
94. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J. Biol. Chem.* **265**, 17,174–17,179.
95. Vigushin, D. M., Ali, S., Pace, P. E., et al. (2001). Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. *Clin. Cancer Res.* **7**, 971–976.
96. Takai, N., Desmond, J. C., Kumagai, T., et al. (2004). Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. *Clin Cancer Res.* **10**, 1141–1149.
97. Chiba, T., Yokosuka, O., Arai, M., et al. (2004). Identification of genes up-regulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells. *J. Hepatol.* **41**, 436–445.
98. Coffey, D. C., Kutko, M. C., Glick, R. D., et al. (2000). Histone deacetylase inhibitors and retinoic acids inhibit growth of human neuroblastoma in vitro. *Med. Pediatr. Oncol.* **35**, 577–581.

99. Carducci, M. A., Nelson, J. B., Chan-Tack, K. M., et al. (1996). Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clin. Cancer Res.* **2**, 379–387.
100. Butler, L. M., Agus, D. B., Scher, H. I., et al. (2000). Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. *Cancer Res.* **60**, 5165–5170.
101. Qiu, L., Burgess, A., Fairlie, D. P., Leonard, H., Parsons, P. G., and Gabrielli, B. G. (2000). Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. *Mol. Biol. Cell.* **11**, 2069–2083.
102. Richon, V. M., Sandhoff, T. W., Rifkind, R. A., and Marks, P. A. (2000). Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc. Natl. Acad. Sci. USA* **97**, 10,014–10,019.
103. Vrana, J. A., Decker, R. H., Johnson, C. R., et al. (1999). Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. *Oncogene* **18**, 7016–7025.
104. Sandor, V., Senderowicz, A., Mertins, S., et al. (2000). P21-dependent g(1)arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228. *Br. J. Cancer* **83**, 817–825.
105. Burgess, A. J., Pavey, S., Warrener, R., et al. (2001). Up-regulation of p21(WAF1/CIP1) by histone deacetylase inhibitors reduces their cytotoxicity. *Mol. Pharmacol.* **60**, 828–837.
106. Johnstone, R. W. (2002). Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat. Rev. Drug Discov.* **1**, 287–299.
107. Mitsiades, C. S., Mitsiades, N. S., McMullan, C. J., et al. (2004). Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. *Proc. Natl. Acad. Sci. USA* **101**, 540–545.
108. Chen, Z., Clark, S., Birkeland, M., et al. (2002). Induction and superinduction of growth arrest and DNA damage gene 45 (GADD45) alpha and beta messenger RNAs by histone deacetylase inhibitors trichostatin A (TSA) and butyrate in SW620 human colon carcinoma cells. *Cancer Lett.* **188**, 127–140.
109. Henderson, C. and Brancolini, C. (2003). Apoptotic pathways activated by histone deacetylase inhibitors: implications for the drug-resistant phenotype. *Drug Resist. Updat.* **6**, 247–256.
110. Henderson, C., Mizzau, M., Paroni, G., Maestro, R., Schneider, C., and Brancolini, C. (2003). Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). *J. Biol. Chem.* **278**, 12,579–12,589.
111. Zhang, X. D., Gillespie, S. K., Borrow, J. M., and Hersey, P. (2003). The histone deacetylase inhibitor suberic bishydroxamate: a potential sensitizer of melanoma to TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis. *Biochem. Pharmacol.* **66**, 1537–1545.
112. Aron, J. L., Parthun, M. R., Marcucci, G., et al. (2003). Depsipeptide (FR901228) induces histone acetylation and inhibition of histone deacetylase in chronic lymphocytic leukemia cells concurrent with activation of caspase 8-mediated apoptosis and down-regulation of c-FLIP protein. *Blood* **102**, 652–658.
113. Facchetti, F., Previdi, S., Ballarini, M., et al. (2004). Modulation of pro- and anti-apoptotic factors in human melanoma cells exposed to histone deacetylase inhibitors. *Apoptosis* **9**, 573–582.
114. Burgess, A., Ruefli, A., Beamish, H., et al. (2004). Histone deacetylase inhibitors specifically kill nonproliferating tumour cells. *Oncogene* **23**, 6693–6701.
115. Rahman, I., Marwick, J., and Kirkham, P. (2004). Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappaB and pro-inflammatory gene expression. *Biochem. Pharmacol.* **68**, 1255–1267.
116. Rosato, R. R., Almenara, J. A., and Grant, S. (2003). The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21CIP1/WAF1 1. *Cancer Res.* **63**, 3637–3645.
117. Rosato, R. R., Wang, Z., Gopalkrishnan, R. V., Fisher, P. B., and Grant, S. (2001). Evidence of a functional role for the cyclin-dependent kinase-inhibitor p21WAF1/CIP1/MDA6 in promoting differentiation and preventing mitochondrial dysfunction and apoptosis induced by sodium butyrate in human myelomonocytic leukemia cells (U937). *Int. J. Oncol.* **19**, 181–191.
118. Chopin, V., Slomianny, C., Hondermarck, H., and Le Bourhis, X. (2004). Synergistic induction of apoptosis in breast cancer cells by cotreatment with butyrate and TNF-alpha, TRAIL, or anti-Fas agonist antibody involves enhancement of death receptors' signaling and requires P21(waf1). *Exp. Cell Res.* **298**, 560–573.
119. Glaser, K. B., Staver, M. J., Waring, J. F., Stender, J., Ulrich, R. G., and Davidsen, S. K. (2003). Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Mol. Cancer Ther.* **2**, 151–163.
120. Zeng, L., Zhang, Y., Chien, S., Liu, X., and Shyy, J. Y. (2003). The role of p53 deacetylation in p21Waf1 regulation by laminar flow. *J. Biol. Chem.* **278**, 24,594–24,599.
121. Vaghefi, H. and Neet, K. E. (2004). Deacetylation of p53 after nerve growth factor treatment in PC12 cells as a post-translational modification mechanism of neurotrophin-induced tumor suppressor activation. *Oncogene* 23, 8078–8087.
122. Bereshchenko, O. R., Gu, W., and Dalla-Favera, R. (2002). Acetylation inactivates the transcriptional repressor BCL6. *Nat. Genet.* **32**, 606–613.
123. Yu, X., Guo, Z. S., Marcu, M. G., et al. (2002). Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. *J. Natl. Cancer Inst.* **94**, 504–513.

124. Cohen, H. Y., Lavu, S., Bitterman, K. J., et al. (2004). Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol. Cell.* **13**, 627–638.
125. Gottlicher, M., Minucci, S., Zhu, P., et al. (2001). Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *Embo J.* **20**, 6969–6978.
126. Cohen, L. A., Marks, P. A., Rifkind, R. A., et al. (2002). Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, suppresses the growth of carcinogen-induced mammary tumors. *Anticancer Res.* **22**, 1497–1504.
127. Deroanne, C. F., Bonjean, K., Servotte, S., et al. (2002). Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. *Oncogene* **21**, 427–436.
128. Kwon, H. J., Kim, M. S., Kim, M. J., Nakajima, H., and Kim, K. W. (2002). Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. *Int. J. Cancer* **97**, 290–296.
129. Qian, D. Z., Wang, X., Kachhap, S. K., et al. (2004). The histone deacetylase inhibitor NVP-LAQ824 inhibits angiogenesis and has a greater antitumor effect in combination with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584. *Cancer Res.* **64**, 6626–6634.
130. Myzak, M. C., Karplus, P. A., Chung, F. L., and Dashwood, R. H. (2004). A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. *Cancer Res.* **64**, 5767–5774.
131. Plumb, J. A., Finn, P. W., Williams, R. J., et al. (2003). Pharmacodynamic response and inhibition of growth of human tumor xenografts by the novel histone deacetylase inhibitor PXD101. *Mol. Cancer Ther.* **2**, 721–728.
132. Butler, L. M., Webb, Y., Agus, D. B., et al. (2001). Inhibition of transformed cell growth and induction of cellular differentiation by pyroxamide, an inhibitor of histone deacetylase. *Clin. Cancer Res.* **7**, 962–970.
133. Phillips, A. C. and Vousden, K. H. (2000). Acetyltransferases and tumour suppression. *Breast Cancer Res.* **2**, 244–246.
134. Carapeti, M., Aguiar, R. C., Goldman, J. M., and Cross, N. C. (1998). A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. *Blood* **91**, 3127–3133.
135. Zheng, Y., Thompson, P. R., Cebrat, M., et al. (2004). Selective HAT inhibitors as mechanistic tools for protein acetylation. *Methods Enzymol.* **376**, 188–199.
136. Lau, O. D., Kundu, T. K., Soccio, R. E., et al. (2000). HATs off: selective synthetic inhibitors of the histone acetyltransferases p300 and PCAF. *Mol. Cell* **5**, 589–595.
137. Balasubramanyam, K., Swaminathan, V., Ranganathan, A., and Kundu, T. K. (2003). Small molecule modulators of histone acetyltransferase p300. *J. Biol. Chem.* **278**, 19,134–19,140.
138. Balasubramanyam, K., Altaf, M., Varier, R. A., et al. (2004). Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *J. Biol. Chem.* **279**, 33,716–33,726.
139. Miller, R. W. and Rubinstein, J. H. (1995). Tumors in Rubinstein-Taybi syndrome. *Am. J. Med. Genet.* **56**, 112–115.
140. Muraoka, M., Konishi, M., Kikuchi-Yanoshita, R., et al. (1996). p300 gene alterations in colorectal and gastric carcinomas. *Oncogene* **12**, 1565–1569.
141. Gayther, S. A., Batley, S. J., Linger, L., et al. (2000). Mutations truncating the EP300 acetylase in human cancers. *Nat. Genet.* **24**, 300–303.
142. Varier, R. A., Swaminathan, V., Balasubramanyam, K., and Kundu, T. K. (2004). Implications of small molecule activators and inhibitors of histone acetyltransferases in chromatin therapy. *Biochem. Pharmacol.* **68**, 1215–1220.
143. Beck, J., Fischer, T., Rowinsky, E., et al. (2004). Phase I pharmacokinetic and pharmacodynamic study of LBH589A: a novel histone deacetylase inhibitor. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology (ASCO), New Orleans, LA, USA.
144. Gore, L., Holden, S. N., Basche, M., et al. (2004). Updated results from a phase I trial of the histone deacetylase inhibitor MS-275 in patients with refractory solid tumors. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology (ASCO).
145. Ryan, Q. C., Headlee, D., Acharya, M., et al. (2005). Phase I and pharmacokinetic study of MS-275, a histone deacetylase inhibitor, in patients with advanced and refractory solid tumors or lymphoma. *J. Clin. Oncol.* **23**, 3912–3922.
146. Carducci, M. A., Gilbert, J., Bowling, M. K., et al. (2001). A Phase I clinical and pharmacological evaluation of sodium phenylbutyrate on an 120-h infusion schedule. *Clin. Cancer Res.* **7**, 3047–3055.
147. Warrell, R. P., Jr., He, L. Z., Richon, V., Calleja, E., and Pandolfi, P. P. (1998). Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J. Natl. Cancer Inst.* **90**, 1621–1625.
148. Vasudev, K., Das, S., Goswami, U., and Tayal, G. (2001). Pharmacokinetics of valproic acid in patients with bipolar disorder. *J. Psychopharmacol.* **15**, 187–190.
149. Yegnasubramanian, S., Kowalski, J., Gonzalgo, M. L., et al. (2004). Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res.* **64**, 1975–1986.
150. Maruyama, R., Toyooka, S., Toyooka, K. O., et al. (2002). Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. *Clin Cancer Res.* **8**, 514–519.
151. Anderson, K. M., Seed, T., Meng, J., and Harris, J. E. (1995). Searle SC41661A, a selective inhibitor of 5-lipoxygenase induces apoptosis in androgen-independent human prostate (PC3) cancer cells. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology (ASCO), Los Angeles, California, USA.

152. Rephaeli, A., Blank-Porat, D., Tarasenko, N., et al. (2005). In vivo and in vitro antitumor activity of butyroyloxymethyl-diethyl phosphate (AN-7), a histone deacetylase inhibitor, in human prostate cancer. *Int. J. Cancer* **116**, 226–235.
153. Kuefer, R., Hofer, M. D., Altug, V., et al. (2004). Sodium butyrate and tributyrin induce in vivo growth inhibition and apoptosis in human prostate cancer. *Br. J. Cancer* **90**, 535–541.
154. Thibault, A. Figg, W. D., Lush, R. M., Myers, C. E., Reed, E., and Samid, D. (1997). A phase II study of 5-aza-2'-deoxycytidine (decitabine) in metastatic, hormone-independent prostate cancer. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, USA.
155. Amrein, P. S., Soulieres, D., Schab, A., Lipscher, E., et al. (2003). Massachusetts General Hospital, Boston, MA; Hopital Notre-Dame, Montreal, Canada; MethylGene, Inc., Montreal, Canada; Epigenomics AG, Berlin, Germany Effect of MG98 treatment on DNA gene promoter CpG island methylation patterns in advanced head and neck carcinoma patients. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, USA.
156. Vidal, L. L., M; Sludden, J; Griffin, MG; et al. (2005). A Phase I and pharmacodynamic study of a 7 day infusion schedule of the DNMT1 antisense compound MG98. In, Annual meeting American Society of Clinical Oncology Proceedings.
157. Siu, L. L. (2000). A Phase I and pharmacokinetic (PK) study of the human DNA methyltransferase (METASE) antisense oligodeoxynucleotide MG98 given as a 21-day continuous infusion every 4 weeks. In: Proceeding of the Annual Meeting of the American Society of Clinical Oncology Proceedings, New Orleans, LA, USA.
158. Matsuyama, A., Shimazu, T., Sumida, Y., et al. (2002). In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *Embo J.* **21**, 6820–6831.
159. Yoshida, M., Nomura, S., and Beppu, T. (1987). Effects of trichostatins on differentiation of murine erythroleukemia cells. *Cancer Res.* **47**, 3688–3691.
160. Gui, C. Y., Ngo, L., Xu, W. S., Richon, V. M., and Marks, P. A. (2004). Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. *Proc. Natl. Acad. Sci. USA* **101**, 1241–1246.
161. Richon, V. M., Emiliani, S., Verdin, E., et al. (1998). A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc. Natl. Acad. Sci. USA* **95**, 3003–3007.
162. Remiszewski, S. W. (2003). The discovery of NVP-LAQ824: from concept to clinic. *Curr. Med. Chem.* **10**, 2393–2402.
163. Atadja, P., Gao, L., Kwon, P., et al. (2004). Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824. *Cancer Res.* **64**, 689–695.
164. Su, G. H., Sohn, T. A., Ryu, B., and Kern, S. E. (2000). A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. *Cancer Res.* **60**, 3137–3142.
165. Kim, Y. B., Lee, K. H., Sugita, K., Yoshida, M., and Horinouchi, S. (1999). Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase. *Oncogene* **18**, 2461–2470.
166. Furumai, R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., and Horinouchi, S. (2001). Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. *Proc. Natl. Acad. Sci. USA* **98**, 87–92.
167. Miller, T. A., Witter, D. J., and Belvedere, S. (2003). Histone deacetylase inhibitors. *J. Med. Chem.* **46**, 5097–5116.
168. Nakajima, H., Kim, Y. B., Terano, H., Yoshida, M., and Horinouchi, S. (1998). FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. *Exp. Cell. Res.* **241**, 126–133.
169. Han, J. W., Ahn, S. H., Park, S. H., et al. (2000). Apicidin, a histone deacetylase inhibitor, inhibits proliferation of tumor cells via induction of p21WAF1/Cip1 and gelsolin. *Cancer Res.* **60**, 6068–6074.
170. Darkin-Rattray, S. J., Gurnett, A. M., Myers, R. W., et al. (1996). Apicidin: a novel antiprotozoal agent that inhibits parasite histone deacetylase. *Proc. Natl. Acad. Sci. USA* **93**, 13,143–13,147.
171. Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* **272**, 408–411.
172. Kijima, M., Yoshida, M., Sugita, K., Horinouchi, S., and Beppu, T. (1993). Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. *J. Biol. Chem.* **268**, 22,429–22,435.
173. Curtin, M. and Glaser, K. (2003). Histone deacetylase inhibitors: the Abbott experience. *Curr. Med. Chem.* **10**, 2373–2392.
174. Saito, A., Yamashita, T., Mariko, Y., et al. (1999). A synthetic inhibitor of histone deacetylase, MS-27-275, with marked in vivo antitumor activity against human tumors. *Proc. Natl. Acad. Sci. USA* **96**, 4592–4597.
175. Kraker, A. J., Mizzen, C. A., Hartl, B. G., Miin, J., Allis, C. D., and Merriman, R. L. (2003). Modulation of histone acetylation by [4-(acetylamino)-N-(2-amino-phenyl) benzamide] in HCT-8 colon carcinoma. *Mol. Cancer Ther.* **2**, 401–408.
176. el-Beltagi, H. M., Martens, A. C., Lelieveld, P., Haroun, E. A., and Hagenbeek, A. (1993). Acetyldinaline: a new oral cytostatic drug with impressive differential activity against leukemic cells and normal stem cells—preclinical studies in a relevant rat model for human acute myelocytic leukemia. *Cancer Res.* **53**, 3008–3014.
177. Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., and Klein, P. S. (2001). Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J. Biol. Chem.* **276**, 36,734–36,741.
178. Huang, L. and Pardee, A. B. (2000). Suberoylanilide hydroxamic acid as a potential therapeutic agent for human breast cancer treatment. *Mol. Med.* **6**, 849–866.

179. Pili, R., Kruszewski, M. P., Hager, B. W., Lantz, J., and Carducci, M. A. (2001). Combination of phenylbutyrate and 13-cis retinoic acid inhibits prostate tumor growth and angiogenesis. *Cancer Res.* **61**, 1477–1485.
180. Yang, X., Ferguson, A. T., Nass, S. J., et al. (2000). Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition. *Cancer Res.* **60**, 6890–6894.
181. Rascle, A., Johnston, J. A., and Amati, B. (2003). Deacetylase activity is required for recruitment of the basal transcription machinery and transactivation by STAT5. *Mol. Cell. Biol.* **23**, 4162–4173.
182. Nimmanapalli, R., Fuino, L., Bali, P., et al. (2003). Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. *Cancer Res.* **63**, 5126–5135.
183. Nimmanapalli, R., Fuino, L., Stobaugh, C., Richon, V., and Bhalla, K. (2003). Cotreatment with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) enhances imatinib-induced apoptosis of Bcr-Abl-positive human acute leukemia cells. *Blood* **101**, 3236–3239.
184. Chen, L., Meng, S., Wang, H., et al. (2005). Chemical ablation of androgen receptor in prostate cancer cells by the histone deacetylase inhibitor LAQ824. *Mol. Cancer Ther.* **4**, 1311–1319.
185. Leoni, F., Zaliani, A., Bertolini, G., et al. (2002). The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proc. Natl. Acad. Sci. USA* **99**, 2995–3000.
186. Piekarz, R., Frye, R., Turner, B. M., et al. (2004). Update on the phase II trial and correlative studies of depsipeptide in patients with cutaneous T-cell lymphoma and relapsed peripheral T-cell lymphoma. In*:* Proceedings of the Annual Meeting of the American Society of Clinical Oncology. New Orleans, LA, USA.
187. Keer, H., Reid, T., and Sreedharan, S. (2002). Pivanex activity in refractory non-small cell lung cancer, a phase II study. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology. Orlando, FL, USA.
188. Chang, S. M., Kuhn, J. G., Robins, H. I., et al. (1999). Phase II study of phenylacetate in patients with recurrent malignant glioma: a North American Brain Tumor Consortium report. *J. Clin. Oncol.* **17**, 984–990.
189. Thibault, A., Cooper, M. R., Figg, W. D., et al. (1994). A phase I and pharmacokinetic study of intravenous phenylacetate in patients with cancer. *Cancer Res.* **54**, 1690–1694.
190. Gilbert, J., Baker, S. D., Bowling, M. K., et al. (2001). A phase I dose escalation and bioavailability study of oral sodium phenylbutyrate in patients with refractory solid tumor malignancies. *Clin. Cancer Res.* **7**, 2292–2300.
191. Rudek, M. A., Zhao, M., He, P., et al. (2005). Pharmacokinetics of 5-azacitidine administered with phenylbutyrate in patients with refractory solid tumors or hematologic malignancies. *J. Clin. Oncol.* **23**, 3906–3911.
192. Kelly, W. K., O'Connor, O. A., Krug, L. M., et al. (2005). Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J. Clin. Oncol.* **23**, 3923–3931.
193. Kelly, W. K., Richon, V. M., O'Connor, O., et al. (2003). Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. *Clin. Cancer Res.* **9**, 3578–3588.
194. Byrd, J. C., Marcucci, G., Parthun, M. R., et al. (2005). A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. *Blood* **105**, 959–967.
195. Kristeleit, R. S., Tandy, D., Atadja, P., et al. (2004). Effects of the histone deacetylase inhibitor LAQ824 on histone acetylation, Hsp70 and c-Raf in peripheral blood lymphocytes from patients wih advanced solid tumours enrolled in a phase I clinical trial. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology, New Orleans, LA, USA.
196. Rowinsky, E. K., de Bono, J., Deangelo, D. J., van Oosterom, A., et al. (2005). Cardiac monitoring in phase I trials of a novel histone deacetylase (HDAC) inhibitor LAQ824 in patients with advanced solid tumors and hematologic malignancies. In: Proceeding of the Annual Meeting of the American Society of Clinical Oncology, Orlando, FL, USA.
197. Atmaca, A., Maurer, A., Heinzel, T., et al. (2004). A dose-escalating phase I study with valproic acid in patinets with advanced cancer. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology, New Orleans, LA, USA.
198. Steele, N., Vidal, L., Plumb, J., Attard, G., et al. (2005). A phase 1 pharmacokinetic (PK) and pharmacodynamic (PD) study of the histone deacetylase (HDAC) inhibitor PXD101 in patients (pts) with advanced solid tumours. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology, Orlando, FL, USA.
199. Siu, L. Carducci, M., Pearce, L., Maclean, M., et al. (2005). Phase I study of isotype-selective histone deacetylase (HDAC) inhibitor MGCD0103 given as three-times weekly oral dose in patients (pts) with advanced solid tumors. In: Proceedings of the NCI/EORTC/AACR Meeting, Philadeplhia, PA, USA.
200. Kalita, A. Maroun, C., Bonfils, C., Gelmon, K., et al. (2005). Pharmacodynamic effect of MGCD0103, an oral isotype-selective histone deacetylase (HDAC) inhibitor, on HDAC enzyme inhibition and histone acetylation induction in Phase I clinical trials in patients (pts) with advanced solid tumors or non-Hodgkin's lymphoma (NHL). In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology Proceedings, Orlando, Florida, USA.
201. Gelmon, K. Tolcher, A., Carducci, M., Reid, G. K., et al. (2005). Phase I trials of the oral histone deacetylase (HDAC) inhibitor MGCD0103 given either daily or 3x weekly for 14 days every 3 weeks in patients (pts) with advanced solid tumors. In: Proceedings of the Annual meeting of the American Society of Clinical Oncology, Orlando, FL, USA.

202. Cote, S., Rosenauer, A., Bianchini, A., et al. (2002). Response to histone deacetylase inhibition of novel PML/RARalpha mutants detected in retinoic acid-resistant APL cells. *Blood* **100**, 2586–2596.
203. Coffey, D. C., Kutko, M. C., Glick, R. D., et al. (2001). The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts in vivo, alone and synergistically with all-trans retinoic acid. *Cancer Res.* **61**, 3591–3594.
204. Demary, K., Wong, L., and Spanjaard, R. A. (2001). Effects of retinoic acid and sodium butyrate on gene expression, histone acetylation and inhibition of proliferation of melanoma cells. *Cancer Lett.* **163**, 103–107.
205. Minucci, S., Horn, V., Bhattacharyya, N., et al. (1997). A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA* **94**, 11,295–11,300.
206. Toma, S., Galmozzi, F., Emionite, L., Grattarola, M., Vergani L. (2003). Effects of HDACIs and retinoids on breast cancer cells. In: Annual meeting American Society of Clinical Oncology, Chicago, IL, USA.
207. Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat. Genet.* **21**, 103–107.
208. Boivin, A. J., Momparler, L. F., Hurtubise, A., and Momparler, R. L. (2002). Antineoplastic action of 5-aza-2'-deoxycytidine and phenylbutyrate on human lung carcinoma cells. *Anticancer Drugs* **13**, 869–874.
209. Zhu, W. G. and Otterson, G. A. (2003). The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. *Curr. Med. Chem. Anti-Canc. Agents* **3**, 187–199.
210. Keen, J. C., Yan, L., Mack, K. M., et al. (2003). A novel histone deacetylase inhibitor, scriptaid, enhances expression of functional estrogen receptor alpha (ER) in ER negative human breast cancer cells in combination with 5-aza 2'-deoxycytidine. *Breast Cancer Res. Treat* **81**, 177–186.
211. Gagnon, J., Shaker, S., Primeau, M., Hurtubise, A., and Momparler, R. L. (2003). Interaction of 5-aza-2'-deoxycytidine and depsipeptide on antineoplastic activity and activation of 14-3-3sigma, E-cadherin and tissue inhibitor of metallo-proteinase 3 expression in human breast carcinoma cells. *Anticancer Drugs* **14**, 193–202.
212. Klisovic, M. I., Maghraby, E. A., Parthun, M. R., et al. (2003). Depsipeptide (FR 901228) promotes histone acetylation, gene transcription, apoptosis and its activity is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells. *Leukemia* **17**, 350–358.
213. Primeau, M., Gagnon, J., and Momparler, R. L. (2003). Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. *Int. J. Cancer* **103**, 177–184.
214. Murakami, J., Asaumi, J., Maki, Y., et al. (2004). Effects of demethylating agent 5-aza-2(')-deoxycytidine and histone deacetylase inhibitor FR901228 on maspin gene expression in oral cancer cell lines. *Oral Oncol.* **40**, 597–603.
215. Bras-Goncalves, R. A., Pocard, M., Formento, J. L., et al. (2001). Synergistic efficacy of 3n-butyrate and 5-fluorouracil in human colorectal cancer xenografts via modulation of DNA synthesis. *Gastroenterology* **120**, 874–888.
216. Rosato, R. R., Almenara, J. A., Yu, C., and Grant, S. (2004). Evidence of a functional role for p21WAF1/CIP1 down-regulation in synergistic antileukemic interactions between the histone deacetylase inhibitor sodium butyrate and flavopiridol. *Mol. Pharmacol.* **65**, 571–581.
217. Almenara, J., Rosato, R., and Grant, S. (2002). Synergistic induction of mitochondrial damage and apoptosis in human leukemia cells by flavopiridol and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). *Leukemia* **16**, 1331–1343.
218. Nguyen, D. M., Schrump, W. D., Chen, G. A., et al. (2004). Abrogation of p21 expression by flavopiridol enhances depsipeptide-mediated apoptosis in malignant pleural mesothelioma cells. *Clin. Cancer Res* **10**, 1813–1825.
219. Yu, C., Rahmani, M., Almenara, J., et al. (2003). Histone deacetylase inhibitors promote STI571-mediated apoptosis in STI571-sensitive and -resistant Bcr/Abl+ human myeloid leukemia cells. *Cancer Res.* **63**, 2118–2126.
220. Yu, C., Rahmani, M., Conrad, D., Subler, M., Dent, P., and Grant, S. (2003). The proteasome inhibitor bortezomib interacts synergistically with histone deacetylase inhibitors to induce apoptosis in Bcr/Abl+ cells sensitive and resistant to STI571. *Blood* **102**, 3765–3774.
221. Kim, J. S., Jeung, H. K., Cheong, J. W., et al. (2004). Apicidin potentiates the imatinib-induced apoptosis of Bcr-Abl-positive human leukaemia cells by enhancing the activation of mitochondria-dependent caspase cascades. *Br. J. Haematol.* **124**, 166–178.
222. Kurz, E. U., Wilson, S. E., Leader, K. B., et al. (2001). The histone deacetylase inhibitor sodium butyrate induces DNA topoisomerase II alpha expression and confers hypersensitivity to etoposide in human leukemic cell lines. *Mol. Cancer Ther.* **1**, 121–131.
223. Bhalla, K., George, P., Gutti, R., et al. (2004). A combination of histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC and AML cells with constitutively active mutant FLT-3 tyrosine kinase. In, Proceedings of the Annual Meeting of the American Society of Clinical Oncology, New Orleans, LA, USA.
224. Rahmani, M., Yu, C., Dai, Y., et al. (2003). Coadministration of the heat shock protein 90 antagonist 17-allylamino-17-demethoxygeldanamycin with suberoylanilide hydroxamic acid or sodium butyrate synergistically induces apoptosis in human leukemia cells. *Cancer Res.* **63**, 8420–8427.

225. Kim, M. S., Blake, M., Baek, J. H., Kohlhagen, G., Pommier, Y., and Carrier, F. (2003). Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. *Cancer Res.* **63**, 7291–7300.
226. Biade, S., Stobbe, C. C., Boyd, J. T., and Chapman, J. D. (2001). Chemical agents that promote chromatin compaction radiosensitize tumour cells. *Int. J. Radiat. Biol.* **77**, 1033–1042.
227. Camphausen, K., Burgan, W., Cerra, M., et al. (2004). Enhanced radiation-induced cell killing and prolongation of gammaH2AX foci expression by the histone deacetylase inhibitor MS-275. *Cancer Res.* **64**, 316–321.
228. Camphausen, K., Scott, T., Sproull, M., and Tofilon, P. J. (2004). Enhancement of xenograft tumor radiosensitivity by the histone deacetylase inhibitor MS-275 and correlation with histone hyperacetylation. *Clin. Cancer Res.* **10**, 6066–6071.
229. Kim, J. H., Shin, J. H., and Kim, I. H. (2004). Susceptibility and radiosensitization of human glioblastoma cells to trichostatin A, a histone deacetylase inhibitor. *Int. J. Radiat. Oncol. Biol. Phys.* **59**, 1174–1180.
230. Zhang, Y., Adachi, M., Zhao, X., Kawamura, R., and Imai, K. (2004). Histone deacetylase inhibitors FK228, N-(2-aminophenyl)-4-[N-(pyridin-3-yl-methoxycarbonyl)amino- methyl]benzamide and m-carboxycinnamic acid bis-hydroxamide augment radiation-induced cell death in gastrointestinal adenocarcinoma cells. *Int. J. Cancer* **110**, 301–308.
231. Chinnaiyan, P., Vallabhaneni, G., Armstrong, E., Huang, S. M., and Harari, P. M. (2005). Modulation of radiation response by histone deacetylase inhibition. *Int. J. Radiat. Oncol. Biol. Phys.* **62**, 223–229.
232. Hernandez, A., Thomas, R., Smith, F., et al. (2001). Butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis. *Surgery* **130**, 265–272.
233. Inoue, H., Shiraki, K., Ohmori, S., et al. (2002). Histone deacetylase inhibitors sensitize human colonic adenocarcinoma cell lines to TNF-related apoptosis inducing ligand-mediated apoptosis. *Int. J. Mol. Med.* **9**, 521–525.
234. Rosato, R. R., Almenara, J. A., Dai, Y., and Grant, S. (2003). Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells. *Mol. Cancer Ther.* **2**, 1273–1284.
235. Guo, F., Sigua, C., Tao, J., et al. (2004). Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells. *Cancer Res.* **64**, 2580–2589.
236. Chinnaiyan, P., Varambally, S., Tomlins, S. A., Huang, S., Chinnaiyan, A. M., and Harari, P. M. (2004). Enhancing the anti-tumor activity of ErbB blockade with histone deacetylase inhibition. In: Proceedings of the Annual Meeting of the American Society of Clinical Oncology, New Orleans, LA, USA.
237. Fuino, L., Bali, P., Wittmann, S., et al. (2003). Histone deacetylase inhibitor LAQ824 down-regulates Her-2 and sensitizes human breast cancer cells to trastuzumab, taxotere, gemcitabine, and epothilone B. *Mol. Cancer Ther.* **2**, 971–984.
238. Marks, P. A., Rifkind, R. A., Richon V. M., et al. (2001). Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer* 1, 194–202.
239. Marks, P. A., Richon, V. M., Miller, T., and Kelly, W. K. (2004). Histone deacetylase inhibitors. *Adv. Cancer Res.* **91**, 137–168.

23

Taxane-Based Chemotherapy for Prostate Cancer

Supriya G. Mohile and Daniel P. Petrylak

Summary

Hormone-refractory prostate cancer (HRPC), defined as progression of disease in the setting castrate testosterone levels, is associated with significant morbidity and a dismal prognosis. Historically, the paradigm for treatment of HRPC focused on palliation because of the lack of available therapies that improved survival. The taxanes, chemotherapeutic agents that stabilize microtubules, thereby preventing further cell division, demonstrated efficacy in the treatment of HRPC in phase I and phase II studies. In addition, taxane-based therapies palliated disease complications with minimal toxicity. Two randomized studies, SWOG 9916 and TAX 327, were the first trials to establish the survival benefit of docetaxel for men with progressive HRPC. As the literature describing the benefit of taxane-based chemotherapy for metastatic disease has grown, the role of these therapies for neoadjuvant and adjuvant treatment of high-risk localized disease are being investigated. The future of taxane-based treatment lies in combination strategies with biological and targeted therapy, including agents targeting angiogenesis, the endothelin receptor, and other signal pathways to help overcome drug resistance and improve clinical outcomes for patients with HRPC.

Key Words: Chemotherapy; docetaxel; estramustine; paclitaxel; palliation; resistance; survival; taxane.

1. INTRODUCTION

The second most common malignancy causing death in men in the United States is prostate cancer. In 2005, 1 in 6 men (more than 230,000 men) will be diagnosed with prostate cancer, and, in the United States, 30,350 men will die of the disease *(1)*. The incidence of prostate cancer rapidly increases with age (from approximately 30% in men older than 50 years to 80% by age 80 years) and the increasing life expectancy of men will result in an increasing number of diagnoses in the new millennium *(2)*.

With the advent of prostate-specific antigen (PSA) screening, the majority of newly diagnosed men with prostate cancer present with localized disease and are candidates for definitive therapies, such as surgery or radiation *(3)*. Despite undergoing therapy for localized disease, a subset of patients will have a high risk of failure as measured by biochemical recurrence or development of metastatic disease after localized therapy. The 10-year actuarial likelihood of biochemical disease recurrence after radical prostatectomy and radiation therapy is approx 25% *(4–6)*. Although definitions for this high-risk subgroup vary, adverse risk factors include Gleason's scores of 8 to 10 on prostatic biopsy, clinical stage T3 or T4 disease, positive lymph nodes, and PSA levels greater than 20 ng/mL at presentation *(7)*.

Surgical or medical androgen ablation is the mainstay of therapy for those patients who ultimately develop osseous or visceral metastasis or who, less commonly, have advanced disease at presentation. Although androgen ablation is a highly effective therapy for advanced hormone-sensitive prostate cancer and is associated with rapid and dramatic responses, almost inevitably the dis-

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

ease progresses to a hormone-refractory state. Men with hormone-refractory prostate cancer (HRPC), defined as progression of disease in the face of castrate testosterone levels, historically have a dismal prognosis, with median survival times of 9 to 12 months *(8)*. In addition, the morbidity associated with hormone-refractory disease is significant and can include bone disease leading to spinal cord compression, fractures, pain, cachexia, anemia, and, ultimately, death.

The management of HRPC in the past has been limited to palliation of symptoms because of a lack of effective treatments. Anti-androgen withdrawal and second-line hormonal therapies, such as corticosteroids, ketoconazole, or estrogen-based agents only achieve transient responses, with a median duration of 3 to 4 months, and no demonstrable survival benefit. Historically, chemotherapy for advanced prostate cancer was viewed as toxic and ineffective. Until recently, HRPC had been considered a chemotherapy-resistant disease with no randomized study demonstrating a survival of chemotherapy *(9,10)*. The combination of mitoxantrone–prednisone was approved by the Food and Drug Administration (FDA) based on palliation of bone pain; three randomized trial also demonstrated modest improvements in time to progression when mitoxantrone combined with corticosteroids was compared with corticosteroids alone *(11–13)*. Two randomized studies, demonstrating that docetaxel-based chemotherapy improves survival in men with HRPC, have shifted the paradigm of chemotherapy from palliation to survival. This review covers the role of taxane-based chemotherapy for different stages of prostate cancer, mechanisms of resistance, and future directions.

2. PRECLINICAL RATIONALE FOR TAXANE-BASED CHEMOTHERAPY

2.1. Rationale for Taxanes

Chemotherapy resistance in prostatic cancer cells is likely multifactorial and may be caused by the lower rates of cycling fraction of these cells and patterns of expression of oncogenes. The *bcl-2* gene family is a class of oncogenes necessary for apoptosis regulation at the level of the mitochondria. Protein derivatives from this family include cell death agonists, such as Bax, or anti-apoptotic proteins, such as Bcl-2. Apoptotic signaling from antiprostate cancer treatments (e.g., androgen withdrawal, chemotherapy, or radiation) can result in the release of cytochrome-c into the cytoplasm through mitochondrial membrane changes initiated by the formation of Bax–Bax complexes. Downstream proteolytic caspases, enzymes that mediate cell death, are activated by the binding of cytochrome-c with Apaf-1 in the cytoplasm. Bcl-2 is a cell death antagonist, preventing apoptotic cell death by binding with Bax, thereby preventing release of cytochrome-c and initiation of the apoptotic cascade. Phosphorylation of Bcl-2 abrogates its anti-apoptotic activity. The balance between cell death antagonists and agonists of the Bcl-2 family of proteins regulates how prostatic cancer cells respond to external stimuli *(14–16)*.

Resistance of prostate cancer cells to cytotoxic treatment has been correlated with overexpression of *bcl-2*. *Bcl-2* expression is increased in clinical biopsy specimens from men with metastatic, androgen-independent disease *(8,17–19)*. In addition, experimental studies have reported that overexpression of *bcl-2* is associated with development of androgen independence and treatment resistence *(20,21)*. For example, there is marked resistance of Dunning-G rat prostate cell lines transfected with bcl-2 to chemotherapy, as compared with control cell lines *(22)*.

Because *bcl-2* overexpression has been correlated with progression to advanced disease states and is associated with the development of resistance, agents that interfere with *bcl-2* regulated pathways may improve outcomes. Taxanes are a class of agents that can induce changes in *bcl-2*. Paclitaxel has been shown to induce apoptosis in PC-3 prostate cancer cells through phosporylation of Bcl-2 *(23)*. Docetaxel has an approx 100-fold higher potency than paclitaxel in inhibiting anti-apoptotic activity of Bcl-2 through phosphorylation *(8,23)*. Taxanes are also theoretically appealing antiprostate cancer agents, because they interfere in the cell cycle with both mitotic and antimitotic effects, and they may have better efficacy than other agents in HRPC because of the inherent low rate of cell cycling in this disease.

2.2. *Rationale for Estramustine*

The approval of estramustine phosphate for HRPC by the Food and Drug administration for HRPC preceded docetaxel by longer than 20 years. This synthetic conjugate of estradiol and nitrogen mustard disrupts the cytoplasmic microtubule apparatus by binding to tubulin and related proteins *(24,25)*. In addition, estramustine inhibits nuclear matrix assembly and p-glycoprotein-controlled drug efflux *(26,27)*. Modest single-agent activity has been demonstrated in HRPC when estramustine is administered orally at total doses of 14 mg/kg/d, with PSA declines of greater than 50% observed in 19% of patients *(28)*. This response rate is tempered by an adverse side effect profile, of gastro-intestinal distress and thromboembolism. In vitro studies demonstrated that if estramustine was combined with other agents that destabilize or stabilize the microtubule apparatus or nuclear matrix, such as vinblastine, paclitaxel, docetaxel, and etoposide, greater than additive or synergistic cytotoxicity was noted in human prostate cancer cell lines *(29,30)*. Clinical evidence of synergistic effects were seen in early phase II studies combining estramustine phophatase with oral etoposide; only 2 of 22 patients demonstrated a response to oral etoposide (VP-16) as a single agent *(31)*, whereas the combination of estramustine and oral etoposide demonstrated PSA declines of greater than 50% in 50% of patients *(32)*. A randomized trial comparing single-agent vinblastine to the estramustine plus vinblastine combination suggested an improved survival with the combination *(33)*.

3. PRELIMINARY STUDIES OF TAXANE-BASED THERAPIES

3.1. *Single-Agent Paclitaxel*

Single-agent paclitaxel has been evaluated for treatment of men with HRPC. In one phase II trial, paclitaxel was administered as a 24-hour continuous infusion at 135 mg/m^2 every 21 days to men with measurable disease *(34)*. One of 23 patients in this study achieved a 50% decline in PSA level and an objective partial response. However, the median survival was only 9 months. The weekly administration of single-agent paclitaxel was administered to 18 patients in a phase II trial of The Eastern Cooperative Oncology Group (ECOG) at 150 mg/m^2 for 6 weeks of an 8-week cycle *(35)*. Of the 18 patients treated (all of whom had measurable disease at trial entry), 4 achieved a major response and 39% of patients had a PSA decline of at least 50%. Of note, toxicities in the two phase II trials were considerably different, which can be attributed to the different modes of administration. Although myelosuppression was common with the 24-hour infusion (administered every 3 weeks), peripheral neurotoxicity was the major high-grade toxicity with the weekly 1-hour infusion.

3.2. *Single-Agent Docetaxel*

A number of trials have been completed with single-agent docetaxel in HRPC using weekly and every 3 weeks schedules (Table 1). Docetaxel at 75 mg/m^2 every 3 weeks was associated with a greater than 50% decline of PSA level in 38% and 46% of patients treated in two separate phase II studies *(36,37)*. Objective partial responses were observed in 17% and 28% of patients with measurable disease in these studies. Weekly docetaxel seemed to have similar activity to weekly paclitaxel with PSA declines of greater than 50% ranging between 20% and 46%, and median survivals between 9 and 14 months *(38–40)*.

Toxicities were predominantly myelosuppression and fatigue when docetaxel was administered every 3 weeks. In a phase II study by Friedland et al. evaluating docetaxel in the every 3 week schedule *(37)*, grade 3 or 4 neutropenia was observed in 86% of patients treated. Myelosuppression was less frequent in the weekly regimen of docetaxel as compared with the 3-week regimen.

3.3. *Taxane Plus Estramustine Combinations*

Results of single-agent trials of taxanes and estramustine in HRPC supported the investigation of the combination of taxane plus estramustine. Results of early phase studies evaluating taxane and estramustine combinations are summarized in Tables 2 and 3.

Table 1
Summary of Single-Agent Docetaxel-Based Treatment of HRPC [a]

Reference	No. of patients	Treatment	≥50% PSA decline (%)	Measurable disease response (%)	Median overall survival (mo)
Picus et al. *(36)*	35	D 75 mg/m^2 q 3 wk	45	28	20
Beer et al. *(38)*	25	D 36 mg/m^2 q wk × 6; rest 2 wk	46	40	10
Berry et al. *(39)*	61	D 36 mg/m^2 q wk × 6; rest 2 wk	41	33	9.4
Friedland et al. *(37)*	16	D 75 mg/m^2 q 3 wk	38	17	NR
Gravis et al. *(40)*	15	D 35 mg/m^2 q wk × 6; rest 2 wk	58	NR	NR

[a] HRPC, hormone-refractory prostate cancer; PSA, prostate-specific antigen; D, docetaxel; q, every; NR, not reported.

3.3.1. Paclitaxel Plus Estramustine

A phase I study combined oral estramustine at 600 mg/m^2 per day with paclitaxel as an infusion during 96 hours in patients with advanced solid tumors *(41)*. Myelosuppression prevented dose escalation of paclitaxel to greater than 120 mg/m^2. Responses were described for prostate cancer and esophageal cancer. The paclitaxel plus estramustine doublet has been evaluated in several phase II studies. In the first phase II trial reported, paclitaxel at 120 mg/m^2 administered by continuous infusion during 96 hours every 3 weeks, in combination with continuous oral estramustine was associated with a PSA decline of greater than 50% in 53% of patients, and a partial response in 44% of men with measurable disease *(42)*. Similar PSA levels and objective response rates were found with the combination of estramustine at 600 mg/m^2 per day and paclitaxel at 225 mg/m^2 administered during 3 hours every 3 weeks *(43)*. PSA decline rates of 42% and 62% were found in two phase II studies of weekly paclitaxel combined with daily oral estramustine *(44,45)*. Other combination regimens of paclitaxel and estramustine with carboplatin and/or etoposide have been evaluated, with PSA responses ranging from 65% to 73%, and measurable disease response rates of 45 to 64% *(46–48)*. Toxicity with these regimens were deemed acceptable, with thromboembolism and neutropenia being most frequently encountered. Of note, a randomized phase II study suggested improved survival of the combination of weekly paclitaxel plus estramustine over paclitaxel alone *(49)*.

3.3.2. Docetaxel Plus Estramustine

Phase I studies of docetaxel plus estramustine found significant PSA declines, responses in measurable disease, and palliation of symptoms associated with HRPC, such as bone pain. In a phase I trial by Petrylak et al. at Columbia Presbyterian Medical Center, men with pretreated androgen-independent prostate cancer received the combination of estramustine (280 mg orally, thrice daily for days 1–5) with docetaxel (40 mg/m^2 escalated to 80 mg/m^2 administered every 3 weeks) *(50)*. Dexamethasone was administered at 20 mg for three doses starting the day before docetaxel. At the dose of 80 mg/m^2, myelosuppression was dose limiting. The recommended dose for future phase II evaluations was 70 mg/m^2 for minimally pretreated patients and 60 mg/m^2 for heavily pretreated patients. PSA declines were noted at all dose levels, with all seven patients treated at the 70 mg/m^2 dose level experiencing a greater than 50% decline of PSA level. In addition, of the 18 patients with bidimensionally measurable disease, 28% achieved an objective response. Median survival time was 23 months with 68% of patients alive at 1 year. In this phase I study, 8 of 15 patients who entered the trial using narcotic analgesics were able to discontinue use of these pain medications for a median of 6 weeks.

Table 2
Phase I Studies Using Docetaxel and Estramustine for HRPC[a]

Reference	No. of patients	≥ 50% PSA decline (%)	>50% tumor reduction	Symptomatic improvement (%)	Median overall survival (mo)	Recommended Phase II regimen
Petrylak *(50)*	MPT: 20	70	28	53	24	D 70 q 3 wk
	EPT: 12	50				D 60 q 3 wk
						E 280 mg tid, day 1–5
Kreis *(51)*	17	82	NR	NR	NR	D 70 q 3 wk
						E 12 mg/kg/d
Natale *(52)*	18	78	67	86	NR	D 35 q wk
						E 280–420 mg tid, day 1–3

[a] HRPC, hormone-refractory prostate cancer; PSA, prostate-specific antigen; MPT: minimal previous therapy (MPT, ≤2 previous chemotherapies, ≤2 previous radiation therapies, no history of radioisotope therapy, no evidence of superscan on bone scan, and no history of whole pelvic radiation therapy); EPT: extensive previous therapy; D, docetaxel; q, every; E: estramustine; tid, thrice daily; NR, not reported.

Table 3
Phase II Studies of Taxane-Estramustine Combinations for HRPC

Combination treatments	Reference	No. of patients	Treatment	>50% PSA decline (%)	Measurable disease response (%)	Median overall survival (mo)
Paclitaxel	Vaughn et al. *(45)*	66	• E 140 tid PO day 1–3 + P 90 mg/m^2 over 1 h q wk × 3	42	15	15.6
	Vaishampayan et al. *(44)*	28	• E 280 mg PO tid day 1–3, 8–10, 15–17 + P 150 mg/m^2 over 1 h day 2, 9, 16 of a 28-day cycle	62	38	13
	Hudes et al. *(42)*	34	• E 600 mg/m^2/d PO + P 120 mg/m^2 over 96 h day 1–4 q 3 wk	53	44	15.9
	Haas et al. *(99)*	24	• E 600 mg/m^2/d PO + P 60–118 mg/m^2 over 3 h q wk	38	46	18.9
	Athanasiadis et al. *(100)*	41	• E 280 mg PO bid + P 60–90 mg/m^2 q wk	56	49	13
	Hudes et al. *(101)*	63	• E 280 mg PO bid q wk × 6 + P 90 mg/m^2/wk over 1 h	58	27	NR
	Kelly et al. *(46)*	56	• E 10 mg/kg/d PO + P 60–100 mg/m^2 over 1 h q wk + C AUC = 6 q 4 wk	67	45	19.9
	Smith et al. *(47)*	37	• E 280 mg PO tid day 1–7 + VP–16 100 mg PO qd day 1–7 + P 135 mg/m^2 over 1 h day 2 q 3 wk	65	45	12.8
	Chay et al. *(48)*	19	• E 280 mg PO tid + VP–16 50 mg/m^2 qd × 7 + P 135 mg/m^2 1 h + C AUC = 5 day 2 q 3 wk	38	17	NR
Docetaxel	Savarese et al. *(54)*	47	• E 10 mg/kg/d PO day 1–5 + D 70 mg/m^2 day 2 q 3 wk + HC 40 mg/d	68	50	20
	Petrylak et al. *(102)*	35	• E 280 mg PO tid day 1–5 + D 70 mg/m^2 d2 q 3 wk	74	57	20
	Sinibaldi et al. *(55)*	32	• E 280 mg PO q 6 h × 5 + D 70 mg/m^2 day 2 q 3 wk	45	23	NR
	Kosty et al. *(56)*	21	• E 140 mg/d day 1–5 + D 43 mg/m^2/wk + DEX 8 mg PO × 3 doses q wk × 3 of 4 wks	71	11	NR
	Sitka Copur et al. *(103)*	30	• E 280 mg PO tid × 3 d wk 1 & 2 + D 35 mg/m^2/wk × 2 q 3 wk	76	58	NR

[a] HRPC, hormone-refractory prostate cancer; PSA, prostate-specific antigen; E: estramustine; tid, thrice daily; PO, by mouth; q, every; P, paclitaxel; NR, not reported; C, carboplatin; AUC, area under the curve; VP-16, etoposide; qd, daily; HC, hydrocortisone; D, docetaxel; DEX, dexamethasone.

A second phase I study in patients with no previous chemotherapy confirmed the dose of docetaxel at 70 mg/m^2 administered every 3 weeks, which was deemed safe with even a higher dose of estramustine (12 mg/kg/d) *(51)*. Estramustine, however, was associated with gastrointestinal distress (nausea and diarrhea) requiring interruptions of therapy. Fatigue was the major dose-limiting toxicity. A phase I study evaluating a weekly dosing schedule of docetaxel with estramustine determined the safest dose at 35 mg/m^2 *(52)*. Both of these phase I studies obtained similarly impressive results to the Petrylak study, with high PSA levels and measurable disease response rates (Table 2).

Several phase II studies support the combination of docetaxel and estramustine for treatment of HRPC *(53–56)*. Overall, PSA response rates ranged from 45 to 74% with measurable disease responses of 11 to 57% (Table 3). Prophylactic anticoagulation decreased the rate of vascular events, including cerebrovascular accidents and deep vein thrombosis, in one phase II study *(53)*. In a Cancer and Leukemia Group B (CALGB) phase II study, the combination of low-dose hydrocortisone plus docetaxel plus estramustine was well-tolerated and efficacious with similar activity to docetaxel plus estramustine alone *(54)*. This study reported a median survival of 20 months. The combination of weekly docetaxel plus dexamethasone plus estramustine also produced high PSA levels and objective response rates and was well-tolerated *(56)*.

Although it was known that corticosteroids, when administered continuously to men with HRPC, can obtain both palliative and PSA responses, the role that intermittent administration of dexamethasone plays in response rates of docetaxel plus estramustine combinations was unclear. Therefore, a phase II lead-in trial was designed to determine the contribution of dexamethasone to the response rates of docetaxel and estramustine *(57)*. Single-agent dexamethasone at 20 mg was administered orally every 6 hours for 3 doses to 12 patients every 3 weeks before starting cytotoxic therapy with docetaxel and estramustine. None of the patients initially treated with dexamethasone alone had a 50% or greater PSA decline. With subsequent treatment with cytotoxic therapy, 92% of patients achieved a PSA decline of greater than 50%, and three of four patients with measurable disease achieved a response. Intermittent dexamethasone, therefore, is not thought to contribute significantly to the PSA response rate of docetaxel in combination with estramustine.

4. PHASE III STUDIES OF DOCETAXEL-BASED THERAPY FOR HRPC

Two phase III trials have compared taxane-based combination regimens with standard mitoxantrone plus prednisone in men with progressive HRPC. These trials were developed to evaluate whether the promising results from phase II studies were caused by real therapeutic interventions, rather than patient selection or patient stage migration. Figure 1 describes the treatment schema and Table 4 describes summary data from these definitive trials.

4.1. Southwest Oncology Group Study 9916

In October of 1999, the Southwest Oncology Group Study (SWOG) initiated an intergroup study with the CALGB, NTTCG, and the CTSU of the National Cancer Institute, comparing randomized men to docetaxel plus estramustine or mitoxantrone plus prednisone *(58)*. The primary end point of this study was survival; the trial was designed to detect a 33% improvement in overall survival between the two treatment arms using a one-sided log-rank test at a p level of 0.025. Secondary end points of SWOG 9916 included progression-free survival, objective response rate, and rate of decline in PSA level. For study entry, patients were required to have androgen-independent prostate cancer (stage M+), with progression documented by a rising PSA level or progression on bone or CT scans. Patients were also required to have undergone castration with orchiectomy or ongoing luteinizing hormone-releasing hormone agonist therapy; at most one previous chemotherapy regimen; no previous treatments with estramustine, taxanes, anthracyclines, or mitoxantrone; and withdrawal of ongoing anti-androgens, chemotherapy, and bisphosphonates.

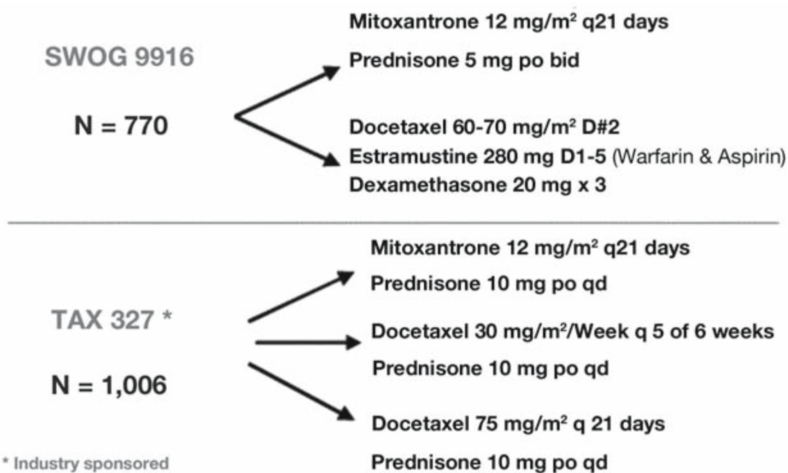


Fig. 1. Treatment design for Southwest Oncology Group Study (SWOG) 9916 and TAX 327.

Men randomized to the taxane arm received estramustine orally at 280 mg thrice daily on days 1 to 5, docetaxel at 60 mg/m^2 iv on day 2 every 21 days, and 60 mg dexamethasone orally in three divided doses before docetaxel. Men randomized to the mitoxantrone arm received mitoxantrone at 12 mg/m^2 iv every 21 days and 5 mg prednisone orally twice daily. Dose escalation to docetaxel 70 mg/m^2 or mitoxantrone 14 mg/m^2 was permitted for those patients who did not experience grade 3 or 4 toxicity in the first cycle of therapy. The protocol was amended in January 2001 to include daily 2 mg Coumadin and 325 mg aspirin in the docetaxel plus estramustine group after decreases in estramustine-related vascular events were noted with prophylactic anticoagulation *(55)*.

Seven hundred seventy patients were enrolled during a 4-year period. Although 12% of patients were found to be ineligible for the study after randomization, primarily because of lack of adequate anti-androgen withdrawal and lack of complete baseline data, results examined by eligibility or intent-to-treat analysis were not different. In the intent-to-treat analysis, docetaxel plus estramustine treatment was associated with an improved median survival (17.5 months compared with 15.6 months; $p = 0.01$), a longer progression-free survival (6.3 months compared with 3.2 months; $p < 0.001$), and more PSA declines (50% as compared with 27%; $p < 0.0001$). Objective responses of measurable soft tissue disease were noted in 17% of patients in the docetaxel arm compared with 11% in the estramustine arm; however, this result was not statistically significant ($p = 0.030$). In addition, palliation of bone pain was not found to be statistically different in the two arms. Overall, the relative risk of death was reduced by 20% with docetaxel and estramustine as compared with mitoxantrone and prednisone Hazard ratio (HR for death, 0.80; 95% confidence interval, 0.67–0.97).

Because PSA declines have been retrospectively associated with improved survival, a secondary analyses was performed evaluating the possible use of PSA as a surrogate marker for survival *(59)*. A 30% decrease at 3 months was found to best meet criteria for surrogacy. Of note, the most commonly reported PSA decline, greater than 50%, barely missed the criteria of surrogacy.

Higher incidence of grade 3 and 4 toxicities was reported in the docetaxel plus estramustine arm when compared with the mitoxantrone plus prednisone arm. The incidence of grade 3 or 4 cardiovascular (15% vs 7%; $p = 0.001$), neurological (7% vs 2%; $p = 0.001$), neutropenic fever (5% vs 2%; $p < 0.001$), gastrointestinal (20% vs 5%; $p < 0.001$), and metabolic disturbances (6% vs 1%; $p < 0.001$) were increased in the experimental arm. However, there was not a higher rate of discontinuation from the study and there was no increase in toxic deaths in the docetaxel plus estramustine arm. A *post hoc*

Table 4
Summary of Definitive Phase III Trials [a]

Study	Treatment regimen	Objective measurable response rate (%)	PSA response rate (%)	% With palliative response	Time to progression	Survival (mo)
SWOG 9916	Docetaxel/estramustine	17	50	17 [b]	6	18
	Mitoxantrone/prednisone	10	27	11	3	16
TAX 327	Docetaxel (q 3 wks)/prednisone	12 [b]	45	35	7.9 [b]	18.9
	Docetaxel (q wk)/prednisone	8 [b]	48	31	8.2 [b]	17.4
	Mitoxantrone/prednisone	7 [b]	32	22	7.8 [b]	16.5

[a] PSA, prostate-specific antigen; SWOG, Southwest Oncology Group Study; TAX, ; q, every.
[b] Did not reach statistical significance.

analysis of toxicity revealed that anticoagulation decreased the rate of cardiac ischemia but not the rate of thrombosis. However, the evaluation of the use of anticoagulation is limited because the trial was not designed to detect a difference in vascular events for patients using anticoagulation as compared with those who did not receive Coumadin and aspirin.

4.2. TAX 327

TAX 327 was an international multicenter study that compared two different dosing schedules of docetaxel plus prednisone with mitoxantrone plus prednisone for metastatic HRPC *(60)*. Men were required to have androgen-independent prostate cancer (stage M+); progressive disease; castrate levels of testosterone (≤50 ng/dL); previous orchiectomy or continued luteinizing hormone-releasing hormone agonist; and no history of any previous chemotherapy except for estramustine. The treatment arms consisted of docetaxel at 75 mg/m^2 every 3 weeks or docetaxel 30 mg/m^2 weekly for 5 of 6 weeks. Patients randomized to the control arm received mitoxantrone at 12 mg/m^2 every 3 weeks. Twice daily oral 5 mg prednisone was administered to patients in all three arms of the study and was continued throughout trial participation. The trial was designed to detect a 33% difference in overall survival of either docetaxel regimen compared with the mitoxantrone plus prednisone arm. The trial was not designed to make comparisons between the two docetaxel treatment arms.

A total of 1006 patients were enrolled in this study. Stratification was performed based on performance levels and pain levels before trial entry. In the intent-to-treat data analysis, the median overall survival rate for the men in the every 3 week docetaxel arm was 18.9 months, whereas the median survival in the control arm was 16.4 months ($p = 0.009$). Weekly docetaxel did not result in a statistically significant survival advantage. When compared with the mitoxantrone plus prednisone group, the reduction in the risk of death was 24% and 9% for the every 3 week and weekly docetaxel arms, respectively. In addition, 45% and 48% of patients in the every 3 week and weekly docetaxel groups had a greater than 50% decline in their PSA as compared with 32% of patients in the mitoxantrone group. There were no significant differences among the three groups in the rate of objective tumor responses. Docetaxel therapy was associated with superior palliation of bone pain (33% and 31% in the docetaxel every 3 weeks and weekly regimens as compared with 21% in the mitoxantrone group). Quality of life, in general, when using the FACT-P instrument was significantly better in the docetaxel groups as compared with the mitoxantrone group.

Neutropenia was more frequent in the every 3 week docetaxel group (32% compared with 21.7% in the mitoxantrone group). Grade 3 and 4 neutropenia occurred in 3% of patients in the docetaxel every 3 week group, with 2.7% experiencing febrile neutropenia. Neuropathy and alopecia were also more frequent in the docetaxel arms; however, the patterns of toxicity were not significantly different between the docetaxel and mitoxantrone groups.

4.3. Conclusions From the Definitive Phase III Trials

Table 4 provides a summary of the definitive phase III trials. SWOG 9916 and TAX 327 were the first trials to demonstrate benefits in survival for men with progressive HRPC. Several considerations should be taken into account for patient care and development of future clinical trials. The median survival of patients in the mitoxantrone arms of the studies were higher than what has been reported in previous phase III trials. Stage migration could account for this discrepancy. Nineteen percent of patients in the SWOG 9916 trial had asymptomatic biochemical progression in the face of metastatic disease; a similar percentage of patients had an asymptomatic PSA rise on TAX 327. These patients may have a longer survival time than patients with progressive symptomatic metastatic disease. In addition, crossover could explain the higher median survival in the mitoxantrone arm. In the SWOG 9916 study, 35% of patients failing mitoxantrone plus prednisone treatment received second-line therapy.

The TAX 327 trial did not find an improved survival benefit with weekly docetaxel as compared with mitoxantrone plus prednisone. The study was not designed to make comparisons between the two docetaxel arms, and statistical variation may account for the lack of benefit obtained. In addition, the dose and schedule of the weekly docetaxel arm may account for the lack of benefit. Despite the lack of survival benefit, weekly docetaxel demonstrated a superior quality of life response when compared with mitoxantrone plus prednisone. This regimen should still be considered for schedule-dependent investigational studies if there is a sound preclinical rationale.

Despite a similar control arm, comparisons between SWOG 9916 and TAX 327 cannot validly be performed because of different entry criteria, follow-up, crossover patterns, and patient selection bias. The role of estramustine in combination with taxanes in the treatment of metastatic HRPC is, therefore, not definitively defined because neither study was designed to evaluate the contribution of the second drug. However, because both trials produced similar hazard ratios for death, and treatment with docetaxel plus prednisone was better tolerated, docetaxel/prednisone in the every 3 week schedule should be considered the standard of care for the treatment of progressive androgen-independent metastatic prostate cancer.

5. OTHER ROLES FOR TAXANE-BASED THERAPIES IN THE TREATMENT OF PROSTATE CANCER

Although most patients with localized disease can be cured with surgery or radiation, there exists a subset of patients that have high risk of failure with standard treatment. Because of the inability of modern-day imaging techniques to detect micrometastases, 30 to 50% of prostate cancer thought to be organ-confined is actually understaged *(61,62)*. Several studies have determined that successful treatment of localized disease with single-agent modalities, such as radiation and surgery, include factors such as PSA levels less than 10 ng/mL, organ-confined prostate cancer, and lack of poorly defined histological features, such as Gleason's score of greater than 7 *(63)*. A significant risk for treatment failure exists for patients with adverse prognostic features because of the likely presence of occult metastases, high volume of the tumor, and/or extraprostatic extension of the tumor. The addition of systemic chemotherapy before (neoadjuvant) or after (adjuvant) to local therapy to potentially eliminate micrometastases or reduce locally advanced prostate tumors may help to improve outcome for high-risk patients. With neoadjuvant therapy, postoperative tissue could be evaluated for efficacy of systemic treatment modalities. Advantages of adjuvant therapy include the lack of delay of definitive local treatment and the sparing of some patients of unnecessary systemic treatment because of better surgical staging.

Androgen-deprivation therapy has been studied in the neoadjuvant setting for patients with high-risk disease. Unfortunately, neoadjuvant hormonal therapy before surgery has failed to demonstrate benefit in biochemical-free, progression-free, or overall survival *(64–67)*. Neoadjuvant hormonal therapy may affect a defined population of androgen-sensitive prostate cancer cells; however, there remains cells that are androgen-insensitive that do not respond. Preoperative hormonal therapy does not reduce the incidence of positive lymph nodes or seminal vesicle involvement *(64–67)*. Neoadjuvant and adjuvant hormonal therapy, however, does have benefit with improvement in progression-free and overall survival for high-risk patients undergoing external beam radiation therapy *(68)*.

As the literature describing the benefit of cytotoxic chemotherapy for metastatic disease has grown, the role of these therapies for neoadjuvant and adjuvant treatment of high-risk disease are starting to be investigated. A few early trials incorporating non-taxane based therapies neoadjuvantly before surgery demonstrated feasibility of the approach but did not demonstrate significant rates of pathological complete remission rates after surgery *(63,69)*. Kelly et al. *(46)* treated 23 high-risk patients with weekly paclitaxel, estramustine, and carboplatin every 4 weeks for 4 cycles before surgery. All patients received androgen ablation. Fifty-five percent of patients remained clinical stage T3 after surgery, although no patients had positive lymph nodes. Early trials incorporating neoadjuvant daily estramustine and 70 mg/m^2 docetaxel every 3 weeks demonstrated negative margins for a high pro-

portion of patients *(70,71)*. The main toxicities associated with these early trials were related to the estramustine, and included nausea, vomiting, and thromboembolic events. In a trial performed at Dana-Farber *(72)*, patients were treated with weekly docetaxel alone for 2 months and then reevaluated with PSA levels and endorectal MRI. Patients with improving or stable disease received an additional 4 months of chemotherapy. No pathological complete remissions were noted in the first 15 patients; however, none had lymph node involvement of disease.

The cooperative groups have designed large randomized adjuvant studies for high-risk prostate cancer patients with clinically localized disease *(73,74)*. The SWOG is randomizing men with high-risk features after prostatectomy to mitoxantrone plus androgen ablation or androgen ablation alone. One randomized trial from the Radiation Therapy Oncology Group (RTOG) attempted to evaluated adjuvant chemotherapy with paclitaxel plus, etoposide plus, and estramustine in patients with high-risk disease treated with radiation therapy and androgen ablation, but closed because of poor accrual. An industry-sponsored trial will compare, in high-risk patients after prostatectomy, adjuvant hormonal therapy to chemohormonal therapy with docetaxel. One novel aspect of this trial's design is that it will compare immediate treatment with treatment at PSA level relapse. Docetaxel neoadjuvant therapy followed by prostatectomy will be compared with prostatectomy alone for high-risk patients in the CALGB. These randomized trials will define the role of chemotherapy in the high-risk prostate cancer patient. However, because these trials will take years to complete accrual and mature, the adjuvant/neoadjuvant use of chemotherapy must still be considered investigational.

6. RESISTANCE TO TAXANE-BASED THERAPIES

Taxanes bind to microtubules and promote their assembly and stabilization, thereby preventing further cell division. The mechanism of taxane-induced cell death involves inhibition of microtubule function but also involves other intracellular pathways. Drug resistance can occur through changes in drug uptake at the cellular level, drug deactivation, suppression of apoptosis, or changes in cellular components that interact with the drug's target *(75)*. Resistance to taxanes and other microtubule-binding agents has been linked to overexpression of the *MDR* gene and its protein derivative, P-glycoprotein. However, it is unlikely that MDR is the primary resistance protein involved in the development of resistance to chemotherapy in prostate cancer, because some studies have demonstrated that this gene is silenced through methylation. Alterations or differences in the cellular levels of other proteins, such as β-tubulin, have been associated with taxane resistance *(76)*. In one study, taxane-resistant clones from the PC-3 and DU-145 prostate cancer cell lines were associated with transient expression of the β-tubulin IV isoform *(75)*. Overexpression of *bcl-2* and a homologous gene, *bcl-xL*, delays taxane-induced apoptosis *(77,78)*. Furthermore, targeting *bcl-2* with oligonucleotides confers cellular sensitivity to taxanes *(79)*. Although taxanes provide a survival benefit, the overall benefit is small and the development of new therapies, in combination or alone, designed to be effective in the face of taxane-resistance are imperative.

7. INVESTIGATIONAL THERAPIES

A number of novel agents are being investigated for combination with docetaxel to improve clinical outcomes for patients with HRPC. A few of the more promising combination regimens are discussed here. Calcitriol, the biologically active form of vitamin D, prevents proliferation of HRPC cell lines at supraphysiological doses. In addition, calcitriol increases cytotoxicity of taxanes independent of bcl-2 *(80,81)*. A PSA response rate of 81% with a median time to progression of 11.4 months was noted in a single institution phase II study combining weekly docetaxel 36 mg/m^2 with pulsed weekly calcitriol *(82)*. Fifty-three percent of patients with measurable disease had at a partial response. A randomized phase II trial, Androgen Independent Prostate Cancer Study of Calcitriol Enhancing Taxotere (ASCENT), compared pulsed high-dose calcitriol, 45 mcg every day plus weekly 36 mg/m^2 docetaxel for 3 of 4 weeks vs docetaxel alone. The primary end point measured a differ-

ence in 50% PSA decline rates at 6 months (power of 85% to detect a difference from 45 to 65%). An adjusted survival analysis demonstrated superiority of the combination over weekly docetaxel. The rates of serious adverse events were significantly lower in the combination arm (27%) vs the docetaxel-only arm (47%). Prospective confirmation is needed to determine whether this is a novel way of reducing docetaxel-based toxicity *(83)*.

Because the growth and metastasis of tumors requires new blood vessel formation, inhibition of angiogenesis is an attractive target for prostate cancer. Vascular endothelial growth factor (VEGF) may regulate the growth of blood vessels in prostate cancer. Serum VEGF is present in both the tissue and serum of prostate cancer patients *(84–86)*. VEGF is present in both localized and metastatic disease, and elevated levels may portend poor prognosis, especially in androgen-independent prostate cancer *(87)*. An analysis of prognostic factors from the CALGB database revealed that, in patients with HRPC, plasma and urine VEGF levels are independently associated with survival in multivariate analyses *(88,89)*. Several trials are evaluating docetaxel with agents that target different aspects of the angiogenic pathway. Thalidomide and its derivatives, which have both immunomodulatory and anti-angiogenic properties, probably work through inhibition of platelet-derived growth factor rather than basic fibroblast growth factor or VEGF *(90)*. Thalidomide demonstrated a response rate of 18% in patients with androgen-independent prostate cancer, some of whom had previous treatment *(91)*. In a randomized phase II trial *(92,93)* evaluating weekly docetaxel plus 200 mg thalidomide per day vs docetaxel alone, median overall survival of the combination arm was 25.9 months compared with 14.7 months in the docetaxel alone arm. Of note, major toxicities included peripheral neurotoxicity and a 23% rate of venous embolism in the combination group. Prophylaxis with low molecular weight heparin prevented further instances of deep venous thromboses. The combination of docetaxel and thalidomide deserves further evaluation given these results. Second generation imides, such as revlamide, are now being evaluated as either single agents or in combination with docetaxel in men with HRPC.

VEGF regulates the growth of blood vessels, and, in prostate cancer, elevated levels are associated with a poor outcome. Bevacizumab, a humanized monoclonal activity directed against VEGF, has been proven active in combination with chemotherapy in advanced colorectal cancer. A trial from the CALGB evaluated 70 mg/m^2 docetaxel every 3 weeks, 280 mg estramustine orally thrice daily on days 1 to 4, and 16 mg/kg bevacizumab every 3 weeks. Seventy-nine percent of patients experienced a PSA response, and 53% of evaluable patients with measurable disease achieved a partial response *(94)*. Median survival had not been reached at 11 months. The toxicity of this approach was generally acceptable, although 53% of patients experienced grade 3 or 4 neutropenia. Based on these results, the CALGB has developed a phase III trial comparing docetaxel plus prednisone with docetaxel plus prednisone plus bevacizumab. The planned accrual, 1020 patients, is powered to detect an improvement in median survival from 19 to 24 months.

Endothelins (ETs) are a family of vasoconstricting proteins that have been associated with progression of advanced prostate cancer. Malignant prostate cancer cells have increased levels of the ET_a receptor and ET-1. ET-1 is involved with growth promotion, inhibition of apoptosis, and promotes the formation of bone lesions in prostate cancer by stimulating mitogenesis of osteoblasts and by decreasing bone resorption by osteoclasts *(4)*. Atrasentan (Abt-627) is a selective ET_a receptor inhibitor and has been evaluated in three randomized clinical trials. A multinational, placebo-controlled, randomized phase II study with 288 participants with HRPC found a trend toward an increased time to disease progression as well as a significant delay in time to PSA progression *(95)*. Atrasentan also stabilized bone turnover markers when administered for 52 weeks, whereas men receiving placebo developed a 58% elevation in mean alkaline phosphatase levels. A phase III study *(96)* randomized 809 patients to oral 10 mg atrasentan daily vs placebo. Again, atrasentan was associated with a trend toward increased time to progression and delayed bone turnover markers. Adverse effects of the experimental arm include headaches, rhinorrhea, edema, and decrease in blood pressure, but, generally, the drug is well-tolerated. Another randomized trial is comparing atrasentan to

placebo in men with androgen-independent prostate cancer whose sole manifestation of relapse is a rising PSA level; the trial has completed accrual and the results have yet to mature. Because of these promising results, this agent will be combined with docetaxel plus prednisone and compared with docetaxel plus prednisone alone in trial headed by SWOG.

Because Bcl-2 protein overexpression is a common manifestation in prostate cancer and may mediate progression to androgen independence, novel therapies targeting this protein may improve clinical outcomes in advanced prostate cancer. Oblimersen, a 18-base synthetic oligodeoxyribonucleotide strand, attaches to the first six codons of the *bcl-2* messenger RNA (mRNA). This hybrid recruits RNase, which cuts off the *blc-2* mRNA and decreases the formation of Bcl-2 protein. In preclinical models, there is enhancement of docetaxel activity against prostate cancer cell lines when combined with oblimersen *(97)*. In a phase I trials of oblimersen and docetaxel *(98)*, 7 of 10 patients had a PSA response. In addition, Bcl-2 protein seemed to be inhibited in tumor tissue cells. In a phase II study *(97)*, 28 patients received 7 mg/kg/d oblimersen administered by continuous intravenous infusion on days 1 to 8 and 75 mg/m^2 docetaxel iv on day 6. PSA responses were observed in 14 (52%) of 27 patients, whereas 4 (33%) of 12 patients with bidimensionally measurable disease had objective responses. The median survival of all patients was 19.8 months. Bcl-2 protein expression decreased a median of 49.9% in peripheral blood mononuclear cells after treatment, but the individual changes did not correlate with response. Based on these promising results, European Organization for Research and Therapy of Cancer (EORTC) has developed a randomized trial evaluating docetaxel and oblimersen vs docetaxel alone.

8. CONCLUSIONS

The improved survival and palliation demonstrated in patients treated with the combination of docetaxel plus prednisone supported approval of the combination by the FDA in May of 2005. In contrast to the preconception that chemotherapy is toxic and ineffective in these men, prostate cancer can now be classified as a chemosensitive disease. The future of taxane-based treatment lies in combination strategies with biological and targeted therapy, including agents targeting angiogenesis, the ET receptor, and other signal pathways. The activity in hormone-refractory disease has provided the impetus for the development of multimodality approaches to the treatment of high-risk localized prostate cancer patients. Large randomized neoadjuvant and adjuvant studies are currently under design.

REFERENCES

1. Jemal, A., Murray, T., Ward, E., et al. (2005). Cancer statistics, 2005. *CA Cancer J. Clin.* **55(1)**, 10–30.
2. Beedassy, A., and Cardi, G. (1999). Chemotherapy in advanced prostate cancer. *Semin. Oncol.* **26(4)**, 428–438.
3. Kupelian, P. A., Elshaikh, M., Reddy, C. A., Zippe, C., and Klein E. A. (2002). Comparison of the efficacy of local therapies for localized prostate cancer in the prostate-specific antigen era: a large single-institution experience with radical prostatectomy and external-beam radiotherapy. *J. Clin. Oncol.* **20(16)**, 3376–3385.
4. Bhandari, M. S., Petrylak, D. P., and Hussain, M. (2005). Clinical trials in metastatic prostate cancer—has there been real progress in the past decade? *Eur. J. Cancer* **41(6)**, 941–953.
5. Coen, J. J., Zietman, A. L., Thakral, H., and Shipley, W. U. (2002). Radical radiation for localized prostate cancer: local persistence of disease results in a late wave of metastases. *J. Clin. Oncol.* **20(15)**, 3199–3205.
6. Han M, Partin AW, Pound CR, Epstein JI, Walsh PC. (2001). Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience. *Urol. Clin. North Am.* **28(3)**, 555–565.
7. D'Amico, A. V., Whittington, R., Malkowicz, S. B., et al. (1998). Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA* **280(11)**, 969–974.
8. Petrylak, D. P. (2002). Chemotherapy for androgen-independent prostate cancer. *Semin. Urol. Oncol.* **20(3 Suppl. 1)**, 31–35.
9. Auclerc, G., Antoine, E. C., Cajfinger, F., Brunet-Pommeyrol, A., Agazia, C., and Khayat, D. (2000). Management of advanced prostate cancer. *Oncologist* **5(1)**, 36–44.
10. Catalona, W. J. (1994). Management of cancer of the prostate. *N. Engl. J. Med.* **331(15)**, 996–1004.

11. Berry, W., Dakhil, S., and Modiano, M. (2002). Phase III study of mitoxantrone plus low dose prednisone versus low dose prednisone alone in patients with asymptomatic hormone refractory prostate cancer. *J. Urol.* **168**, 2439–2443.
12. Tannock, I. F., Osoba, D., Stockler, M. R., et al. (1996). Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. *J. Clin. Oncol.* **14(6)**, 1756–1764.
13. Kantoff, P. W., Halabi, S., Conaway, M., et al. (1999). Hydrocortisone with or without mitoxantrone in men with hormone-refractory prostate cancer: results of the cancer and leukemia group B 9182 study. *J. Clin. Oncol.* Aug; **17(8)**, 2506–2513.
14. Chi, K. N. (2005). Targeting Bcl-2 with oblimersen for patients with hormone refractory prostate cancer. *World J. Urol.* **23(1)**, 33–37.
15. Reed, J. C. (1997). Double identity for proteins of the Bcl-2 family. *Nature* **387(6635)**, 773–776.
16. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. *Proc. Natl. Acad. Sci. USA* **95(9)**, 4997–5002.
17. Colombel, M., Symmans, F., Gil, S., et al. (1993). Detection of the apoptosis-suppressing oncoprotein bc1-2 in hormone-refractory human prostate cancers. *Am. J. Pathol.* **143(2)**, 390–400.
18. McDonnell, T. J., Navone, N. M., Troncoso, P., et al. (1997). Expression of bcl-2 oncoprotein and p53 protein accumulation in bone marrow metastases of androgen independent prostate cancer. *J. Urol.* **157(2)**, 569–574.
19. McDonnell, T. J., Troncoso, P., Brisbay, S. M., et al. (1992). Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res.* **52(24)**, 6940–6944.
20. Bubendorf, L., Sauter, G., Moch, H., et al. (1996). Prognostic significance of Bcl-2 in clinically localized prostate cancer. *Am. J. Pathol.* **148(5)**, 1557–1565.
21. Raffo, A. J., Perlman, H., Chen, M. W., Day, M. L., Streitman, J. S., and Buttyan, R. (1995). Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. *Cancer Res.* **55(19)**, 4438–4445.
22. Tu, S. M., McConnell, K., and Marin, M. C. (1995). Combination adriamycin and suramin induces apoptosis in bcl-2 expressing prostate carcinoma cells. *Cancer Lett.* **93**, 147–155.
23. Haldar, S., Chintapalli, J., and Croce, C. M. (1996). Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res.* **56(6)**, 1253–1255.
24. Dahllof, B., Billstrom, A., Cabral, F., and Hartley-Asp, B. (1993). Estramustine depolymerizes microtubules by binding to tubulin. *Cancer Res.* **53(19)**, 4573–4581.
25. Stearns, M. E. and Tew, K. D. (1985). Antimicrotubule effects of estramustine, an antiprostatic tumor drug. *Cancer Res.* **45(8)**, 3891–3897.
26. Pienta, K. J. and Lehr, J. E. (1993). Inhibition of prostate cancer growth by estramustine and etoposide: evidence for interaction at the nuclear matrix. *J. Urol.* **149(6)**, 1622–1625.
27. Speicher, L. A., Barone, L. R., Chapman A. E., et al. (1994). P-glycoprotein binding and modulation of the multidrug-resistant phenotype by estramustine. *J. Natl. Cancer Inst.* **86(9)**, 688–694.
28. Yagoda, A. and Petrylak, D. (1993). Cytotoxic chemotherapy for advanced hormone-resistant prostate cancer. *Cancer* **71(3 Suppl.)**, 1098–1109.
29. Speicher, L. A., Barone, L., and Tew, K. D. (1992). Combined antimicrotubule activity of estramustine and taxol in human prostatic carcinoma cell lines. *Cancer Res.* **52(16)**, 4433–4440.
30. Kreis, W., Budman, D. R., and Calabro, A. (1997). Unique synergism or antagonism of combinations of chemotherapeutic and hormonal agents in human prostate cancer cell lines. *Br. J. Urol.* **79(2)**, 196–202.
31. Hussain, M. H., Pienta, K. J., Redman, B. G., Cummings, G. D., and Flaherty, L. E. (1994). Oral etoposide in the treatment of hormone-refractory prostate cancer. *Cancer* **74(1)**, 100–103.
32. Pienta, K. J., Redman, B., Hussain, M., et al. (1994). Phase II evaluation of oral estramustine and oral etoposide in hormone-refractory adenocarcinoma of the prostate. *J. Clin. Oncol.* **12(10)**, 2005–2012.
33. Hudes, G., Einhorn, L., Ross, E., et al. (1999). Vinblastine versus vinblastine plus oral estramustine phosphate for patients with hormone-refractory prostate cancer: a Hoosier Oncology Group and Fox Chase Network phase III trial. *J. Clin. Oncol.* **17(10)**, 3160–3166.
34. Roth, B. J., Yeap, B. Y., Wilding, G., Kasimis, B., McLeod, D., and Loehrer, P. J. (1993). Taxol in advanced, hormone-refractory carcinoma of the prostate. A phase II trial of the Eastern Cooperative Oncology Group. *Cancer* **72(8)**, 2457–2460.
35. Trivedi, C., Redman, B., Flaherty, L. E., et al. (2000). Weekly 1-hour infusion of paclitaxel. Clinical feasibility and efficacy in patients with hormone-refractory prostate carcinoma. *Cancer* **89(2)**, 431–436.
36. Picus, J. and Schultz, M. (1999). Docetaxel (Taxotere) as monotherapy in the treatment of hormone-refractory prostate cancer: preliminary results. *Semin. Oncol.* **26(5 Suppl. 17)**, 14–18.
37. Friedland D., Cohen J., Miller R., Jr., et al. (1999). A phase II trial of docetaxel (Taxotere) in hormone-refractory prostate cancer: correlation of antitumor effect to phosphorylation of Bcl-2. *Semin. Oncol.* **26(5 Suppl. 17)**, 19–23.
38. Beer, T. M., Pierce, W. C., Lowe, B. A., and Henner, W. D. (2001). Phase II trial of weekly docetaxel in symptomatic androgen-independent prostate cancer. *Ann. Oncol.* **12**, 1273–1279.

39. Berry, W., Dakhil, S., Gregurich, M. A., and Asmar, L. (2001). Phase II trial of single-agent weekly docetaxel in hormone-refractory, symptomatic, metastatic carcinoma of the prostate. *Semin. Oncol.* **28(Suppl. 15)**, 8–15.
40. Gravis, G., Bladou, F., Salem, N., et al. (2003). Weekly administration of docetaxel for symptomatic metastatic hormone-refractory prostate carcinoma. *Cancer* **98(8)**, 1627–1634.
41. Hudes, G. R., Obasaju, C., Chapman, A., Gallo, J., McAleer, C., and Greenberg, R. (1995). Phase I study of paclitaxel and estramustine: preliminary activity in hormone-refractory prostate cancer. *Semin. Oncol.* **22(3 Suppl. 6)**, 6–11.
42. Hudes, G. R., Nathan, F., Khater, C., et al. (1997). Phase II trial of 96-hour paclitaxel plus oral estramustine phosphate in metastatic hormone-refractory prostate cancer. *J. Clin. Oncol.* **15(9)**, 3156–3163.
43. Kuzel, T. M., Kies, M. S., Wu, N., Hsieh, Y. C., and Rademaker, A. W. (2002). Phase I trial of oral estramustine and 3-hr infusional paclitaxel for the treatment of hormone refractory prostate cancer. *Cancer Invest.* **20(5–6)**, 634–643.
44. Vaishampayan, U., Fontana, J., Du, W., and Hussain, M. (2002). An active regimen of weekly paclitaxel and estramustine in metastatic androgen-independent prostate cancer. *Urology* **60(6)**, 1050–1054.
45. Vaughn, D. J., Brown, A. W., Jr., and Harker, W. G., et al. (2004). Multicenter Phase II study of estramustine phosphate plus weekly paclitaxel in patients with androgen-independent prostate carcinoma. *Cancer* **100(4)**, 746–750.
46. Kelly, W. K., Curley, T., Slovin, S., et al. (2001). Paclitaxel, estramustine phosphate, and carboplatin in patients with advanced prostate cancer. *J. Clin. Oncol.* **19(1)**, 44–53.
47. Smith, D. C., Esper, P., Strawderman, M., Redman, B., and Pienta, K. J. (1999). Phase II trial of oral estramustine, oral etoposide, and intravenous paclitaxel in hormone-refractory prostate cancer. *J. Clin. Oncol.* **17(6)**, 1664–1671.
48. Chay, C. and Smith, D. C. (2001). Adjuvant and neoadjuvant therapy in prostate cancer. *Semin. Oncol.* **28(1)**, 3–12.
49. Berry, W. R., Hathorn, J. W., Dakhil, S. R., et al. (2004). Phase II randomized trial of weekly paclitaxel with or without estramustine phosphate in progressive, metastatic, hormone-refractory prostate cancer. *Clin. Prostate Cancer* **3(2)**, 104–111.
50. Petrylak, D. P., Macarthur, R. B., O'Connor, J., et al. (1999). Phase I trial of docetaxel with estramustine in androgen-independent prostate cancer. *J. Clin. Oncol.* **17(3)**, 958–967.
51. Kreis, W., Budman, D. R., Fetten, J., Gonzales, A. L., Barile, B., and Vinciguerra, V. (1999). Phase I trial of the combination of daily estramustine phosphate and intermittent docetaxel in patients with metastatic hormone refractory prostate carcinoma. *Ann. Oncol.* **10(1)**, 33–38.
52. Natale, R. B. and Zaretsky, S. L. (1999). Phase I/II trial of estramustine (E) and Taxotere (T) in patients with metastatic hormone-refractory prostate cancer (HRPC). *Pr. Am. Soc. Clin. Oncol.* **348a(18)**, 1343 (abstr).
53. Petrylak, D., Shelton, G., England-Owen, C., Pfaff, C., Bagliella, E., and Diuleo, R. (2000). Response and preliminary survival results of a phase II study of docetaxel (D) + estramustine phosphate (EMP) in patients (Pts) with hormone refractory prostate cancer (HRPC): updated preliminary analysis. *Pr. Am. Soc. Clin. Oncol.* **19(334a)**, 1312 (abstr).
54. Savarese, D., Taplin, M. E., Halabi, S., Hars, V., Kreis, W., and Vogelzang, N. (1999). A phase II study of docetaxel (Taxotere), estramustine, and low-dose hydrocortisone in men with hormone-refractory prostate cancer: preliminary results of cancer and leukemia group B Trial 9780. *Semin. Oncol.* **26(5 Suppl. 17)**, 39–44.
55. Sinibaldi, V. J., Carducci, M. A., Moore-Cooper, S., Laufer, M., Zahurak, M., and Eisenberger, M. A. (2002). Phase II evaluation of docetaxel plus one-day oral estramustine phosphate in the treatment of patients with androgen independent prostate carcinoma. *Cancer* **94(5)**, 1457–1465.
56. Kosty, M. P., Ferreira, A., and Byrntesen, T. (2001). Weekly docetaxel and low-dos estramustine phosphate in hormone-refractory prostate cancer: a phase II study. *Proc. Am. Soc. Clin. Oncol.* **20(152b)** 2360 (abstr).
57. Weitzman, A. L., Shelton, G., Zuech, N., et al. (2000). Dexamethasone does not significantly contribute to the response rate of docetaxel and estramustine in androgen independent prostate cancer. *J. Urol.* **163(3)**, 834–837.
58. Petrylak, D. P., Tangen, C. M., Hussain, M. H., et al. (2004). Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N. Engl. J. Med.* **351(15)**, 1513–1520.
59. Crawford, E. D., Pauler, D. K., and Tangen, C. M. (2004). Three-month change in PSA as a surrogate endpoint for mortality in advanced hormone-refractory prostate cancer (HRPC): data from Southwest Oncology Group Study S9916. *Pr. Am. Soc. Clin. Oncol.* **23(4505)**, 382.
60. Tannock, I. F., de Wit, R., Berry, W. R., et al. (2004). Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N. Engl. J. Med.* **351(15)**, 1502–1512.
61. Smith, D. S., Humphrey, P. A., and Catalona, W. J. (1997). The early detection of prostate carcinoma with prostate specific antigen: the Washington University experience. *Cancer* **80(9)**, 1852–1856.
62. Soh, S., Kattan, M. W., Berkman, S., Wheeler, T. M., and Scardino, P. T. (1997). Has there been a recent shift in the pathological features and prognosis of patients treated with radical prostatectomy? *J. Urol.* **157(6)**, 2212–2218.
63. Pettaway, C. A., Pisters, L. L., Troncoso, P., et al. (2000). Neoadjuvant chemotherapy and hormonal therapy followed by radical prostatectomy: feasibility and preliminary results. *J. Clin. Oncol.* Mar ;18(5), 1050–1057.
64. Scolieri, M. J., Altman, A., and Resnick, M. I. (2000). Neoadjuvant hormonal ablative therapy before radical prostatectomy: a review. Is it indicated? *J. Urol.* **164(5)**, 1465–1472.
65. Soloway, M. S., Sharifi, R., Wajsman, Z., McLeod, D., Wood, D. P., Jr., and Puras-Baez, A. (1995). Randomized prospective study comparing radical prostatectomy alone versus radical prostatectomy preceded by androgen blockade

in clinical stage B2 (T2bNxM0) prostate cancer. The Lupron Depot Neoadjuvant Prostate Cancer Study Group. *J. Urol.* **154(2 Pt 1)**, 424–428.
66. Goldenberg, S. L., Klotz, L. H., Srigley, J., et al. (1996). Randomized, prospective, controlled study comparing radical prostatectomy alone and neoadjuvant androgen withdrawal in the treatment of localized prostate cancer. Canadian Urologic Oncology Group. *J. Urol.* **156(3)**, 873–877.
67. Aus, G., Abrahamsson, P. A., Ahlgren, G., et al. (1998). Hormonal treatment before radical prostatectomy: a 3-year followup. *J. Urol.* **159(6)**, 2013–2016; discussion 2016–2017.
68. D'Amico, A. (2001). Radiation and hormonal therapy for locally advanced and clinically localized prostate cancer. *Urology* **58(2 Suppl. 1)**, 78–82.
69. Clark, P. E., Peereboom, D. M., Dreicer, R., Levin, H. S., Clark, S. B., and Klein, E.A. (2001). Phase II trial of neoadjuvant estramustine and etoposide plus radical prostatectomy for locally advanced prostate cancer. *Urology* **57(2)**, 281–285.
70. Hussain, M., Smith, D. C., El-Rayes, B. F., et al. (2003). Neoadjuvant docetaxel and estramustine chemotherapy in high-risk/locallyadvanced prostate cancer. *Urology* **61(4)**, 774–780.
71. Ko, Y. J., Dewolf, W., Olumi, A., Sesterhenn, I. A., Upton, M., and Bubley, G. J. (2002). Neoadjuvant chemo-hormonal therapy followed by radical prostatectomy for high risk prostate cancer. *Am. Soc. Clin. Oncol.* **21**, 2645.
72. Oh, W. K. (2003). The evolving role of chemotherapy and other systemic therapies for managing localized prostate cancer. *J. Urol.* **170**, S28–S34.
73. Smith, D. C., Chay, C. H., Dunn, R. L, et al. (2003). Phase II trial of paclitaxel, estramustine, etoposide, and carboplatin in the treatment of patients with hormone-refractory prostate carcinoma. *Cancer* **98(2)**, 269–276.
74. Vaishampayan, U. and Hussain, M. (2000). Adjuvant chemo-/hormonal therapy trials for locally advanced prostate cancer. *Curr. Oncol. Rep.* **2(5)**, 402–408.
75. Makarovskiy, A. N., Siryaporn, E., Hixson, D. C., and Akerley, W. (2002). Survival of docetaxel-resistant prostate cancer cells in vitro depends on phenotype alterations and continuity of drug exposure. *Cell Mol. Life Sci.* **59(7)**, 1198–1211.
76. Ranganathan, S., Dexter, D. W., Benetatos, C. A., Chapman, A. E., Tew, K. D., and Hudes, G. R. (1996). Increase of beta(III)- and beta(IVa)-tubulin isotopes in human prostate carcinoma cells as a result of estramustine resistance. *Cancer Res.* **56(11)**, 2584–2589.
77. Wahl, A. F., Donaldson, K. L., Fairchild, C., et al. (1996). Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. *Nat. Med.* **2(1)**, 72–79.
78. Lebedeva, I., Rando, R., Ojwang, J., Cossum, P., and Stein, C. A. (2000). Bcl-xL in prostate cancer cells: effects of overexpression and down-regulation on chemosensitivity. *Cancer Res.* **60(21)**, 6052–6060.
79. Gleave, M. E., Miayake, H., Goldie, J., Nelson, C., and Tolcher, A. (1999). Targeting bcl-2 gene to delay androgen-independent progression and enhance chemosensitivity in prostate cancer using antisense bcl-2 oligodeoxynucleotides. *Urology* **54(6A Suppl)**, 36–46.
80. Blutt, S. E., McDonnell, T. J., Polek, T. C., and Weigel, N. L. (2000). Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. *Endocrinology* **141(1)**, 10–17.
81. Hershberger, P. A., Yu, W. D., Modzelewski, R. A., Rueger, R. M., Johnson, C. S., and Trump, D. L. (2001). Calcitriol (1,25-dihydroxycholecalciferol) enhances paclitaxel antitumor activity in vitro and in vivo and accelerates paclitaxel-induced apoptosis. *Clin. Cancer Res.* **7(4)**, 1043–1051.
82. Beer, T. M., Eilers, K. M., and Garzotto, M. (2003). Weekly high-dose calcitriol and docetaxel in metastatic androgen-independent prostate cancer. *J. Clin. Oncol.* **21**, 123–128.
83. Beer, T. M., Ryan, C. W., Venner, P. M., et al. (2005). Interim results from ASCENT: A double-blinded randomized study of DN-101 (high-dose calcitriol) plus docetaxel vs. placebo plus docetaxel in androgen-independent prostate cancer (AIPC). *Proc. Am. Soc. Clin. Oncol.* 4516 (abstract).
84. Ferrer, F. A., Miller, L. J., Lindquist, R., et al. (1999). Expression of vascular endothelial growth factor receptors in human prostate cancer. *Urology* **54(3)**, 567–572.
85. Ferrer, F. A., Miller, L. J., Andrawis, R. I., et al. (1998). Angiogenesis and prostate cancer: in vivo and in vitro expression of angiogenesis factors by prostate cancer cells. *Urology* **51(1)**, 161–167.
86. Ferrer, F. A., Miller, L. J., Andrawis, R. I., et al. (1997). Vascular endothelial growth factor (VEGF) expression in human prostate cancer: in situ and in vitro expression of VEGF by human prostate cancer cells. *J. Urol.* **157(6)**, 2329–2333.
87. Duque, J. L., Loughlin, K. R., Adam, R. M., Kantoff, P. W., Zurakowski, D., and Freeman, M. R. (1999). Plasma levels of vascular endothelial growth factor are increased in patients with metastatic prostate cancer. *Urology* **54(3)**, 523–527.
88. Bok, R. A., Halabi, S., Fei, D. T., et al. (2001). Vascular endothelial growth factor and basic fibroblast growth factor urine levels as predictors of outcome in hormone-refractory prostate cancer patients: a cancer and leukemia group B study. *Cancer Res.* **61(6)**, 2533–2536.
89. George, D. J., Halabi, S., Shepard, T. F., et al. (2001). Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. *Clin. Cancer Res.* **7(7)**, 1932–1936.

90. Ng, S. S., MacPherson, G. R., Gutschow, M., Eger, K., and Figg, W. D. (2004). Antitumor effects of thalidomide analogs in human prostate cancer xenografts implanted in immunodeficient mice. *Clin. Cancer Res.* **10(12 Pt 1)**, 4192–4197.
91. Figg, W. D., Dahut, W., Duray, P., et al. (2001). A randomized phase II trial of thalidomide, an angiogenesis inhibitor, in patients with androgen-independent prostate cancer. *Clin. Cancer Res.* **7(7)**, 1888–1893.
92. Figg, W. D., Arlen, P., Gulley, J., et al. (2001). A randomized phase II trial of docetaxel (taxotere) plus thalidomide in androgen-independent prostate cancer. *Semin. Oncol.* **28(4 Suppl. 15)**, 62–66.
93. Retter, A. S., Ando, Y., Price, D. K., et al. (2005). Follow-up analysis of a randomized phase II study of docetaxel (D) and thalidomide (T) in androgen-independent prostate cancer (AIPC): updated survival data and stratification by CYP2C19 mutational status. *Proc. Am. Soc. Clin. Oncol.* **265** (2005 Prostate Cancer Symposium abstract).
94. Picus, J., Halabi, S., and Rini, B. I. (2004). The use of bevacizumab. *Am. Soc. Clin. Oncol.* **22**, 1578A.
95. Carducci, M. A., Padley, R. J., Breul, J., et al. (2003). Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. *J. Clin. Oncol.* **21(4)**, 679–689.
96. Carducci, M. A., Nelson, J. B., and Saad, F. (2004). Effects of atrasentan on disease progression and biological markers in men with metastatic hormone-refractory prostate cancer: phase III study. *Pr. Am. Soc. Clin. Oncol.* **222(41S)**, abstr 4508.
97. Tolcher, A. W., Chi, K., Kuhn, J., et al. (2005). A phase II, pharmacokinetic, and biological correlative study of oblimersen sodium and docetaxel in patients with hormone-refractory prostate cancer. *Clin. Cancer Res.* **11(10)**, 3854–3861.
98. Tolcher, A. W., Kuhn, J., Schwartz, G., et al. (2004). A Phase I pharmacokinetic and biological correlative study of oblimersen sodium (genasense, g3139), an antisense oligonucleotide to the bcl-2 mRNA, and of docetaxel in patients with hormone-refractory prostate cancer. *Clin. Cancer Res.* **10(15)**, 5048–5057.
99. Haas, N., Roth, B., Garay, C., et al. (2001). Phase I trial of weekly paclitaxel plus oral estramustine phosphate in patients with hormone-refractory prostate cancer. *Urology* **58(1)**, 59–64.
100. Athanasiadis, A., Tsavdaridis, D., Rigatos, S. K., Athanasiadis, I., Pergantas, N., and Stathopoulos, G. P. (2003). Hormone refractory advanced prostate cancer treated with estramustine and paclitaxel combination. *Anticancer Res.* **23(3C)**, 3085–3088.
101. Hudes, G. R., Nathan, F. E., Khater, C., et al. (1995). Paclitaxel plus estramustine in metastatic hormone-refractory prostate cancer. *Semin. Oncol.* **22(5 Suppl. 12)**, 41–45.
102. Petrylak, D. P., Macarthur, R., O'Connor, J., et al. (1999). Phase I/II studies of docetaxel (Taxotere) combined with estramustine in men with hormone-refractory prostate cancer. *Semin. Oncol.* **26(5 Suppl 17)**, 28–33.
103. Sitka Copur, M., Ledakis, P., Lynch, J., et al. (2001). Weekly docetaxel and estramustine in patients with hormone-refractory prostate cancer. *Semin Oncol.* **28(4 Suppl. 15)**, 16–21.

24

Co-Targeting Therapeutics for Hormone Refractory Prostate Cancer

Vasily Assikis

Summary

For many decades therapeutic strategies for advanced solid tumors have focused almost exclusively on the cancer cell itself. This approach fails to address the symbiotic relationship of the malignant cell and its surrounding stroma. An emerging line of evidence supports the notion that benign stroma may be not an innocent bystander, but rather, a facilitator of invasion, metastasis, and growth of a malignant cell from a distant tissue of origin. Although the exact mechanisms vary greatly among different tumor types, is it safe to say that more than one mechanisms/pathways are in play in this complex process. As it pertains to prostate cancer and its known predilection for bone as its primary landing zone, efforts to therapeutically manipulate the tumor–stroma relationship have focused on ways to hinder local invasion and early metastasis, angiogenesis, and deprivation of key local growth factors/cytokines that mediate homing, attachment, and growth of the prostate cancer cell in the metastatic echelon. This chapter will review some basic strategies of co-targeting cancer and stroma in advanced prostate cancer that have been tested in the clinic.

Key Words: Angiogenesis; bone metastases; bone microenvironment; novel therapeutics; stroma.

1. INTRODUCTION

Traditionally, anticancer therapies have focused on exploiting the weaknesses of the high proliferation rate of neoplastic cells. The idea is simple: if one can decipher the genetic blueprint of the cancer cell (in this case the epithelial prostate cancer cell) one will be able to modulate the malignant phenotype in various therapeutic manners. Ultimately, the goal is to eradicate all malignant cells and silence all neoplastic potential. Often, such a cancer cell-centered line of thinking fails to appreciate the crucial role stroma plays in the emergence and fruition of neoplasia as a process. Cancer cells do not grow in a vacuum. On the contrary, they need to invade surrounding tissues and eventually devise ways to escape the tissue of origin and metastasize. Metastasis is a nonrandom but rather poorly efficient process *(1)*.

Our view of the surrounding supporting structures at a cellular level (hereafter referred to as stroma), historically, has been as an innocent victim: in its effort to proliferate, the epithelial cancer cell will overcome the cell–cell and cell–matrix barriers and invade the adjacent stroma. Only recently have we come to appreciate that, instead of an innocent bystander, surrounding stroma is, in fact, a key promoter of the malignant process *(2–4)*. Probed by the presence of early neoplasia, stroma is genetically and phenotypically reactive and its nature is altered *(5,6)*. Reactive stroma can permit and support, rather than restrict, further growth and invasion by maintaining a reciprocal interaction with the proliferating epithelial cells. This ongoing cross talk is the *sine qua non* of efficient invasion at a

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

local level and is also prerequisite for distant metastasis. A comprehensive view of cancer as a disease of both the affected tissue of origin and the surrounding stroma allows an integrated therapeutic approach that targets both the "seed" and the "soil" *(7)*.

For prostate cancer, the bone microenvironment is the favored metastatic focus. The mechanisms responsible for bone tropism are beyond the scope of this chapter but can be briefly summarized as:

1. Targeting: pairs of chemokines and their respective receptors *(8,9)*.
2. Attachment: vascular zip codes that guide circulating prostate cancer cells to specific binding motifs in target tissues *(10,11)*.
3. Invasion: prostate cancer cell osteomimicry, the ability to switch on bone-like genes that allow it to "blend into" the bone milieu *(12,13)*.
4. Sustained growth: the abundance of growth factors in the bone microenvironment that will facilitate sustained growth *(14,15)*.

This chapter reviews preclinical and early clinical data on stroma-targeting strategies in advanced prostate cancer. In this setting, the cancer cells have already accumulated multiple genetic alterations. Despite that, they are still dependent on epithelial–stromal communications to sustain their invasive phenotype. In many cases, the mechanism for uncontrolled growth is shared by the epithelial and the stroma cell (for example, survival signaling through soluble growth factors). Neoplasia is a very complex process, and the heterogeneity of prostate cancer, especially in the metastatic setting, may allow the interplay of a number of interwoven signaling pathways. The goal of stroma-targeting therapeutic approaches is to develop complimentary strategies that will form the foundation of an integrated approach to improving cytotoxic therapy, which, to date, still remains the backbone of systemic therapy for hormone-refractory prostate cancer (Fig. 1).

2. TUMOR–STROMA INTERACTION IN PROSTATE CANCER PROGRESSION

The role of tumor–stroma interaction was only recently recognized as a crucial biological process in cancer development. Whereas abnormal epithelial carcinoma cells make up what is defined as "tumor," the surrounding structures (extracellular matrix [ECM], fibroblasts, immune and inflammatory cells, and vascular components) comprise what is defined as "tumor stroma."

Cross talk between epithelial and stromal elements is an ongoing process during fetal prostate development, postnatal prostate maturation, differentiation, and hormonal responsiveness *(16,17)*. In prostate cancer, input from the host microenvironment accounts for accelerated local tumor invasion *(18,19)* as well as distant metastasis *(20)*. Moreover, these reciprocal interactions enhance tumor genetic instability *(21)* and underlie the most crucial biological event in advanced prostate cancer: the transition to a castration-refractory state *(20,22,23)*. Recent data have shed light on the molecular processes governing this interaction.

1. The osteomimicry of prostate cancer cells—their ability to turn on the expression of bone genes *(12)*.
2. Tissue plasticity: the capability of epithelial cells for mesenchymal transition and *vice versa*. This is a phenomenon that allows the epithelial cancer cell to express properties that are prerequisite for invasion and metastasis *(24,25)*.
3. Mutagenicity of the stroma: transplantation of immortalized human prostate cells along with reactive myofibroblasts can lead to a malignant phenotype with specific stable genetic alterations, presumably mediated through oncogenic signaling from the reactive fibroblasts *(5,6,26,27)*.
4. The "reactive stroma": cancer cells alter their adjacent stroma to create a permissive and supportive environment for further progression. This intricate intercellular communication is mediated by cell–cell, cell–ECM and cell–soluble growth factors/cytokines interaction.

3. INTERCELLULAR COMMUNICATION BETWEEN CARCINOMA AND STROMA: FROM BIOLOGY TO NOVEL THERAPEUTICS

3.1. Cell–Cell and Cell–ECM Interactions

Expanding carcinoma populations require increasing cell motility. This is often achieved through modulation of the expression or use of certain adhesion receptors, including those of the integrin family. Metastatic potential has been correlated with a predilection for specific integrins in prostate cancer models *(28–31)*. Early preclinical data support the potential for integrating integrin-targeted therapies with currently available options *(32,33)*. An a_vb_3 integrin monoclonal antibody (Vitaxin™, MedImmune, MD) is currently in phase II clinical trials in metastatic androgen-independent prostate cancer (AIPCa).

3.2. Cell-Soluble Factors

Cancer cells produce a number of stroma-modulating factors (vascular endothelial growth factor [VEGF], fibroblastic growth factor [FGF], platelet-derived growth factor [PDGF], transforming growth factor [TGF]-β, and epidermal growth factor [EGF]) and cytokines (interleukin [IL]-2, IL-6). These factors act as paracrine mediators that induce stromal reactions such as angiogenesis *(34)* and inflammation *(35)*. In addition, they activate stromal cellular elements, such as fibroblasts, smooth muscle cells, and adipocytes, which, in turn, secrete similar soluble growth factors that are shed in the microenvironment and act on the carcinoma compartment. This well-orchestrated "ping-pong" interaction leads to a sustained proliferative advantage as well as disruption of tissue homeostasis (Dvorak's concept of a tumor as a wound that never heals; ref. *36*).

Prostate cancer cells express variable amounts of EGF receptor (EGFR). The expression increases proportionally to total tumor burden, with the highest levels found in hormone-refractory metastatic disease *(37)*. The ligands for the receptor, TGF and EGF, are both secreted by both tumor stroma and tumor itself and can act in an autocrine and paracrine manner. Despite encouraging preclinical results with inhibitors of the EGFR tyrosine kinase *(38)*, efficacy in the clinical setting has not yet been shown *(39)*.

Her-2/neu (ErbB2) is reportedly expressed in discordant percentages of samples (0–100% depending on the technique used) *(40,41)*. Similar to EGFR inhibitors, early clinical experience with the Her-2/neu inhibitor, trastuzumab, is not encouraging *(42)*. More recently, interest has grown in the use of 2C4, a monoclonal antibody that sterically hinders ErbB2 recruitment into Erb-B ligand complexes *(43)*. Inhibition of ligand-dependent ErbB2 signaling by 2C4 occurs in both low and high ErbB2-expressing systems. This approach is currently in clinical trials in AIPCa *(44)*.

Hepatocyte growth factor/scatter factor is secreted by tumor stroma and binds to its cognate epithelial surface receptor c-met, a tyrosine kinase that mitigates pleiotropic signaling *(45)*. This pathway is emerging as a novel target for anti-invasion strategies *(46)*.

The family of FGFs is abundantly expressed by tumor stroma. Prostate cancer cells have four types of receptors for FGF (FGFR)-1 to -4 *(47)*. The effects of increased FGFR signaling are wide-ranging and involve both the cancer cells and surrounding stroma, including the vasculature *(48)*. A number of approaches that could target FGFR and/or FGFR signaling in prostate cancer are currently being developed.

One of the seminal features of "reactive fibroblasts" is their elevated expression of TGF-β *(49)*. On the other hand, TGF-β1 is a known stimulator of stromal myodifferentiation *(50)*. Therefore, TGF-β excites some interest as a novel target for disruption of the stromal–epithelial loop *(51)*.

One of the most interesting growth factor/receptor pairs in prostate cancer is insulin-like growth factor (IGF)-1/IGF-1 receptor (IGF-1R). A number of experimental models have shown that signal-

ing through this pathway is particularly operational in advanced androgen-independent states *(52,53)*. A number of therapeutic strategies against IGF-1R have been investigated *(54)*. Most recently, interest has been drawn to small molecule inhibitors *(55)* and, in fact, some have argued that sufficient blockade of IGF-1R mediated signaling can even overcome the therapy refractoriness seen in end-stage disease *(14)*.

The PI3-K/Akt pathway is downstream to a number of the aforementioned cell membrane targets, and, depending on the system studied, this pathway mitigates a large proportion of the surface-to-nucleus signaling. Although clinical investigation of the Akt pathway in prostate cancer is in its infancy *(56,57)*, its role in tumor–stroma signaling is just starting to be appreciated *(58)*.

Soluble cytokines exert signaling effects in prostate cancer. IL-6 is involved in regulation of immune reaction and cell growth and differentiation. It causes multifunctional responses ranging from inhibition of proliferation to promotion of cell survival. IL-6 is produced by androgen-independent prostate cancer cell lines and has been shown to act in an autocrine and/or paracrine manner to stimulate their growth, but it suppresses the growth of androgen-dependent cell lines. It is thought that both the cancer cell as well as the reactive stroma contribute to the production of IL-6. IL-6 is a candidate for targeted therapy in prostate cancer because of its association with morbidity *(59,60)*. TNF is another cytokine often found elevated in patients with advanced prostate cancer and cachexia *(61)*. Although not clearly understood, cachexia is thought to be the cause of death for a larger percentage of prostate cancer patients. With the availability of anti-TNF agents for autoimmune disorders, clinical investigations are expected in advanced prostate cancer.

3.3. *Cell–ECM*

A number of proteolytic enzymes are dispersed in the microcirculation during invasion. Their role is the degradation of the ECM, which will, in turn, expose protein fragments that will further propagate the pro-migratory and pro-invasion signaling *(62)*. Key players in this process are the matrix metalloproteinases (MMPs). Encouraging results in preclinical models led to clinical trials *(63)*. Early results in other tumor types have been disappointing *(64,65)*. MMP inhibitors are currently in clinical development for metastatic AIPCa *(66)*. Clearly, further clinical development of this class of drugs requires an innovative approach. The goal should be retardation of tumor growth to achieve a new level of homeostasis, rather than the conventional cytoreduction *(67)*. Other molecules of interest in this category include urokinase inhibitors, which have been shown to confer antitumor effects in preclinical models *(68,69)*. Finally, other metalloproteinases (a disintegrin and metalloprotease, and tissue inhibitor of metalloproteinases) are emerging as molecules of interest *(70,71)*.

4. TARGETING THE NEOVASCULATURE

The prerequisite formation of neovasculature has been the target of novel therapeutic approaches in various solid tumors. The success of such strategies in other common epithelial malignancies (breast, lung, and colon) raises hopes that their incorporation in treatment schemas will lead to incremental advances in advanced prostate cancer as well.

Most of the early clinical work focused on VEGF and its receptors. Bevacizumab, a monoclonal antibody against VEGF, has not shown significant activity as a single agent in advanced prostate cancer, but is currently under investigation in conjunction with chemotherapy or immunotherapy *(72–74)*. Recently, the role of the PDGF receptor and its regulation of pericytes and agents that disrupt them is emerging as an area of interest *(75)*. Imatinib mesylate, a multikinase inhibitor, is being actively studied in advanced prostate cancer. Its therapeutic effects in prostate cancer are thought to be exerted by targeting the PDGF receptor. After the first report of possible synergy with docetaxel, the role of imatinib in conjunction with cytotoxic chemotherapy is currently being addressed in an ongoing multicenter placebo-controlled trial *(76)*.

Thalidomide is a pleiotropic drug that is thought to confer some of its therapeutic effects through anti-angiogenic properties. Although as a single agent it has modest activity in metastatic AIPCa *(77)*, in conjunction with taxane chemotherapy, it has been reported to confer significant clinical benefits *(78)*.

A totally different approach to target the neovasculature in metastatic foci comes from the identification of specific "vascular addresses" *(10)*. Vasculature in different organs has different binding sites that are encoded by specific repetitive peptide motifs. Phage display can lead to identification of such "vascular zip codes" which, in turn, can be used as homing devices for delivery of therapy *(79–81)*.

5. CAN IMMUNOTHERAPY AFFECT THE STROMA?

One of the stromal elements that can be manipulated to fight epithelial cancer cells is the effector immune cell. The emergence of solid tumors is "permitted" by the tolerance of the immune system. Active vaccine immunotherapy has long been studied in prostate cancer. Although a review of such strategies is beyond the scope of this chapter, we will review some recent findings that raise the issue of the importance of bone microenvironment as a co-target of vaccine strategies. GVAX™ (Cell-Genesys, CA) is an allogeneic prostate cancer vaccine *ex vivo* transduced to secrete granulocyte macrophage-colony stimulating factor. In reports of its early clinical development, it was noted that type I carboxy-terminal telopeptide, a marker of osteoclast activity, was stable or reduced in 62% of patients receiving the vaccine *(82)*. This raises the issue of yet unappreciated mechanisms through which activation of the cellular and humoral effector arms may affect the bone environment. To what extent this accounts for the vaccine's clinical effects remains to be seen.

6. BONE-TARGETED THERAPY

It has long been appreciated that the bone microenvironment modulates prostate cancer cell survival and proliferation. In addition, much of the morbidity associated with metastatic disease is caused by perturbation of normal bone architecture. The bone itself has, thus, become a therapeutic target. Improvement of our understanding of normal bone physiology and the alterations imposed by metastatic prostate cancer has led to the identification of various targets within the bone. Although in the most advanced settings, prostate cancer is a primarily osteoblastic disease, it is now well-understood that the opposing osteoclasts are also activated *(83)*.

In fact, targeting strategies against osteoclasts, such as the use of high-potency bisphosphonates, have been shown to offer significant clinical benefit, in terms of palliation *(84)*. The possible antitumor role of bisphosphonates has been reported in some, but not all, preclinical models, but not further elucidated in the clinical setting *(85–87)*.

Palliation can also be achieved with radiopharmaceuticals that target the prostate cancer cell–bone interface *(88,89)*. Interestingly, bone-targeted therapy with a radiopharmaceutical as a consolidation strategy in chemosensitive advanced disease has been reported to prolong survival in a prospective randomized phase II study *(90)*.

Another target of great therapeutic potential is the endothelin (ET) receptor A (ET_A). ETs are peptides with pleiotropic activities. ET-1 is the primary circulating form, and its binding to the ET receptor has been shown to regulate angiogenesis, blood flow, nociception, and bone deposition *(91)*. It is thought that the activation of the ET-1/ ET_A pathway serves as an autocrine and paracrine loop for tumor growth. Moreover, ET-1 is a potent mitogen for osteoblasts. Atrasentan is an orally bioavailable drug that blocks ET_A selectively *(92)*. A randomized, placebo-controlled phase II trial in patients with AIPCa and bone metastases showed that atrasentan prolonged the median time to progression (TTP) (from 137 to 183 days; $p = 0.13$) and the median time to prostate-specific antigen progression (from 71 to 155 days; $p = 0.002$) *(93)*. A larger phase III trial with an identical design

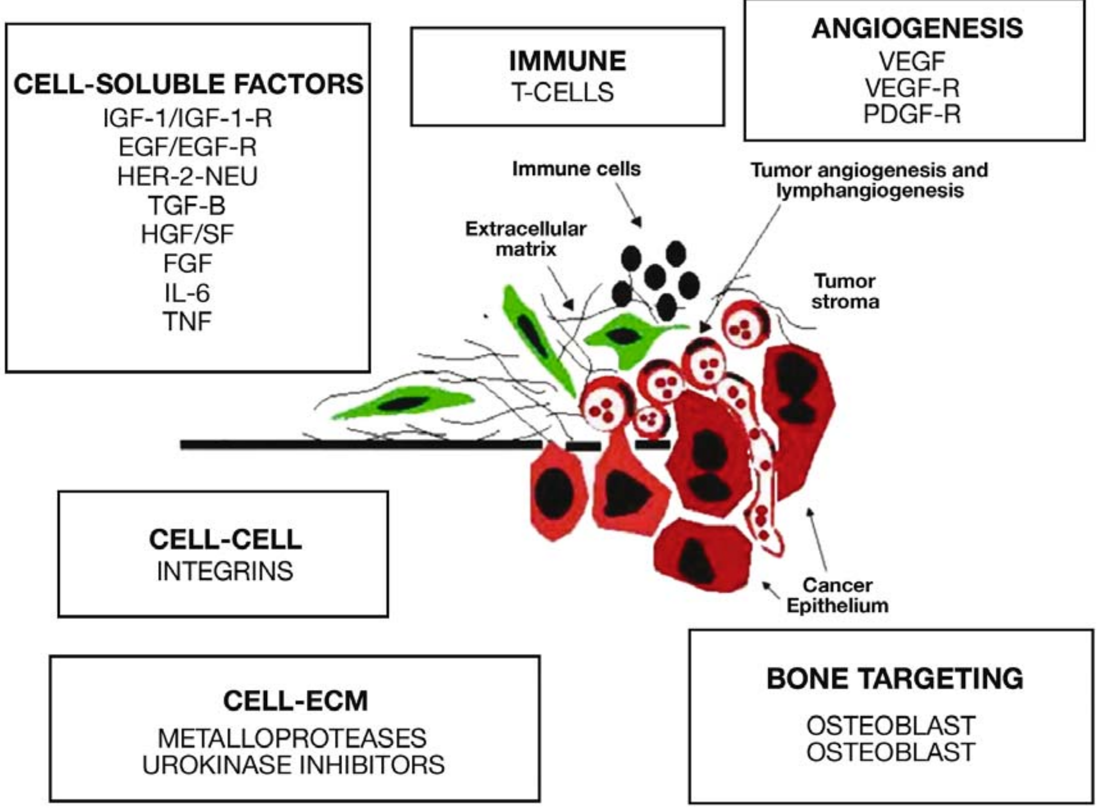


Fig. 1. Tumor–stroma targets in prostate cancer. IGF, insulin-like growth factor; R, receptor; EGF, epidermal growth factor; TGF, transforming growth factor; HGF/SF, hepatocyte growth factor/scatter factor; FGF, fibroblast growth factor; IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; ECM, extracellular matrix.

found a nonsignificant trend in delayed TTP (log–rank $p = 0.091$; hazard ratio, 1.14; 95% confidence interval, 0.98–1.34) *(94)*. A meta-analysis of the two trials combined exhibited a statistically significant hazard ratio of 1.189 ($p = 0.014$) in favor of atrasentan in delaying TTP. The role of atrasentan in earlier stages of the disease is currently under investigation.

7. CO-TARGETING OF TUMOR-STROMA WITH GENE THERAPY

Work by Leland Chung and colleagues has led the foundation for gene therapy strategies using tumor-restrictive and tumor-specific promoters that govern expression of genes in prostate cancer epithelium and bone *(95,96)*. Experimental evidence exists to show that prostate cancer cells become metastatic after acquiring and expressing osteomimetic properties (synthesis, secretion, and deposition of bone matrix proteins such as osteopontin, osteocalcin [OC], osteonectin, and bone sialoprotein) *(12,97)*. Expression of such bone markers has been reported in human specimens of localized and metastatic prostate cancer *(98)*.Using the OC promoter as a tumor-restrictive tool, adenoviral vectors were constructed to deliver the herpes simplex virus thymidine kinase (*HSV-TK*) gene. When expressed in target tissues, HSV-TK can convert antiviral prodrugs (acyclovir, valacyclovir) to their active phosphorylated form that can effectively block cell replication. Preclinical work has shown that the

OC promoter strategy results in prostate-specific expression of the vector *(95,98)*. The success of the preclinical model led to the first phase I prostate cancer/stroma co-targeting gene therapy trial in men *(99)*. The adenoviral vector Ad-OC-TK/Val was administered twice during a 1-week period directly into the metastatic sites of 11 men with metastatic AIPCa (lymph node or bone metastases) followed by a 21-day course of oral valacyclovir. There were no serious adverse events and there was extensive cellular death in the treated lesions as assessed by TUNEL assays. This proof-of-concept trial demonstrated the feasibility and safety of a co-targeted gene therapy approach.

More recently, the same group of investigators targeted the tumor endothelium. They demonstrated that human umbilical vein endothelial cells (HUVEC) and human C4-2 androgen-independent prostate cancer cells, when infected with an antiangiogenic adenoviral (Ad)-Flk1-Fc vector secreting a soluble form of Flk1, showed dramatically inhibited proliferation, migration, and tubular formation of HUVECs *(100)*. In a subcutaneous C4-2 tumor xenograft model, tumor volume was decreased by 40 to 60% in animals treated with Ad-Flk1-Fc or Ad-hOC-E1 plus vitamin D3 alone, and by 90% in a combined treatment group, showing that co-targeting strategies of tumor and tumor endothelium are feasible *(100)*.

8. CONCLUSIONS

Future prostate cancer therapies will focus on the interruption of the reciprocal interaction of stroma with the epithelial component. The list of therapeutic targets will continue to expand as our understanding of the bone microenvironment improves. Angiogenesis, osteoblasts, osteoclasts, inflammatory cells, and local milieu soluble factors will serve as the initial testing ground. Even with the currently known targets of interest, rational combinations can lead to incremental advances in clinical outcome. The challenge lies in prioritization and rapid execution of proof-of-principle trials that will test hypotheses and provide a “go or no-go” decision for further development. This will require synergy and an overall global strategy so human (and economic) resources are maximally used. Finally, integration of all available therapeutic strategies holds the realistic promise of incremental, but definite, improvements in clinical benefit, both in terms of quality of life and overall survival.

REFERENCES

1. Fidler, I. J. and Kripke, M. L. (1977). Metastasis results from preexisting variant cells within a malignant tumor. *Science* **19**, 893–895.
2. DeWever, O. and Mareel, M. (2003). Role of tissue stroma in cancer cell invasion. *J. Pathol.* **200**, 429–444.
3. Liotta, L. A. and Kohn, E. C. (2001). The microenvironment of the tumor-host interface. *Nature* **411**, 375–379.
4. Mueller, M. M. and Fusenig, N. E. (2004). Friends or foe-Bipolar effects of the tumour stroma in cancer. *Nat. Rev. Cancer* **4**, 839–849.
5. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., and Cunha, G. R. (1999). Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res.* **59(19)**, 5002–5011.
6. Cunha, G. R., Hayward, S. W., Wang, Y. Z., and Ricke, W. A. (2003). Role of the stromal microenvironment in carcinogenesis of the prostate. *Int. J. Cancer* 107(1), 1–10.
7. Paget S. (1889). The distribution of secondary growths in cancer of the breast. Lancet **1**, 571–573.
8. Muller, A., Homey, B., Soto, H., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410(6824)**, 50–56.
9. Taichman, R.S., Cooper, C., Keller, E.T., Pienta, K.J., Taichman, N.S., and McCauley, L.K. (2002). Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res.* **62(6)**, 1832–1837.
10. Arap, W., Haedicke, W., Bernasconi, M., et al. (2002). Targeting the prostate for destruction through a vascular address. *Proc. Natl. Acad. Sci. USA* **99(3)**, 1527–1531.
11. Ruoslahti, E. (2002).Specialization of tumour vasculature. *Nat. Rev. Cancer* **2(2)**, 83–90.
12. Koeneman, K. S., Yeung, F., and Chung, L. W. K. (1999). Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* **39(4)**, 246–261.
13. Sikes, R. A., Nicholson, B. E., Koeneman, K. S., et al. (2004). Cellular interactions in the tropism of prostate cancer to bone. *Int. J. Cancer* **110(4)**, 497–503.

14. Bogdanos, J., Karamanolakis, D., Tenta, R., et al. (2003). Endocrine/paracrine/autocrine survival factor activity of bone microenvironment participates in the development of androgen ablation and chemotherapy refractoriness of prostate cancer metastasis in skeleton. *Endocr. Relat. Cancer* **10(2)**, 279–289.
15. Chung, L. W. K. (2003). Prostate carcinoma bone-stroma interaction and its biologic and therapeutic implications. *Cancer* **97(3 Suppl)**, 772–778.
16. Chung, L. W. K., Gleave, M. E., Hsieh, J. T., and Zhau, H. E. (1991). Reciprocal mesenchymal-epithelial interaction affecting prostate tumor growth and hormonal responsiveness. *Cancer Surv.* **11**, 91–121.
17. Cunha, G. R., Chung, L. W., Shannon, J. M., Taguchi, O., and Fujii, H. (1983). Hormone-induced morphogenesis and growth: role of mesenchymal-epithelial interactions. *Recent Prog. Horm. Res.* **39**, 559–598.
18. Camps, J. L., Chang S. M., Hsu, T. C., et al. (1990). Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. *Proc. Natl. Acad. Sci. USA* **87(1)**, 75–79.
19. Chung, L. W. K. and Davis, R. (1996). Prostate epithelial differentiation is dictated by its surrounding stroma. *Mol. Biol. Rep.* **23**, 13–19.
20. Thalmann, G., Anezinis, P., Chang, S. M., et al. (1994). The LNCaP mouse model of human prostate cancer: Androgen independent cancer progression and osseous metastasis. *Cancer Res.* **54**, 2577–2581.
21. Tlsty, T. D. (1998). Cell-adhesion dependent influences on genomic instability and carcinogenesis. *Curr. Opin. Cell Biol.* **10**, 647–653.
22. Thalmann, G. N., Sikes, R. A., Wu, T. T., et al. (2000). The LNCaP progression model of human prostate cancer: androgen independence and osseous metastasis. *Prostate* **44**, 91–103.
23. Gleave, M. E., Hsieh, J. T., von Eschenbach, A. C., and Chung, L. W. K. (1992). Prostate and bone fibroblasts induce human prostate cancer growth in vivo: implications for bidirectional tumor-stromal cell interaction in prostate carcinoma growth and metastasis. *J. Urol.* **147**, 1151–1159.
24. Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat.* **154**, 8–20.
25. Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2**, 442–454.
26. Tlsty, T. D. (2001). Stromal cells can contribute oncogenic signals. *Semin. Cancer Biol.* **11**, 97–104.
27. Phillips, J. L., Hayward, S. W., Wang, Y., et al. (2001). The consequences of chromosomal aneuploidy on gene expression profiles in a cell line model for prostate carcinogenesis. *Cancer Res.* **61**, 8143–8149.
28. Dedhar S., Saulnier R., Nagel R., and Overall C. M. (1993). Specific alterations in the expression of $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins in highly invasive and metastatic variants of human prostate carcinoma cells selected by in vitro invasion through reconstituted basement membrane. *Clin. Exp. Metastasis.* **11**, 391–400.
29. Edlund, M., Miyamoto, T., Sikes, R. A., et al. (2001). Integrin expression and usage by prostate cancer cell lines on laminin substrata. *Cell Growth Differ.* **12(2)**, 99–107.
30. Edlund, M., Sung, S. Y., and Chung, L. W. (2004). Modulation of prostate cancer growth in bone microenvironments. *J. Cell Biochem.* **91(4)**, 686–705.
31. Witkowski, C. M., Rabinovitz, I., Nagle, R. B., Affinito, K-S. D., and Cress, A. E. (1993). Characterization of integrin subunits, cellular adhesion and tumorigenicity of four human prostate cell lines. *J. Cancer Res. Clin. Oncol.* **119**, 637–644.
32. Nemeth, J. A., Cher, M. L., Zhou, Z., Mullins, C., Bhagat, S., and Trikha, M. (2003). Inhibition of alpha(v)beta3 integrin reduces angiogenesis, bone turnover, and tumor cell proliferation in experimental prostate cancer bone metastases. *Clin. Exp. Metastasis* **20(5)**, 413–420.
33. Janssen, M.L., Oyen, W.J., Dijkgraaf, I., et al. (2002). Tumor targeting with radiolabeled alpha(v)beta(3) integrin binding peptides in a nude mouse model. *Cancer Res.* **62(21)**, 6146–6151.
34. Bergers, G. and Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* **3**, 401–410.
35. Coussens, L. M. and Werb, Z. (2002). Inflammation and cancer. *Nature* **420**, 860–867.
36. Dvorak, H. F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* **315**, 1650–1659.
37. Di Lorenzo, G., Tortor, G., D'Armiento, F. P., et al. (2002). Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin. Cancer Res.* **8**, 3438–3444.
38. Sirotna, F. M., She, Y., Lee, F., Chen, J., and Scher, H. I. (2002). Studies with CWR22 xenografts in nude mice suggest that ZD1839 may have a role in the treatment of both androgen-dependent and androgen-independent human prostate cancer. *Clin. Cancer Res.* **8**, 3870–3876.
39. Canil, C. M., Moore, M. J., Winquist, E., et al. (2005). Randomized phase II study of two doses of gefitinib in hormone-refractory prostate cancer: a trial of the National Cancer Institute of Canada-Clinical Trials Group. *J. Clin. Oncol.* **23(3)**, 455–460.
40. Sanchez, K. M., Sweeney, C. J., Mass, R., et al. (2002). Evaluation of HER-2/neu expression in prostatic adenocarcinoma: a requested for a standardized, organ specific methodology. *Cancer* **95**, 1650–1655.
41. Calvo, B., Levine, A., Marcos, M., et al. (2003). Human epidermal receptor-2 expression in prostate cancer. *Clin. Cancer Res.* **9**, 1087–1097.

42. Ziada, A., Barqawi, A., Glode, L. M., et al. (2004). The use of trastuzumab in the treatment of hormone refractory prostate cancer; phase II trial. *Prostate* **60(4)**, 332–337.
43. Agus, D. B., Akita, R. W., Fox, W. D., et al. (2002). Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell.* **2(2)**, 127–137.
44. Agus, D. B., Gordon, M., Taylor, C., et al. (2003). Clinical activity in a phase I trial of HER-2-targeted rhuMAb 2C4 (pertuzumab) in patients with advanced solid malignancies (AST). *Proc. Am. Soc. Clin. Oncol.* (abstr 771).
45. You, X., Yu, H. M., Cohen-Gould, L., et al. (2003). Regulation of migration of primary prostate epithelial cells by secreted factors from prostate stromal cells. *Exp. Cell Res.* **288(2)**, 246–256.
46. Davies, G., Watkins, G., Mason, M. D., and Jiang, W. G. (2004). Targeting the HGF/SF receptor c-met using a hammerhead ribozyme transgene reduces in vitro invasion and migration in prostate cancer cells. *Prostate* **60(4)**, 317–324.
47. Foster, B. A., Kaplan, P. J., and Greenberg, N. M. (1999). Characterization of the FGF axis and identification of a novel FGFR1iiic isoform during prostate cancer progression in the TRAMP model. *Prostate Cancer Prostatic Dis.* **2(2)**, 76–82.
48. Kwabi-Addo, B., Ozen, M., and Ittmann, M. (2004). The role of fibroblast growth factors and their receptors in prostate cancer. *Endocr. Relat. Cancer* **11(4)**, 709–724.
49. San Francisco, I. F., DeWolf, W. C., Peehl, D. M., and Olumi, A. F. (2004). Expression of transforming growth factor-beta 1 and growth in soft agar differentiate prostate carcinoma-associated fibroblasts from normal prostate fibroblasts. *Int. J. Cancer* **112(2)**, 213–218.
50. Gerdes, M. J., Larsen, M., Dang, T. D., Ressler, S. J., Tuxhorn, J. A., and Rowley, D. R. (2004). Regulation of rat prostate stromal cell myodifferentiation by androgen and TGF-beta1. *Prostate* **58(3)**, 299–307.
51. Tuxhorn, J. A., McAlhany, S. J., Yang, F., Dang, T. D., and Rowley, D. R. (2002). Inhibition of transforming growth factor-beta activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. *Cancer Res.* **62(21)**, 6021–6025.
52. Krueckl, S. L., Sikes, R. A., Edlund, N. M., et al. (2004). Increased insulin-like growth factor I receptor expression and signaling are components of androgen-independent progression in a lineage-derived prostate cancer progression model. *Cancer Res.* **64(23)**, 8620–8629.
53. Nickerson, T., Chang, F., Lorimer, D., Smeekens, S. P., Sawyers, C. L., and Pollak, M. (2001). In vivo progression of LAPC-9 and LNCaP prostate cancer models to androgen independence is associated with increased expression of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR). *Cancer Res.* **61(16)**, 6276–6280.
54. Zhang, H. and Yee, D. (2004). The therapeutic potential of agents targeting the type I insulin-like growth factor receptor. *Expert Opin. Investig. Drugs* **13(12)**, 1569–1577.
55. Mitsiade, C. S., Mitsiades, N. S., McMullan, C. J., et al. (2004). Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell.* **5(3)**, 221–230.
56. Majumder, P. K., Febbo, P. G., Bikoff, R., et al. (2004). mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat. Med.* **10(6)**, 594–601.
57. Tolcher, A. W. (2004). Novel therapeutic molecular targets for prostate cancer: the mTOR signaling pathway and epidermal growth factor receptor. *J. Urol.* **171**, S41–43.
58. Su, J. D., Mayo, L. D., Donner, D. B., and Durden, D. L. (2003). PTEN and phosphatidylinositol 3'-kinase inhibitors up-regulate p53 and block tumor-induced angiogenesis: evidence for an effect on the tumor and endothelial compartment. *Cancer Res.* **63(13)**, 3585–3592.
59. George, D. J., Halabi, S., Shepard, T. F., et al. (2005). The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: results from cancer and leukemia group B 9480. *Clin. Cancer Res.* **11(5)**, 1815–1820.
60. Culig, Z., Steiner, H., Bartsch, G., and Hobisch, A. (2005). Interleukin-6 regulation of prostate cancer cell growth. *J. Cell Biochem.* **95(3)**, 497–505.
61. Pfitzenmaier, J., Vessella, R., Higano, C. S., Noteboom, J. L., Wallace, D., Jr, and Corey E. (2003). Elevation of cytokine levels in cachectic patients with prostate carcinoma. *Cancer* **97(5)**, 1211–1216.
62. Nemeth, J. A., Yousif, R., Herzog, M., et al. (2002). Matrix metalloproteinase activity, bone matrix turnover, and tumor cell proliferation in prostate cancer bone metastasis. *J. Natl. Cancer Inst.* **94(1)**, 17–25.
63. Lokeshwar, B. L. (1999). MMP inhibition in prostate cancer. *Ann. NY Acad. Sci.* **878**, 271–289.
64. Shepherd, F. A., Giaccone, G., Seymour, L., et al. (2002). Prospective, randomized, double-blind, placebo-controlled trial of marimastat after response to first-line chemotherapy in patients with small-cell lung cancer: a trial of the National Cancer Institute of Canada-Clinical Trials Group and the European Organization for Research and Treatment of Cancer. *J. Clin. Oncol.* **20(22)**, 4434–4439.
65. Moore, M., Hamm, J., Dancey, J., et al. (2003). Comparison of gemcitabine versus the matrix metalloproteinase inhibitor BAY 12-9566 in patients with advanced or metastatic adenocarcinoma of the pancreas: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J. Clin. Oncol.* **21(17)**, 3296–3302.

66. Lara, P. N., Longmate, J., Stadler, W., et al. (2004). Angiogenesis inhibition in metastatic hormone refractory prostate cancer (HRPC): a randomized phase II trial of two doses of the matrix metalloproteinase inhibitor (MMPI) BMS-275291. *J. Clin. Oncol.* **22**, 4647.
67. Hess, K. R. and Abbruzzese, J. L. (2001). Matrix metalloproteinase inhibition of pancreatic cancer: matching mechanism of action to clinical trial design. *J. Clin. Oncol.* **19(15)**, 3445–3446.
68. Soff, G. A., Sanderowitz, J., Gately, S., et al. (1995). Expression of plasminogen activator inhibitor type 1 by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis, and metastasis to lung and liver in an athymic mouse model. *J. Clin. Invest.* **96(6)**, 2593–2600.
69. Jankun, J., Keck, R. W., Skrzypczak-Jankun, E., and Swiercz, R. (1997). Inhibitors of urokinase reduce size of prostate cancer xenografts in severe combined immunodeficient mice. *Cancer Res.* **57(4)**, 559–563.
70. Karan D., Lin, F. C., Bryan, M., et al. (2003). Expression of ADAMs (a disintegrin and metalloproteases) and TIMP-3 (tissue inhibitor of metalloproteinase-3) in human prostatic adenocarcinomas. *Int. J. Oncol.* **23(5)**, 1365–1371.
71. McCulloch, D. R., Akl, P., Samaratunga, H., Herington, A. C., and Odorico, D. M. (2004). Expression of the disintegrin metalloprotease, ADAM-10, in prostate cancer and its regulation by dihydrotestosterone, insulin-like growth factor I, and epidermal growth factor in the prostate cancer cell model LNCaP. *Clin. Cancer Res.* **10(1 Pt 1)**, 314–323.
72. Reese, D., Frohlich, M., Bok, R., et al. (1999). Phase II trial of humanized monoclonal anti-vascular endothelial growth factor antibody (rhuMAbVEGF) in hormone refractory prostate cancer. *Proc. Am. Soc. Clin. Oncol.* (abstr 1355). Atlanta, GA.
73. Picus, J., Halab, S. I., Rini, B., et al. (2003). Small E. The use of bevacizumab (B) with docetaxel (D) and estramustine (E) in hormone refractory prostate cancer (HRPC): initial results of CALGB 90006. *Proc. Am. Soc. Clin. Oncol.* (abstr 1578). Chicago, IL.
74. Rini, B., Weinberg, V., Fong, L., and Small, E. (2005). A phase 2 study of prostatic acid phosphatase-pulsed dendritic cells (APC8015; Provenge) in combination with bevacizumab in patients with serologic progression of prostate cancer after local therapy. ASCO Prostate Cancer Symposium (abstr 251). Orlando, FL.
75. Pietras, K. and Hanahan, D. (2005). A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. *J. Clin. Oncol.* **23(5)**, 939–952.
76. Mathew, P., Thall, P. F., Jones, D., et al. (2004). Logothetis C. Platelet-derived growth factor receptor inhibitor imatinib mesylate and docetaxel: a modular phase I trial in androgen-independent prostate cancer. *J. Clin. Oncol.* **22(16)**, 3323–3329.
77. Figg, W. D., Dahut, W., Duray, P., et al. (2001). A randomized phase II trial of thalidomide, an angiogenesis inhibitor, in patients with androgen-independent prostate cancer. *Clin. Cancer Res.* **7(7)**, 1888–1893.
78. Dahut, W. L., Gulley, J. L., Arlen, P. M., et al. (2004). Randomized phase II trial of docetaxel plus thalidomide in androgen-independent prostate cancer. *J. Clin. Oncol.* **22(13)**, 2532–2539.
79. Arap, M. A., Lahdenranta, J., Mintz, P. J., et al. (2004). Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. *Cancer Cell.* **6(3)**, 275–284.
80. Pasqualini, R. and Arap, W. 2002. Translation of vascular diversity into targeted therapeutics. *Ann. Hematol.* **81(Suppl. 2)**, S66–67.
81. Zurita, A. J., Troncoso, P., Cardo-Vila, M., Logothetis, C. J., Pasqualini, R., and Arap, W. (2004). Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer. *Cancer Res.* **64(2)**, 435–439.
82. Small, E., Higano, C., Smith, D., et al. (2004). A phase 2 study of an allogeneic GM-CSF gene-transduced prostate cancer cell line vaccine in patients with metastatic hormone-refractory prostate cancer. *J. Clin. Oncol.* **22**, 4565.
83. Smith, M. R., Eastham, J., Gleason, D. M., et al. (2003). Randomized controlled trial of zoledronic acid to prevent bone loss in men receiving androgen deprivation therapy for nonmetastatic prostate cancer. *J. Urol.* **169(6)**, 2008–2012.
84. Saad, F., Gleason, D. M., Murray, R., et al. (2002). Zoledronic Acid Prostate Cancer Study Group. A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. *J. Natl. Cancer Inst.* **94(19)**, 1458–1468.
85. Montague, R., Hart, C. A., George, N. J., Ramani, V. A., Brown, M. D., and Clarke, N. W. (2004). Differential inhibition of invasion and proliferation by bisphosphonates: anti-metastatic potential of Zoledronic acid in prostate cancer. *Eur. Urol.* **46(3)**, 389–401.
86. Lee, Y. P., Schwarz, E. M., Davies, M., et al. (2002). Use of zoledronate to treat osteoblastic versus osteolytic lesions in a severe-combined-immunodeficient mouse model. *Cancer Res.* **62(19)**, 5564–5570.
87. Lee, M. V., Fong, E. M., Singer, F. R., and Guenette, R. S. (2001). Bisphosphonate treatment inhibits the growth of prostate cancer cells. *Cancer Res.* **61(6)**, 2602–2608.
88. Lewington, V. J., McEwan, A. J., Ackery, D. M., et al. (1991). A prospective, randomised double-blind crossover study to examine the efficacy of strontium-89 in pain palliation in patients with advanced prostate cancer metastatic to bone. *Eur. J. Cancer* **27(8)**, 954–958.
89. Sartor, O., Reid, R. H., Hoskin, P. J., et al. (2004). Quadramet 424Sm10/11 Study Group Samarium-153-Lexidronam complex for treatment of painful bone metastases in hormone-refractory prostate cancer. *Urology* **63(5)**, 940–945.

90. Tu, S. M., Millikan, R. E., Mengistu, B., et al. (2001). Bone-targeted therapy for advanced androgen-independent carcinoma of the prostate: a randomised phase II trial. *Lancet* **357(9253)**, 336–341.
91. Wu-Wong, J. R., Dixon, D. B., Chiou, W. J., et al. (1999). Pharmacology of A-216546: a highly selective antagonist for endothelin ET(A) receptor. *Eur. J. Pharmacol.* **366(2–3)**, 189–201.
92. Nelson, J. B., Nguyen, S. H., Wu-Wong, J. R., et al. (1999). New bone formation in an osteoblastic tumor model is increased by endothelin-1 overexpression and decreased by endothelin A receptor blockade. *Urology* **53(5)**, 1063–1069.
93. Carducci, M. A., Padley, R. J., Breul, J., et al. (2003). Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. *J. Clin. Oncol.* **21(4)**, 679–689.
94. Carducci, M., Nelson, J. B., Saad, F., et al. (2004). Effects of atrasentan on disease progression and biological markers in men with metastatic hormone-refractory prostate cancer: Phase 3 study. *J. Clin. Oncol.* **22**, 4508.
95. Gardner, T. A., Ko, S. C., Kao, C., Shirakawa, T., Cheon, J., and Gotoh A. (1998). Exploiting stromal-epithelial interaction for model development and new strategies of gene therapy for prostate cancer and osteosarcoma metastases. *Gene Ther. Mol. Biol.* **2**, 41–58.
96. Ko, S. C., Cheon, J., Kao, C., et al. (1996). Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res.* **56**, 4614–4619.
97. Waltregny, D., Bellahcene, A., Van Riet, I., et al. (1998). Prognostic value of bone sialoprotein expression in clinically localized human prostate cancer. *J. Natl. Cancer Inst.* **90**, 1000–1007.
98. Koeneman, K. S., Kao, C., Ko, S. C., et al. (2000). Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. *World J. Urol.* **18(2)**, 102–110.
99. Kubo, H., Gardner, T. A., Wada, Y., et al. (2003). Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum. Gene Ther.* **14(3)**, 227–241.
100. Jin, F., Xie, Z., Kuo, C. J., Chung, L. W., and Hsieh, C. L. (2005). Cotargeting tumor and tumor endothelium effectively inhibits the growth of human prostate cancer in adenovirus-mediated antiangiogenesis and oncolysis combination therapy. *Cancer Gene Ther.* **12(3)**, 257–267.

25

Intercellular Targets of Prostate Cancer

Young E. Whang and Daniel J. George

Summary

Prostate cancer is a hormonally driven disease with a relatively long natural history and predictable pattern of metastasis. Intercellular growth factor signals play an important role in the progression of prostate cancer in both the hormone-intact setting as well as disease progression in the setting of androgen deprivation therapy. Many intercellular growth factors have been implicated in prostate cancer based on various levels of evidence, from epidemiological studies to preclinical models. In this chapter, we review some of the most prominent intercellular growth factor pathways involved in prostate cancer, particular in the so-called hormone-refractory setting. In particular, we focus on those pathways for which targeted therapeutics have been developed and are being tested in clinical trials for patients with advanced prostate cancer. In the future, insights into the biology of these intercellular targets may lead to patient selection for clinical trials based upon biomarker or molecular profiles in order to enrich for reponse to targeted therapy.

Key Words: Prostate cancer; epidermal growth factor; vascular endothelial growth factor; platelet-derived growth factor.

1. INTRODUCTION

Testosterone, dihydrotestosterone, and the androgen receptor represent the most studied and interrogated extracellular growth factor/hormonal pathway in prostate cancer. Since the work of Charles Huggins and others, inhibition of androgen signaling has represented the most striking proof of concept that targeted therapy can profoundly affect the clinical outcomes of patients with prostate cancer. However, targeting this pathway is clearly not enough to completely abrogate growth, and eventually many patients develop evidence of disease progression despite castrate levels of testosterone. With further biological insights into the mechanisms of disease progression in this setting, we hope to further delay or inhibit the progression of disease in these patients.

Normal prostate epithelial cells are dependent on a variety of factors for survival and proliferation, including cell-to-cell and cell-to-matrix interactions, growth factors, and hormones. Understanding how cellular proliferation, differentiation, and apoptosis are regulated by these microenvironmental signals is central to understanding of the biology of prostate cancer and may represent potential novel therapeutic targets in the setting of androgen-independent disease progression. Growth factors, such as epidermal growth factor (EGF) and other ligands related to the EGF superfamily, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), endothelins, insulin-like growth factors (IGF), fibroblast growth factors (FGF), and other cytokines and their cognate receptors have been implicated in the development and progression of prostate cancer (see Table 1). Therefore, selective inhibition of these signaling pathways has attracted much interest as a potential therapeutic strategy.

From: *Contemporary Cancer Research: Prostate Cancer:*
Biology, Genetics, and the New Therapeutics, Second Edition
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

Table 1
Growth Factor Pathways Implicated in Prostate Cancer [a]

• TGF-β family	• PDGF (A and B)	• KGF family
• FGF (bFGF, aFGF)	• NGF family	• HGF/SF
• EGFβ family (TGF-α, amphiregulin)	• VEGF	• IL-6
• HER-2/neu	• ET-1, ET-2	• TNF-α
• IGFβ (I and II)		

[a] TGFβ, transforming growth factor-β; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; EGF, epidermal growth factor; HER, human EGF receptor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; NGF, nerve growth factor; VEGF, vascular endothelial growth factor; KGF, keratinocyte growth factor; HGF/SF, hepatocyte growth factor/scatter factor; IL, interleukin-6; TNF, tumor necrosis factor-α; ET-1, -2, Endothelin-1, -2.

2. EGF SUPERFAMILY

The family of receptor tyrosine kinases related to the EGF receptor (EGFR) is one of the best characterized and intensely studied signaling systems because of its involvement in growth and differentiation of normal epithelial cells and their postulated role in many human epithelial malignancies *(1,2)*. This family is made up of four members: EGFR (also known as ErbB-1, from its isolation as a second oncogene transduced by chicken erythroblastosis retrovirus), human EGF receptor (HER-2, or ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4). These four members share general structural similarities with regions composed of an extracellular portion that contains the ligand-binding domain, a single hydrophobic transmembrane domain, and an intracellular cytoplasmic tail that contains the intrinsic tyrosine kinase domain and tyrosine residues that are phosphorylated after stimulation with ligands. Binding of ligands to these receptors causes dimerization and activation of the intracellular tyrosine kinase domain and leads to autophosphorylation of their cytoplasmic tails and initiation of multiple signaling pathways that ultimately result in changes in gene expression and cellular phenotypes.

Complexity in the EGFR signaling system arises from the fact that there are multiple ligands with affinity for one member over another and induction of both homodimers and heterodimers of EGFR family members *(1,2)*. For example, ligands such as EGF, amphiregulin, and transforming growth factor (TGF)-α only bind to EGFR, whereas other members of the EGF ligand family (i.e., betacellulin, heparin-binding EGF, and epiregulin) can bind both to EGFR and HER-4. Heregulin (also known as neuregulin and neu differentiation factor) binds to HER-3 and HER-4, but not to EGFR. Many ligands capable of binding to these receptors have been identified, but no ligand binding to HER-2 has been identified. After ligand binding, each receptor may form either homodimers or heterodimers. In general, heterodimer formation is preferred over homodimerization, and HER-2 seems to be the preferred dimerization partner for other family members (i.e., EGFR/HER-2, HER-2/HER-3, and HER-2/HER-4). The intracellular cytoplasmic tail of HER-3 is unique among EGFR family members in that, despite homology with kinase domains of other family members, substitutions in critical amino acids lead to complete impairment of kinase activity of HER-3. Therefore, HER-3 may only become phosphorylated and activate downstream signaling when it dimerizes and acts as a ligand-binding partner with another EGFR family member (most frequently with HER-2). In addition, the intracellular domain of HER-3 uniquely encodes many tyrosine residues, which, after phosphorylation, efficiently couple to activation of the phosphatidylinositol (PI)-3 kinase pathway, which, in turn, activates potent mitogenic and antiapoptotic kinases, such as Akt and mammalian target of rapamycin (mTOR). The HER-2/HER-3 heterodimer may initiate strong oncogenic

signals because of its ability to activate the PI-3 kinase/Akt/mTOR signaling pathway *(3)*. Recent structural studies of extracellular domains of EGFR family members have provided new insights into the basis for homodimerization and heterodimerization *(4)*. Binding of EGF to the EGF receptor causes conformational changes in the ligand-binding domain that bring the region termed dimerization arm to the surface. Exposure of the dimerization arm facilitates binding of one receptor molecule to another receptor with the exposed dimerization arm. On the other hand, the extracellular ligand-binding domain of HER-2 has already adopted a conformation with the exposed dimerization arm without any ligand bound. Therefore, HER-2 is already in an "activated" conformation ready to dimerize without need for ligand binding. This structure may underlie the propensity of HER-2 to become constitutively activated with homodimer formation when gene amplification causes overexpression, found in a subset of breast cancer.

In the growth and differentiation of normal prostatic epithelial cells, paracrine interaction between stromal and epithelial cells involving EGFR and its ligands EGF and TGF-α has been postulated to play a central role *(5)*. EGF is produced in the apical membrane of epithelial cells and secreted into the prostatic fluid at concentrations higher than any other body fluid *(6)*. TGF-α is secreted under stimulation of androgen in the stromal cells and may activate EGFR found on the basolateral surface of prostate epithelial cells. During transformation and progression of prostate cancer, there is evidence for a shift from the paracrine to autocrine stimulation of the EGFR signaling loop. Prostate cancer cell lines synthesize TGF-α in vitro *(7,8)*. Expression of TGF-α and EGF messenger RNA (mRNA) is significantly higher in prostate cancer tumors than in benign prostate cells *(9)*. Immunohistochemistry of primary tumors show that TGF-α production is associated with loss of differentiation in localized tumors and with progression to the metastatic androgen-independent stage *(10,11)*. These findings imply that autocrine activation of EGFR may play a role in progression of prostate cancer. However, because of a lack of suitable reagents, the activation or phosphorylation status of EGFR in prostate cancer has not yet been extensively characterized. These data in aggregate suggest that EGFR is a potential target in advanced prostate cancer. Therefore, the efficacy of gefitinib (Iressa, Astra Zeneca) *(12)*, a specific kinase inhibitor of EGFR, was tested on the prostate cancer xenograft tumor CWR22. Administration of gefitinib inhibited the growth of androgen-dependent and androgen-independent CWR22 xenograft tumors and enhanced the inhibitory activity of bicalutamide and chemotherapeutic agents *(13)*. Therefore, the clinical activity of gefitinib as monotherapy was tested in a clinical trial organized by the National Cancer Institute of Canada *(14)*. Forty patients with chemotherapy-naïve hormone-refractory prostate cancer (HRPC) were randomized between 250 mg or 500 mg daily gefitinib. However, there was no response in prostate-specific antigen (PSA) or objective measurable disease at either dose. Currently, it seems that gefitinib is not active as a single agent when it is tested in an unselected group of HRPC patients with unknown molecular characteristics of tumors.

HER-2 has been important in oncology because it was one of the earliest examples of the oncogenes driving human cancer, and targeted therapy against HER-2 has served as a paradigm in drug development. Slamon and his colleagues have demonstrated that HER-2 was overexpressed by gene amplification in approx 20 to 30% of breast cancer tumors and that HER-2 overexpression carried a poor prognosis *(15)*. Trastuzumab (Herceptin), a humanized monoclonal antibody specific for HER-2, is effective as a single agent in treating patients with HER-2 overexpressing breast cancer, producing a response rate of 26% *(16)*. When trastuzumab was administered in combination with chemotherapy with patients with HER-2-positive metastatic breast cancer, it demonstrated a survival advantage in patients administered trastuzumab *(17)*. The potential role of HER-2 in prostate cancer was also stimulated by a report that showed increased expression of HER-2 during progression of the prostate xenograft tumor model to androgen independence and that enforced overexpression of HER-2 converted androgen-dependent sub-lines to androgen independence *(18)*. Several publications that examined the expression levels of HER-2 in primary tumors before and after androgen ablation reported that HER-2 expression increased during progression to the hormone-refractory stage, impli-

cating a role of HER-2 in this process *(19,20)*. However, the frequency of HER-2 overexpression in primary prostate cancer is a controversial subject. A recent comprehensive survey of 18 published studies that used 9 different antibodies and varying definitions of overexpression found that the reported overexpression rate of HER-2 by immunohistochemistry varied from 0 to 100% *(21)*. Most recent reports have found that HER-2 overexpression is rare in prostate cancer *(21,22)*. Although there may not be consensus on the frequency of HER-2 overexpression by immunohistochemistry, prostate cancer does not seem to show gene amplification of *HER-2* detected by the fluorescence in situ hybridization method commonly used for breast cancer *(21)*. There are three publications that report on the current experience of HER-2 targeted therapy in androgen-independent prostate cancer using trastuzumab *(23–25)*. In all of these clinical studies, the level of HER-2 expression in primary tumor specimens was analyzed by immunohistochemistry. Approximately 10% of the specimens tested expressed HER-2 at the 2+ or 3+ level and was considered to be HER-2 positive. One study demonstrated the presence of HER-2 gene amplification in 0 of 34 specimens *(23)*. Because of the infrequent overexpression of HER-2 found on screening, two of three trials included both patients with HER-2-positive and HER-2-negative tumors *(24,25)*. All three reports concluded that trastuzumab as a single agent is completely ineffective in unselected HRPC patients, with a PSA response rate of 0%.

Despite the lack of efficacy of trastuzumab in prostate cancer, HER-2 remains a valid target in prostate cancer. Although the mechanisms by which trastuzumab inhibits HER-2 signaling are likely multifactorial and need further elucidation *(1)*, the clinical activity of trastuzumab in breast cancer requires high level expression of HER-2 from gene amplification *(16)*. This situation is unlikely to be associated with prostate cancer. Alternative means of HER-2 inhibition may be more effective in tumors without HER-2 overexpression but constitutively activated HER-2 (i.e., through autocrine or paracrine mechanisms). For example, heregulin is not expressed in benign prostate cells but only expressed by prostate tumor cells, suggesting a possibility of the presence of the HER-2/HER-3 autocrine activation loop *(26)*. There are novel agents that target HER-2 activated by heterodimerization with HER-3. Pertuzumab (also known as 2C4 or Omnitarg) is an antibody binding to the dimerization arm of HER-2, thereby inhibiting ligand-activated heterodimer formation of HER-2 and HER-3 *(27)*. Administration of this antibody potently inhibited growth of prostate xenograft tumors, providing strong support for the existence of the HER-2/HER-3 autocrine loop in prostate cancer that is necessary for the maintenance of prostate cancer growth. The phase I trial of pertuzumab has demonstrated the feasibility and tolerability of administration every 3 weeks *(28)*. The clinical activity of pertuzumab in prostate cancer remains to be elucidated. There are several small molecule inhibitors undergoing clinical development that are capable of inhibiting HER-2 kinase activity by competitive binding of the ATP pocket of the kinase domain *(1)*. PKI-166 is a dual inhibitor of EGFR and HER-2 and it inhibits growth of prostate cancer xenograft tumors *(29)*. Mellinghoff and their colleagues have subsequently shown that PKI-166 partly acts by inhibiting the transcriptional function of the androgen receptor, which plays a key role in maintaining the viability of HRPC *(30)*. Lapatinib (GW572016) is also a dual inhibitor of EGFR and HER-2 that inhibits growth of HER-2-overexpressing breast xenograft tumors in animal models *(31)*. Two recent studies have shown that lapatinib is a more potent inhibitor of in vitro proliferation of prostate cancer cells than gefitinib, and that lapatinib inhibits the transcriptional function of the androgen receptor in prostate cancer cells *(32,33)*. It remains to be seen whether these HER-2 targeting agents will have clinically meaningful activity in prostate cancer.

3. VASCULAR ENDOTHELIAL GROWTH FACTOR

VEGF is a pleiotrophic glycoprotein with autocrine, paracrine, and endocrine functions *(31,32,34)*. VEGF is a homodimeric cytokine that might also function in the early development and progression of prostate cancer. VEGF was originally identified for its effects on endothelial cell proliferation and vascular permeability *(35,36)*. Since its discovery, VEGF has been shown to bind to the surface of

endothelial cells on two tyrosine kinase receptors, Flt-1 and KDR (or VEGFR-1 and -2, respectively) and to stimulate physiological and pathophysiological angiogenesis *(37)*. Four alternatively spliced isoforms of VEGF have been identified, which differ in their cellular location and in heparin binding *(37)*. Two of these isoforms are detectable in circulating fluids, including plasma and serum. In addition, a variety of VEGF-related peptides have been identified, including placental growth factor, and VEGF-B, C, and D, that either alone or in concert with VEGF demonstrate angiogenic activity *(37)*. Nonetheless, despite potential redundancy in VEGF signaling, a single allelic knockout of the VEGF gene in mice is lethal *(38)*. These results suggest that VEGF is critical to embryogenesis, but may have roles in addition to angiogenesis. For instance, the KDR tyrosine kinase receptor for VEGF is present on early pluripotent angioblasts and may function in the proliferation and differentiation of hematopoietic and endothelial stem cells *(39)*. Finally, VEGF is produced in many normal tissues where it might function to promote the early growth and progression of primary tumors. Of note, one of the richest sources of VEGF production in humans is the prostate gland *(40)*.

Normal prostatic growth and survival seems to depend on angiogenesis mediated by the VEGF axis. Support for this finding comes from studies of castrated animals, in which the prostate gland shrinks down in size and glandular epithelial cells undergo apoptosis, leaving behind stromal and basal cells. Interestingly, VEGF is under androgen regulation in the prostate, and loss of prostatic VEGF expression occurs within hours of castration *(41,42)*. When testosterone is restored, VEGF expression resumes first, followed by endothelial cell regrowth, epithelial cell growth, and, finally, an increase in prostate gland size. This chronology suggests that prostatic regrowth is preceded and dependent on VEGF-mediated vasculature regrowth *(41,42)*.

Similar to the normal prostate gland, growth of prostate cancer may also be VEGF dependent. In mice bearing the human prostate cancer xenograft LNCaP, castration results in a precipitous and immediate loss of VEGF expression in the tumor, followed by selective ablation of small microvessels and, eventually, tumor shrinkage *(43)*. In clinical prostate cancer specimens, microvessel density is an independent prognostic factor for prostate cancer disease progression and survival *(44–47)*. More recently, VEGF expression in primary prostate specimens has been shown to correlate to microvessel density and to prognosis *(48)*.

We measured VEGF levels in plasma prospectively collected from patients enrolled onto Cancer and Leukemia Group B (CALGB) 9480, an intergroup study of suramin in patients with HRPC, to determine whether these levels had prognostic significance *(49)*. Plasma VEGF levels in this population ranged from 4 to 885 pg/mL, with a median level of 83 pg/mL. As a continuous variable, plasma VEGF levels inversely correlated with survival time ($p = 0.002$). Using various exploratory cut points, plasma VEGF levels seemed to correlate with survival. In multivariate models in which other prognostic factors (serum PSA level, alkaline phosphatase level, evidence of measurable disease, and hemoglobin) were included, plasma VEGF level was significant at various cut points tested, particularly at a cut point of at least 260 pg/mL. Similar results were observed with urine VEGF levels in a smaller subset of patients *(50)*. Although these data are exploratory and need to be confirmed in an independent data set, these do suggest that VEGF may have clinical significance in patients with HRPC.

Preclinical studies suggest that inhibition of the VEGF pathway may result in an antitumor response. Several studies suggest that monoclonal antibodies to VEGF can cause tumor regression in preclinical animal prostate tumor models *(51,52)*. In addition, several small molecules targeted against the cell surface tyrosine kinase receptors for VEGF (VEGFR1-3) have demonstrated antitumor effects in prostate tumor models *(53,54)*. In a phase I clinical trial of the pan-VEGFR inhibitor, PTK787/ZK 222584, we studied the effect of a high-fat diet on drug absorption in patients with HRPC. Although no clinically significant food effect was observed on drug absorption or pharmacokinetics, we did observe persistent reductions in serum PSA levels of 40% or greater from baseline in 4 of 19 patients *(55)*. RhuMAb VEGF (bevacizumab) is a humanized murine monoclonal antibody that neutralizes VEGF activity and has shown promise in animal tumor models *(56–58)*.

A phase I study of the safety and efficacy of single-agent rhuMAb VEGF in metastatic HRPC was undertaken at the University of California, San Francisco *(59)*. Fifteen patients were treated with 10 mg/kg rhuMAb VEGF every 14 days for six infusions (one cycle) followed by additional treatment for selected patients exhibiting a response or stable disease. After one cycle, none of the 15 patients evaluable for tumor response had an objective complete or partial response. Three possible mixed responses were observed. No patient achieved an at least 50% decrease in serum PSA level after one cycle, although 4 patients (27%) had a PSA decline of less than 50%. The median time to objective progression was 3.9 months, and the median time to PSA progression was 2 months. Toxicity was generally mild, with asthenia present in 6 of the 15 patients (40%). Two patients developed severe hyponatremia, although the association with rhuMAb VEGF was unclear. The conclusion was that single-agent rhuMAb VEGF in this dose and schedule did not produce significant objective responses in HRPC. However, this trial was not designed to test the cytostatic effect of the drug, which, in retrospect, may be the most important feature of this class of agents.

Based on these data, the CALGB investigated the role of bevacizumab with estramustine and docetaxel in patients with progressive castrate metastatic prostate cancer in CALGB 90006 *(60)*. Seventy-nine patients were treated with this combination (280 mg estramustine orally thrice daily on days 1–5; 70 mg/m^2 docetaxel iv on day 2; 15 mg/kg bevacizumab on day 2) every 21 days. Typical premedication for docetaxel was administered, and 2 mg Coumadin daily was administered to help prevent any thromboembolic disease related to the estramustine. Patients tolerated the therapy well. There was one death caused by mesenteric vein thrombosis, one death caused by a perforated sigmoid colon diverticulum, two patients with a CNS bleed, and two patients each with pulmonary embolism and deep venous thrombosis. Although these thromboembolic events are of concern, the overall incidence was not dramatically higher than what has been observed with estramustine and docetaxel without bevacizumab. The other toxicities were similar to what was observed in published studies from Savarese and colleagues using a combination of estramustine, docetaxel, and hydrocortisone. The combination regimen resulted in a 50% or greater PSA decline in 58 (81%) of 72 patients. Median time to objective disease progression was 9.7 months, median time to PSA failure was 9.9 months, and overall median survival was 21 months.

These results compare favorably with previous phase II combination studies within CALGB and support the randomized study of bevacizumab in docetaxel and prednisone in and ongoing intergroup study (CALGB 90401). Correlative science investigations within this study will prospectively confirm the prognostic significance of plasma VEGF levels in HRPC patients and evaluate this marker in conjunction with other prognostic factors.

4. PLATELET-DERIVED GROWTH FACTOR

The PDGF pathway may be one such novel pathway to target in the treatment of early prostate cancer *(61–65)*. PDGF stimulates proliferation in both normal and malignant cells *(64)*. Prostate cancer cells express both PDGF receptor (PDGFR) and ligand, and dysregulation of PDGFR signaling in tumors leads to autocrine and paracrine stimulation of cell growth *(63,65)*. PDGF may function early in prostatic carcinogenesis because it has also been shown to be mitogenic to human cells derived from patients with prostatic hyperplasia *(65)*. More specifically, it seems that the b-subunit of PDGFR is primarily responsible for many of the cellular functions associated with PDGF biology in tumors *(66–68)*.

Expression of PDGFR has been reported to be as high as 88% of prostate cancer samples tested by immunohistochemistry *(63)* and also in prostatic hyperplasia, suggesting that PDGFR expression is observed early in carcinogenesis . In addition, gene expression profiles containing PDGFR-b along with four other genes were found to be associated with a higher PSA failure rate in men treated by radical prostatectomy for clinically localized prostate cancer *(69)*. In follow-up of these results, we tested imatinib mesylate [Gleevec, Novartis Pharmaceuticals, East Hanover, NJ] in 13 patients before prostatectomy *(70)*. Although no pathological changes constituted a significant clinical response,

decreases in blood flow as measured by dynamic contrast-enhanced MRI suggested a possible stromal effect. In addition, gene expression profiling on laser capture microdissected epithelial tumor cells from pretreatment biopsies and posttreatment prostatectomy samples suggested an expression pattern associated with Gleevec treatment *(71)*.

Thus far, three phase I to II trials have investigated the benefit of single-agent PDGFR inhibitors in the treatment of prostate cancer *(70,72,73)* and a fourth study combining Gleevec with Docetaxel chemotherapy is completed but not yet reported. Unfortunately, results from these studies to date demonstrate in both hormone-naïve and HRPC patients little evidence of antitumor effect. In more recent work, the frequency of PDGFR-b expression was reexamined in a wide range of prostate cancer samples, and in association with important pathologic and clinical parameters *(74)*. They demonstrate that expression of PDGFR-b is rarely robust and not associated with poor prognostic factors, questioning the unselected use of PDGFR inhibition as a strategy in prostate cancer.

5. ENDOTHELINS

Endothelins are peptides that are produced by both endothelial cells as well as epithelial cells in tissues such as the prostate *(75,76)*. Endothelin-1 binds to endothelin receptor A, and is thought to be the predominant endothelial signaling pathway in normal prostate tissue, as well as in prostate cancer tissue. Patients with metastatic HRPC have an increased expression of endothelin-1 when compared with normal prostatic epithelium and localized prostate cancer *(77,78)*. Endothelin-1 may function both to induce prostate cancer cell proliferation and as a mitogen for osteoblasts and to inhibit osteoclasts *(75)*.

Atrasentan (Xinlay, Abbott Laboratories, Abbott Park, IL) is a highly potent (Ki 0.034 n*M*) and selective (1800-fold) endothelin receptor A antagonist, which is being developed for the treatment of prostate cancer and other malignancies *(79)*. To evaluate atrasentan as a treatment for HRPC, two multicenter, randomized, double-blinded phase II clinical trials were conducted, M96-594 and M96-500 [24,25]. In each trial, subjects were randomly selected in a double-blinded fashion to receive placebo, 2.5, or 10 mg of atrasentan on a once-a-day dosing schedule. Study M96-594 was conducted on subjects with asymptomatic, metastatic, HRPC who were followed to the primary end point of clinical disease progression. Study M96-500 was conducted in subjects with metastatic HRPC who were symptomatic with disease-related pain. The primary end point assessed during the 12 weeks of the M96-500 study was a reduction in pain or a reduction in the use of opioid analgesics.

A dose–response effect was observed using several clinical measures of disease progression in both studies. In study M96-594, the median time to disease progression was delayed 34 to 52% in subjects treated with 10 mg atrasentan. Likewise, more than a 100% delay in the median time to PSA progression was observed for the 10 mg atrasentan group. Treatment with 10 mg atrasentan also attenuated bone metabolism, as measured by serum and bone alkaline phosphatase, markers of bone synthesis, and urine N-telopeptides, a marker of bone resorption. Attenuation in the radiographic progression of disease, as measured by bone scan indices, was also observed. In subjects treated with 2.5 mg atrasentan, delays in the median time to disease progression and PSA progression, as well as effects on bone metabolism were consistently smaller than those observed in subjects treated with 10 mg atrasentan. In the M96-500 study, trends in the reduction of pain for either atrasentan group (2.5 or 10 mg) compared with placebo were observed, although these were not statistically significant. We are currently conducting a phase I/II study of 10 mg atrasentan daily combined with docetaxel, and the Southwest Oncology Group is planning a phase III study comparing docetaxel prednisone atrasentan to docetaxel prednisone alone in men with HRPC and bone metastases.

6. INSULIN-LIKE GROWTH FACTOR

IGF-I and IGF-II are single-chain 7-kDa polypeptides that are produced predominantly by the liver and released into the circulation in high concentration. Although nearly every mammalian cell type can also synthesize and export IGF-I and IGF-II and the IGF regulation system in each organ is

tissue specific, all of the IGF regulation systems share certain components, including ligands (IGF-I and IGF-II), IGF binding proteins (IGFBPs)-1 to -6, IGF receptors (type I and II), and IGFBP-specific proteases *(80)*. Although both IGFs are mitogens, IGF-I is the principle regulator of growth after birth, acting systemically as well as locally via endocrine, paracrine, and autocrine mechanisms *(80)*. In vivo, tissue IGF-I bioactivity is determined not only by circulating IGF-I and IGFBP-3 levels, but also by other IGFBPs (such as IGFBP-1), local production of IGFs, IGFBPs, and possibly IGFBP proteases (such as PSA) that enhance IGF-I availability by cleaving IGFBPs.

Epidemiological data supports a role for IGF-I in the carcinogenesis of prostate cancer. Using the Physicians' Health Study, a large, prospectively collected database, Chen et al. *(9)* demonstrated that men in highest quartile of plasma IGF-I concentrations (within the normal physiological range) had a 4.3-fold higher risk of prostate cancer compared with those in the lowest quartile *(81)*. This association was independent of baseline PSA level, suggesting that IGF-I might be an independent predictor of prostate cancer risk. These findings were subsequently confirmed by other case–control studies *(82,83)*. Further epidemiological studies suggest a functional role of IGF-1 in prostate cancer disease progression, and preclinical models support the IGF-1 axis as a target for therapeutic intervention. Specific inhibitors of this pathway are under development and may be available for clinical testing in the near future.

7. FIBROBLAST GROWTH FACTOR

FGFs make up a large family of 23 related polypeptide ligands and four FGF receptors with highly conserved amino-acid sequences *(84,85)*.

FGFs and their receptors are important in the prostate formation and possibly in the carcinogenesis of prostate cancer *(86–89)*. Of the various ligands, FGF-7 (or keratinocyte growth factor) may mediate the effects of androgen on prostate growth *(90)*. FGF-7 expression is limited to stromal cells in a variety of tissues and it is mitogenic for epithelial cells *(91)*. In the prostate, production of FGF-7 is similarly restricted to stromal cells and is induced by androgens *(90)*. In normal prostate, and in prostate malignancies as well, it seems that the interaction between FGF-7 and this receptor plays a part in maintaining androgen-responsive growth and differentiation of epithelial cells *(90)*.

FGFs may also play an important role in the transformation of prostate cancer from an androgen-dependent to an androgen-independent state. Using the transplantable Dunning R3327 rat prostate tumor model, Yan et al. demonstrated that transformation from the indolent R3327 phenotype to the aggressive androgen-insensitive R3327AT3 phenotype is associated with a change in the alternative splicing pattern of the epithelial cell FGF-R2 receptor *(92)*. Normal prostate epithelia, as well as R3327 tumor cells, express exclusively the FGF-R2 (IIIb) isoform. In contrast, AT3 tumors express the FGF-R2 (IIIc) variant. Carstens et al. demonstrated a similar pattern in human prostate cancer xenografts as well as primary cultures *(93)*. Loss of responsiveness to FGF-7 may render these cells independent of stromal derived factors and set up autocrine loops between FGF ligands and their receptors, allowing autonomous growth and progression to a more malignant phenotype *(92)*. Such findings support the investigation of future targeted therapeutics against the FGF-7/ FGF-R2 axis in prostate cancer.

8. OTHER GROWTH FACTOR TARGETS

A plethora of other growth factors have also been implicated in prostate cancer, ranging from epidemiological links to expression on preclinical cell lines and clinical specimens to in depth functional studies. Although the list is long and ever changing, some of the more notable examples include the family of neurotrophins, interleukin (IL)-6, hepatocyte growth factor/scatter factor, and the integrin family of proteins. The neurotrophin family (including nerve growth factor, brain-derived growth factor, NT-3 and NT-4) seemed to be a promising target in preclinical models *(94,95)*, and clinical studies of the prototype nerve growth factor receptor inhibitor (CEP-701, Cephalon Pharmaceuticals,

West Chester, PA) have completed phase I testing *(96)*. IL-6 seems to be expressed in advanced HRPC and elevated plasma and serum levels of IL-6 are an independent prognostic factor for survival in several studies *(97,98)*. Antibodies to IL-6 are currently being tested in phase II studies in HRPC. Finally, integrins represent a relatively new anti-angiogenic target in cancer, with $\alpha_3\beta_5$ representing the most well-characterized *(99,100)*. Antibodies to integrins, such as vitaxin (Medimmune Inc, Gaithersburg, MD) have shown promise in early phase studies *(101)*.

9. CONCLUSIONS

In summary, there is strong evidence implicating signaling by EGFR family members in the physiology of normal and malignant prostate epithelial cells. There is evidence supporting the validity of the EGFR family of tyrosine kinases as therapeutic targets in prostate cancer, and we now have growing understanding of the signaling network by EGFR family members. There are intense ongoing efforts to develop agents targeting EGFR family members for clinical applications in prostate cancer and in other malignancies. In addition, the rationale, preclinical, and early clinical results targeting VEGF seem promising as we await the completion of the intergroup phase III trial. Other targets, including PDGF, neurotrophins, and yet to be tested growth factors may require more difficult trial designs that enrich for patients with tumors that demonstrate overexpression or even activation of these pathways. Other more ubiquitous targets, such as endothelins, may require combinations with more biologically linked targets, such as other paracrine growth factor pathways in bone, to demonstrate more robust clinical benefit. Ultimately, a better understanding of molecular phenotypes underlying sensitivity to each of these novel targets and development of predictive markers for response are challenges facing clinical development of agents specifically targeted against them.

REFERENCES

1. Hynes, N. E. and H. A. Lane. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer* **5**, 341–354.
2. Yarden, Y. and M. X. Sliwkowski. (2001). Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* **2**, 127–137.
3. Holbro, T., et al. (2003). The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc. Natl. Acad. Sci. USA* **100**, 8933–8938.
4. Burgess, A. W., et al. (2003). An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Mol. Cell* **12**, 541–552.
5. Kim, H. G., et al. (1999). EGF receptor signaling in prostate morphogenesis and tumorigenesis. *Histol. Histopathol.* **14**, 1175–1182.
6. Elson, S. D., C. A. Browne, and G. D. Thorburn. (1984). Identification of epidermal growth factor-like activity in human male reproductive tissues and fluids. *J. Clin. Endocrinol. Metab* **58**, 589–594.
7. Hofer, D. R., et al. (1991). Autonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor alpha. *Cancer Res.* **51**, 2780–2785.
8. Wilding, G., et al. (1989). Role of transforming growth factor-alpha in human prostate cancer cell growth. *Prostate* **15**, 1–12.
9. Ching, K. Z., et al. (1993). Expression of mRNA for epidermal growth factor, transforming growth factor-alpha and their receptor in human prostate tissue and cell lines. *Mol. Cell Biochem.* **126**, 151–158.
10. Harper, M. E., et al. (1993). An immunocytochemical analysis of TGF alpha expression in benign and malignant prostatic tumors. *Prostate* **23**, 9–23.
11. Scher, H. I., et al. (1995). Changing pattern of expression of the epidermal growth factor receptor and transforming growth factor alpha in the progression of prostatic neoplasms. *Clin. Cancer Res.* **1**, 545–550.
12. Wakeling, A. E., et al. (2002). ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res.* **62**, 5749–5754.
13. Sirotnak, F. M., et al. (2002). Studies with CWR22 xenografts in nude mice suggest that ZD1839 may have a role in the treatment of both androgen-dependent and androgen-independent human prostate cancer. *Clin. Cancer Res.* **8**, 3870–3876.
14. Canil, C. M., et al. (2005). Randomized phase II study of two doses of gefitinib in hormone-refractory prostate cancer: a trial of the National Cancer Institute of Canada-Clinical Trials Group. *J. Clin. Oncol.* **23**, 455–460.
15. Slamon, D. J., et al. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177–182.

16. Vogel, C. L., et al. (2002). Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol.* **20**, 719–726.
17. Slamon, D. J., et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**, 783–792.
18. Craft, N., et al. (1999). A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat. Med.* **5**, 280–285.
19. Osman, I., et al. (2001). HER-2/neu (p185neu) protein expression in the natural or treated history of prostate cancer. *Clin. Cancer Res.* **7**, 2643–2647.
20. Signoretti, S., et al. (2000). Her-2-neu expression and progression toward androgen independence in human prostate cancer. *J. Natl. Cancer Inst.* **92**, 1918–1925.
21. Calvo, B. F., et al. (2003). Human epidermal receptor-2 expression in prostate cancer. *Clin. Cancer Res.* **9**, 1087–1097.
22. Reese, D. M., et al. (2001). HER2 protein expression and gene amplification in androgen-independent prostate cancer. *Am. J. Clin. Pathol.* **116**, 234–239.
23. Lara, P. N., Jr., et al. (2004). Trastuzumab plus docetaxel in HER-2/neu-positive prostate carcinoma: final results from the California Cancer Consortium Screening and Phase II Trial. *Cancer* **100**, 2125–2131.
24. Carducci, M. A., Padley, R. J., Bruel, J., Vogelzang, N. J., Jonnenberg, B. A., Daliani, D. D., et al. (2003) Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. *J. Clin. Oncol.* **21(4)**, 679–689.
25. Nelson, J. B., Nabulsi, A. A., Vogelzang, N. J., Bruel, J., Zonnenberg., B. A., Daliani, D. D., et al. (2003) Suppression of prostate cancer induced bone remodeling by the endothelin receptor A antagonist atrasentan. *J. Urol.* **169(3)**, 1143–1149.
26. Leung, H. Y., et al. (1997). A potential autocrine loop between heregulin-alpha and erbB-3 receptor in human prostatic adenocarcinoma. *Br. J. Urol.* **79**, 212–216.
27. Agus, D. B., et al. (2002). Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* **2**, 127–137.
28. Agus, D. B., et al. (2005). Phase I clinical study of pertuzumab, a novel HER dimerization inhibitor, in patients with advanced cancer. *J. Clin. Oncol.* **23**, 2534–2543.
29. Mellinghoff, I. K., C. Tran, and C. L. Sawyers. (2002). Growth inhibitory effects of the dual ErbB1/ErbB2 tyrosine kinase inhibitor PKI-166 on human prostate cancer xenografts. *Cancer Res.* **62**, 5254–5259.
30. Mellinghoff, I. K., et al. (2004). HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell* **6**, 517–527.
31. Rusnak, D. W., et al. (2001). The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol. Cancer Ther.* **1**, 85–94.
32. Gregory, C. W., et al. (2005). Heregulin-induced activation of HER2 and HER3 increases androgen receptor transactivation and CWR-R1 human recurrent prostate cancer cell growth. *Clin. Cancer Res.* **11**, 1704–1712.
33. Liu, Y., et al. (2005). Inhibition of HER-2/neu kinase impairs androgen receptor recruitment to the androgen responsive enhancer. *Cancer Res.* **65**, 3404–3409.
34. Hicklin, D. J. and L. M. Ellis. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J. Clin. Oncol.* **23**, 1011–1027.
35. Keck, P. J., et al. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246**, 1309–1312.
36. Leung, D. W., et al. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**, 1306–1309.
37. Ferrara, N. and T. Davis-Smyth. (1997). The biology of vascular endothelial growth factor. *Endocr. Rev.* **18**, 4–25.
38. Gale, N. W. and G. D. Yancopoulos. (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev.* **13**, 1055–1066.
39. Ziegler, B. L., et al. (1999). KDR receptor: a key marker defining hematopoietic stem cells. *Science* **285**, 1553–1358.
40. Brown, L. F., et al. (1995). Vascular permeability factor (vascular endothelial growth factor) is strongly expressed in the normal male genital tract and is present in substantial quantities in semen. *J. Urol.* **154(Pt 1)**, 576–579.
41. Franck-Lissbrant, I., et al. (1998). Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. *Endocrinology* **139**, 451–456.
42. Shabisgh, A., et al. (1999). Early effects of castration on the vascular system of the rat ventral prostate gland. *Endocrinology* **140**, 1920–1926.
43. Benjamin, L. E., et al. (1999). Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J. Clin. Invest* **103**, 159–165.
44. Borre, M., et al. (1998). Microvessel density predicts survival in prostate cancer patients subjected to watchful waiting. *Br. J. Cancer* **78**, 940–944.
45. Lissbrant, I. F., et al. (1997). Vascular density is a predictor of cancer-specific survival in prostatic carcinoma. *Prostate* **33**, 38–45.

46. Silberman, M. A., et al. (1997). Tumor angiogenesis correlates with progression after radical prostatectomy but not with pathologic stage in Gleason sum 5 to 7 adenocarcinoma of the prostate. *Cancer* **79**, 772–779.
47. Weidner, N., et al. (1993). Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am. J. Pathol.* **143**, 401–409.
48. Borre, M., B. Nerstrom, and J. Overgaard. (2000). Association between immunohistochemical expression of vascular endothelial growth factor (VEGF), VEGF-expressing neuroendocrine-differentiated tumor cells, and outcome in prostate cancer patients subjected to watchful waiting. *Clin. Cancer Res.* **6**, 1882–1890.
49. George, D. J., et al. (2001). Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. *Clin. Cancer Res.* **7**, 1932–1936.
50. Bok, R. A., et al. (2001). Vascular endothelial growth factor and basic fibroblast growth factor urine levels as predictors of outcome in hormone-refractory prostate cancer patients: a cancer and leukemia group B study. *Cancer Res.* **61**, 2533–2536.
51. Borgstrom, P., et al. (1998). Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. *Prostate* **35**, 1–10.
52. Melnyk, O., et al. (1999). Neutralizing anti-vascular endothelial growth factor antibody inhibits further growth of established prostate cancer and metastases in a pre-clinical model. *J. Urol.* **161**, 960–963.
53. Nicholson, B., et al. (2004). Combination antiangiogenic and androgen deprivation therapy for prostate cancer: a promising therapeutic approach. *Clin. Cancer Res.* **10**, 8728–8734.
54. Wood, J. M., et al. (2000). PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res.* **60**, 2178–2189.
55. George, D. J. Phase I study of the novel, oral angiogenesis inhibitor PTK787/ZK 222584 (PTK/ZK): Evaluating the pharmacokinetic effect of a high-fat meal in patients with hormone-refractory prostate cancer (HRPC). Oh, W., Gilligan, T., Masson, E., Souppart, C., Wang, Y., Ho, Y., Lebwohl, D., Laurent, D., and Kantoff, P. *J. Clin. Oncol.* **22(14S)**, 4689. 7-15-0004.
56. Kim, K. J., et al. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **362**, 841–844.
57. Mordenti, J., et al. (1999). Efficacy and concentration-response of murine anti-VEGF monoclonal antibody in tumor-bearing mice and extrapolation to humans. *Toxicol. Pathol.* **27**, 14–21.
58. Warren, R. S., et al. (1995). Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J. Clin. Invest* **95**, 1789–1797.
59. Reese, D. (2005). A phase II trial of humanized monoclonal anti-vascular endothelial growth factor antibody (rhuMAB VEGF) in hormone refractory prostate cancer. Frohlich, M., Bok, R., Corry, M., Novonty, W., Holmgren, E., and Small, E. *Proc. Am. Soc. Clin. Oncol.* **18**, 1999.
60. Picus, J. (2005). The use of bevacizumab (B) with docetaxel (D) and estramustine (E) in hormone refractory prostate cancer (HRPC): Initial results of CALGB 90006. Halabi, S., Rini, B., Vogelzang, N., Whang, Y. E., Kaplan, E., Kelly, W., and Small, E. *Proc. Am. Soc. Clin. Oncol.* **22**, 1578.
61. Fudge, K., C. Y. Wang, and M. E. Stearns. (1994). Immunohistochemistry analysis of platelet-derived growth factor A and B chains and platelet-derived growth factor alpha and beta receptor expression in benign prostatic hyperplasias and Gleason-graded human prostate adenocarcinomas. *Mod. Pathol.* **7**, 549–554.
62. Kim, H. E., et al. (1997). Platelet-derived growth factor (PDGF)-signaling mediates radiation-induced apoptosis in human prostate cancer cells with loss of p53 function. *Int. J. Radiat. Oncol Biol. Phys.* **39**, 731–736.
63. Ko, Y. J., et al. (2001). A multi-institutional phase ii study of SU101, a platelet-derived growth factor receptor inhibitor, for patients with hormone-refractory prostate cancer. *Clin. Cancer Res.* **7**, 800–805.
64. Sitaras, N. M., et al. (1988). Constitutive production of platelet-derived growth factor-like proteins by human prostate carcinoma cell lines. *Cancer Res.* **48**, 1930–1935.
65. Vlahos, C. J., et al. (1993). Platelet-derived growth factor induces proliferation of hyperplastic human prostatic stromal cells. *J. Cell Biochem.* 52.4 (): 404–413.
66. Claesson-Welsh, L. (1994). Platelet-derived growth factor receptor signals. *J. Biol. Chem.* **269**, 32,023–32,026.
67. Crosby, J. R., et al. (1998). Chimaeric analysis reveals role of PDGF receptors in all muscle lineages. *Nat. Genet.* **18**, 385–388.
68. Lindahl, P., et al. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277**, 242–245.
69. Singh, D., et al. (2002). Gene expression correlates of clinical prostate cancer behavior. *Cancer Cell* **1**, 203–209.
70. George, D. J. (2002). A pilot study of STI571 in patients prior to prostatectomy. Febbo, P., Weinstein, M., Oh, W., Gilligan, T., Tempany, C., Badawi, R., Van Den Abeele, A., Richie, J., Kantoff, P., and Bubley, G. 8.
71. Febbo, P. (2005). The application of oligonucleotide microarrays to assess the biological effects of neoadjuvant imatinib mesylate treatment for localized prostate cancer. Thorner, A., Rubin, M. A., Loda, M., Kantoff, P., Oh, W., Golub, T. R., and George, D. *J. Cancer Res.* in press.

72. Mathew, P., et al. (2004). Platelet-derived growth factor receptor inhibitor imatinib mesylate and docetaxel: a modular phase I trial in androgen-independent prostate cancer. *J. Clin. Oncol.* **22**, 3323–3329.
73. Rao, K., et al. (2005). A phase II trial of imatinib mesylate in patients with prostate specific antigen progression after local therapy for prostate cancer. *Prostate* **62**, 115–122.
74. Hofer, M. D., et al. (2004). Expression of the platelet-derived growth factor receptor in prostate cancer and treatment implications with tyrosine kinase inhibitors. *Neoplasia* **6**, 503–512.
75. Guise, T. A. and K. S. Mohammad. (2004). Endothelins in bone cancer metastases. *Cancer Treat. Res.* **118**, 197–212.
76. Nelson, J., et al. (2003). The endothelin axis: emerging role in cancer. *Nat. Rev. Cancer* **3**, 110–116.
77. Nelson, J. B., et al. (1995). Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat. Med.* **1**, 944–949.
78. Nelson, J. B., et al. (1996). Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res.* **56**, 663–668.
79. Norman, P. (2002). Atrasentan Abbott. *Curr. Opin. Investig. Drugs* **3**, 1240–1248.
80. Jones, J. I. and D. R. Clemmons. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev.* **16**, 3–34.
81. Chan, J. M., et al. (1998). Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* **279**, 563–566.
82. Wolk, A., et al. (1998). Insulin-like growth factor 1 and prostate cancer risk: a population-based, case-control study. *J. Natl. Cancer Inst.* **90**, 911–915.
83. Yu, H., et al. (1999). Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. *J. Natl. Cancer Inst.* **91**, 151–156.
84. Ornitz, D. M. and N. Itoh. (2001). Fibroblast growth factors. *Genome Biol.* **2**, REVIEWS3005.
85. Powers, C. J., S. W. McLeskey, and A. Wellstein. (2000). Fibroblast growth factors, their receptors and signaling. *Endocr. Relat. Cancer* **7**, 165–197.
86. Cunha, G. R., et al. (1987). The endocrinology and developmental biology of the prostate. *Endocr. Rev.* **8**, 338–362.
87. Dorkin, T. J., et al. (1999). FGF8 over-expression in prostate cancer is associated with decreased patient survival and persists in androgen independent disease. *Oncogene* **18**, 2755–2761.
88. Leung, H. Y., et al. (1996). Over-expression of fibroblast growth factor-8 in human prostate cancer. *Oncogene* **12**, 1833–1835.
89. Ropiquet, F., et al. (2000). Increased expression of fibroblast growth factor 6 in human prostatic intraepithelial neoplasia and prostate cancer. *Cancer Res.* **60**, 4245–4250.
90. Yan, G., et al. (1992). Heparin-binding keratinocyte growth factor is a candidate stromal-to-epithelial-cell andromedin. *Mol. Endocrinol.* **6**, 2123–2128.
91. Finch, P. W., et al. (1989). Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* **245**, 752–755.
92. Yan, G., et al. (1993). Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptor genes in prostate epithelial cells accompany stromal independence and malignancy. *Mol. Cell Biol.* **13**, 4513–4522.
93. Carstens, R. P., et al. (1997). Alternative splicing of fibroblast growth factor receptor 2 (FGF-R2) in human prostate cancer. *Oncogene* **15**, 3059–3065.
94. George, D. J., et al. (1999). Sustained in vivo regression of Dunning H rat prostate cancers treated with combinations of androgen ablation and Trk tyrosine kinase inhibitors, CEP-751 (KT-6587) or CEP-701 (KT-5555). *Cancer Res* **59**, 2395–2401.
95. Weeraratna, A. T., et al. (2000). Rational basis for Trk inhibition therapy for prostate cancer. *Prostate* **45**, 140–148.
96. Marshall, J. L., et al. (2005). Phase I trial of orally administered CEP-701, a novel neurotrophin receptor-linked tyrosine kinase inhibitor. *Invest New Drugs* **23**, 31–37.
97. George, D. J., et al. (2005). The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: results from cancer and leukemia group B 9480. *Clin. Cancer Res.* **11**, 1815–1820.
98. Nakashima, J., et al. (2000). Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin. Cancer Res* **6**, 2702–2706.
99. Fornaro, M., T. Manes, and L. R. Languino. (2001). Integrins and prostate cancer metastases. *Cancer Metastasis Rev.* **20**, 321–331.
100. Slack-Davis, J. K. and J. T. Parsons. (2004). Emerging views of integrin signaling: implications for prostate cancer. *J. Cell Biochem.* **91**, 41–46.
101. McNeel, D. G., et al. (2005). Phase I trial of a monoclonal antibody specific for alphavbeta3 integrin (MEDI-522) in patients with advanced malignancies, including an assessment of effect on tumor perfusion. *Clin. Cancer Res.* **11**, 7851–7860.

26

Novel and Efficient Translational Clinical Trial Designs in Advanced Prostate Cancer

André Rogatko and Mourad Tighiouart

Summary

We propose a new paradigm for the clinical evaluation of new cancer therapies. It entails adjusting the search for the optimal dose on the basis of measurable patient characteristics that may be predictive of adverse responses to treatment, and extending this search beyond phase I and into phases II and III. We provide examples of how the fine-tuning of dose may involve use of patient-specific attributes to obtain a personalized treatment regimen; and how novel methods for phase I design can be used to update the working dose for the conduct of phase II and III cancer clinical trials.

Key Words: Bayesian design; dose escalation; escalation with overdose control; EWOC; overdose control; phase I design.

1. INTRODUCTION

Clinical trials of new anticancer therapies are widely used, critically important tools in the search for more effective cancer treatments. Cancer trials typically proceed through several distinct phases. The major objective in phase I trials is to identify a working dose for subsequent studies, whereas the major end point in phase II and III trials is treatment efficacy. Ideally, from a therapeutic perspective, clinical trials should be designed to maximize the number of patients receiving an optimal dose. Consequently, more patients would be treated with therapeutic doses of promising new agents, and fewer patients would have to suffer the deleterious effects of toxic doses.

With cytotoxic agents, it is a long-accepted assumption of cancer chemotherapy that toxicity is a prerequisite for optimal antitumor activity and that efficacy increases with dose. It could be argued that the paradigm used for cytotoxic drugs will not apply for newer molecularly targeted therapies. It has been assumed that inhibition of protein function, rather than toxicity, will determine the optimal dose of such therapies. However, early experience with EGF and VEGF receptor antagonists has shown that target inhibition in tumor tissue is difficult to quantify. Inhibition of the target is usually associated with toxicity, which influences the selection of an optimal dose. Therefore, it is unlikely that future therapies will be completely free of toxicity constraints. Furthermore, targeted agents, such as angiogenesis inhibitors or growth factor receptor antagonists are likely to be used in combination with cytotoxic agents. For these reasons, the paradigm used for cytotoxic drugs should be applicable and relevant to molecularly targeted therapies.

Phase I studies assume that dose is the most significant determinant of toxicity. Our analysis of multiple phase I and early phase II trials revealed that dose is not always the most significant predictor of toxicity *(1)*. Even with conventional patient selection criteria that included the requirement for normal or near-normal hepatic and renal function, patient characteristics had greater predictive value

From: *Contemporary Cancer Research: Prostate Cancer: Biology, Genetics, and the New Therapeutics, Second Edition*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons © Humana Press Inc., Totowa, NJ

than dose for the toxicity for several agents. Thus, the current eligibility criteria for most phase I and II trials do not provide populations that are uniform enough to conclude that differences in toxic response are primarily dose related.

Identification of the optimal dose is usually restricted to the phase I setting, although the number and type of patients evaluated is very limited. Typically, the dose selected for phase II may be based on the toxicity experiences of a few heavily pretreated patients with limited life expectancy. The assumption that the dose selected in such a patient population will be optimal for other populations may well be incorrect. Furthermore, as experience accumulates with a new therapy, it often becomes apparent that certain groups of patients tolerate greater or lesser doses because of differences in age, sex, organ function, and genetic profile that may affect clearance and metabolism. An example is the topoisomerase inhibitor, irinotecan, which, at recommended doses often causes unacceptable and unpredictable gastrointestinal and hematological toxicity, a risk that increases with hepatic dysfunction *(2,3)*. Another example is capecitabine, a widely used fluoropyrimidine that requires dose reduction for elderly patients and those with mild-to-moderate renal insufficiency *(4)*. The recommended starting dose of docetaxel, a microtubule inhibitor used in the treatment of lung, breast, ovarian, and other cancers, was reduced from the approved recommended dose based on subsequent clinical experience in larger and older populations *(5,6)*. These observations raise concerns for the dosing recommendations based on limited patient cohorts typically treated in phase I and II trials. Consequently, the limited information available from phase I trials is frequently insufficient for an accurate determination of the phase II dose, which should accommodate patient heterogeneity in susceptibility to adverse effects of treatment.

According to the current paradigm for the clinical evaluation of new cancer therapies, (A) the dose of a therapeutic agent is not adjusted to accommodate individual patient differences, and (B) the identification of the working dose of new cancer therapies is mainly restricted to phase I trials. We propose that (A') the dose should be fine-tuned using patient-specific attributes, and (B') the search for the optimal dose should be extended beyond phase I and into phases II and III. We provide examples of how phase I design methods can be used to update the working dose for phases II and III and how fine-tuning the dose may involve using patient-specific attributes to obtain a personalized treatment regimen. We present these examples to motivate the development of new and improved methods.

2. CANCER PHASE I TRIAL

The primary objective of a phase I clinical trial is to determine the dose of a new drug or combination of drugs for subsequent clinical evaluation of efficacy. The dose sought is typically referred to as the maximum tolerated dose (MTD) and its definition depends on the treatment under investigation, the severity and reversibility of its side effects, and on clinical attributes of the target patient population. Because it is generally assumed that toxicity is a prerequisite for optimal antitumor activity, the MTD of a cytotoxic agent typically corresponds to the highest dose associated with a tolerable level of toxicity. More precisely, the MTD is defined as the dose expected to produce some degree of medically unacceptable, dose-limiting toxicity (DLT) in a specified proportion θ of patients. Thus, if a population of patients is treated at the MTD, a proportion θ of them will manifest DLT. The value chosen for the target probability θ would depend on the nature and consequences of the DLT; it would be set relatively high when the DLT is a transient, correctable, or nonfatal condition, and low when it is lethal or life threatening *(7)*.

Table 1 summarizes the attributes that an optimal dose-finding procedure should have to achieve the goals (A') and (B'). Although none of these characteristics are possessed by the usual modified Fibonacci phase I design, each can be addressed within a Bayesian framework *(8)*.

In the next section, we outline a Bayesian dose-escalation scheme permitting precise determination of the therapeutic working dose while directly controlling the likelihood of an overdose. The

Table 1
Optimal Dose-Finding Procedure Attributes[a]

Attribute	Description
• Freedom to choose any target probability θ	Because cancer phase I and II patient populations typically differ with respect to treatment tolerance, a different target probability θ might be chosen in each case. Consequently, a method for fine-tuning the working dose should allow any value of θ to be targeted and permit its change during phases II and III
• Patient-specific dosing	The method should allow the dose level to be adjusted according to patient characteristics associated with susceptibility to treatment.
• Assess the precision of the MTD estimate	To decide if and when to adjust the phase II/III dose, the method should provide an estimate of how precisely the MTD has been estimated
• Use all available information	The method used to adjust the dose should be fully adaptive. That is, it should be able to exploit all of the information accumulated during both the current and previous phases of clinical evaluation
• Assess patient risk	Both the investigator and patient might benefit if updated assessments of the risk of toxicity were available during the trial

[a]Attributes that an optimal dose-finding procedure should possess to achieve the goals: (A') the dose should be fine-tuned using patient-specific attributes, and (B') the search for the optimal dose should be extended beyond phase I and into phases II and III. MTD, maximum tolerated dose.

method, known as EWOC (escalation with overdose control), has been used to design phase I clinical trials at the Fox Chase Cancer Center and at the Sylvester Comprehensive Cancer Center, University of Miami School of Medicine. Zacks et al. *(9)* discuss statistical properties of the method, and a comparison of EWOC with alternative phase I design methods is given in Babb et al. *(7)*. Babb and Rogatko *(10)* provide a summary of Bayesian phase I design methods and Tighiouart et al. *(11)* studied the performance of EWOC under a rich class of previous distributions for θ. EWOC will be the basis for a more general method to be used beyond phase I trials. A computer program implementation of the EWOC method for Windows environment is available free of charge. Version 2 is a user-friendly, dialog-based, stand-alone application and the self-extracting file can be downloaded from the web site, http://www.sph.emory.edu/BRI-WCI/software.html.

3. THE EWOC METHOD

EWOC was the first dose-finding procedure to directly incorporate the ethical constraint of minimizing the chance of treating patients at unacceptably high doses. Its defining property is that the expected proportion of patients treated at doses above the MTD is equal to a specified value α, the feasibility bound. This value is selected by the clinician and reflects their level of concern regarding overdosing. Zacks et al. *(9)* showed that among designs with this defining property, EWOC minimizes the average amount by which patients are underdosed. This means that EWOC approaches the MTD as rapidly as possible, while keeping the expected proportion of patients overdosed less than the value α. Zacks et al. *(9)* also showed that, as a trial progresses, the dose sequence defined by EWOC approaches the MTD (i.e., the sequence of recommended doses converges in probability to the MTD). Eventually, all patients beyond a certain time would be treated at doses sufficiently close to the MTD.

The value selected for the feasibility bound determines the rate of change in dose level between successive patients. Low values result in a cautious escalation scheme with relatively small increments in dose, whereas high values produce a more aggressive escalation. In actual application, the

value of the feasibility bound is initially set at a small value ($\alpha = 0.25$, for instance) and then allowed to increase in a predetermined manner in the absence of unacceptable toxicity. The rationale behind this approach is that uncertainty regarding the MTD is highest at the onset of the trial and a small value of α affords protection against the possibility of administering dose levels much greater than the MTD. As the trial progresses, uncertainty regarding the MTD declines and the likelihood of selecting a dose level significantly above the MTD becomes smaller. Consequently, a relatively high probability of exceeding the MTD ($\alpha = 0.5$, for instance) can be tolerated near the conclusion of the trial because the magnitude by which any dose exceeds the MTD is expected to be small.

4. PERSONALIZING THE PHASE I DOSE—EWOC WITH COVARIATES

A key assumption implied by the definition of the phase I target dose (MTD) is that every subgroup of the patient population has the same MTD. That is, it is assumed that the patient population is homogeneous in terms of treatment tolerance and every patient should be treated at the same dose. As a result, no allowance is made for individual patient differences in susceptibility to treatment *(12)*. Recently, however, improvements in our understanding of the pharmacokinetics and the pharmacogenetics of drug metabolism have led to the development of new treatment paradigms that accommodate individual patient needs *(13,14)*. For example, the observation that impaired renal function can result in reduced clearance of carboplatin, led to the development of dosing formulas based on renal function that permit careful control over individual patient exposure *(15)*. Additionally, the National Cancer Institute (NCI) accounts for the contribution of previous therapy by establishing separate phase II doses for heavily pretreated and minimally pretreated patients. For these reasons, the EWOC method was extended to permit the incorporation of patient-specific characteristics into the statistical design of phase I clinical trials. The extension of EWOC to include covariates will be illustrated in the context of a Food and Drug Administration (FDA)-approved phase I study of PNU-214565 (PNU) involving patients with advanced adenocarcinomas of gastrointestinal origin *(10)*. PNU is a murine Fab fragment of the monoclonal antibody 5T4 fused to a mutated superantigen staphylococcal enterotoxin A (SEA). Preclinical testing demonstrated that the action of PNU is moderated by the neutralizing capacity of antibodies to anti-SEA. Consequently, dose levels were adjusted during the trial according to each patient's pretreatment plasma concentration of antibodies to SEA.

It was assumed that the probability of DLT is an increasing function of dose (for fixed anti-SEA) and, because anti-SEA has a neutralizing effect on PNU, a decreasing function of anti-SEA (for fixed dose). Because the probability of DLT depends on both the PNU dose level and the anti-SEA concentration, the dose-toxicity model was chosen to be a logistic function of pretreatment anti-SEA and PNU dose level. The MTD was defined as a function of the anti-SEA concentration. Specifically, the MTD was defined, for patients with pretreatment anti-SEA concentration equal to a specific value a, as the dose level of PNU (ng/kg) that produces DLT in a proportion $\theta = 0.1$ of the patients with anti-SEA equal to a at baseline. The small value chosen for θ reflects the severity of the treatment-induced toxicities (e.g., myelosuppression) observed in previous studies. A detailed clinical report on the PNU trial is given by Cheng et al *(16)*.

The amount of information gained during the trial was evidenced by the magnitude of change in the recommended dose levels. At the onset, the recommended PNU dose started as 25 ng/kg for patients with anti-SEA values ranging from 1 to 1800 pmol/mL. By trial termination, after 78 patients had been treated, the recommended PNU dose changed to 43.59 ng/kg for patients with anti-SEA value of 1 pmol/mL, and to 1088.25 ng/kg for patients with anti-SEA value of 1500 pmol/mL. Source code in Visual Fortran 90 used to implement EWOC with covariates as described in Babb et al. *(10)* and Cheng et al. *(16)* is available from the authors. Figure 1 shows the PNU doses recommended by EWOC as a function of anti-SEA concentration, both at the onset of the trial and after 78 patients had been treated. The large impact of the covariate on the dosing scheme is indicated by the slope of the upper curve ($n = 78$), and the magnitude of information accumulated from the trial is evidenced by the difference between the two curves.

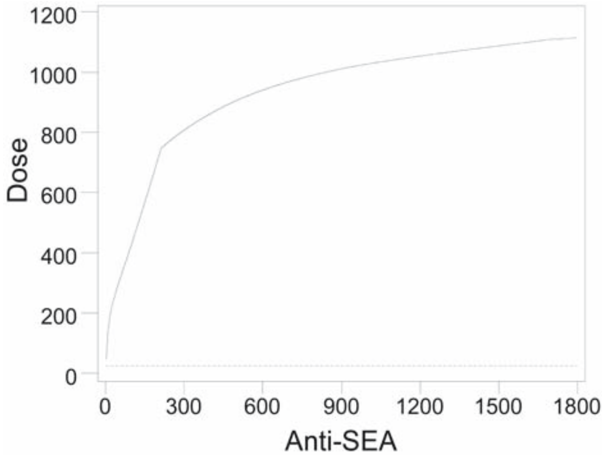


Fig. 1. PNU-214565 doses recommended by Escalation With Overdose Control (EWOC) (ng/kg) as a function of pre-treatment anti-staphylococcal enterotoxin A (SEA) concentration (pmol/mL) at the beginning (*dotted line*) and end of the trial (*solid line*).

5. IS THE PHASE I MTD THE APPROPRIATE DOSE FOR PHASES II AND III?

Although the major end point in phase II trials is efficacy, the search for the optimal dose is usually restricted to the phase I setting. In this case, the phase II dose determination may be based on the data accumulated from a patient population with very different characteristics than the target phase II population. The implicit assumption that the dose selected in one patient population will be optimal for other populations may be incorrect. In a phase II trial, the frequent occurrence of toxicity leads generally to dose reduction. In contrast, dose increase because of lack of toxicity is much less common, because such treatments are generally considered "well-tolerated." Because maximizing dose intensity is still regarded as an important condition to achieve an optimal therapeutic effect, failure to increase the dose in the absence of toxicity may result in patients being treated at subtherapeutic dose levels.

EWOC was applied retrospectively to dose-toxicity data from two trials completed at the Fox Chase Cancer Center. In the phase I study *(17)*, 26 patients with a wide variety of malignancies and varying exposure to cytotoxic therapy and radiation were treated with paclitaxel and estramustine. Based on toxicity observed in the phase I population, the phase II dose of paclitaxel was chosen to be 120 mg/m^2. Applying EWOC analysis to this data set yields $\theta = 0.2$ for a dose of 120 mg/m^2, indicating that approximately 1 in 5 patients would be expected to have DLT at the phase II dose of paclitaxel. In the phase II trial *(18)*, 34 patients with hormone refractory prostate cancer and no previous chemotherapy received the recommended phase II dose, using the same treatment regimen and same definition of DLT as for the phase I trial. Only 2 of the 34 patients in the phase II trial experienced first cycle DLT, and only 3 other patients required dose reduction of paclitaxel over subsequent cycles. Because the incidence of DLT was lower in the phase II trial, EWOC analysis results in a lower value of 2 for this population. Figure 2 shows, for each trial, the recommended EWOC dose of paclitaxel for selected values of the target probability of DLT (θ) and dose for the phase I and phase II populations. The recommended EWOC dose of paclitaxel for any selected value of the target probability of DLT (θ) is higher for the phase II population. For this analysis, the feasibility-bound α was set to 0.5, implying that both underdosing and overdosing are of equal concern. If the object of the phase II trial had been to administer a paclitaxel dose to achieve $\theta = 0.2$, then more aggressive dosing would have been possible. Although we analyzed this example retrospectively,

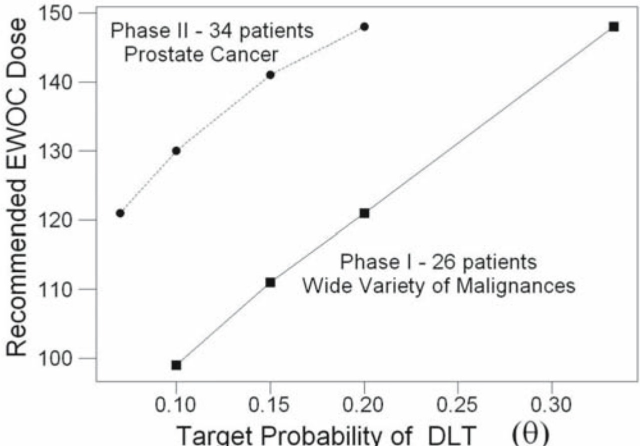


Fig. 2. Recommended Escalation With Overdose Control (EWOC) doses of paclitaxel (mg/m^2) for selected values of the target probability of dose-limiting toxicity (DLT) (θ) were calculated using dose-toxicity data from phase I and II studies of paclitaxel in combination with 600 mg/m^2 estramustine, with $\alpha = 0.5$.

Table 2
Phase II Trial Design Allowing Dose Modifications Based on Number and Type of Toxicities Observed [a]

Number of DLTs in 14 patients	Probability when $\theta = 0.2$	Action
0	0.044	Increase radiation from 45 to 60 Gy
1, 2, 3, or 4	0.826	None
5 or 6	0.118	Decrease taxol from 50 to 40 mg/m^2
7 or more	0.012	Terminate trial

[a] Rule used for dose modifications in a phase II trial of a combination of radiation and taxol. The design allows the dose to be adjusted according to an assessment of whether a desired level of toxicity is achieved. DLT, dose-limiting toxicity.

one could envision a similar analysis conducted during the phase II trial after accrual of an initial cohort of 10 to 12 patients to adjust the dose using relationships similar to those in Fig. 2.

One sees that different doses are required in the two patient populations to obtain the same targeted probability of DLT. This example shows how EWOC can be used to aid the clinician in selecting a working dose for a cytotoxic treatment. Once the clinician decides on a target probability of DLT, θ, the phase II (or III) dose can be determined.

A phase II (or III) trial can be designed to permit dose modifications based on the number and type of toxicities observed during the trial. As a specific example, the following is the design of a phase II trial involving a combination of radiation and taxol. The design allows the dose to be adjusted according to an assessment of whether a desired level of toxicity is achieved. A portion of the protocol is given below and summarized in Table 2.

> The adequacy of the selected dose will be assessed after 14 patients have been treated. We target the dose such that the probability of DLT, θ, is 0.2. If no DLT's are observed among the first 14 patients (assuming that $\theta = 0.2$, the probability of this outcome is 0.044), the radiation will be increased from 45 Gy to 60 Gy. If one, two, three or four DLT's are observed the treatment will remain unchanged. If five or six DLT's are observed, the taxol dose will be decreased from 50

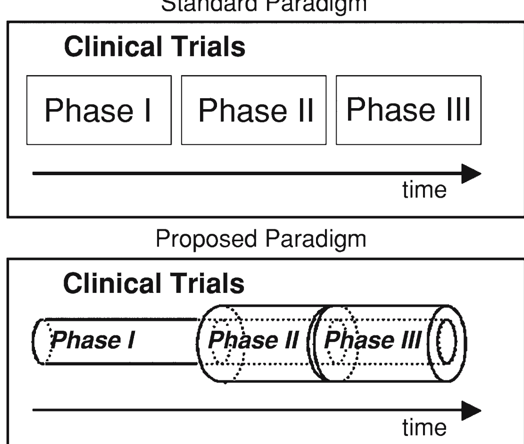


Fig. 3. The never-ending phase I trial. The standard paradigm of clinical evaluation of new cancer therapies restricts the determination of dose to the initial phase of the process (top). The proposed paradigm entails extending the search for the optimal dose beyond phase I and into phases II and III (bottom).

mg/m^2 to 40 mg/m^2 (assuming that $\theta = 0.2$, the probability of this outcome is 0.118). If seven or more DLT's are observed the trial will be terminated. When $\theta = 0.2$ the probability of observing 7 or more DLT's and incorrectly terminating the trial is 0.012.

The same design could be implemented to adjust the dose in the phase III setting. The main distinction between phase II and III trials is that larger samples are typically used in phase III, thereby permitting further refinement of the working dose and better adjustment to individual patient needs. This example should be interpreted as an enticement for the development of new methods to implement item (B') of the proposed new paradigm, that is, to extend the search for the optimal dose beyond phase I and into phases II and III.

6. DOSE-FINDING BEYOND PHASE I TRIALS

The standard paradigm of clinical evaluation of new cancer therapies restricts the dose determination to the initial phase of the process (Fig. 3, top). Progress in DNA-array technology and pharmacogenetics undeniably argues against the concept of "one dose fits all." At the same time, it would be unreasonable to design phase I trials with sufficient power to distinguish the important patient-specific characteristics for a given therapy.

One solution is to continue with the quest for determining the best dose throughout phases II and III (Fig. 3, bottom). Hence, clinical trials might progress as follows. First, a phase I trial is conducted to characterize the toxicity profile of the treatment and determine a starting dose for phase II investigation. Subsequently, the phase II and III trials can be designed in stages with the data from each stage used to determine if and what adjustment of the dose is needed. Dose modification would continue until either a specific number of patients have been treated or the dose has converged to the MTD according to some criterion (such as the posterior variance of the estimated MTD).

In the previous sections, it was shown that methodologies are available to improve the design and analysis of cancer clinical trials. We highlighted the importance of targeting θ, the proportion of patients treated at the MTD expected to manifest DLT, during all phases of treatment evaluation. We also presented methods that permit the incorporation of personal information, thereby allowing dose

levels to be tailored to the individual patient according to his or her attributes. It is important to note that, for any phase of the evaluation process, the working dose should be adjusted on the basis of accumulated toxicity and efficacy information. Therefore, each patient at each stage will be provided with the best dose possible, more patients will be treated with therapeutic doses of a promising new agent, and fewer patients will be overdosed and suffer from toxic effects.

There has been a substantial effort from the cancer research and treatment community to translate basic science and laboratory findings faster into clinical trials in an attempt to accelerate the improvement in cancer patients' survival and quality of life. Research biostatisticians dedicated to the discovery and improvement of clinical trial design play a fundamental role in the process of drug discovery and treatment. As in any science, research biostatisticians generate new methods that are published in specialized journals using a language typical to the profession. Most of these new methods are rarely used, although they may offer advantages over the standard methods *(19,20)*. Despite a compelling rationale for their use, EWOC and other Bayesian methods are not used commonly in phase I trials. Some of the reasons include:

1. Lack of awareness and/or understanding of Bayesian methods among clinical investigators and industry sponsors involved in drug development.
2. Fear that newer methods are complex, may cause delay or loss of information, rather than increase efficiency and information gained. This is partly a consequence of 1.
3. Change from the status quo. If nothing else, the "modified Fibonacci" 3+3 has conformity among its adherents.

The "modified Fibonacci" 3+3 dose escalation design remains a favored design for phase I trials despite the important limitations described above and by others *(21–23)*. The consequence of using ineffective methods, such as the "modified Fibonacci" 3+3 dose escalation in clinical practice is that more patients are treated with doses outside the therapeutic window *(24,25)*.

It is our responsibility to not only develop new and better designs, but also to shepherd new approaches into clinical practice. This will require effective communication with clinical colleagues, and active representation on scientific review boards and clinical research committees. The main goals of this paper are to encourage additional work in the realm of cancer clinical trial design and to urge biostatisticians to actively participate in translating new methods into the real world of clinical trials.

REFERENCES

1. Rogatko, A., Babb, J. S., Wang, H., Slifker, M. J., and Hudes, G. R. (2004). Patient characteristics compete with dose as predictors of acute treatment toxicity in early phase clinical trials. *Clin. Cancer Res.* **10**, 4645–4651.
2. Raymond, E., Boige, V., Faivre, S., et al. (2002). Dosage adjustment and pharmacokinetic profile of irinotecan in cancer patients with hepatic dysfunction. *J. Clin. Oncol.* **20**, 4303–4312.
3. Meyerhardt, J. A., Kwok, A., Ratain, M. J., McGovren, J. P., and Fuchs, C. S. (2004). Relationship of baseline serum bilirubin to efficacy and toxicity of single-agent irinotecan in patients with metastatic colorectal cancer. *J. Clin. Oncol.* **22**, 1439–1446.
4. Poole, C., Gardiner, J., Twelves, C., et al. (2002). Effect of renal impairment on the pharmacokinetics and tolerability of capecitabine (Xeloda) in cancer patients. *Cancer Chemother. Pharmacol.* **49**, 225–234.
5. Valero, V., Holmes, F. A., Walters, R. S., et al. (1995). Phase II trial of docetaxel: a new, highly effective antineoplastic agent in the management of patients with anthracycline-resistant metastatic breast cancer. *J. Clin. Oncol.* **13**, 2886–2894.
6. Salminen, E., Bergman, M., Huhtala, S., and Ekholm, E. (1999). Docetaxel: standard recommended dose of 100 mg/m(2) is effective but not feasible for some metastatic breast cancer patients heavily pretreated with chemotherapy-A phase II single-center study. *J. Clin. Oncol.* **17**, 1127.
7. Babb, J., Rogatko, A., and Zacks, S. (1998). Cancer Phase I clinical Trials: efficient dose escalation with overdose control. *Stat. Med.* **17**, 1103–1120.
8. Thall, P. F., Sung, H. G., and Choudhury, A. (2001). Dose-finding based on feasibility and toxicity in T-cell infusion trials. *Biometrics* **57**, 914–921.
9. Zacks, S., Rogatko, A., and Babb, J. (1998). Optimal Bayesian-feasibile dose escalation for cancer phase I trials. *Stat. Prob. Ltrs.* **38**, 215–220.

10. Babb, J. S. and Rogatko, A. (2001). Patient specific dosing in a cancer phase I clinical trial. *Stat. Med.* **20**, 2079–2090.
11. Tighiouart, M., Rogatko, A., and Babb, J. (2005). Flexible Bayesian methods for phase I clinical trials: Dose Escalation With Overdose Control. *Stat. Med.* **24**, 2183–2196.
12. Dillman, R. O. and Koziol, J. A. (1992). Phase I cancer trials: limitations and implications. *Mol. Biother.* **4**, 117–121.
13. Piantadosi, S. and Liu, G. (1996). Improved designs for dose escalation studies using pharmacokinetic measurements. *Stat. Med.* **15**, 1605–1618.
14. Wijesinha, M. C. and Piantadosi, S. (1995). Dose-response models with covariates. *Biometrics* **51**, 977–987.
15. Newell, D. R. (1994). Pharmacologically based phase I trials in cancer chemotherapy. *Hematol. Oncol. Clin. North Am.* **8**, 257–275.
16. Cheng, J. D., Babb, J. S., Langer, C., et al. (2004). Individualized patient dosing in phase I clinical trials: The role of EWOC in PNU-214936. *J. Clin. Oncol.* **22**, 602–609.
17. Hudes, G. R., Obasaju, C., Chapman, A., Gallo, J., McAleer, C., and Greenberg, R. (1995). Phase I study of paclitaxel and estramustine: preliminary activity in hormone-refractory prostate cancer. *Semin. Oncol.* **22**, 6–11.
18. Hudes, G. R., Nathan, F., Khater, C., et al. (1997). Phase II trial of 96-hour paclitaxel plus oral estramustine phosphate in metastatic hormone-refractory prostate cancer. *J. Clin. Oncol.* **15**, 3156–3163.
19. Dent, S. F. and Eisenhauer, E. A. (1996). Phase I trial design: are new methodologies being put into practice? *Ann. Oncol.* **7**, 561–566.
20. Eisenhauer, E. A., O'Dwyer, P. J., Christian, M., and Humphrey, J. S. (2000). Phase I clinical trial design in cancer drug development. *J. Clin. Oncol.* **18**, 684–692.
21. Shen, L. Z. and O'Quigley, J. (1996). Consistency of continual reassessment method under model misspecification. *Biometrika* **83**, 395–405.
22. Ahn, C. (1998). An evaluation of phase I cancer clinical trial designs. *Stat. Med.* **17**, 1537–1549.
23. Storer, B. (2001). Choosing a phase I design. In: Handbook of Statistics in Clinical Oncology (Crowley, J., ed.). Marcel Dekker, New York, NY, pp. 73–91.
24. O'Quigley, J. and Chevret, S. (1991). Methods for dose finding studies in cancer clinical trials: a review and results of a Monte Carlo study. *Stat. Med.* **10**, 1647–1664.
25. Smith, T. L., Lee, J. J., Kantarjian, H. M., Legha, S. S., and Raber, M. N. (1996). Design and results of phase I cancer clinical trials: three-year experience at M.D. Anderson Cancer Center. *J. Clin. Oncol.* **14**, 287–295.

Index

A

AAV, *see* Adeno-associated virus
ABCA5, expression and Gleason score correlation, 252
ABX-EGF, immune therapy for prostate cancer, 405
Acetate, molecular imaging in prostate cancer, 197
Actin, lipid rafts and polymerization, 129, 130
Adeno-associated virus (AAV),
gene delivery for molecular imaging, 221
gene therapy vector, 143
Adenovirus,
gene delivery for molecular imaging, 220
gene therapy vector, 140–143
African Americans,
androgen receptor polymorphisms, 361, 362
complementary and alternative medicine use, 358
diet, 358, 359
healthcare system interactions,
early detection behavior, 357
overview, 356, 357
treatment decisions, 358
mortality from prostate cancer, 355, 356
population stratification, 363, 364
prostate cancer susceptibility genes,
familial cancer, 363
overview, 362, 363
racial differences in prostate and cancer,
androgen receptor, 365–267
prostate-specific antigen, 364
tissue androgens, 365
Akt, targeting for telomerase inhibition, 335
Androgen, telomerase effects, 331, 332
Androgen deprivation therapy, radiation combination therapy,
biological considerations, 385–387
clinical trials, 388
duration, 387, 389
radiation-induced side effect alterations, 390
sequencing, 389, 390
Androgen independence, xenograft models of progression,
CWR22, 9
LAPC-4, 9
LAPC-9, 9
LNCaP, 9
LuCaP series, 10–13
PC-346, 9
Androgen receptor (AR),
activation, 88
coactivators and prostate cancer, 92–94
consensus element, 90
gene, 88
nonsteroidal activation in prostate cancer progression, 95–97
racial differences, 361, 362, 365–367
refractory prostate cancer features,
autonomous activation, 90
expression increase, 90, 276
mutations, 90–92, 276
therapeutic targeting, 97, 98
structure, 88, 89
Angiogenesis,
quantum dot analysis, 239, 240
thalidomide targeting, 457, 467
vasculogenic mimicry of cancer cells, 78
Antisense,
Bcl-2 targeting, 458
telomerase inhibition, 334
APC, hypermethylation in prostate cancer, 305, 308
AR, *see* Androgen receptor

ARA54, androgen receptor coactivation, 94, 95
ARA70, androgen receptor coactivation, 92, 93, 95
ATBF1, loss of heterozygosity, 271
Atrasentan, prostate cancer trial, 457, 458, 467, 468, 481
Atrophy, see Prostate atrophy
5-Aza-cytidine, DNA methyltransferase inhibition, 309, 418
5-Aza-deoxycytidine, DNA methyltransferase inhibition, 309, 418

B

BAG-1L, androgen receptor coactivation, 93
BALB/c Nu/nu mouse, xenograft models, 4
Bcl-2,
  antisense targeting, 458
  taxane effects, 446
Bevacizumab, immune therapy for prostate cancer, 406, 457, 466, 479, 480
BGN, expression and Gleason score correlation, 252, 253
Bioluminescence imaging, molecular imaging, 204, 205, 208
Bisphosphonates, cancer-associated osteolysis management, 182, 183
Bone,
  anatomy, 176, 177
  metastasis, see Metastasis
  osteoclastogenesis, 177, 178
  remodeling, 178
Bone metastasis, *see* Metastasis
Bone sialoprotein (BSP), osteomimicry of cancer cells, 76
Brachytherapy, *see* Radiation therapy, prostate cancer
BRCA1,
  androgen receptor coactivation, 95
  prostate cancer susceptibility studies, 294, 295
  telomerase effects, 331
BRCA2, prostate cancer susceptibility studies, 294, 295
BSP, *see* Bone sialoprotein

C

E-Cadherin, loss of heterozygosity, 271
Calydon virus, oncolytic virus therapy, 149
Cancer stem cell, *see* Prostate stem cells
CaP, *see* Prostate cancer
Carcinoembryonic antigen (CEA), molecular imaging in prostate cancer, 197
Cathepsin K, osteoclastogenesis regulation, 178
Caveolin,
  loss of heterozygosity, 271, 272
  racial differences, 366
CBP, *see* CREB-binding protein
CEA, *see* Carcinoembryonic antigen
CGH, *see* Comparative genomic hybridization
CHEK2, prostate cancer susceptibility studies, 295
Chemokines, prostate cancer cell homing, 81
ChL6, immune therapy for prostate cancer, 405
Cholesterol,
  lipid rafts,
    prostate cancer progression role,
      actin polymerization, 129, 130
      prospects for study, 131
      receptor-mediated signaling, 127, 129
    signaling overview, 125, 128
    structure and function, 125–127
  prostate cancer promotion studies, 120–122
  reduction and prostate cancer prevention, 119, 122, 123
  statin pleiotropic effects, 123, 124
Choline, molecular imaging in prostate cancer, 197
Chromatin remodeling, *see* Epigenetics
Chromosome instability (CIN),
  human cancers, 318, 319
  prostate carcinogenesis, 338–340
  telomerase stabilization in cancer, 323
  telomere link, 320
CIN, *see* Chromosome instability
Clinical trial design,
  dose-finding after phase I, 493, 494

maximum tolerated dose in phase II and phase III trials, 491–493
phase I trial,
dose optimization, 487–489
goals, 488
escalation with overdose control method,
with covariates, 490
overview, 489, 490
phases, 487
COL1A2, expression and Gleason score correlation, 252, 253
Comparative genomic hybridization (CGH),
array-based comparative genomic hybridization, 274, 275
chromosome 7 studies, 273, 274
prostate cancer stage and genetic aberrations, 270, 272–274
Complementary and alternative medicine, racial differences in use, 358
CpG island, *see* DNA methylation
CREB, activation in cancer, 76, 77
CREB-binding protein (CBP), prostate cancer levels, 94
CWR22, *see* Xenograft models

D

DC, *see* Dendritic cell
Dendritic cell (DC),
immunotherapy, 154
telomerase targeting with immunotherapy, 337
Desert Hedgehog, *see* Hedgehog
Diffuse/hormonal atrophy, *see* Prostate atrophy
DNA methylation,
cancer defects,
overview, 303, 304
prostate cancer,
detection for biomarker use, 305, 307–309
genes affected, 305, 306
GSTP1 hypermethylation, 276, 304, 305, 308
therapeutic targeting, 309–312
CpG islands, 301, 302
DNA methyltransferases,
inhibitors, 309, 310, 418–421
types and function, 302–304
hTERT, 335
DNA microarray,
laser capture microdissection of precursor lesions, 261
principles, 248–250
prostate cancer studies,
aggressive tumor identification, 253, 256, 257
biologically-important changes, 262–264
Gleason score correlates, 252, 253
limitations, 257, 259, 260
metastasis studies, 253, 254, 256, 257
overview, 250, 252
prospects, 260, 261
recurrence prediction, 254–256
stromal contamination in tumor expression profiling, 261, 262
Docetaxel, *see* Taxane chemotherapy, prostate cancer
Dopamine-2 receptor, indirect molecular imaging in prostate cancer, 201, 202
Dose escalation, *see* Clinical trial design

E

EGF, *see* Epidermal growth factor
EGFR, *see* Epidermal growth factor receptor
EIF3S3, comparative genomic hybridization studies, 272, 273
ELAC2, linkage studies of prostate cancer susceptibility, 288, 289
Elongin C, expression in prostate cancer, 273
EMT, *see* Epithelial-to-mesenchymal transition
Endothelin,
prostate cancer expression, 457
therapeutic targeting, 457, 467, 468, 481
EPHB2, mutation in prostate cancer, 277
Epidermal growth factor (EGF), androgen receptor interactions, 96
Epidermal growth factor receptor (EGFR), *see also* Her-2/neu,
classification, 476
ligands, 477

lung cancer studies, 263, 264
prostate cancer cell expression, 465
racial differences, 367
signaling, 476, 477
Epigenetics,
chromatin remodeling,
chromatin-binding domains,
bromodomains, 413, 414
chromodomains, 414
SANT domains, 414
complexes, 413
DNA methyltransferase inhibitors, 309, 310, 418–421
environmental modulation, 411, 412
histone acetyltransferases,
domains, 416, 417
GNAT superfamily, 417
inhibitors, 426, 427
MYST superfamily, 417
p300/CBP superfamily, 417, 418
histone code, 414
histone deacetylases,
classification, 415, 416
inhibitors,
activity in vivo, 425, 426
antitumor activity, 427, 428
apoptosis induction, 424, 425
biomarkers for evaluation, 428, 432
cell cycle effects, 424
classes, 421–423
clinical trials, 429–431
combination therapy, 432–434
gene expression effects, 425
mechanisms of action, 424, 432
nonhistone protein effects, 425
pharmacology, 429
toxicity, 428
nucleosome structure, 412
Epithelial-to-mesenchymal transition (EMT), cancer cells, 78
Escalation with overdose control (EWOC), dose optimization,
with covariates, 490
overview, 489, 490
Estramustine,
bevacizumab combination therapy, 457, 480
hormone-refractory prostate cancer treatment rationale, 447
taxane combination therapy,
docetaxel, 448–451
paclitaxel, 448, 450
toxicity, 455, 456, 480
Estrogen, telomerase effects, 332, 333
EWOC, *see* Escalation with overdose control

F

FAK, *see* Focal adhesion kinase
FGF, *see* Fibroblast growth factor
Fibroblast, plasticity in stroma, 74, 75
Fibroblast growth factor (FGF),
prostate cancer cell expression, 465
prostate cancer role, 482
types, 482
Focal adhesion kinase (FAK), vasculogenic mimicry of cancer cells, 78
Focal atrophy, *see* Prostate atrophy

G

Gene therapy,
goals, 211
molecular imaging studies,
preclinical studies, 209
reporter gene linking to therapeutic gene
bi-cistronic approach, 216, 217
bi-directional transcriptional approach, 217
co-vector administration approach, 217
fusion approach, 217
tissue-specific promoter evaluation, 212–216
prospects, 155, 210
prostate cancer approaches,
corrective therapy,
MDM2, 148
p53, 146
PTEN, 148p16, 148
cytotoxic gene therapy,
herpes simplex virus-thymidine kinase, 152
TRAIL use, 153
immunotherapy,
dendritic cell immunotherapy, 154
in vivo immunotherapy, 154, 155

tumor vaccines, 154
oncolytic viral therapy,
adenovirus constructs using osteocalcin promoter, 149, 150
adenovirus constructs using prostate-specific enhancing sequence, 150
Calydon virus, 149
host immune response control, 150, 151
ONYX-015, 149
prospects, 151
overview, 147
prostate-specific promoters,
osteocalcin, 145
prostate-specific antigen, 144
prostate-specific enhancing sequence, 144, 145
prostate-specific membrane antigen, 144
telomerase, 145
telomerase targeting, 336
tumor-stroma targeting, 468, 469
vectors,
adeno-associated virus, 143
adenovirus, 140–143
overview, 140
Genetic instability, *see* Chromosome instability
Genistein, telomerase inhibition, 334
Gli, regulation of Hedgehog expression, 109, 110
Glutathione *S*-transferase (GST),
expression in prostate atrophy, 43, 44
hypermethylation of *GSTP1*, 276, 304, 305, 308
loss in prostate cancer, 80
GNAT, histone acetyltransferase superfamily, 417
GRN163, telomerase inhibition, 334
GST, *see* Glutathione *S*-transferase

H

HAT, *see* Histone acetyltransferase
HDAC, *see* Histone deacetylase
Hedgehog (Hh),
Gli regulation of expression, 109, 110
processing, 10
prostate cancer,
signaling activity, 114, 115
tumor growth, 115–117
prostate development role, 110, 111, 113
prostate regeneration role, 114
signaling, 108, 109
types, 107
Her-2/neu,
prognostic value, 477
prostate cancer cell expression, 465
signaling, 476, 477
therapeutic targeting, 477, 478
Herpes simplex virus-thymidine kinase,
cytotoxic gene therapy, 152
indirect molecular imaging in prostate cancer, 198, 199, 201
molecular imaging, 198, 199, 201
Hh, *see* Hedgehog
Histone acetyltransferase (HAT),
domains, 416, 417
GNAT superfamily, 417
inhibitors, 426, 427
MYST superfamily, 417
p300/CBP superfamily, 417, 418
Histone deacetylase (HDAC),
classification, 415, 416
inhibitors,
activity in vivo, 425, 426
antitumor activity, 427, 428
apoptosis induction, 424, 425
biomarkers for evaluation, 428, 432
cell cycle effects, 424
classes, 421–423
clinical trials, 429–431
combination therapy, 432–434
gene expression effects, 425
mechanisms of action, 424, 432
nonhistone protein effects, 425
overview, 310, 311
pharmacology, 429
toxicity, 428
HMG-CoA reductase inhibitors, *see* Statins
Hormone-refractory prostate cancer,
androgen receptor features,
autonomous activation, 90
expression increase, 90, 276
mutations, 90–92, 276
therapeutic targeting, 97, 98

chemotherapy, *see* Estramustine; Taxane chemotherapy, prostate cancer
Host–cancer interactions,
epithelial-to-mesenchymal transition, 78
host stem cells, 81
infiltrating inflammatory cells, 79, 80
osteomimicry, 76–78
plasticity of cancer and stromal fibroblast, 74, 75
therapeutic targeting, 81, 82
vasculogenic mimicry, 78

I

IGF, *see* Insulin-like growth factor
IL-6, *see* Interleukin-6
Imatinib mesylate, prostate cancer studies, 466, 480, 481
Immunohistochemistry, telomerase expression studies in prostate cancer, 327, 328
Immunotherapy, *see* Dendritic cell; Monoclonal antibodies, prostate cancer immune therapy
Indian Hedgehog, *see* Hedgehog
Inflammation, *see* Prostate inflammation
In situ hybridization, telomerase expression studies in prostate cancer, 327, 328
Insulin-like growth factor (IGF),
prostate carcinogenesis role, 482
receptor and prostate cancer cell expression, 465, 466
types, 481
Integrins, prostate cancer cell homing, 81
Interleukin-6 (IL-6),
androgen receptor interactions, 96, 97
osteoclastic bone metastasis role, 181, 182
prostate cancer cell expression, 466
therapeutic targeting, 483

J

J591, immune therapy for prostate cancer, 401–406

K

Keratin, expression in prostate atrophy, 42
Ki-67, expression in prostate atrophy, 43
KLF6, defects in prostate cancer, 277

L

LAPC-4, *see* Xenograft models
LAPC-9, *see* Xenograft models
Laser capture microdissection (LCM), prostate cancer precursor lesions and gene profiling, 261
LCM, *see* Laser capture microdissection
Linkage studies, prostate cancer,
alternative approaches, 294, 295
difficulties, 290, 291
familial clustering evidence, 286, 287
genome-wide scanning, 291–293
ICPCG study, 291, 292
prospects, 295, 296
susceptibility genes,
ELAC2, 288, 289
MSR1, 289, 290
overview, 287, 288
RNASEL, 289
search, 285, 286
Lipid raft,
prostate cancer progression role
actin polymerization, 129, 130
prospects for study, 131
receptor-mediated signaling, 127, 129
signaling overview, 125, 128
structure and function, 125–127
LNCaP, *see* Xenograft models
LOH, *see* Loss of heterozygosity
Loss of heterozygosity (LOH),
ATBF1, 271
caveolin, 271, 272
E-cadherin, 271
NKX3.1, 271
prostate cancer stage and genetic aberrations, 270–272
PTEN, 271
LuCaP series, *see* Xenograft models

M

Magnetic resonance imaging (MRI), molecular imaging, 208, 209
MAPK, *see* Mitogen-activated protein kinase
Matrix metalloproteinases (MMPs), osteoclastic bone metastasis role, 180, 181

MBD2, therapeutic targeting, 311, 312
MDM2, prostate cancer gene therapy, 148
MDX-010, immune therapy for prostate cancer, 406
MeCP2, therapeutic targeting, 311
Metastasis,
bone tropism mechanisms, 464
gene expression studies, 253, 254, 256, 257
osteoblastic bone metastasis, 178, 179
osteoclastic bone metastasis, 179–182
osteolysis management, 182, 183
osteomimicry of cancer cells, 76–78
sites in prostate cancer, 175
xenograft models,
bone metastasis,
bone injection, 17
clinical significance, 15, 16
human adult bone model, 17, 19, 20
intravenous and cardiac injection, 17
intratibial injection, 20, 23, 25
SCID-hu model, 17
non-osseous metastasis,
LNCaP, 14
LuCaP series, 15
PC3, 14, 15
overview, 13, 14
preclinical use of bone metastasis models,
bone injection models, 25, 26
cardiac injection model, 26
human adult bone model, 24, 25
SCID-hu model, 24
Mitogen-activated protein kinase (MAPK), androgen receptor interactions, 95, 96
MMPs, *see* Matrix metalloproteinases
Molecular imaging, *see also* Quantum dots,
bioluminescence imaging, 204, 205, 208
direct imaging, 195, 197, 198
gene delivery vectors,
adeno-associated virus, 221
adenovirus, 220
nonviral vectors, 221, 222
retrovirus, 221
gene therapy studies,
preclinical studies, 209
reporter gene linking to therapeutic gene,
bi-cistronic approach, 216, 217
bi-directional transcriptional approach, 217
co-vector administration approach, 217
fusion approach, 217
tissue-specific promoter evaluation, 212–216
indirect imaging,
herpes simplex virus-thymidine kinase, 198, 199, 201
receptor-based approach, 201–203
magnetic resonance imaging, 208, 209
molecular interaction studies, 217–219
multimodal imaging, 220
overview, 193, 194
positron emission tomography, 195
rationale, 194
single-photon emission computed tomography, 203, 204
strategies, 194, 195
Monoclonal antibodies, prostate cancer immune therapy,
development, 398, 399
overview, 397
phage display, 399
prospects, 407, 408
radiolabels, 399, 400
targets and clinical trials,
ABX-EGF, 405
bevacizumab, 406
ChL6, 405
MDX-010, 406
prostate-specific membrane antigen,
J591, 401–406
overview, 399–401
trastuzumab, 405, 406
transgenic mouse for production, 398, 399
MRI, *see* Magnetic resonance imaging
MSR1,
candidate gene in familial prostate cancer, 80
linkage studies of prostate cancer susceptibility, 289, 290

MYST, histone acetyltransferase superfamily, 417

N

Nerve growth factor (NGF), telomerase effects, 331
Neurotrophins, therapeutic targeting, 482
NGF, *see* Nerve growth factor
NKX3.1,
  expression in prostate atrophy, 43
  loss of heterozygosity, 271
NMRI/Nu mouse, xenograft models, 5
NOD/SCID mouse, xenograft models, 5
Nucleosome, *see* Epigenetics

O

Obesity, cancer risks, 359, 360
Oblimersen, Bcl-2 targeting, 458
Oncolytic viruses, *see* Gene therapy
ONYX-015, oncolytic virus therapy, 149
Osteocalcin,
  oncolytic virus therapy using promoter, 149, 150
  osteomimicry of cancer cells, 76
  promoter use for gene therapy, 145
Osteomimicry, cancer cells, 76–78
Osteopontegerin,
  osteoclastic bone metastasis role, 180
  osteoclastogenesis role, 177
  osteomimicry of cancer cells, 76

P

p16, prostate cancer gene therapy, 148
p27, expression in prostate atrophy, 43
p53,
  mutation in prostate cancer, 275
  prostate cancer gene therapy, 146
  racial differences, 366
p300/CBP, histone acetyltransferase superfamily, 417, 418
Paclitaxel, *see* Taxane chemotherapy, prostate cancer
Parathyroid hormone-related peptide (PTHrP), osteoclastic bone metastasis role, 181
PC-346, *see* Xenograft models
PDGF, *see* Platelet-derived growth factor
PET, *see* Positron emission tomography
Phage display, therapeutic antibodies, 399
PIAS, androgen receptor coactivation, 93
PinX1, loss of heterozygosity, 330
Platelet-derived growth factor (PDGF),
  prostate cancer cell expression, 480
  receptor therapeutic targeting, 466, 467, 480, 481
Positron emission tomography (PET), molecular imaging, 195
Prenylation, statin therapy effects, 124
Procainamide, DNA methyltransferase inhibition, 310
Prostate atrophy,
  classification,
    overview, 33
    proliferative inflammatory atrophy, 37, 38
    Working Group Classification
      focal atrophy, 35–37
      normal epithelium, 34, 35
  diffuse/hormonal atrophy,
    distribution, 38, 39
    features, 38
    history of study, 39, 40
    versus focal atrophy, 39
  focal atrophy,
    versus diffuse/hormonal atrophy, 39
    distribution, 38, 39
    features, 38
  gene expression,
    glutathione *S*-transferase, 43, 44
    keratin, 42
    Ki-67, 43
    NKX3.1, 43
    p27, 43
  injury and regeneration theory of prostate carcinogenesis, 44
  molecular biology, 42
Prostate cancer (CaP),
  chemotherapy, *see* Estramustine; Taxane chemotherapy, prostate cancer
  chromosomal aberrations, *see* Chromosome instability; Comparative genomic
  hybridization; Loss of heterozygosity
  diet effects on progression, 120
  epidemiology, 3, 139, 445
  gene expression, *see* DNA microarray

gene therapy, *see* Gene therapy
metastasis, *see* Metastasis
racial differences, *see* African Americans
radiation therapy, *see* Radiation therapy, prostate cancer
susceptibility genes, *see* Linkage studies, prostate cancer
tissue microarray, *see* Tissue microarray
xenograft, *see* Xenograft models, prostate cancer
Prostate gland, embryology, 74, 110, 111, 113
Prostate inflammation,
acute, 40, 41
chronic, 40
granulomatous, 41
injury and regeneration theory of prostate carcinogenesis, 44
overview, 40
Prostate stem cell,
cancer stem cells,
characterization in cancer and metastasis, 68, 70
features, 63, 64
ontogeny, 66
epithelial stem cells,
differentiation, 65, 66
identification and isolation, 66, 67
immunophenotype, 64, 65
markers, 63
Prostate-specific antigen (PSA),
promoter use for gene therapy, 144
screening, 248, 445
Prostate-specific enhancing sequence (PSES),
gene therapy use, 144, 145
oncolytic virus therapy, 150
Prostate-specific membrane antigen (PSMA),
molecular imaging in prostate cancer, 197, 198
monoclonal antibody immune therapy for prostate cancer,
J591, 401–406
overview, 399–401
promoter use for gene therapy, 144
structure, 399, 400
Prostate stem cell antigen (PSCA),
comparative genomic hybridization studies, 273
molecular imaging in prostate cancer, 198
PSA, *see* Prostate-specific antigen
PSCA, *see* Prostate stem cell antigen
PSES, *see* Prostate-specific enhancing sequence
PSMA, *see* Prostate-specific membrane antigen
PTEN,
loss of heterozygosity, 271
mutation in prostate cancer, 275, 276
prostate cancer gene therapy, 148
PTHrP, *see* Parathyroid hormone-related peptide

Q

Quantum dots,
advantages in imaging, 231
angiogenesis analysis, 239, 240
cellular imaging and tracking, 236, 237
optical properties, 233–236
probe development, 232, 233
prostate cancer applications,
in vivo targeting and imaging, 240, 241
molecular profiling of specimens, 237–239
sentinel lymph node mapping, 239, 240
size, 231
toxicity and potential clinical use, 241

R

Racial differences, *see* African Americans
RAD21, comparative genomic hybridization studies, 272, 273
Radiation therapy, prostate cancer,
androgen deprivation therapy combination therapy,
biological considerations, 385–387
clinical trials, 388
duration, 387, 389
radiation-induced side effect alterations, 390
sequencing, 389, 390
dose-escalated radiotherapy,
clinical trials, 379.380

cooperative group trial, 380
high-dose rate brachytherapy, 382–384
low-dose rate brachytherapy, 383, 385
randomized trials, 380, 381
rationale, 378
retrospective analysis, 381, 382
historical perspective, 377
WPRT for occult nodal metastasis, 391, 392
RAG, knockout mouse for xenograft model, 5
RANK ligand,
osteoclastic bone metastasis role, 180
osteoclastogenesis role, 177
osteomimicry of cancer cells, 76
RASSF1α, hypermethylation in prostate cancer, 305, 308
Rb, androgen receptor coactivation, 95
Retinoic acid, telomerase effects, 331, 335
Retrovirus, gene delivery for molecular imaging, 221
Reverse transcriptase-polymerase chain reaction (RT-PCR), telomerase expression studies in prostate cancer, 326–328
RNASEL,
candidate gene in familial prostate cancer, 80
linkage studies of prostate cancer susceptibility, 289
RT-PCR, *see* Reverse transcriptase-polymerase chain reaction

S

SCID mouse, *see* Severely combined immunodeficient mouse
SCID-hu model, *see* Xenograft models
Sentinel lymph node, quantum dot mapping, 239, 240
Severely combined immunodeficient (SCID) mouse, xenograft models, 5
Sex hormone-binding globulin (SHBG), racial differences, 365
SHBG, *see* Sex hormone-binding globulin
Single-photon emission computed tomography (SPECT), molecular imaging, 203, 204
Smads, transforming growth factor-β signaling, 166
Sodium iodide symporter, indirect molecular imaging in prostate cancer, 202
Somatostatin receptor, indirect molecular imaging in prostate cancer, 203
Sonic Hedgehog, *see* Hedgehog
SPARC, expression and Gleason score correlation, 252, 253
SPECT, *see* Single-photon emission computed tomography
SRC-1, androgen receptor coactivation, 93
Statins,
cancer protection studies, 122, 123
pleiotropic effects, 123, 124
Stem cells, *see* Prostate stem cells
Stroma,
contamination in prostate cancer gene expression profiling, 261, 262
fibroblast plasticity, 74, 75
immunotherapy effects, 467
prostate tumor interactions in progression,
cell–cell interactions, 465
cell–extracellular interactions, 465, 466
gene therapy targeting, 468, 469
growth factors, 465, 466
overview, 464

T

Taxane chemotherapy, prostate cancer,
calcitriol combination therapy, 456, 457
clinical trials,
docetaxel, 447, 451–455
estramustine combination therapy
docetaxel, 448–451
paclitaxel, 448, 450
toxicity, 455, 456
paclitaxel, 447
hormone-refractory prostate cancer, 446
rationale, 446
resistance, 456
thalidomide combination therapy, 457

Telomerase,
chromosome restabilization in cancer, 323
promoter methylation, 335
promoter use for gene therapy, 145
prostate cancer,
activity and expression studies, 324–328
diagnostic value, 328–330
regulation, 330–333
structure, 323
therapeutic targeting,
direct inhibition, 333, 334
gene therapy, 336
immunotherapy, 337
indirect inhibition, 334, 335
oncolytic virus therapy, 336, 337
overview, 333
Telomere,
chromosome instability link, 320
features, 319, 320
prostate carcinogenesis modeling, 338–341
shortening,
prostate cancer,
early tumorigenesis studies, 322, 323
observations in cells, 320, 321
prognostic value, 321
tumor suppressive cellular responses, 320
therapeutic targeting, 337, 338
Testosterone, *see* Androgen
TGF-β, *see* Transforming growth factor-β
Thalidomide,
angiogenesis inhibition, 467
taxane combination therapy, 457
Tip60, androgen receptor coactivation, 94
Tissue microarray,
analysis, 57, 59
archived patient specimens, 50
cell line utilization, 56
commercial microarrays, 58, 59
construction, 51–53
frozen tissue, 56
principles, 50
prostate cancer research,
needle biopsies, 56
outcome studies, 55
overview, 53, 54
progression studies, 54, 55
specialized microarrays, 55, 56
TRAIL, cytotoxic gene therapy, 153
Transforming growth factor-β (TGF-β),
activation, 166
immunosuppression, 168, 169
immunotherapy,
autoimmune disease and insensitivity, 169
clinical prospects, 170, 171
insensitive bone marrow transplant and tumor cell elimination, 169, 170
insensitive cytotoxic T cell transfer and tumor eradication, 170
prostate cancer cell expression, 465
receptors, 166
signaling,
normal signaling, 167
prostate cancer
overexpression, 167, 168
sensitivity downregulation, 167
Smads, 166
superfamily, 165, 166
tumor expression and host immunity effects, 168
tumor immunosurveillance effects, 169
tumor progression role, 75
Trastuzumab, HER-2 targeting, 405, 406, 477, 478
Tumor vaccine, immunotherapy, 154

V

Vascular endothelial growth factor (VEGF),
isoforms, 479
prostate cancer levels and role, 479
receptors, 479
therapeutic targeting, 457, 466, 479, 480
Vasculogenic mimicry, cancer cells, 78
VEGF, *see* Vascular endothelial growth factor
Vitamin D3,
taxane combination therapy, 456, 457
telomerase effects, 331, 335

W

WPRT, *see* Radiation therapy, prostate cancer

X

Xenograft models, prostate cancer,
androgen independence progression,
CWR22, 9
LAPC-4, 9
LAPC-9, 9
LNCaP, 9
LuCaP series, 10–13
PC-346, 9
cell line and tissue sources, 6, 7
common types, 7, 8
host selection,
BALB/c Nu/nu mouse, 4
NMRI/Nu mouse, 5
non-obese diabetic/SCID mouse, 5
RAG knockout mouse, 5
severely combined immunodeficient mouse, 5
implantation
orthotopic, 6
sub-renal capsule, 6
subcutaneous, 5
metastasis studies,
bone metastasis,
bone injection, 17
clinical significance, 15, 16
human adult bone model, 17, 19, 20
intravenous and cardiac injection, 17
intratibial injection, 20, 23, 25
SCID-hu model, 17
non-osseous metastasis,
LNCaP, 14
LuCaP series, 15
PC3, 14, 15
overview, 13, 14
preclinical use of bone metastasis models,
bone injection models, 25, 26
cardiac injection model, 26
human adult bone model, 24, 25
SCID-hu model, 24
prospects, 27