Breast Cancer: Cellular and Molecular Biology

Cancer Treatment and Research

WILLIAM L MCGUIRE, *series editor*

Livingston RB (ed): Lung Cancer 1. 1981. ISBN 90-247-2394-9.
Bennett Humphrey G, Dehner LP, Grindey GB, Acton RT (eds): Pediatric Oncology 1. 1981. ISBN 90-247-2408-2.
DeCosse JJ, Sherlock P (eds): Gastrointestinal Cancer 1. 1981. ISBN 90-247-2461-9.
Bennett JM (ed): Lymphomas 1, including Hodgkin's Disease. 1981. ISBN 90-247-2479-1.
Bloomfield CD (ed): Adult Leukemias 1. 1982. ISBN 90-247-2478-3.
Paulson DF (ed): Genitourinary Cancer 1. 1982. ISBN 90-247-2480-5.
Muggia FM (ed): Cancer Chemotherapy 1. ISBN 90-247-2713-8.
Bennett Humphrey G, Grindey GB (eds): Pancreatic Tumors in Children. 1982. ISBN 90-247-2702-2.
Costanzi JJ (ed): Malignant Melanoma 1. 1983. ISBN 90-247-2706-5.
Griffiths CT, Fuller AF (eds): Gynecologic Oncology. 1983. ISBN 0-89838-555-5.
Greco AF (ed): Biology and Management of Lung Cancer. 1983. ISBN 0-89838-554-7.
Walker MD (ed): Oncology of the Nervous System. 1983. ISBN 0-89838-567-9.
Higby DJ (ed): Supportive Care in Cancer Therapy. 1983. ISBN 0-89838-569-5.
Herberman RB (ed): Basic and Clinical Tumor Immunology. 1983. ISBN 0-89838-579-2.
Baker LH (ed): Soft Tissue Sarcomas. 1983. ISBN 0-89838-584-9.
Bennett JM (ed): Controversies in the Management of Lymphomas. 1983. ISBN 0-89838-586-5.
Bennett Humphrey G, Grindey GB (eds): Adrenal and Endocrine Tumors in Children. 1983. ISBN 0-89838-590-3.
DeCosse JJ, Sherlock P (eds): Clinical Management of Gastrointestinal Cancer. 1984. ISBN 0-89838-601-2.
Catalona WJ, Ratliff TL (eds): Urologic Oncology. 1984. ISBN 0-89838-628-4.
Santen RJ, Manni A (eds): Diagnosis and Management of Endocrine-related Tumors. 1984. ISBN 0-89838-636-5.
Costanzi JJ (ed): Clinical Management of Malignant Melanoma. 1984. ISBN 0-89838-656-X.
Wolf GT (ed): Head and Neck Oncology. 1984. ISBN 0-89838-657-8.
Alberts DS, Surwit EA (eds): Ovarian Cancer. 1985. ISBN 0-89838-676-4.
Muggia FM (ed): Experimental and Clinical Progress in Cancer Chemotherapy. 1985. ISBN 0-89838-679-9.
Higby DJ (ed): The Cancer Patient and Supportive Care. 1985. ISBN 0-89838-690-X.
Bloomfield CD (ed): Chronic and Acute Leukemias in Adults. 1985. ISBN 0-89838-702-7.
Herberman RB (ed): Cancer Immunology: Innovative Approaches to Therapy. 1986. ISBN 0-89838-757-4.
Hansen HH (ed): Lung Cancer: Basic and Clinical Aspects. 1986. ISBN 0-89838-763-9.
Pinedo HM, Verweij J (eds): Clinical Management of Soft Tissue Sarcomas. 1986. ISBN 0-89838-808-2.
Higby DJ (ed): Issues in Supportive Care of Cancer Patients. 1986. ISBN 0-89838-816-3.
Surwit EA, Alberts DS (eds): Cervix Cancer. 1987. ISBN 0-89838-822-8.
Jacobs C (ed): Cancers of the Head and Neck. 1987. ISBN 0-89838-825-2.
MacDonald JS (ed): Gastrointestinal Oncology. 1987. ISBN 0-89838-829-5.
Ratliff TL, Catalona WJ (eds): Genitourinary Cancer. 1987. ISBN 0-89838-830-9.
Nathanson L (ed): Basic and Clinical Aspects of Malignant Melanoma. 1987. ISBN 0-89838-856-2.
Muggia FM (ed): Concepts, Clinical Developments, and Therapeutic Advances in Cancer Chemotherapy. 1987. ISBN 0-89838-879-5.

Breast Cancer: Cellular and Molecular Biology

edited by

MARC E. LIPPMAN

and

ROBERT B. DICKSON
National Cancer Institute

1988 KLUWER ACADEMIC PUBLISHERS
BOSTON / DORDRECHT / LONDON

Distributors

for the United States and Canada: Kluwer Academic Publishers, 101 Philip Drive, Assinippi Park, Norwell, MA 02061
for the UK and Ireland: Kluwer Academic Publishers, Falcon House, Queen Square, Lancaster LA1 1RN, UK
for all other countries: Kluwer Academic Publishers Group, Distribution Center, P.O. Box 322, 3300 AH Dordrecht, The Netherlands

Library of Congress Cataloging in Publication Data

Breast cancer: cellular and molecular biology/edited by Marc E. Lippman, Robert B. Dickson.
p. cm. — (Cancer treatment and research)
Includes bibliographies and index.
ISBN-13: 978-1-4612-8975-3 e-ISNB-13: 978-1-4613-1733-3
DOI: 10.1007/978-1-4613-1733-3
1. Breast—Cancer. 2. Breast—Pathophysiology. 3. Breast—Cytopathology. I. Lippman, Marc E., 1945– II. Dickson, Robert B. (Robert Brent), 1952– . III. Series.
[DNLM: 1. Breast Neoplasms—physiopathology. 2. Cell Transformation, Neoplastic. 3. Epithelium—physiopathology. 4. Estrogens—physiology. 5. Growth Substances—physiology. W1 CA693/WP 870 B8232]
RC280 B8B6657 1988
616.99'44907—dc19
DNLM/DLC
for Library of Congress

87-38322
CIP

Contents

Cancer Treatment and Research

Foreword

Where do you begin to look for a recent, authoritative article on the diagnosis or management of particular malignancy? The few general oncology textbooks are generally out of date. Single papers in specialized journals are informative but seldom comprehensive; these are more often preliminary reports on a very limited number of patients. Certain general journals frequently publish good in-depth reviews of cancer topics, and published symposium lectures are often the best overviews available. Unfortunately, these reviews and supplements appear sporadically, and the reader can never be sure when a topic of special interest will be covered.

Cancer Treatment and Research is a series of authoritative volumes which aim to meet this need. It is an attempt to establish a critical mass of oncology literature covering virtually all oncology topics, revised frequently to keep the coverage up to date, easily available on a single library shelf or by a single personal subscription.

We have approached the problem in the following fashion. First, by dividing the oncology literature into specific subdivisions such as lung cancer, genitourinary cancer, pediatric oncology, etc. Second, by asking eminent authorities in each of these areas to edit a volume on the specific topic on an annual or biannual basis. Each topic and tumor type is covered in a volume appearing frequently and predictably, discussing current diagnosis, staging, markers, all forms of treatment modalities, basic biology, and more.

In Cancer Treatment and Research, we have an outstanding group of editors, each having made a major commitment to bring to this new series the very best literature in his or her field. Kluwer Academic Publishers has made an equally major commitment to the rapid publication of high quality books, and worldwide distribution.

Where can you go to find quickly a recent authoritative article on any major oncology problem? We hope that Cancer Treatment and Research provides an answer.

WILLIAM L. MCGUIRE
Series Editor

Preface

Marc E. Lippman, M.D., and Robert B. Dickson, Ph.D.

The past few years have witnessed a revolution in our understanding of the biology of breast cancer. In part, this is due to the availability of biochemical and molecular biological techniques to allow identification, characterization, and experimental perturbation of potent regulatory molecules such as receptors, proteases, growth factors, and oncogenes. A considerable number of researchers utilize fresh human clinical material, normal and human breast cancer cell lines in vitro, and xenograft tumors in vivo in the athymic (or nude) mouse. Others, however, employ a mouse model system whereby a tumor virus known as mouse mammary tumor virus (MMTV), carried in the milk or through the germ line, triggers mouse mammary cancer. In this volume we present both areas of investigation: first, studies on human mammary cancer, then second, work on mouse breast cancer induced by MMTV. We believe that by presenting both kinds of studies we provide a book suitable for a wide readership of postdoctoral and medical fellows and researchers.

The first chapter, by Martha Stampfer and Jack Bartley, presents the state of the art in culture of normal human mammary epithelial cells. Studies on cancer should always use normal tissue as a reference point in order to highlight tumor-specific versus simply proliferation-specific cellular characteristics. This chapter also presents information on properties of carcinogen-treated mammary epithelial cells. The second chapter, continuing in the theme of human breast cancer, studies rearrangements in human genes which might be associated with loss of cancer repressor gene. In addition to possible importance of exposure to chemical carcinogens, breast cancer risk is known to be associated with a significant genetic component. Igbal Unissa Ali and Robert Callahan propose that inherited rearrangements of chromosome 11 could be one of these genetic factors. The third chapter, by Matthias Kraus and co-workers continues the theme of genetic alterations in cancer patients by reporting on the specific oncogenes found activated in breast cancers themselves. Alterations in c-H-*ras* and *erb*B-2 oncogenes may be involved in progression of breast cancer to more malignant forms. In the fourth chapter, Bernd Groner and co-workers explore other functions of activated oncogene expression, This chapter analyzes the effects of oncogenes on the differentiated state of breast tissue. Oncogenes have proven powerful switches to dediffer-

entiate or despecialize the secretory epithelium. The fifth chapter, by Adrian Harris and Stewart Nicholson reports on studies of the receptor for epidermal growth factor (EGF) in breast cancer. This is also a proto-oncogene known as c-*erb*B, and its expression appears to be a new tumor marker for the poor prognosis breast cancer patients. The sixth chapter, by ourselves, addresses the possible role of polypeptide growth factors in the local autocrine and paracrine control of breast cancer. Growth factors TGFα, IGF-I, and PDGF and the growth inhibitory TGFβ are under regulation by estrogen and anti-estrogen control in hormone responsive breast cancer. The seventh chapter, by Robert Shiu, extends the scope of endocrine control of breast cancers to include direct effects by pituitary hormones such as IGF-II and prolactin. The thesis of this chapter is that host factors, in addition to circulating estrogen, control the progression of breast cancer. The eighth chapter, by Gary Stack and co-workers, addresses the molecular biology of the estrogen receptor itself and of a major estrogen regulated protein known as pS2. The studies of this group have begun to define the biochemical and molecular details of estrogen receptor-gene interactions. The ninth chapter, by Henri Rochefort and co-workers, examines another estrogen receptor regulated protein, a secreted cathepsin. This protein may have a role in degrading basement membrane which encapsulates the tumor. The tenth chapter, by Lance Liotta and Mary Stracke further addresses this area of tumor-host interactions. They summarize data demonstrating that metastasis requires a cancer cell to bind to the basement membrane through its receptors for laminin. Next, elaboration of various proteolytic enzymes allows the cell to chew its way through the basement membrane, and seed other bodily tissues in advanced stages of the disease. The eleventh chapter, by Erik Thompson and co-workers, extends this theme by reporting in detail on the structure of laminin and on an in vitro basement membrane model system for analysis of the invasive characteristics of tumor cells.

The twelfth chapter begins an in-depth consideration of the mouse as a model system for breast cancer. Barbara Vonderhaar describes the organogenesis of breast tissue. She points out that growth factors such as EGF and TGFα, and the expression of the EGF receptor may be critical regulators of normal development of the mouse mammary gland. The thirteenth chapter, by Gordon Hagar, characterizes the MMTV virus itself. This virus integrates into the host genome and promotes transcription of host genes. Roel Nusse, in the fourteenth chapter, further examines some of these cellular genes activated by MMTV. They appear to be a new class of oncogenes, at least one of which is closely related to a fibroblast growth factor. In the fifteenth chapter, Philippa Darbre and Roger King address the question of loss of hormonal controls during the malignant progression of mouse mammary cancer cells in vitro. They note that loss of hormonal control can occur by other means than by loss of the steroid hormone receptors. In particular, methylation of genes may make them refractory to hormonal control. Takami Oka and co-workers, in the sixteenth chapter, continues analysis of the role of

growth factors in mouse mammary tumors. He reports that salivary gland-derived EGF is a critical, host supplied hormonal factor which is necessary for spontaneous tumor formation. Finally, David Salomon and William Kidwell, in the seventeenth chapter, note that rodent mammary tumors themselves produce growth factors, in striking similarity to human breast cancer. They further note that growth factor production may be a normal function of mammary epithelial cells since a plethora of growth factors are found in milk.

We believe that, taken together, these chapters provide a comprehensive, up-to-date synthesis of current thinking on the cellular and molecular biology of breast cancer. We hope this volume provides a stimulus for future basic and clinical research in breast cancer. Perhaps, treatments of the future will be developed out of some of the newly elucidated concepts in growth control presented herein.

Marc E. Lippman, M.D.
Robert B. Dickson, Ph.D

List of Contributors

AARONSON, Stuart A., Laboratory of Cellular and Molecular Biology, National Institutes of Health, Building 37, Room 1E24, Bethesda, Maryland 20892 U.S.A.

ALBINI, A., Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, Building 6, Room 224, Bethesda, Maryland 20892 U.S.A.

ALI, I.U., Laboratory of Tumor Immunology and Biology, Building 10, Room 8B07, National Cancer Institute, Bethesda, Maryland 20892 U.S.A.

ANDRES, A.C., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern SWITZERLAND

AUGEREAU, Patrick, INSERM Unit 148, 60, rue de Navacelles, 34100 Montpellier, FRANCE

BALL, R., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern SWITZERLAND

BARTLEY, Jack C., Lawrence Berkeley Laboratory, Building 934, Berkeley, California 94720 U.S.A.

BELLOCQ, J.P., Institut de Chimie Biologique, Faculte de Medecine, 11, rue Humann, 67085 Strasbourg Cedex FRANCE

BERRY, M., Institut de Chimie Biologique, Faculte de Medecine, 11, rue Humann, 67085 Strasbourg Cedex FRANCE

CALLAHAN, Robert, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Building 10, Room 8B07, Bethesda, Maryland 20892 U.S.A.

CAPONY, Francoise, INSERM Unit 148, 60, rue de Navacelles, 34100 Montpellier FRANCE

CAVAILLES, Vincent, INSERM Unit 148, 60, rue de Navacelles, 34100 Montpellier FRANCE

CHAMBON, Pierre, Laboratorie de Genetique Moleculaire des Cucaryotes du CNRS, Unite 184 de Biologie Moleculaire et de Genie Genetique de l'INSERM, Institut de Chimie Biologique, Faculte de Medecine, 11, rue Humann, 67085 Strasbourg-Cedex FRANCE

DARBRE, Philippa, D., Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX UNITED KINGDOM

DEMBINSKI, Thomas C., Department of Physiology, Faculty of Medicine,

University of Manitoba, Winnipeg, Manitoba R3E 0W3 CANADA
DICKSON, Robert, Medical Breast Cancer Section, Medicine Branch, National Cancer Institute, Building 10, Room 12N226, Bethesda, MD 20892 U.S.A.
DI FIORE, Pier Paolo, Molecular Biology Section, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892 U.S.A.
FREISS, Gilles, Unite Hormones et Cancer (U 148) INSERM, University of Montpellier — 60, rue de Navacelles, 34090 Montpellier FRANCE
GAIRARD, B., Service Gynecologique et Obstetrical, Hopital de Hautepierre, 67091 Strasbourg FRANCE
GARCIA, Marcel, INSERM Unit 148, 60, rue de Navacelles, 34100 Montpellier FRANCE
GREEN, S., Institut de Chimie Biologique, Faculte de Medecine, 11, rue Humann, 67085 Strasbourg-Cedex FRANCE
GRONER, Bernd, Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern SWITZERLAND
HAGER, Gordon, L., Hormone Action and Oncogenesis Section, Laboratory of Experimental Carcinogenesis, National Cancer Institute, Building 37, Room 3C19, Bethesda, Maryland 20892 U.S.A.
HARRIS, Adrian, L., University Departments of Clinical Oncology and Surgery, Cancer Research Unit, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP UNITED KINGDOM
HYNES, N.E., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern, SWITZERLAND
IWASIOW, B., Department of Physiology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3 CANADA
KIDWELL, William R., Laboratory of Tumor Immunology and Biology, Division of Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Building 10, Room 5B39, Bethesda, Maryland 20892 U.S.A.
KING, Robert J.B., Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, ENGLAND
KOEHL, C., Laboratoire de Genetique Molecular des Eucaryotes du CNRS, Unite 148, de Biologie Moleculaire et de Genie Genetique de l'INSERM Institut de Chimie Biologique, Faculte de Medecine, 67085 Strasbourg, Cedex FRANCE
KOZMA, S., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern, SWITZERLAND
KRAUS, Matthias H., Molecular Biology Section, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892 U.S.A.
KUMAR, V., Institut de Chimie Biologique, Faculté de Medecine, 11, rue Humann, 67085 Strasbourg-Cedex FRANCE

KURACHI, Hirohisa, Department of Obstetrics and Gynecology, Osaka University Medical School, Osaka, JAPAN

LIDEREAU, R., Centre Rene Huguenin, 5 rue Gaston Latouche, 92211 St. Cloud, FRANCE

LIOTTA, Lance A., Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Building 10, Room 2A33, Bethesda, Maryland 20892

LIPPMAN, Marc E., Medical Breast Cancer Section, Medicine Branch, National Cancer Institute, Building 10, Room 12N226, Bethesda, Maryland 20892 U.S.A.

MARTIN, George R., Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, Building 30, Room 416, Bethesda, Maryland 20892 U.S.A.

MORISSET, Muriel, INSERM Unit 148, 60, rue de Navacelles, 34100 Montpellier, FRANCE

MURPHY, L.C., Department of Physiology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3 CANADA

MYAL, Y., Department of Physiology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3 CANADA

NICHOLSON, S.L., Cancer Research Unit and Department of Clinical Oncology, University of Newcastle upon Tyne, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, UNITED KINGDOM

NUNEZ, A.M., Institut de Chimie Biologique, Faculte de Medecine, 11, rue Humann, 67085 Strasbourg-Cedex FRANCE

NUSSE, Roel, Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, THE NETHERLANDS

OKA, Takami, Laboratory of Molecular and Cellular Biology, National Institute of Diabetes, Digestive and Kidney Diseases, Building 8, Room 304, Bethesda, Maryland 20892 U.S.A.

OKAMOTO, Shigeru, Department of Pathology, Osaka University Medical School, Osaka, JAPAN

PIERCE, Jacalyn H., Molecular Biology Section, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892 U.S.A.

PONGLIKITMONGKOL, M., Laboratorie de Genetique Molecular des Eucaryotes du CNRS, Unite 184, de Biologie Moleculaire et de Genie Genetique de l'INSERM, Institute de Chimie Biologique, Faculte de Medecine, 67085, Strasbourg, Cedex FRANCE

REDMOND, S., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern, SWITZERLAND

REICH, R., Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, Building 30, Room 414, Bethesda, Maryland 20892

REICHMANN, E., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern, SWITZERLAND

RENAUD, R., Service Gynecologique et Obstetrical, Hôpital Central, 67091 Strasbourg, FRANCE

RIO, M.C., Institut de Chimie Biologique, Faculté de Medecine, 11, rue Humann, 67085 Strasbourg-Cedex FRANCE

ROBERTS, M., Yale University, Department of Biology, Kline Biology Tower, New Haven, Connecticut 06510

ROCHEFORT, Henri, Unite d'Endocrinologie Cellulaire et Moleculaire, (U 148) INSERM and University of Montpellier, 60, Rue de Navacelles, 34100 Montpellier FRANCE

SALOMON, David S., Laboratory of Tumor Immunology and Biology, Division of Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Building 10, Room 5B43, Bethesda, Maryland 20892 U.S.A.

SAURER, S., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern, SWITZERLAND

SCHMITT-NEY, M., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern SWITZERLAND

SCHONENBERGER, C., Ludwig Institute for Cancer Research, Bern Branch, Inselspital, CH-3010 Bern SWITZERLAND

SHIU, Robert P.C., Department of Physiology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3 CANADA

STACK, G., Institut de Chimie Biologique, Faculte de Medecine, 11, rue Humann, 67085 Strasbourg-Cedex FRANCE

STAMPFER, Martha R., Lawrence Berkeley Laboratory, Building 934, Berkeley Laboratory, Building 934, Berkeley, California 94720 U.S.A.

STRACKE, Mary L., Laboratory of Pathology, National Cancer Institute, Building 10, Room B1B47, Bethesda, Maryland 20892

THOMPSON, E.W., Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, Building 30, Room 414, Bethesda, Maryland 20892

TSUTSUMI, Osamu, Department of Obstetrics and Gynecology, Faculty of Medicine, Tokyo University, Tokyo JAPAN

TSUYUKI, D., Department of Physiology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3 CANADA

VIGNON, Francoise, ISERM Unit 148, 60, rue de Navacelles, 34100 Montpellier FRANCE

VONDERHAAR, Barbara K., Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Building 10, Room 5B56, Bethesda, Maryland 20892 U.S.A.

1. Human mammary epithelial cells in culture: differentiation and transformation

Martha R. Stampfer, and Jack C. Bartley

1. Introduction

The development of human mammary epithelial cell (HMEC) tissue culture systems has permitted these cells to be examined under controlled experimental conditions. Since recourse to *in vivo* studies is not possible with humans, the fidelity with which the culture system reflects the *in vivo* situation is particularly important, if the information obtained *in vitro* is to have meaning for understanding the normal and perturbed functions of the human breast. We describe here our results in developing a system for long term growth of human mammary epithelial cells in monolayer culture [1–4]. While this system is still far from providing an accurate representation of the many different stages of functional differentiation which may occur in the breast, it is a useful beginning to understand control of normal differentiated states, and to examine the multiple steps in the progression from normal to malignantly transformed cells.

2. Processing of human mammary cells for culture

Unlike most other human organs, large quantities of normal mammary epithelial tissues are readily available as discard material from the surgical procedure of reduction mammoplasty. Although the fat content of this tissue is greatly increased, the epithelial cell content appears similar to that found in smaller breasts: pathology evaluation indicates no abnormalities in the majority of cases, with mild or moderate fibrocystic disease present in a significant fraction of specimens. Thus, tissue acquisition is not at all limiting for experimental studies of normal HMEC, and specimens from many individuals of all ages are available. However, unlike the situation with most other organs and with animal model systems, reduction mammoplasty tissue can be obtained only from non-pregnant, non-lactating individuals, and therefore represents from the outset a relatively uninduced state of funtional differentiation. Human breast tissues are also readily available from biopsies and mastectomies (including both the tumor and non-tumor areas of the removed

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

breast). These surgical procedures provide atypical and malignant HMEC, although primary tumors are a very heterogeneous population and the cells which grow out in culture may not include the most invasive, malignant cell type [5].

Our method for processing of the surgically derived material for tissue culture involves, first, coarse dissection of the specimen to separate the epithelial material from the fat and stroma, followed by enzymatic digestion with collagenase and hyaluronidase to yield nearly pure epithelial clumps. This material is then passed through filters of fixed pore size to separate the epithelial clumps (termed organoids) from the single cells and small clumps of the digested stromal tissue [1, 4]. Due to the large quantities of starting discard surgical material, especially from the reduction mammoplasties, frozen storage of multiple ampoules of the mammary organoids is possible from each individual specimen donor. Consequently, repetition of experiments with the same person's cell pool can be conducted over an indefinite time span, and questions can be asked about individual differences. Fibroblastic cells from the digested filtrate can also be stored frozen, and are useful for comparisons of epithelial versus fibroblastic cell behavior from the same individual.

3. Growth capacity of HMEC

For HMEC to achieve widespread usage in the study of human cellular physiology and carcinogenesis, it is necessary that large quantities of actively proliferating cells be available, and that the culture systems should be modulatable to reflect *in vivo* differentiated states. We have developed culture systems that provide the first of these prerequisites—long term active proliferation; work on development of modulatable differentiated states is still in progress. Our laboratory has utilized two main media to produce active HMEC growth (Table 1). Our original medium, MM [2], contains fetal bovine serum, conditioned media from other cell lines, and several growth factors. In it, cells from premenopausal tissues proliferate rapidly for 3–5 passages, with doubling times of 18–30 hrs. They maintain a cobblestone epithelial morphology until senescence, when the cell population becomes more heterogeneous, with large, vacuolated cells. Approximately 40 such specimens have been examined. The 7 post-menopausal reduction mammoplasty specimens obtained have shown active growth for fewer subcultures. The 40 tumor-derived specimens examined have displayed greater heterogeneity in growth pattern, ranging from equal to that of the normal cells, to poor growth even at second passage.

HMEC have also been grown in a serum-free medium, MCDB170, developed specifically to promote their proliferation [3]. Thus far, 23 specimens from 19 individuals have been tested. In MCDB170, cells initially show rapid growth for 2–3 passages, followed by what appears to be terminal

Table 1. Contents of HMEC cell culture media.

MM Medium	
Ham's F12	30%
Dulbecco's Modified Eagles Medium (DME)	30%
Fetal Bovine Serum	0.5%
Conditioned Media from Human Cell Lines*	
Adult Bladder (Hs767Bl) and/or	
Fetal Intestine (fHsInt)	30%
Adult Myoepithelial (Hs578Bst)	9%
Insulin	10 μg/ml
Hydrocortisone	0.1 μg/ml
Epidermal Growth Factor (EGF)	5 ng/ml
Triiodothyronine	10^{-8} M
Estradiol	10^{-9} M
Cholera toxin	1 ng/ml
MCDB170 Medium	
Basal MCDB170	100%
Insulin	5 μg/ml
Hydrocortisone	0.5 μg/ml
Epidermal Growth Factor (EGF)	10ng/ml
Ethanolamine	10^{-4} M
Phosphoethanolamine	10^{-4} M
Transferrin	10 μg/ml
Bovine Pituitary Extract	70 μg/ml
(Isoproterenol)	10^{-5} M

*Cells fed with 1:1 Ham's F12/DME, 5% fetal bovine serum, 5 μg/ml insulin.

differentiation of the majority cell population. The initial cobblestone epithelial morphology gradually changes to produce large, flat, striated cells with irregular edges, and limited proliferative capacity. The small subpopulation which maintains the typical epithelial cobblestone morphology retains active proliferation (doubling times of 18–30 hours), reestablishing itself as the uniform cell type within 1–2 passages. For some individual specimen donors, a cAMP stimulator is required during this 'self-selection' period to maintain this actively growing epithelial population. Depending upon the individual, rapid growth continues after this selection period for another 6–16 passages, yielding a total of about 45–70 population doublings. This long-term growth in MCDB170 permits frozen storage of large cell pools at passages 5–8, which consequently allows use of a standard cell population for numerous experiments. At senescence, the cells maintain viability and some mitotic activity, but show no net gain in cell number. Increased vacuolization and heterogeneity in cell size are observed, while the cells retain the smooth-edged cobblestone epithelial appearance. Since MCDB170 was originally formulated by use of clonal assays, it is also capable of supporting clonal growth of HMEC in

the absence of any additional factors or feeder layers, with colony forming efficiencies of 10–50%.

We have carefully followed growth to senescence of the 23 different specimens placed in MCDB170 during the past four years (Figure 1). In general, these data indicate that, even given the vagaries of cell culture, cells from a given individual tend to senesce around the same passage, regardless of when or if they have been stored frozen. Since the MCDB170 medium was optimized using cells from only one individual (specimen 161), it is possible that the differences observed in the number of population doublings to senescence may simply reflect interindividual differences in optimal growth requirements relative to the nutritional formulation of this medium. We have not seen any significant differences in cell morphology or growth behavior relative to donor age or tissue pathology. However, there are consistant individual differences in the morphology of the cultured cell populations, particularly in the amount of cell-cell contact and patchiness of the growing cells, and in the degree of cell alignment of the cells in patches, which, at the extreme, produces a swirling, 'thumbprint' morphology (Figure 2).

The data in Figure 1 also provide an indication of the quantities of HMEC that can be generated from a single individual, as well as the apparent absence of any viability loss due to frozen storage. For specimens 161 and 184, frozen cells from the same individual have been repeatedly examined. These include: (1) different ampoules of the originally frozen primary organoids (typically 15–50 ampoules are generated from reduction mammoplasties); (2) preselection cell pools frozen at second passage ($\sim 2-3 \times 10^7$ total cells generated/primary ampoule); (3) post-selection cell pools frozen at 4–18 passage (multiple pools of $>2 \times 10^7$ cells each possible at 5–8 passages). Consequently, a virtually unlimited supply of normal, proliferating HMEC can be obtained.

Of particular interest is the fact that all the samples which have thus far been examined always do senesce. This result is in striking contrast to that seen with most rodent model systems, where spontaneous transformation to immortality is often observed, and emphasizes the stability of human cells in culture. While these data do not rule out the possibility that spontaneous

---

Figure 1. Growth capacity of HMEC in MCDB170 medium. Primary cultures obtained from reduction mammoplasties (top two rows) and mastectomies (T = tumor tissue, P = non-tumor tissue from tumor-bearing breast) were initiated and subcultured as described [4], with about 8-10 fold amplification per passage. A few of the reduction mammoplasty specimens were grown in MCDB202, a closely related medium [3]. Bottom horizontal lines indicate passage level of initiation of frozen ampoules. Top horizontal lines indicate passage level of no net increase in cell numbers. Internal horizontal lines indicate that cultures were frozen and reinitiated at that passage. Continuous hatched lines indicate cells derived from the same "selection". Asterisks indicates cells exposed to a cAMP stimulator during selection. For specimen 184, × indicates cultures initiated from the same primary ampoule but taken through selection with three different cAMP stimulators (cholera toxin, isoproterenol, prostaglandin E_1). In a few cases, indicated by ⊟, the tumor cultures were grown in MM in primary culture.

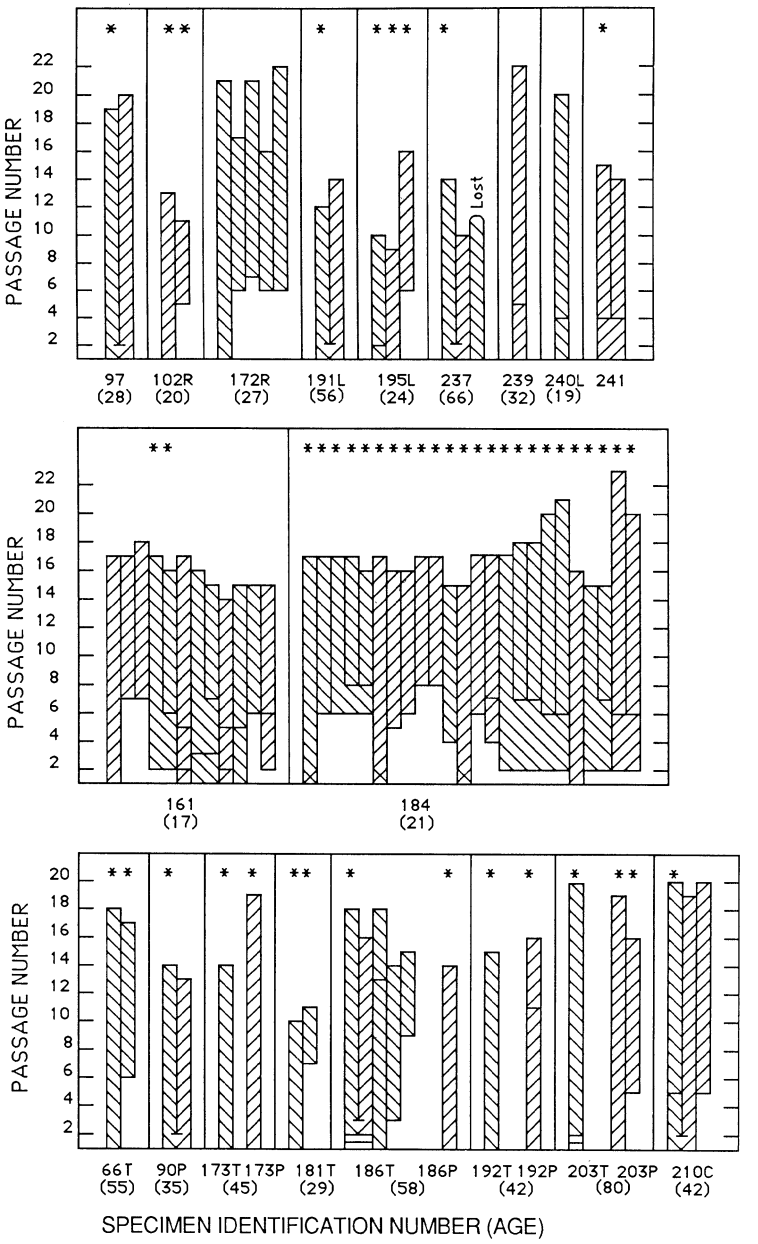

PASSAGE NUMBER
22
20
18
16
14
12
10
8
6
4
2
Lost
97 (28)
102R (20)
172R (27)
191L (56)
195L (24)
237 (66)
239 (32)
240L (19)
241
161 (17)
184 (21)
66T (55)
90P (35)
173T
173P
181T (29)
186T (58)
186P
192T (42)
192P
203T (80)
203P
210C (42)
SPECIMEN IDENTIFICATION NUMBER (AGE)

Figure 1.

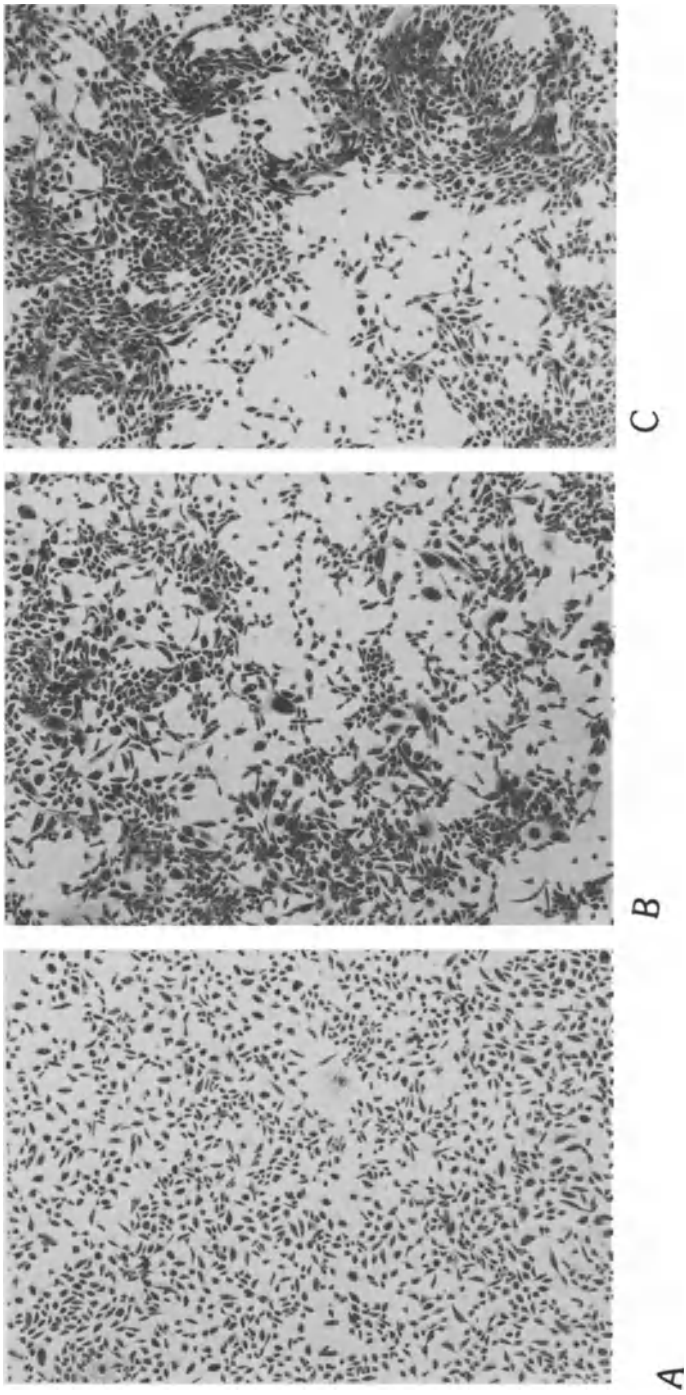


Figure 2.

transformation to immortality may occur, they do provide evidence that such an event must be extremely rare in these normal human epithelial cells.

4. Identification of cultured HMEC

The mammary epithelial nature of the cells cultured in MCDB170 and MM can be demonstrated with a variety of markers. Morphologically, in addition to the cobblestone epithelial appearance in the light microscope, the cells examined in the electron microscope show the epithelial characteristics of tonofilaments, desmosomes, and microvilli [1]. Indirect immunofluorescence and immunoperoxidase assays have shown that the cells are 100% positive for epithelial keratins and the mammary epithelial specific enzyme, thioesterase II [6, 7], and that they have the epithelial pattern of powdery cell-associated fibronectin [8]. The mammary epithelial milk fat globule antigens are also expressed on some of the cells; these will be discussed later as markers of differentiation and transformation. Most of these markers are stable with continued culture in MCDB170, except that the cells undergoing terminal differentiation during the selection period display large quantities of fibrillar fibronectin. This property, as well as their striated appearance, suggests that these cells may be differentiating along the pathway of myoepithelial cells. This possibility is currently being examined by assaying for a larger number of antigenic markers. All the reduction mammoplasty derived cell cultures tested have shown a normal diploid karyotype, including both pre- and post-selection cells in MCDB170 [3, 9].

5. Metabolism of carcinogens

One of the main questions we wished to address with a human mammary epithelial system was the effect of potential carcinogenic agents. Ultimately, the problem is to resolve what agents, in what combinations, are capable of causing carcinogenesis in human epithelial cells in vivo. However, in practice, with human tissues we are limited to experimentation with cells in culture conditions, and the ensuing uncertainty about the extent to which the cells in culture reflect what may occur *in vivo*. In an effort to approximate what might be occuring during carcinogenesis, one of our goals with the human mammary epithelial cell system was to use carcinogens to obtain transformation *in vitro*, and then to investigate the effects of different carcinogens, and possible modifiers of carcinogenesis, on this transformation. The data obtained from these experiments with human cells could complement the data obtained

Figure 2. Morphology of normal HMEC grown in MCDB170. Giemsa stained cultures from (A) 184, passage 7; (B) 172, passage 13; (C) 161, passage 9.

from animal model systems, where *in vivo/in vitro* comparisons can be performed, but where the uncertainty rests in the extrapolation of data from one species to another.

For our initial studies we chose to examine the effects of the chemical carcinogen, benzo(a)pyrene (BaP), because: (1) it belongs to the class of compounds, polycyclic aromatic hydrocarbons, known to be excellent inducers of mammary cancer in rodents [10]; (2) previous research has done much to characterize the metabolism of BaP in rodent and human systems; (3) it is one of the major environmental pollutants resulting from the incomplete combustion of fossil fuels and organic material. BaP is an inactive procarcinogen, requiring a series of metabolic steps for conversion to the active, ultimate carcinogenic form, the 7,8-diol-9,10-epoxide [11]. It has been thought that formation of adducts between this diol-epoxide and DNA may be a necessary step for carcinogenesis. Since the extent and pattern of BaP metabolism can vary greatly among species, as well as among different individuals and cell types within one specie [12, 13], we examined the rate and route of BaP metabolism in human mammary epithelial and fibroblastic cells from many specimen donors [14–16]. Our results indicate that the HMEC readily metabolize BaP through the pathways leading to the 7,8-diol-9,10-epoxide, and form adducts with the deoxyguanosine of DNA at levels similar to that found in cells transformed by BaP. In contrast, the same concentration of BaP given to fibroblast cells from the same individual yields a much slower rate of metabolism, mainly through pathways that do not yield the diol-epoxide, and a much lower production of DNA adducts. Comparisons of interindividual BaP metabolite yields from 22 different specimen donors [16] show around a 5-fold range of values; the data also suggest that adduct production is higher in cells derived from tumor tissues compared to reduction mammoplasties.

Additionally, we have found that culture conditions can significantly influence the metabolites formed. Normal cells grown for 8–10 passages in MCDB170 showed a 10-fold reduction in water soluble conjugates and a 3-fold reduction in organosoluble metabolites compared to second passage cells; however, the amount of DNA adduct formation was unchanged. These data suggest a loss of specific enzyme activities as a result of prolonged life in culture. When the metabolite yield from freshly fed, subconfluent second passage cells was compared to cell populations that were confluent and freshly fed, or confluent and unfed for the prior 72 hours (and thus in overly acidic media), it was found that the less optimal culture conditions led to an overall decrease in metabolite yield specifically involving the organosoluble products. These results point out the extent to which data obtained *in vitro* can vary depending upon the particulars of the experimental conditions, and emphasize again the need for well-characterized and optimized culture conditions.

Preliminary studies [Leadon, S.A., Stampfer, M.R., Bartley, J.C., unpublished] have been initiated to determine the possible role of oxidative damage resulting from BaP metabolism (as potentially distinct from damage due to the bulky diol-epoxide-DNA adducts). To test this possibility, we have

utilized a monoclonal antibody specific to thymine glycols on DNA [17] to measure the induction of this product of free radical oxidation of DNA. We have found that the extent of thymine glycols formed exceeded that from the bulky adducts. Superoxide dismutase provided similar protection against both the lethal effects of the BaP and the induction of oxidative damage, while having no effect on BaP metabolites or adducts. These results suggest a possible role of oxidative damage in carcinogenesis, and the need for further investigations.

6. Transformation of HMEC in culture

The development of a system for transformation in vitro of human epithelial cells could provide an experimental means to systematically observe the progressive changes which occur during carcinogenesis. However, there are two major problems in accomplishing such a goal. One is that transformation of any human cells in culture has been extremely difficult. Unlike most rodent model cell systems, spontaneous transformation to either immortality or malignancy does not occur; even transformation induced by tumor viruses or oncogenes, irradiation, or chemical carcinogens is rare [18, 19]. Differences between human and rodent cells in chromosomal stability, the action of tumor viruses and oncogenes, and possible tumor suppressor mechanisms, may account for this dramatic difference. The other problem encountered lies in identifying and quantifying transformed human epithelial cells. Ideally, markers which permit easy selection or detection of small numbers of transformed cells within a large cell population are most useful for *in vitro* assays. However, unlike fibroblasts, transformed human epithelial cells do not show the morphological changes that yield easily quantifiable foci. Unlike rodent systems, there is no syngeneic host in which to test for tumorigenicity. Consequently, markers for malignant transformation are approximations which are often difficult to quantify.

Some markers that have been useful in detecting abnormal human epithelial cells in culture include anchorage independent growth (AIG), tumorigenicity in nude mice, altered antigenicity or lectin binding, reduced requirements for growth factors, escape from TGF-β or Ca^{++} induced terminal differentiation, and an extended lifespan or escape from senesence [6, 18–27]. In our original studies, we decided to use an extended lifespan (EL) in culture as our initial screen for carcinogen-induced changes. This marker is convenient because of its strong selective ability, although its correlation to events *in vivo* is unclear. It is possible that the extended proliferative potential commonly seen *in vitro* may be related to the hyperplasia commonly seen *in vivo* in abnormal breast and other epithelial tissues; this hyperplasia is likely the result of some alterations in normal growth control. Transformation to an indefinite lifespan is also observed in some human epithelial tumor derived (but not normal) cells in culture, so this marker is also associated with car-

cinogenesis. However, such transformation to immortality is usually rare, and more likely to be found in cells from metastatic tumors, rather than primary tumor tissues, particularly in breast cancer. Thus although transformation to established cell lines is an extremely useful tool for *in vitro* transformation, providing the most convenient means of further cellular and molecular characterization, the cells thereby selected may represent only a limited spectrum of possible transformation pathways.

The first series of experiments we performed utilized cells from one individual, specimen 184, normal reduction mammoplasty tissue from a 21 year old women [28]. Medium MM was used because the HMEC have only a 15–25 population doubling lifespan in it, permitting early detection of cells displaying an extended lifespan. Actively proliferating primary cultures received two or three exposures of 1 μg/ml of BaP. These flasks were then subjected to repeated partial trypsinizations (PT), wherein about 50% of the cells in the flask are removed, and the remaining cells allowed to regrow. After some PT, the removed cells were seeded into secondary cultures and their fate followed. The results of three such experiments, using three different primary ampoules, are outlined in Figure 3. In all three experiments, populations of EL cells and changes in morphology were found in the cultures exposed to BaP. These treated cells grew both for longer times in the primary cultures, and for more subcultures. Widespread heterogeneity in growth patterns and morphology were displayed by these EL cultures, both among the different experiments and also among cells seeded from the same or different PT within one experiment.

Although numerous different EL cultures were generated by the exposure to BaP, these eventually senesced after about a 2–3 fold increase in population doublings compared to the control cells grown in MM. However, in two separate instances cell growth continued, leading to two different immortally transformed cell lines, 184A1 and 184B5. The emergence of these lines is outlined in Figure 4. In the case of 184A1, a single EL patch at passage 5 gave rise to a uniformly growing EL population designated 184Aa. As 184Aa senesced around passage 9, another cell population with a distinctive morphology (small cells growing as singlets) was observed (Figure 5A). These cells, designated 184A1, have maintained indefinite growth; they have been carried to passage 101 in MCDB170 with approximately 20 fold amplification per passage and a 24–36 hour doubling time. We have subsequently placed frozen stocks of 184Aa at seventh and eighth passage back into MM and

---

Figure 3. Growth pattern of HMEC exposed to BaP in MM. Primary cultures of specimen 184 were exposed to 1 μg/ml BaP at the indicated times in three separate experiments (designated A, B, C) [28]. The fate of both the subcultured cells and the cells in the primary flasks was then observed. Since several dishes were plated at each subculture, and if growing, their lineages followed independently, more than one kind of growth pattern could be observed at a given passage level. In experiment 184C, cholera toxin was inadvertantly omitted from the medium until 22 days after seeding (passage 5 of subculture C1, 4 of C2, 3 of C3).

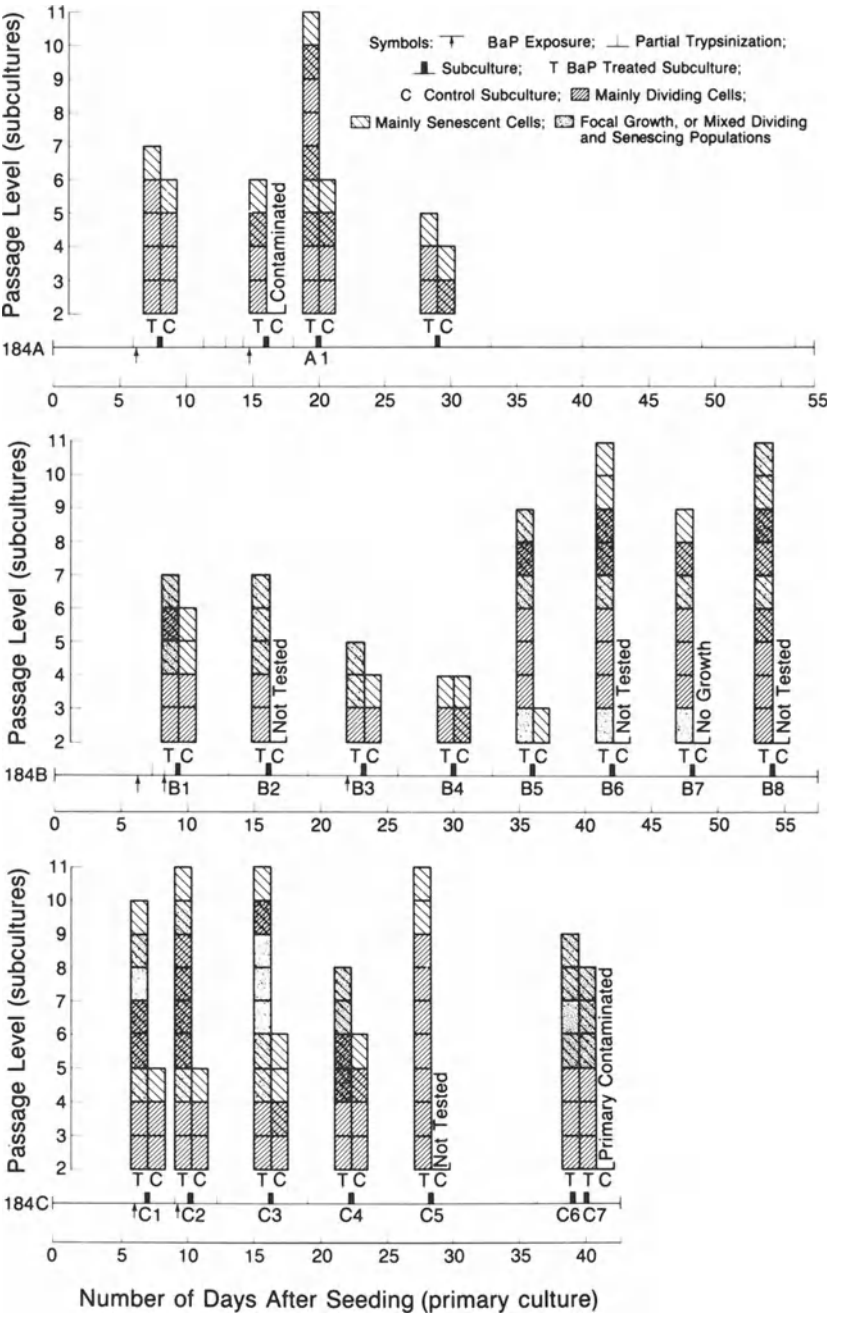

Symbols: BaP Exposure; Partial Trypsinization;
Subculture; T BaP Treated Subculture;
C Control Subculture; Mainly Dividing Cells;
Mainly Senescent Cells; Focal Growth, or Mixed Dividing and Senescing Populations
Passage Level (subcultures)
184A
A1
Contaminated
184B
B1
B2
B3
B4
B5
B6
B7
B8
Not Tested
No Growth
184C
C1
C2
C3
C4
C5
C6
C7
Primary Contaminated
Number of Days After Seeding (primary culture)

Figure 3.

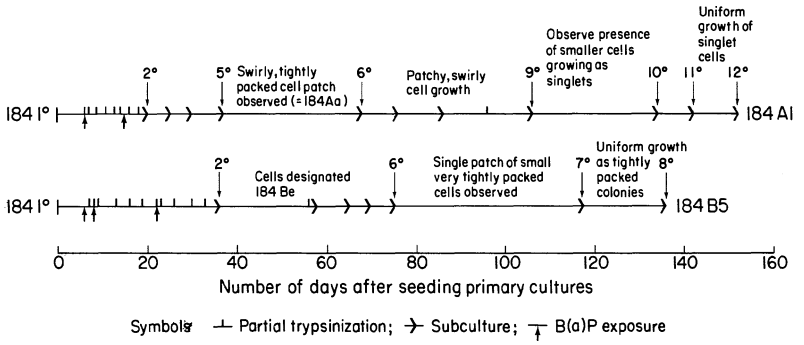


Figure 4. Development of established cell lines following BaP exposure of HMEC specimen 184.

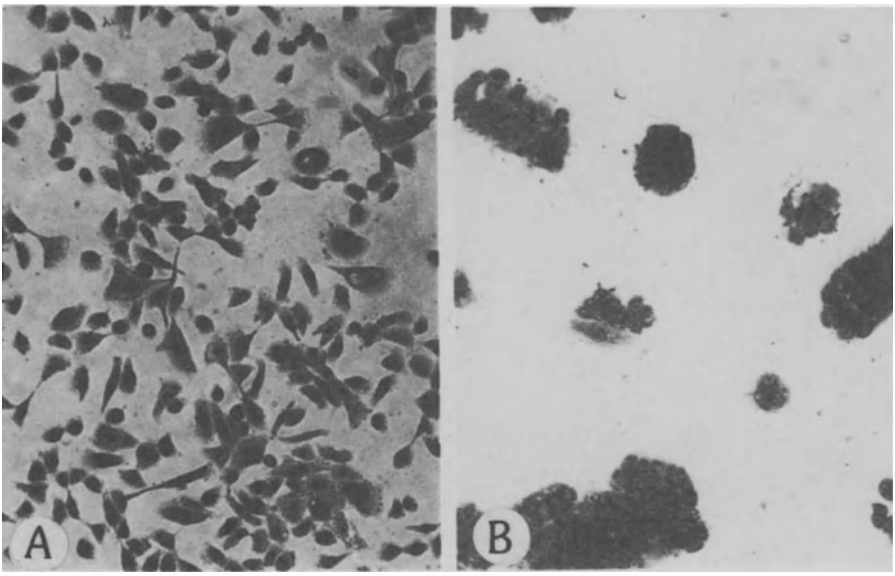


Figure 5. Giemsa stained established cell lines grown in MCDB170. (A) 184A1, passage 42; (B) 184B5, passage 11.

MCDB170, and have not seen an immortal population emerge. Since early passage 184A1 cells all show the same few chromosomal abnormalities, the immortalization event presumably took place in a single cell during the original eighth or ninth passage culture. In the case of 184B5, an EL culture designated 184Be, which displayed three areas of focal growth in second passage, grew uniformly actively to passage 6, when focal growth reappeared. One small patch with a very distinctive tightly packed morpholgy (Figure 5B) was noticed, and maintained indefinite growth potential. This line, 184B5, has been carried to passage 100 in MCDB170, with about 30 fold amplification per passage and a 24 hour doubling time. Subsequent seeding of a fourth passage frozen stock of 184Be did not lead to emergence of immortal cells.

The established cell lines 184A1 and 184B5 have been characterized by a variety of criteria (see Table 2 and below). As part of this characterization, it was observed that neither line formed tumors in adult or newborn nude mice, and that little or no anchorage independent growth was displayed. Therefore, these lines probably represented immortal but not malignant transformants. Attempts were then made to determine if these lines could be further transformed to malignancy. Our initial studies, as decribed below, have used tumor viruses and oncogenes to induce malignant transformation. While the resulting transformants are valuable in providing fully malignant HMEC populations to examine, it is important to remember that their correlation to malignant transformation *in vivo* is again questionable. Further experiments are ongoing to induce malignant transformation without the use of known tumor viruses and oncogenes.

In the first set of experiments [Stampfer, M.R., Arnstein, P., unpublished], 184B5 cells at passage 21 were exposed to Kirsten sarcoma virus (KSV) with a baboon pseudotype [23]. Injection of the infected passage 23 cells into nude mice produced tumor in 100% of the exposed mice. These tumors were classified as poorly differentiated epidermoid carcinoma, and grew to 2–5 cm

Table 2. Characterization of normal and transformed HMEC

Specimen identity	% AIG[a]	Tumors in nude mice[b]	Karyology[c]	Growth in DME + 10% Fetal bovine serum
Normal				
184	≤0.001	–	normal diploid	–
Extended Life				
184Aa	≤0.001	–	normal diploid	–
184Be	≤0.001	–	normal diploid	–
Immortal				
184A1	≤0.001	–	near diploid	–
184A1N4	≤0.001	–	near triploid	–
184B5	0.001–0.008	–	near diploid	–
Oncogene Exposed				
184A1N4-HSV	0.003	+/–	NT[d]	+
184A1N4-T	0.006	–	NT	+
184A1N4-mos	≤0.001	+/–	NT	+
184A1N4-HSV-mos	0.002	+	NT	+
184A1N4-HSV-T	0.028	++	near tetraploid	+
184B5-KSV	≤0.001	+	NT	NT

[a] 10^5 cells where seeded/5 ml methocel/60 mm dish as described [7, 29]. Colonies ≥100 μ diameter were counted after three weeks.
[b] 5×10^6 cells were injected intraperitoneally/mouse as described [23].
[c] 50–100 interphases were examined/cell type by Giemsa staining as described [23].
[d] NT = not tested

in 1–3 months. The tumors then remained without further growth. Cells recovered from the tumors show the same morphology as the initial infected population, but have not yet been further characterized except to show that they have the same or less AIG than the uninfected 184B5 cells.

In a separate series of experiments, 184A1N4, a subclone of 184A1, was exposed to infection with Harvey sarcoma virus (HSV), and/or retroviral vectors carrying the mos or the SV40-TT oncogene [29]. 184A1N4 was originally selected on the basis of its slightly reduced nutritional requirements (growth in the absence of the conditioned medium in MM), and was subsequently shown to have a near triploid karyology. In this case, exposure to all of these oncogenes enabled 184A1N4 cells to grow in an extremely selective medium (DME +10% fetal bovine serum). However, only weak tumorigenicity was conferred by exposure to HSV or mos alone, and none by the exposure to the T oncogene. Transformants bearing both the ras oncogene and T oncogene produced tumors in 100% of injected nude mice. These were undifferentiated carcinomas of 2–3 cm by 1 month, which proceeded to grow and kill the animals. Transformants containing the ras and mos oncogenes also produced progressively growing tumors in 3 of 8 injected nude mice.

7. Characterization of the HMEC transformed in culture

We have used our *in vitro* system of transformed HMEC to compare the properties of the normal parental cells, the EL cells, the two immortal cell lines, and the cell lines transformed with oncogenes, all from the same individual. The origin of the cell lines from the parental 184 cells was first demonstrated by comparing their profile of seven different polymorphic enzymes; all three cell types had an identical profile [28]. The mammary epithelial nature of the cell lines was demonstrated by the presence of epithelial specific keratins, an epithelial pattern of powdery cell associated fibronectin (Figure 6), and expression of human mammary milk fat globule antigens (see Table 4).

We have assayed for three properties associated with malignant transformation: AIG, tumorigenicity in nude mice, and karyotypic abnormalities [30]. Table 2 summarizes some of these results. As has been observed in other epithelial systems, there was not an exact correlation between expression of AIG and tumorigenicity, although the most tumorigenic transformants showed the greatest amount of AIG. Normal diploid karyotypes were displayed by the parental 184 cells and the two EL cultures examined. Early passage (11th) cultures of both 184A1 and 184B5 were near diploid, each containing a set of chromosomal aberrations present in all the cells analyzed, thus confirming their clonal origins. In the case of 184A1, these consisted of 2 deletions and a monosomy; for 184B5, these consisted of several rearrangements, duplications, and deletions. The 11th passage 184B5 populations also contained cells with chromosomal markers in addition to the stem cell aberrations. The

karyotypes of 184A1 and 184B5 have been followed as a function of passage in culture. Examination of uncloned populations after approximately 150 population doublings has shown the acquisition of additional chromosomal abnormalities. However, these involved only 3–5 chromosomes, and were all identifiable. Thus, compared to human epithelial cell lines obtained from tumors, or immortalized with oncogenes, these lines show a relatively stable karyotype. The karyotype of only one of the oncogene exposed cell lines been determined [29], namely the cells obtained from a nude mouse tumor formed from 184A1N4-HSV-T. Surprisingly, these cells show few differences from the parental 184A1N4 cells, which are near triploid, containing only one translocation in addition to the 184A1 set of chromosomal aberrations. 184A1N4-HSV-T are near tetraploid, lacking the 184A1N4 translocation, and containing no additional clonal chromosomal changes.

We have begun to test these different HMEC populations for their response to and synthesis of various growth factors. The 184A1 and 184B5 lines have been compared to the parental 184 cells for their requirements of the growth factors present in the MCDB170 medium (Table 3). While several differences are observed, we have not yet determined the causes of these varying nutritional requirements. The observed variation in the effect of EGF has been followed more carefully. Normal 184 HMEC seeded in mass culture at 7th passage in MCDB170 plus isoproterenol and minus EGF, showed only a slight decrease in doubling time compared to the plus EGF controls, and senesced only one passage earlier. However, cells seeded at clonal density displayed almost no growth minus EGF (data not shown). In contrast, both 184A1 and 184B5 seeded in mass culture without EGF showed severe growth inhibition; only a few areas of growing cells were observed. Continued passage of this selected population eventually (within about 5–6 passages) produced cell populations capable of good growth independent of EGF. Preliminary experiments [31] have examined these cells, as well as the oncogene exposed cell lines, for the presence of mRNA for the EGF receptor and TGF-α. Large amounts of both mRNA species were found in all the examined cells, including the normal 184. This production of TGF-α by the normal cells may acount for the great difference seen in their requirements for EGF in mass culture vs. clonal growth.

The nutritional requirements of all the 184A1N4 oncogene exposed cells are much less stringent than the cell lines or normal 184, since they are capable of growth in media consisting of just DME and 10% fetal bovine serum. This difference may be due to both an absence of specific nutritional requirements, as well as an escape from growth factor inhibitors present in the serum. A possible role for TGF-β inhibition in this result has been tested by looking at the effect of TGF-β on HMEC growth (Figure 7). As has been described for other human epithelial cell systems [26, 27], TGF-β inhibits normal cell growth. However, the uncloned 184B5 cells maintain growth, although with somewhat decreased doubling times, even in 30 ng/ml of

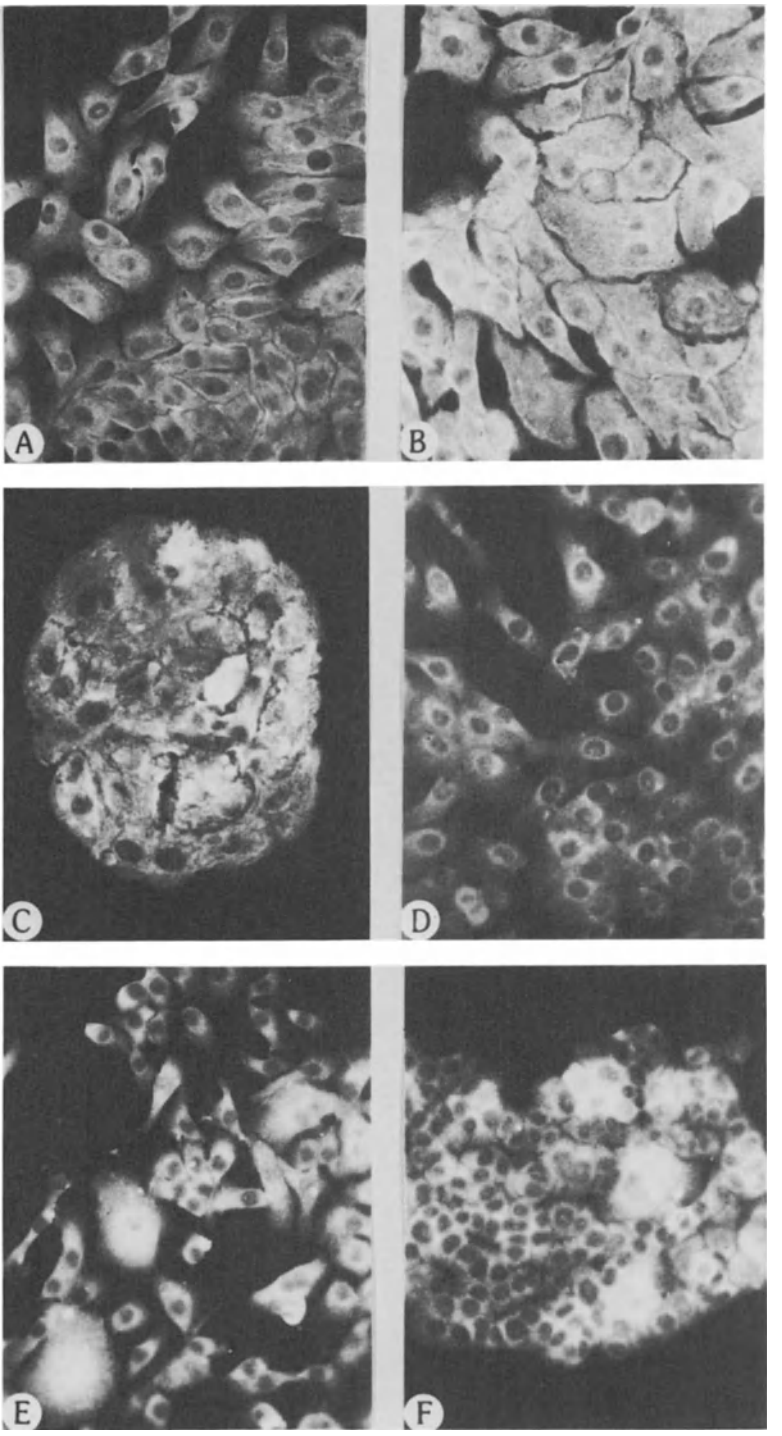

A
B
C
D
E
F

Table 3. Growth factor requirements of normal and transformed HMEC in MCDB170

	Percentage of control cell growth		
Medium	*184*	*184A1*	*184B5*
Complete MCDB170	100	100	100
Minus I[a]	24	14	100
Minus HC	40	62	27
Minus EGF	43	13	62
Minus BPE	10	29	4
Plus IP	133	98	136
Plus IP minus I	65	11	35
Plus IP minus HC	48	82	25
Plus IP minus EGF	114	20	16
Plus IP minus BPE	20	21	22

[a] Abbreviations used: I = insulin, HC = hydrocortisone, EGF = epidermal growth factor, BPE = bovine pituitary extract, IP = isoproterenol.
Normal 184 cells were tested at 11th passage, 184A1 and 184B5 were tested between 17 and 20th passage. Cells grown in complete MCDB170 were subcultured into duplicate 35 mm dishes (5×10^4 dish) in the indicated medium. When the control cultures were subconfluent or just confluent, all the cultures were trypsinized and the cells counted by hemocytometer.

TGF-β. The uncloned 184A1 population, while still sensitive to TGF-β inhibition, also contains a subpopulation resistant to TGF-β (including the 184A1N4 subclone). Thus the difference in TGF-β response alone can not explain the selective growth of the oncogene exposed cells. We are currently exploring the possible use of such growth in selective media as a possible screen for progressive transformation changes of the 184A1 and 184B5 cells further exposed to chemical carcinogens.

8. Expression of differentiated properties of normal and transformed HMEC

The mammary gland *in vivo* is capable of expressing many widely different states of functional differentiation; puberty, normal post-menarch cycling, pregnancy, lactation, involution, and post-menopause. Unlike certain other epithelial systems, e.g., keratinocytes, the pathway of HMEC terminal differentiation does not necessarily coincide with expression of properties of functional differentiation; some functionally differentiated states may never occur for any of a given individual's HMEC. Also, in practice, human tissues available as surgical discard material are almost always from either post-menarch cycling or post-menopausal non-cycling glands (which may, or may

Figure 6. Indirect immunofluorescence assay of normal and transformed HMEC specimen 184. (A, B, C) Binding of monoclonal antibody to stratum corneum keratin; (D, E, F), binding of monoclonal antibody to fibronectin. (A, D) normal 184; (B, E) 184A1; (C, F) 184B5.

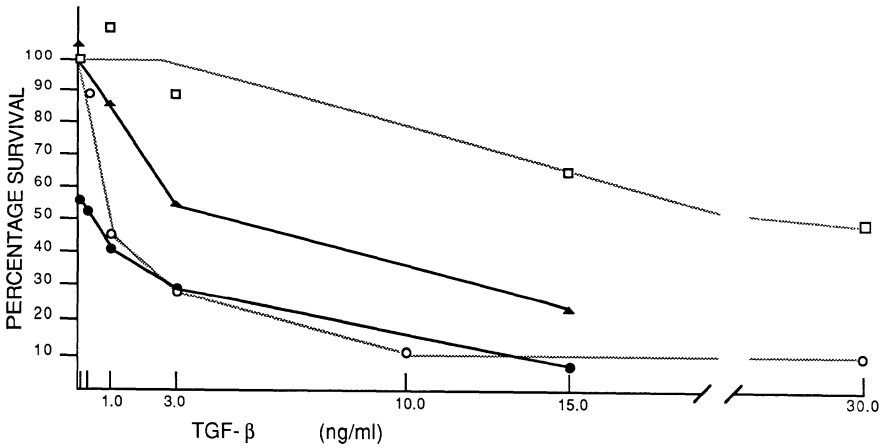


Figure 7. Effect of TGF-β on growth of normal and transformed HMEC specimen 184. Cells grown in MCDB170 were subcultured into duplicate 35 mm dishes (4×10^4/dish) containing the indicated concentration of TGF-β. When control cultures were subconfluent or just confluent, all the cultures were trypsinized and the cells counted by hemocytometer.
▲ = 184, passage 8
□ = 184B5
○ = 184A1
● = 184Aa

not have pregnancy/lactation histories). Consequently, examining HMEC in culture for differentiated properties is not simple. The advantage is that so many different markers of differentiated states exist; the disadvantage is that there are so many different differentiated states, and the tissues obtained were not (and may never have been) expressing what is considered the most functionally differentiated states. Adding to this complexity is the fact that expression of differentiated properties by many epithelial cells is intimately connected to their three dimensional architecture. *In vivo*, this can be seen in the polarized nature of columnar, secretory epithelial cells: nourishment and innervation come from the side next to the basement membrane, while secretion occurs at the other, usually microvilli covered end, facing a lumen. *In vitro* growth of HMEC as squamous appearing cells on plastic may not be conducive to expression of normal secretory products. Recent studies with rodent mammary epithelial model systems have demonstrated the importance of both basement membrane substrates, and three dimensional shape, for maximal expression of products of the most differentiated states (pregnancy/lactation) [32–34].

We have begun explorations of the factors controling HMEC differentiation with the realization that the system is extremely complex, and that each approach represents an approximation in an attempt to dissect many interacting variables. Thus far, we have examined the properties described below as possible markers for HMEC differentiation *in vitro*:

(1) Pattern of glucose metabolites. Previous studies with rodent model systems [35] have shown that mammary cells derived from virgin, pregnant, and lactating glands have characteristically different quantitative yields of glucose metabolic products; for example, glycogen and lactate synthesis are highest in pregnant cells, while lactose synthesis is high in lactating cells.

(2) Human mammary milk fat globule antigens (HMFGA). Monoclonal antibodies recognizing different HMFGA epitopes are available for analysis of HMEC populations [36–38]. Specific antibodies have been shown to bind in greatest amounts to cells from lactating tissues, and/or from tumor tissues. These antibodies may therefore be useful in defining both differentiated and transformed populations.

(3) Milk components. α-lactalbumin and caseins are synthesized in vivo by lactating cells. The production of these proteins in culture would be indicative of induction of a more differentiated state.

(4) Apolipoproteins. Preliminary work in our laboratory (Stampfer, M.R., Forte, G., Bartley, J.C., unpublished) indicates that HMEC secrete specific apolipoproteins. We are currently exploring whether such synthesis may be modulated by the state of functional differentiation.

(5) Thioesterase II. This enzyme is responsible for the synthesis of medium chain fatty acids found only in the mammary cells of mammals. Its synthesis in rodents is constituitive [39] (and thus its usefulness as a marker of mammary epithelia), but since the levels of this enzyme are greatly increased during pregnancy/lactation it may also serve as a marker of differentiation.

In addition to studying HMEC differentiation as a means of elucidating control of gene expression in normal human epithelial cells, such information is also important for understanding carcinogenesis. The multistep progression to cancer involves pertubations in the normal cellular mechanisms of growth and terminal differentiation. Carcinoma cells usually display aberrations in expression of normal functional differentiation properties. Cancer may be considered a disease of aberrant differentiation [40]. Additionally, the differentiated state of a cell may effect how it responds to potential carcinogenic insults. For example, in both rodent and human mammary glands there is evidence that chemical carcinogens and/or radiation is most likely to result in mammary cancer when exposure occurs during the time of 'puberty' [10]. We have begun comparing our normal, tumor derived, and *in vitro* transformed HMEC for expression of the properties listed above. Future protocols for inducing transformation in culture may involve modulation of the differentiated state of the exposed cells.

Table 4 presents a general summary of our results to date on examination of differentiated properties. Included in these data are preliminary experiments assessing the effects of different cell matrices. We have found thus far that maximal expression of some differentiated properties occurs when cells are placed on self-matrices, that is the extracellular matrix laid down by cells actively proliferating in MCDB170. Other substrates tested include the

Table 4. Differentiated properties of normal and transformed HMEC.

Specimen	184		184Aa		184Be	184A1		184B5	
PROPERTY									
Glucose Metabolites[a]	*MM*	*170*	*MM*	*170*	*MM*	*MM*	*170*	*MM*	*170*
Glycogen	142	12	6	8	11	8	4	20	1
Lactate	995	358	855	374	1275	917	157	1184	85
HMFGA[b]									
P_2A_1	++++	++	NT		NT	+	+	++++	++++
P_3B_1	+	0/+	NT		NT	+	+	++	++
P_2C_1	0/+	0/+	NT		NT	+	+	++	+
Apolipoprotein-A1[c]	NT	yes	NT		NT	NT	yes	NT	yes
Milk Proteins[d]	*MM**	*MM*	*170*			*MM*	*170*	*MM*	*170*
α-lactalbumin	+	−	−			−	−	−	−
β-casein	++	+	−			−	−	−	−

[a]Cells were grown in the indicated medium and analyzed for glucose metabolites by two dimensional chromatography as described [35]. 184 and 184Aa were tested at passage 8; the MM values for 184 were determined by switching cells grown in MCDB170 to MM 72 hr prior to analysis. 184A1 values are from passage 42 and 184B5 from passage 11. Values are presented as nMol/mg protein.

[b]Cells were grown in the indicated medium and assayed by indirect immunofluorescence. The monoclonal antibodies to HMFGA were raised as described [38].

[c]Cells were grown in MCDB170 in the presence of S^{35}-methionine and assayed for apolipoproteins A1, A2, B, and E by Western blotting followed by autoradiography.

[d]Cells were assayed for α-lactalbumin and β-casein by Western blotting using a monoclonal antibody to casein and a polyclonal antibody to milk (Parry, G., Bartley, J.C., unpublished). The cells were continuously grown in MCDB170 (170), switched to MM for 48 hrs prior to assay (MM), or seeded onto extracellular matrices deposited by heavily confluent cultures of cells grown in MM or MCDB170 and removed by non-ionic detergents. MM* indicates cells placed on a MCDB170 matrix and grown in MM without EGF plus prolactin.

matrix laid down by cells grown in MM, collagen type I, and EHS and PFHR9 extracellular matrices [41, 42]. The different media we have examined for modulation of differentiated state are MCDB170 and MM, without or without specific factors such as EGF and prolactin. In general, we have observed that the MM medium, particularly without EGF and with prolactin, produces the most differentiated phenotype. This can be seen in the glucose metabolite pattern, which most closely resembles that found in mammary cells from virgin mice when HMEC are grown in MCDB170, while resembling that found in the cells from pregnant mice when the HMEC are grown in MM. Growth in MM also increased expression of some HMFGA. Placement in MM minus EGF plus prolactin resulted in expression of α-lactalbumin and β-casein if the HMEC were on a extracellular martix produced in MCDB170.

In the cases where we have compared the immortalized cells with their parental cells, some differences have been observed, such as in glucose metabolites, HMFGA expression, and the absence of β-casein secretion (Table 4). In particular, 184B5 showed increased levels of HMFGA, even in MCDB170, whereas 184A1 had decreased levels. The synthesis of apoplipoprotein was similar in all cases examined. In other experiments, where the secreted proteins of cells grown in MM and MCDB170 were analyzed by two dimensional gel electrophoresis, we have seen that both 184A1 and 184B5 have much less material than the parental cells in the position of fibronectin. Also, the pattern of proteins secreted by 184B5, in either medium, closely resembles that of the parental cells grown in MM, whereas 184A1 displayed a more limited protein profile. These results suggest that the 184B5 cell line has a more differentiated phenotyope than 184A1.

Another concern related to expression of differentiated properties in culture, is the extent to which a given phenotype remains stable upon continued time and passage *in vitro*, and how it may be influenced by changes in the culture conditions. Loss of differentiated properties is commonly observed when cells are placed in culture, however, in some cases it has been shown that these properties are not irreversibly lost since they can be re-expressed if the cells are again placed *in vivo* [43, 44]. We have examined glucose metabolism and BaP metabolism for effect of continued culture [45]. No significant changes were seen in the glucose metabolite pattern in early (2–3) compared to later (6–10) passage cells. In contrast, as mentioned earlier, the BaP metabolite pattern showed major changes. Consequently, use of these cells to examine a given enzymatic activity may first require testing that activity for possible effects of passage in culture. As regards expression of differentiated properties, we have found that cells grown for 6–10 passages in MCDB170, which are not expressing certain phenotypes such as high glycogen and lactate synthesis, or secretion of β-casein, may be rapidly induced to the more differentiated phenotype by placement in MM medium. Thus these properties have not been irreversibly lost. These results provide encouragement that culture conditions can be found which will support functional differentiation, and point to the need to define more precisely what those conditions are.

9. Summary

Large quantities of normal and malignant human mammary epithelial tissues are readily available as surgical discard material. We have developed culture conditions that permit long term, active proliferation of these HMEC in a serum-free medium. Thus, large pools HMEC can be stored frozen for repetition of experiments from the same individual's cell population, and for use of the same cell pool by multiple investigators. Of all the specimens that we have thus far examined, we have observed no instances of spontaneous transfor-

mation to immortality, nor any karyotypic abnormalities in the cells derived from reduction mammoplasties. However, exposure of normal HMEC to the chemical carcinogen, benzo(a)pyrene did lead to expression of an extended life in culture, and two instances of transformation to immortality. These two established cell lines contain some chromosomal abnormalities, yet retain a relatively stable karyotype upon continued passage in culture. Transformation to malignancy was achieved by exposing these cell lines to tumor viruses and oncogenes. Both the normal HMEC, and the HMEC transformed in vitro, are now being utilized to understand the factors controlling expression of mammary specific properties, response to and production of various growth factors, and the nature of the progressive events leading to malignancy. The maximal usefulness of this, and other human epithelial cell systems, for elucidating the mechanisms of normal and diseased human cellular physiology will require continued efforts to optimize the culture conditions so that they resemble as closely as possible the processes occuring in humans in vivo.

Acknowledgments

We acknowledge the excellent technical assistance of Annie Pang, Linda Hayashi, Kari Koszdin, and Gerri Levine. This work was supported by CA24844, CA30028, and CA38889 from the National Institutes of Health, and the Director, Office of Energy Research, Office of Health and Environmental Research of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098.

References

1. Stampfer M, Hallowes RC, and Hackett AJ: Growth of normal human mammary cells in culture. In Vitro 16: 415–425, 1980.
2. Stampfer MR: Cholera toxin stimulation of human mammary epithelial cells in culture. In Vitro 18: 531–537, 1982.
3. Hammond SL, Ham RG, Stampfer MR: Serum-free growth of human mammary epithelial cells. Rapid clonal growth in defined medium and extended serial passage with pituitary extract. Natl Acad Sci USA 81: 5435–5439, 1984.
4. Stampfer MR: Isolation and growth of human mammary epithelial cells. J Tissue Cult Methd 9: 107–115, 1985.
5. Smith HS, Wolman SR, Auer G, Hackett AJ: Cell culture studies: a perspective on malignant progression of human breast cancer. In: Breast Cancer: Origins, Detection, and Treatment Rich MA, Hager JC, Taylor-Papdimitriou J, (eds.) Boston, Martinus Nijhoff Publishing, 1985, pp. 75–89.
6. Stampfer MR, Hackett AJ, Smith HS, Hancock MC, Leung JP, Edgington TS: Growth of human mammary epithelium in culture and expression of tumor specific properties. Cold Spring Harbor Conferences on Cell Proliferation 9: 819–829, 1982.
7. Smith S, Pasco D, Thompson BJ, Stampfer M, Nandi, S: Thioesterase II, a new marker enzyme for human cells of breast epithelial origin. J Nat Cancer Inst 73: 323–329, 1984.
8. Stampfer MR, Vlodavsky I, Smith HS, Ford R, Becker F, Riggs J: Fibronectin production by

human mammary cells. J Nat Cancer Inst 67: 253–261, 1981.
9. Wolman SR, Smith HS, Stampfer M, Hackett AJ: Growth of diploid cells from breast cancers. Cancer Genet Cytogenet 16: 49–64, 1985.
10. Meranze DR, Gruenstein M, Shimkin MD: Effect of age and sex on the development of neoplasms in Wistar rats receiving a single intragastric instillation of 7,12-dimethylbenzy(a) anthracene. Int J Cancer 4: 480–484, 1969.
11. Levin W, Wood AW, Chang RL, Slaga TJ, Yagi H, Jerina DM, Conney AH: Marked differences in the tumor initiating activity of optically pure(+)– and (–)-trans-7,8,dihydroxy-7,8-dihydrobenzo(a)pyrene on mouse skin. Cancer Res 37: 2721–2725, 1977.
12. Stowers SJ, Anderson MW: Formation and persistence of benzo(a)pyrene metabolite-DNA adducts. Environ Health Perspect 62: 31–40, 1985.
13. Autrup H, Harris CC: Metabolism of chemical carcinogens by cultured human tissues. In: Human Carcinogenesis, Harris CC and Autrup H (eds). New York, Academic Press, 1983, pp 169–194.
14. Stampfer MR, Bartholomew JC, Smith HS, Bartley J: Metabolism of benzo(a)pyrene by human mammary epithelial cells: toxicity and DNA adduct formation. Proc Natl Acad Sci USA 78: 6251–6255, 1981.
15. Bartley JC, Bartholomew JC, Stampfer MR: Metabolism of benzo(a)pyrene in human mammary epithelial and fibroblast cells: metabolite pattern and DNA adduct formation. J Cell Biochem 18: 135–148, 1982.
16. Bartley JC, Stampfer MR: Factors influencing benzo(a)pyrene metabolism in human mammary epithelial cells in culture. Carcinogenesis 6: 1017–1022, 1985.
17. Leadon SA, Hanawalt PC: Monoclonal antibody to DNA containing thymine glycol. Mutat Res 112: 191–200, 1983.
18. DiPaolo JA: Relative difficulties in transforming human and animal cells in vitro. J Natl Cancer Inst 70: 3–8, 1983.
19. Harris CC: Human tissues and cells in carcinogenesis research. Cancer Res 47: 1–10, 1987.
20. Chang SE, Keen J, Lane EB, Taylor-Papdimitriou J: Establishment and characterization of SV40 transformed human breast epithelial cell lines. Cancer Res 42: 2040–2053, 1982.
21. Rhim JS, Gilbert J, Arnstein P, Price FM, Sanford KK, Aaronson SA: Neoplastic transformation of human epidermal keratinocytes by the combined action of adeno12-SV40 virus and Kirsten murine sarcoma virus. Science 1250–1252, 1985.
22. Yoakum GH, Lechner JF, Gabrielson EW et al. Transformation of human bronchial epithelial cells transfected by Harvey ras oncogene. Science 227: 1174–1179, 1985.
23. Walen KH, Arnstein P: Induction of tumorigenesis and chromosomal abnormalities in human amniocytes infected with simian virus 40 and Kirsten sarcoma virus. In Vitro 22: 57–65, 1986.
24. Rhim JS, Fujita J, Arnstein P, Aaronson SA: Neoplastic conversion of human keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. Science 232: 385–388, 1986.
25. Arklie J, Taylor-Papadimitriou J, Bodmer W, Egan M, Millis R: Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. Int J Cancer 28: 23–29, 1981.
26. Masui T, Wakefield LM, Lechner JF, LaVeck MA, Sporn MB, Harris, CC Type β transforming growth factor: a differentiation-inducing serum factor for normal human bronchial epithelial cells. Proc Natl Acad Sci USA 83: 2438–2442, 1986.
27. Shipley GD, Pittelkow MR, Wille JJ, Scott RE, Moses, HL: Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. Cancer Res 46: 2068–2071, 1986.
28. Stampfer MR, Bartley JC: Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo(a)pyrene. Proc Natl Acad Sci USA 82: 2394–2398, 1985.
29. Clark R, Stampfer MR, Milley B, O'Rourke E, Walen K, Kreigler M, Kopplin J, McCormick, F: Transformation of human mammary epithelial cells by oncogenic retroviruses. Cancer Research, in press.

30. Walen KH, Stampfer MR: Karyotypic changes in human mammary epithelial cells transformed in vitro. Submitted to Cancer Genet Cytogenet.
31. Bates SE, Valverius E, Salomon D, Stampfer M, Lippman ME, Dickson RB: Expression and estrogen regulation of transforming growth factor-α mRNA in human breast cancer. Proc Am Assoc Cancer Res 28: 233, 1987.
32. Wicha MS, Lowrie G, Kohn E, Bayavandoss P, Mahn T: Extracellular matrix promotes mammary epithelial growth and differentiation in vitro. Proc Natl Acad Sci USA 79: 3213–3217, 1982.
33. Lee EY-H, Lee WH, Kaetzel CS, Parry G, Bissell MJ: Interaction of mouse mammary epithelial cells with collagen substrata: Regulation of casein gene expression and secretion. Proc Natl Acad Sci USA 82: 1419–1423, 1985.
34. Emerman JT, Bartley JC, Bissell MJ: Glucose metabolite patterns as markers of functional differentiation in freshly isolated and cultured mouse mammary epithelial cells. Exp Cell Res 134: 241–250, 1981.
35. Parry G, Cullen B, Kaetzel CS, Kramer R, Moss L: Regulation of differentiation and polarized secretion in mammary epithelial cells maintained in culture: Extracellular matrix and membrane polarity influences. Submitted to J Cell Biol 1987.
36. Arklie J, Taylor-Papadimitriou J, Bodmer W, Egan M, Millis R: Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. Int J Cancer 28: 23–29, 1981.
37. Ceriani R, Peterson J, Blank E: Variability in surface antigen expression of human breast epithelial cells cultured from normal breast, normal tissue peripheral to breast carcinomas, and breast carcinomas. Cancer Research 44: 3033–3039, 1984.
38. Moss L, Greenwalt D, Cullen B, Dinh N, Ranken R, Parry G: Cell-to-cell heterogeneity in the expression of carbohydrate based epitopes of a mucin-type glycoprotein on the surface of human mammary carcinoma cells. Submitted to J Cell Physiol, 1987.
39. Smith S, Pasco D, Nanci S: Biosynthesis of medium-chain fatty acids by mammary epithelial cells from virgin rats. Biochem J 212: 155–159, 1983.
40. Sachs Leo: Cell differentiation and bypassing genetic defects in the suppression of malignancy. Cancer Res 47: 1981–1986, 1987.
41. Kleinman HK, McGarvey ML, Hassel JR, Star VL, Cannon FB, Laurie GW, Martin GR: Basement membrane complexes with biological activity. Biochemistry 25: 312–318, 1986.
42. Kramer RH, Bensch KG, Davidson PM, Karasek MA: Basal lamina formation by cultured microvascular endothelial cells. J Cell Biol 99: 692–698, 1984.
43. Janss DH, Hillman EA, Malan-Shibley LB, Ben TL: Methods for the isolation and culture on normal human breast epithelial cells. Methods in Cell Biology, Vol 21B, Prescott DM (ed). New York, Academic Press, 1980, pp 107–134.
44. Terzaghi M, Nettesheim P, Williams ML: Repopulation of denuded tracheal grafts with normal, preneoplastic, and neoplastic epithelial cell populations. Cancer Res 38: 4546–4553, 1978.
45. Stampfer MR, Bartley JC: Development of human mammary epithelial cell culture systems and their use for studies of carcinogenesis and differentiation. In: In Vitro Models for Cancer Research, Weber M, Sekely L, (eds). Boca Raton, CRC Press, 1986, Vol. 3, pp 11–30.

2. Heterogeneity of genetic alterations in primary human breast tumors

Iqbal Unnisa Ali, Rosette Lidereau, and Robert Callahan

1. Introduction

The etiology of human breast cancer is thought to involve a complex interplay among genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host [1, 2]. As a consequence, several different types of breast tumors can be distinguished by histopathological criteria, chromosomal abnormalities, hormone receptor status, and other biochemical characteristics. However, attempts to derive a cohesive picture of how the various factors participate in the etiology of breast cancer have been confounded by a lack of information on specific genetic mutations associated with the initiation or progression of the disease. Three general approaches have been used to attempt to identify genetic mutations associated with breast cancer.

1.1 Cytogenetic abnormalities

The majority of primary and metastatic breast cancers contain an abnormal karyotype that includes unidentified marker chromosomes, homogeneous staining regions, and extrachromosomal double minutes [3–6]. Cytogenetic analyses using chromosome banding techniques were carried out mostly on tissue culture cells derived from primary breast tumors. In one study of primary breast tumors, chromosomes 8, 13, and 16 were found to be frequently lost [7]. Trisomy and translocations affecting chromosome 1q were also observed, although there were no specific breakpoints or translocation partner chromosomes involved [8]. Cytogenetic abnormalities of chromosomes 6, 7, and 11 have also been reported in primary breast tumors [9]. Chromosome 6 abnormalities were found in 30% of the tumors examined. In a study of eight breast tumor cell lines, chromosomes 1 and 11 were frequently involved in translocations [10]. However, at the present time, there is no information on whether these chromosome abnormalities are associated with specific cellular genes. Moreover, the limited number of primary tumor samples that have been studied precludes any firm conclusions on the frequency of specific abnormalities or their association with particular aspects of the disease.

M.E. Lippman and R.B. Dickson (ed.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

1.2 Gene transfer

Transfection of murine NIH 3T3 fibroblasts or mammary gland epithelial cell lines with cellular DNA from human breast tumor-derived tissue culture cell lines has been the primary biological assay. In this assay, an activated oncogene causes morphological changes as well as an increased tumorigenic potential of the tissue culture cells. This assay system has identified an amino acid 12-point mutation in the c-H-*ras*-l proto-oncogene in a breast carcinosarcoma cell line [11]. Also, identification of the N-*ras* oncogene and other uncharacterized genes was reported by two other laboratories using DNAs from breast cancer cell lines and from a human breast tumor metastasis in similar transfection assays [12, 13]. However, there have been at least two consistent difficulties with this approach. First, primary breast tumor DNA does not routinely score positive in this assay. This may reflect the inability of the relevant activated proto-oncogene to transform the target cell line to the tumorigenic phenotype. Second, foci of transformed tissue culture cells can arise as a function of amplification and/or recombination events occurring during or after gene transfer and are unrelated to genetic mutations in the parental tumor DNA.

The gene transfer technique was also used to explore the concept that genetically altered proto-oncogenes contribute to tumor growth or metastasis by activating the expression of other previously silent cellular genes or by circumventing the need for certain growth requirements. For example, transfection of NIH 3T3 cells with c-*myc* recombinant plasmid leads to a high level of expression of the v-*erb* A-related 1,25-dihydroxyvitamin protein D3 receptor [14]. Introduction of v-H-*ras* into the MCF-7 breast tumor cell line eliminates the requirement of estrogen for cell growth [15]. Although the *in vitro* experiments are instructive, their relevance to the evolution of breast tumors *in vivo* remains unclear.

1.3 Genetic alterations in breast tumor cell lines

The third approach has been to survey breast tumor cell lines for genetic alterations of specific proto-oncogenes. The N-*ras* proto-oncogene has been shown to be amplified in the MCF-7 breast tumor cell line [16]. Similarly, c-*myc* is amplified in the SKBR-3 cell line [17] and in tumors induced in nude mice by the SW6B-S breast tumor cell lines [18]. Amplification of another proto-oncogene c-*erb* B-2, which is related to the epidermal growth factor receptor c-*erb* B proto-oncogene, has been reported in a primary breast tumor [19]. A serious drawback of this approach in most cases is our inability to distinguish between genetic alterations that have been selected during passage in tissue culture and those that occurred in the parental tumor.

This chapter summarizes the status of another strategy that is aimed at determining, at a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant correlation with aspects of the

patient's history, characteristics of the tumor, or the patient's prognosis. As a starting point, we and others have begun to examine the genetic integrity of cellular proto-oncogenes in primary breast tumors. The aim of this type of study is 2-fold: (1) to identify mutations that frequently occur in primary breast tumors and are thus candidates for further experimental studies and (2) to attempt to develop molecular markers that are clinically useful in the management of the disease.

2. Involvement of proto-oncogenes in breast cancer

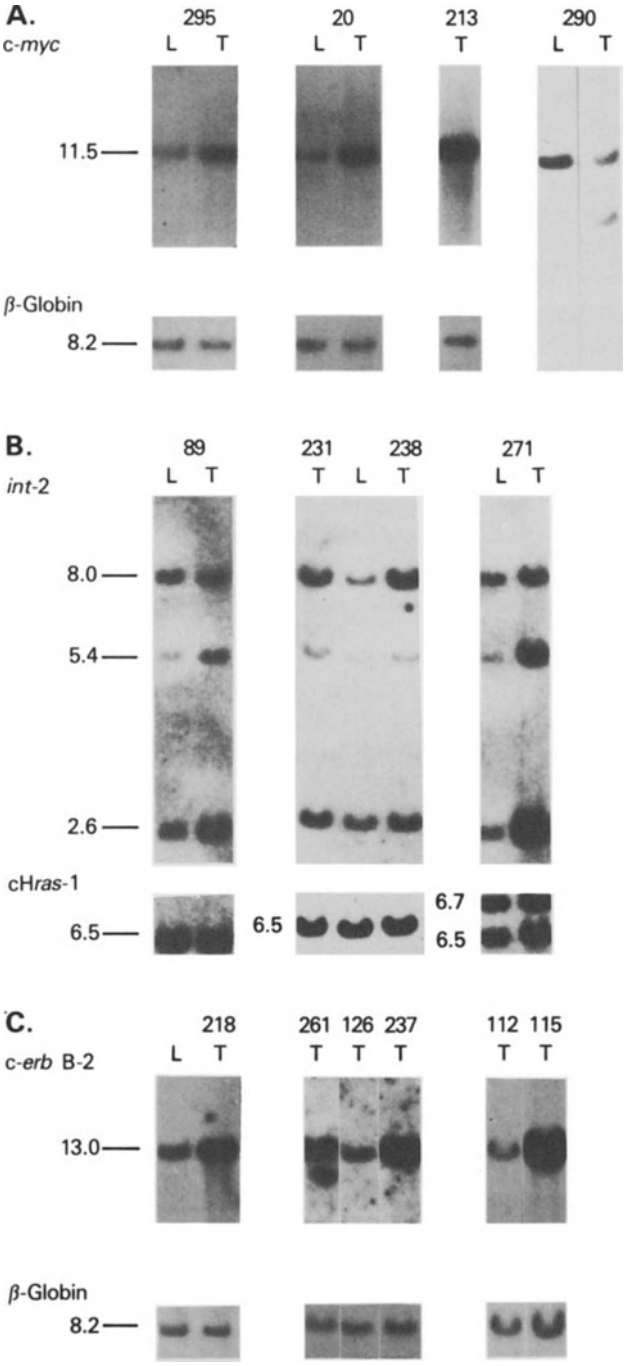
There is a large body of circumstantial evidence that implicates protooncogenes in the genesis of tumors [20, 21], In general, alterations of the *ras* and *myc* proto-oncogene families appear to occur frequently in human tumors. The types of alterations encountered in these and other protooncogenes include point mutations, amplification, rearrangement, and deletion or insertion of genetic material. A systematic analysis of protooncogenes in primary breast tumor DNA has revealed that amplification of the c-*myc*, *int*-2, and c-*erb* B-2 genes frequently occurs in different subsets of tumors.

2.1 Amplification and Rearrangement of c-myc

Amplified c-*myc* proto-oncogene was detected mostly in cell lines derived from various cancers [22, 23]. These cell lines include human colon carcinoma COLO 320 [24], promyelocytic leukemia HL-60 [25], several human neuroblastomas [26] and small cell lung carcinomas [27], and a breast carcinoma [17]. Amplification of c-*myc* and L-*myc* is frequently observed in highly malignant cell lines derived from variants of small cell lung carcinomas [28]. A more compelling correlation between alteration of c-*myc* and breast cancer is provided by two strains of transgenic mice carrying a MMTV/c-*myc* fusion gene [29]. These mice develop a high incidence of mammary adenocarcinomas, suggesting that constitutional deregulation of c-*myc* might contribute to the development of breast cancer.

A study of the c-*myc* locus in primary breast tumor DNAs from 121 patients revealed two types of genetic alterations [30]. In 32% of the tumors, there was a 2- to 15-fold amplification of the gene (Figure 1A). The amplification was very likely specific for c-*myc* because the c-*mos* proto-oncogene, also located on chromosome 8, was not amplified [31]. In five other tumors, a rearrangement of c-*myc* was detected (Figure 1A). Further analysis of one of these DNAs indicated that the breakpoint could be near the 3′ end of c-*myc* exon 3. Current efforts are focused on the isolation of recombinant DNA clones containing the breakpoint to further characterize the nature of this genetic lesion and determine its effect on c-*myc* expression.

Amplification of the c-*myc* proto-oncogene was also reported in another study of primary breast tumors where 2 of the 10 tumors examined were


A.
c-myc
295
L T
20
L T
213
T
290
L T
11.5
β-Globin
8.2
B.
int-2
89
L T
231
T L T
238
271
L T
8.0
5.4
2.6
cHras-1
6.5
6.5
6.7
6.5
C.
c-erb B-2
218
L T
261 126 237
T T T
112 115
T T
13.0
β-Globin
8.2

found to contain a 3- to 8-fold amplification of c-*myc* [32]. In the study of Escot *et al.* [30], genetic alteration of c-*myc* had a significant correlation ($P < 0.02$) with tumors from patients ≥51 years of age. The patients comprising this group were either postmenopausal, had a previous hysterectomy (25–30 years of age), or were males, suggesting that there may be a link between their hormonal milieu and selection for deregulation of c-*myc* expression.

Amplification of c-*myc* and also of other proto-oncogenes is generally associated with enhanced levels of gene expression. Slamon et al. [33], using dot blot analysis of RNA from primary breast tumors, noted the frequent expression of c-*myc* RNA. In our study of 14 breast tumor RNAs by Northern blot analysis (Figure 2), 10 were found to express high levels of c-*myc* RNA [30]. However, only 6 of the 10 tumors contained an amplified c-*myc* gene. It seemed possible that the other four tumors contained mutations outside the restriction fragments examined or contained small undetected mutations that promote c-*myc* RNA expression. A similar phenomenon has been observed in primary colon carcinomas in which c-*myc* appears unaltered, but the tumor expresses high levels of c-*myc* RNA [34]. One difficulty in evaluating gene expression in total RNA extracted from primary breast tumors is the variable extent of cellular heterogeneity. To circumvent this problem, we have examined c-*myc* expression at the cellular level in frozen sections of breast tumors by use of the RNA:RNA in situ hybridization technique [35]. Postive c-*myc* hybridization signals were associated with carcinoma cells in all cases, including tumors with no apparent alteration of the c-*myc* locus. High levels of c-*myc* expression were observed in four of seven tumors that contained an amplified c-*myc*. Moreover, high levels of c-*myc* RNA were also detected in two of nine cases that had an apparently normal c-*myc* locus but comparatively low cellularity. In addition to carcinoma cells, dense clusters of infiltrating lymphocytes present in three tumors contained c-*myc* RNA. These results, taken together, suggest: (1) the extent and frequency of c-*myc* amplification has probably been underestimated because of heterogeneous cellularity; (2) c-*myc* amplification is related to high level expression, but other unknown factors may also play a role; and (3) c-*myc* RNA in total RNA from biopsy samples may be contributed by infiltrating lymphocytes.

Figure 1. Amplification and/or rearrangement of c-*myc*, *int*-2, and c-*erb* B-2 in primary breast tumor DNAs. DNA (10 μg) from tumors and/or lymphocytes was restricted with *Hin*dIII (A), *Bam*HI (B), or *Eco*RI (C). (A) DNAs from tumors 295 (2-fold amplification), 20 (6-fold amplification), 213 (>10-fold amplification), and 290 (rearrangement) and corresponding lymphocytes (except tumor 213) were hybridized first with the c-*myc* probe (pRyc 7.4) and then the β globin probe (CHB19). (B) DNAs from tumors 89 (2-fold amplification), 231 (5-fold amplification), 238 (5-fold amplification), and 271 (16-fold amplification) and corresponding lymphocytes (except for tumors 231 and 238) were hybridized first with *int*-2 probe (SS-6) and then the c-H-*ras*-1 probe (J77). (C) DNAs from tumors 218 (5-fold amplification), 261 (5-fold amplification), 126 (no amplification), 237 (>15-fold amplification), 112 (no amplification), and 115 (>15-fold amplification) and the corresponding lymphocytes to tumor 218 were hybridized first with the c-*erb* B-2 probe (MAC 117) and then the β globin probe (CHB19).

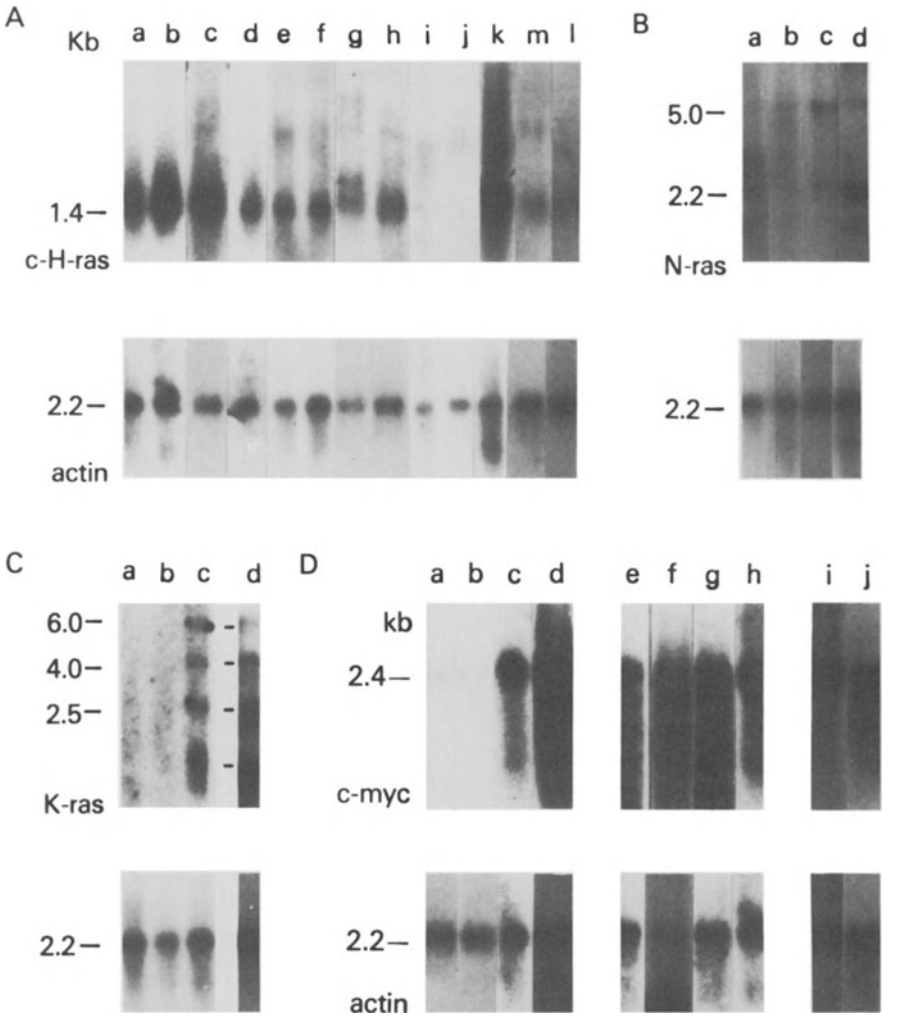


Figure 2. mRNA expression of the three *ras* genes and c-*myc* in primary human breast tumors and human tissue cluture cell lines. Polyadenylated RNA (4 μg) was analyzed by Northern blotting. RNA blots were hybridized with c-H-*ras*-1 (A), N-*ras* (exons 1 and 2) (B), K-*ras*-2 (C), and c-*myc* (pRyc 7.4) (D). Each of the blots were then hybridized with the actin probe. (A) The RNAs were from tumors 87, 114, 292, 68, 368, 361, 307, 375, and 377 (lanes a-j); the bladder carcinoma cell line T24 and breast tumor cell lines T47-D and MCF-7 (lanes k-m). (B) RNAs were from tumors 368 (lane a) and 361 (lane b) and cell lines T47-D (lane c) and MCF-7 (lane d). (C) RNAs were from tumors 368 (lane a) and 361 (lane b) and cell lines MCF-7 (lane c) and colon carcinoma LS-174 (lane d). (D) RNAs were from tumors 305, 307, 87, 293, 239, 121, 182, and 114 (lanes a-h, respectively) and cell lines T47-D (lane i) and MCF-7 (lane j).

2.2 *Amplification of the* int-2 *Locus*

The study of experimentally induced mammary tumors has focused, in part, on high-incidence strains of mice infected with the mouse mammary tumor virus (MMTV) [36, 37], which acts as an insertional mutagen in infected tissue [38]. Recent studies have identified three cellular genetic loci (designated *int*-1, *int*-2, and *int*-3), located on different mouse chromosomes, which are frequently occupied by an MMTV genome in mammary tumor DNA [39–41]. One consequence of viral insertion at these loci is the activation of expression of an adjacent cellular gene. Normally *int*-1 and *int*-2 are only expressed during embryonic development [42–44]. The frequency with which these genes are activated in MMTV-induced tumors suggests that their expression plays a role in mammary tumorigenesis. In the BR-6 mouse strain, activation of *int*-2 expression by MMTV appears to occur early in mammary tumor development [45]. In addition, *in vitro* studies of the biological activity of the recombinant *int*-1 gene shows that it confers certain characteristics of the malignant phenotype on tissue culture cells [46, 47].

These considerations have led us to investigate whether the human homologues of the *int* genes are genetically altered in primary human breast tumors [48]. Recombinant clones of human DNA containing *int*-2-related sequences have recently been obtained and shown to be located on human chromosome 11q13 [49]. A survey of 110 primary infiltrating ductal carcinoma DNAs showed a 2- to 15-fold amplification of the *int*-2 locus in 18 tumor DNAs (Figure 1B). In each of these cases, other loci on chromosome 11p were not amplified, indicating that the amplification was not a result of polyploidy. Examination of the patient's history, the characteristics of the tumor, and the patient's prognosis showed that amplification of *int*-2 had a significant association ($P < 2 \times 10^{-6}$) with the patients who subsequently developed a distal metastasis. Although, at the present time, we have no information on the spectrum of normal human tissues in which *int*-2 is expressed nor the effect of amplification on its expression, it seems probable that genetic alteration of this gene in murine and human breast tumors is more than coincidence.

2.3 *Amplification of* c-erb *B-2*

The oncogene *neu* was originally identified in chemically induced neuroglioblastomas of rat [50]. Nucleotide sequence analysis showed it to be related to the c-*erb* B proto-oncogene that encodes the epidermal growth factor receptor [51]. The human homologue has been independently identified by several laboratories and has been designated either HER-2 or c-*erb* B-2 [19, 53, 54]. The c-*erb* B-2 proto-oncogene is located on chromosome 17q21–22 [55]. Amplification of this gene has been detected in a primary human breast tumor cell line [19], a gastric carcinoma cell line (MKN-7), and a primary salivary gland adenocarcinoma [54].

In a study of 103 primary breast carcinomas, Slamon *et al.* [56], found that

18% of the breast tumors contained a 2- to >20-fold amplification of c-*erb* B-2. Statistical analysis of the data did not show a significant association ($P < 0.11$) between amplification of c-*erb* B-2 and patients with greater than three involved axillary lymph nodes. Slamon *et al.*, then performed a similar analysis on 86 primary breast tumors from patients with one or more involved lymph nodes who had been followed post-surgery for 24–86 months. Amplification of c-*erb* B-2 was found in 40% of these tumors and there was an association ($P < 0.06$) between patients with greater than three involved lymph nodes and amplification of the gene. When the results of the two studies were combined, the association was highly significant ($P < 0.002$). Moreover, a highly statistically significant association was found between the degree of gene amplification and the time until relapse ($P < 0.0001$) as well as survival ($P < 0.0011$). This observation has a precedence. In neuroblastoma, a similar correlation was found between the level of amplification of N-*myc* and very aggressive form of tumors [57].

Recently, Ali *et al.* [58, 59] and van de Vijver *et al.* [60], have independently examined c-*erb* B-2 in separate panels of primary breast tumor DNAs. In our study, we found 12 of 122 tumor DNAs (10%) in which c-*erb* B-2 was amplified 2- to >15-fold (Figure 1C). In five of these tumor DNAs, the gene was amplified 2- to 5-fold, in another 5 tumors 6- to 15-fold, and in two tumors >15-fold (Figure 1C). Van de Vijver *et al.* [59], examined 95 primary breast tumor DNAs and found 2- to 5-fold amplification of c-*erb* B-2 in 15 tumors. Another proto-oncogene, c-*erb* A-1, is located very near c-*erb* B-2 on chromosome 17q21 [61]. Previously, a survey of 84 of the primary breast tumor DNAs used in the present study for genetic alterations of c-*erb* A-1 found no evidence for amplification or rearrangement of this gene [62]. However, van de Vijver *et al.* [59], have found that 6 of 10 tumors containing an amplified c-*erb* B-2 were also amplified for c-*erb* A-1. No tumors were found to be amplified for c-*erb* A-1 alone. To distinguish between amplification of c-*erb* B-2 and increased copy number of chromosome 17, another marker, the p53 tumor-associated gene located on 17p [62, 63], was examined. There was no evidence for the amplification of this gene [58–60], suggesting that the amplification of c-*erb* B-2 is not a function of ploidy. It was also shown that amplification of c-*erb* B-2 was associated with increased levels of c-*erb* B-2 RNA but not c-*erb* A-1 RNA [59], suggesting that amplification of c-*erb* A-1 is a fortuitous event due to its close proximity to c-*erb* B-2.

One important aspect in which our study [58] and that of van de Vijver *et al.* [59], differs from Slamon *et al.* [56], is that in neither study was there a significant association between amplification of c-*erb* B-2 and lymph node status of the patient. In addition, the patients in our study have been monitored for a median follow-up period of 53 months (4–113 months) post-surgery for disease status. At the present time, 39 of the 122 patients have had a local recurrence or have developed a distal metastasis. Among the 12 patients whose tumor DNA contained an amplified c-*erb* B-2, only 2 have had either a local recurrence or distal metastasis. Thus, amplification of c-*erb*

B-2 does not appear in this study to represent a significant indicator of the patient's prognosis. Statistical analysis of the distribution of other clinical parameters also shows no association between c-*erb* B-2 amplification and the histopathological grade, the estrogen/progesterone receptor status of the tumor, or the age/menopausal status of the patient. Clearly, additional studies are warranted to clarify these issues and to attempt to confirm the potentially exciting findings of Slamon *et al.* [56].

*2.4 Rare alleles of c-H-*ras*-1 and breast cancer patients*

The c-H-*ras*-1 proto-oncogene is distinguished by several restriction fragment length polymorphisms (RFLP) due to the variable tandem reiteration (VTR) of a 28-base pair (bp) sequence located 3′ to the last coding exon [64]. Krontiris *et al.* [65], initially reported that leukocyte and tumor DNAs from patients with a variety of different malignancies, as well as tumor-derived cell lines, contain a high frequency of rare c-H-*ras*-1 alleles not found in unaffected populations. Lidereau *et al.* [66], compared the c-H-*ras*-1 allele frequency in 104 breast cancer patients with that of 56 unaffected individuals with no familial history of breast cancer. In the combined populations, four common (6.5, 7.0, 7.6, and 8.0 kb) and 16 rare (5.9 to 8.7 kb) alleles were observed in *Bam*HI-digested lymphocyte or tumor DNA. The distribution of these alleles differed significantly ($P < 0.001$) between the two populations. In the unaffected population, the four common alleles represented 91% of the total alleles, whereas in the breast cancer population they represented only 59% of the total. This difference resulted from a significant decrease in the 6.5-kb ($P < 0.001$) and 8.0-kb ($P < 0.02$) common alleles accompanied with an increased frequency of rare alleles in breast cancer patients. In fact, the 6.3-kb rare allele had a significant ($P < 0.05$) association with breast cancer patients. The high frequency of rare alleles in the breast cancer patients was also associated with a significantly higher frequency of genotypes composed of two rare alleles ($P < 0.001$) in this population compared with the unaffected population.

At the present time, little is known about the molecular and biological consequences of the high frequency of rare c-H-*ras*-l alleles in breast cancer patients. In this context, Krontiris *et al.* [65], have claimed that deletion of the VTR in the EJ *ras* oncogene decreases its transforming potential 5- to 10-fold. This suggests that the VTR affects the regulation of c-H-*ras*-l expression. Horan Hand *et al.* [67], have shown by immunohistochemical techniques that 60% of the primary breast tumors express *ras* p21. It has been shown by Northern blot analysis that primary breast tumors express primarily c-H-*ras* RNA and that this expression is not associated with amplification or rearrangement of the gene [68, 69]. Using immunohistochemical techniques, Lidereau *et al.* [70], assayed paraffin sections of 104 breast tumors from patients whose c-H-*ras*-l genotype had previously been determined. The results showed a significant association between *ras* p21 expression and (1)

patients with two rare alleles or one common and one rare allele ($P < 0.037$) and (2) postmenopausal patients with two rare alleles ($P < 0.05$). In a separate study, Ohuchi *et al.* [71], also found higher levels of *ras* p21 expression in breast tumors associated with postmenopausal or nulliparous patients. In spite of these correlations, the relationship between expression and c-H-*ras*-l genotype is complex. For instance, while some tumors that are homozygous for a particular allele express *ras* p21, other tumors homozygous for the same alleles do not. Moreover, there is no correlation between the size of the VTR and *ras* p21 expression. This suggests that, although the VTR in certain rare alleles may contribute to aberrant expression of c-H-*ras*-l, other factors are also involved. Further studies designed to determine the manner in which the VTR regulates c-H-*ras*-l expression may provide further insight into this phenomenon.

Another unresolved issue is whether the high frequency of rare alleles observed by Krontiris [65] is a generalized phenomenon associated with cancer patients per se or is associated with specific types of cancer. Several recent studies have begun to address this question. Theillet *et al.* [72], examined 32 colon adenocarcinoma patients at c-H-*ras*-l and found a significant ($P < 0.05$) increase in the frequency of rare alleles. Heighway *et al.* [73] compared c-H-*ras*-l in 132 lung carcinoma patients and 104 normal individuals. They found a significant increase in the frequency of the 6-kb common allele of c-H-*ras*-l in non-small cell lung carcinoma patients compared with small cell lung carcinoma patients ($P < 0.004$) or controls ($P < 0.05$). Similar analysis of c-H-*ras*-l in patients with myodysplasia [74] and melanoma [75] has failed, however, to reveal differences in the allele frequency from that observed in normal populations. Taken together, these data suggest that the high frequency of rare c-H-*ras*-l alleles may be associated with specific types of cancer.

3. Possible recessive mutations in breast cancer

The idea that germinal mutations predispose to hereditary cancers emerged from the study of pediatric tumors. The presence of the germline mutations does not by itself give rise to a malignant phenotype in a heterozygous state. Evolution of tumor requires a 'second hit' [76], which is mostly achieved by an effective loss of the normal allele, thereby unmasking the original recessive mutation. In the nonhereditary form of these cancers, both of these events are believed to be somatic in origin.

The identification and mapping of recessive mutations and normal cellular sequences that might possibly include regulatory genes with suppressor functions have been greatly facilitated by RFLP analysis. Using this and other techniques, researchers have detected chromosomal deletions in several pediatric malignancies, such as retinoblastoma, hepatoblastoma, rhabdomyosarcoma, and Wilms' tumor [77–84], as well as in adult cancers. For instance,

deletion of normal cellular sequences on chromosomes 11, 22, and 2 has been detected in bladder carcinoma [85], acoustic neuroma [86], and uveal melanoma [87], respectively. Other candidates for genetic predisposing mutations may be cancers with hereditary elements [88], such as polyposis of the colon, colon carcinoma, neurofibromatosis, familial meningioma, familial medullary carcinoma of thyroid, and breast carcinoma, just to name a few.

3.1 Delection of sequences on chromosome 11 in primary breast tumors

Allelic loss of the c-H-*ras*-1 gene located on chromosome 11p15 has been reported in pediatric malignancies [80–84], including hepatoblastoma, rhabdomyosarcoma, and Wilms' tumor, as well as in adult bladder carcinoma [85]. In a survey of 104 breast cancer patients, deletions of a c-H-*ras*-1 allele (Figure 3) was detected in 27% of the tumor DNAs of patients constitutionally heterozygous at this locus [68]. Statistical analysis showed a significant association between allele loss and histopathological grade III tumors ($P < 0.02$), estrogen and/or progesterone receptor-negative tumors ($P < 0.01$), and subsequent development of distal metastasis ($P < 0.05$).

To determine the extent of deletion on chromosome 11 in these tumors, four other markers (γ globin, PTH, calcitonin, and catalase) on 11p and the *int*-2 locus on 11q were examined in matched sets of lymphocyte and tumor DNAs [89]. RFLP analyses using these polymorphic markers demonstrated that the deletions in breast tumors included, in addition to the c-H-*ras*-1 locus, several other loci on chromosome 11. Also, the deletions were of variable length and lacked any apparent common breakpoint at either end (Figure 4). However, the region of chromosome 11p that might be critical in these tumors was suggested by the genotypes of the tumors from patients 180 and 223. Patient 180 was constitutionally heterozygous at c-H-*ras*-1 locus, the γ globin locus of the β globin cluster, and the PTH locus. The tumor DNA from this patient was reduced to homozygosity at the c-H-*ras*-1 and γ globin loci, but not at the PTH locus. Conversely, the tumor DNA from patient 223 had lost one PTH allele but maintained heterozygosity at the γ globin locus.

The maintenance of heterozygosity at the PTH locus in the tumor DNA of patient 180 and at the calcitonin locus in the tumor DNA of another patient (408) with deletions proximal to these loci suggests that a putative breast tumor locus is different from the WGAR (Wilms' tumor, aniridia, genitourinary malformation, and mental retardation) locus which has been mapped between the catalase and the β subunit of follicle-stimulating hormone [90–93]. Clearly, an expanded study of additional tumors is warranted to focus on further characterization of the region between the β globin and PTH loci.

Taken together, 20 of 99 tumor DNAs examined in our studies [68, 89] exhibited deletions of various markers on chromosome 11p. These deletions were found to have a significant association with histopathological grade III ($P < 0.006$), estrogen ($P < 0.02$), and progesterone ($P < 0.002$) receptor-

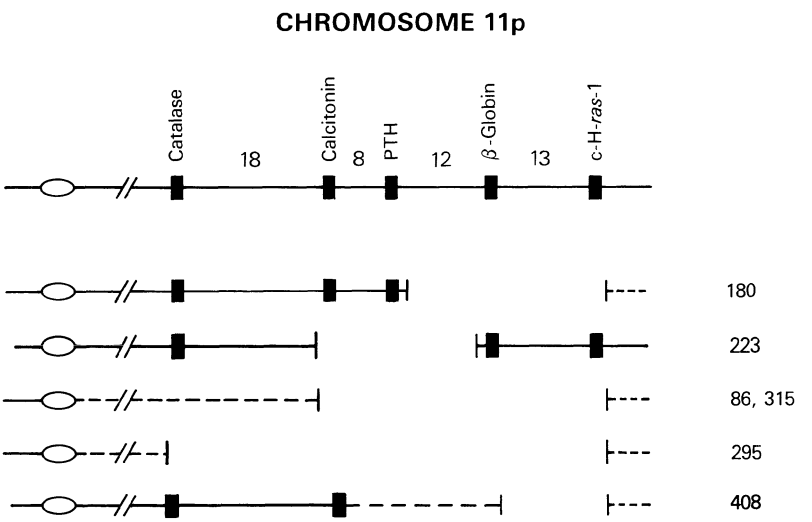


Figure 3. A schematic representation of the deletions on the short arm of chromosome 11 in breast cancer patients. The order and distance between the markers on chromosome 11p are according to the recombination percentages listed in human gene mapping 8 (HGM8) [129]. Numbers between genetic loci refer to recombination percentages or distances in centimorgans. Vertical lines mark the beginning or end of the deletion at a heterozygous locus. Broken lines indicate that the markers were either not informative (catalase in patients 86 and 315; γ-globin and PTH in patient 408) or not done (covering the region between catalase and centromere and the region between c-H-*ras*-1 and telomere), and therefore, the deletion could theoretically extend beyond these loci. Patient 223 was homozygous for the c-H-*ras*-1 and catalase loci, but the densitometric scanning of the autoradiogram suggested that the deletion region did not include the catalase locus in this patient.

negative tumors. Other studies have shown that patients with progesterone receptor negative tumors respond poorly to adjuvant endocrine therapy [94]. Consistent with this poor prognosis is the significant association ($P < 0.05$) between chromosome 11p deletions and patients who later develop distal metastasis.

The development of homo- or hemizygosity of certain regions of different chromosomes appears to be an important mechanism contributing to tumor development. How do tumor cells achieve loss of constitutional heterozygosity of certain genes? Several abnormal somatic segregation events might bring about homozygosity, including: (1) mitotic recombination between a particular locus and the centromere; (2) mitotic nondisjunction with the loss of one chromosomal homologue and in some cases reduplication of the other chromosome; (3) interstitial deletions; (4) gene conversion [77, 95, 96]. Evidence for some of these mechanisms, especially mitotic nondisjunction followed by reduplication, has been observed in pediatric malignancies [77, 80–84]. In the case of the primary breast tumors, a comparison of the hybridization signals of the remaining allele in tumor DNA with that in lymphocyte DNA is consistent with reduction to homozygosity by deletions of portions of chromosome 11. The tumor from patient 253 represented one exception. In

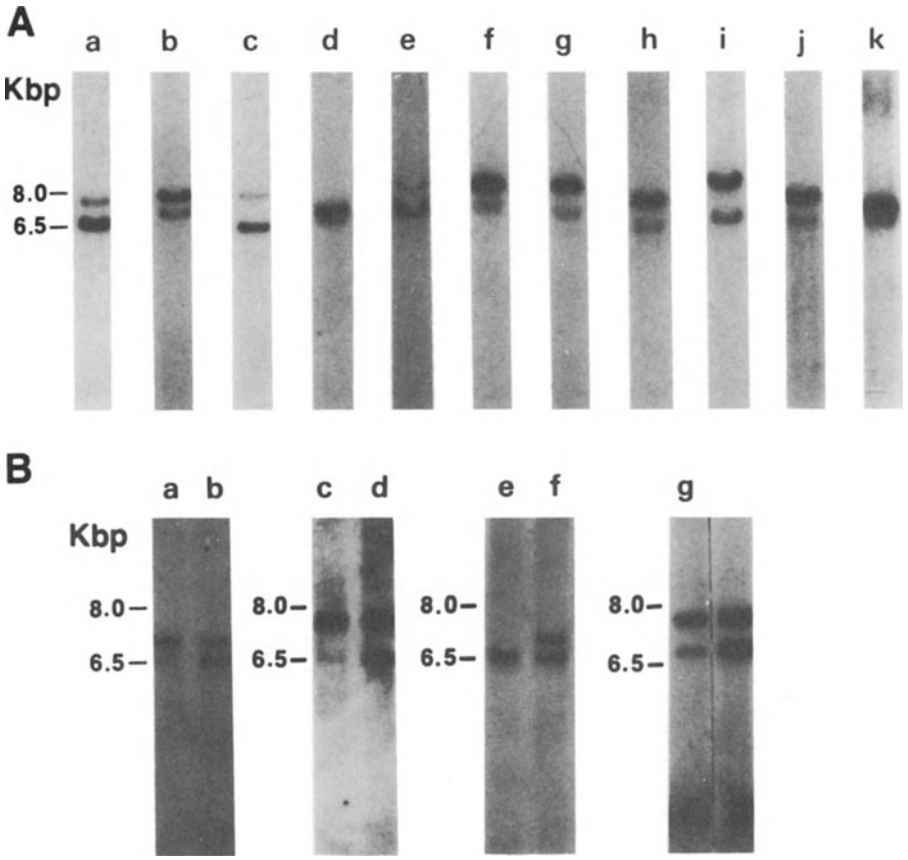


Figure 4. Allelic loss of c-H-*ras*-1 in primary breast tumor DNAs. DNAs were restricted by *Bam*HI and analyzed by the Southern blot technique. (A) DNAs were from tumors 53, 86, 105, 166, 169, 174, 180, 182, 239, 253, and 315 (lanes a-k, respectively). (B) Matched sets of tumor and lymphocyte DNAs, respectively, were from patients 166 (lanes a and b), 253 (lanes c and d), 295 (lanes e and f), and 86 (lanes g and h).

this tumor DNA, one allele each of c-H-*ras*-1 and γ globin on 11p and *int*-2 on 11q was lost, suggesting the loss of an entire chromosomal homologue.

The deletion of cellular sequences could be a nonspecific event, as it appears to occur at several loci throughout the tumor genome in melanoma cell lines [97]. It could thus reflect a generalized instability of the genome in these tumor cell lines. However, in several other tumors, including bladder carcinoma, acoustic neuroma, and uveal melanoma [85–87], reduction to homozygosity appears to be specific for loci on particular chromosomes. In our study of primary breast tumors, we have examined seven markers located on six different chromosomes for evidence of allele loss in the tumor DNAs containing a chromosome 11p deletion. We have found only two tumors with an additional deletion of either c-*erb* A-2 (chromosome 3) or c-*myb* (chromosome 6).

The deletion of sequences on chromosome 11 occurring in breast cancer and other malignancies of epithelial origin suggests the presence of regulatory gene(s) with possible 'suppressor' functions on this chromosome [98, 99]. Various somatic cell hybrid experiments have provided evidence that implicates sequences on the short arm of chromosome 11 in the suppression of tumorigenicity of HeLa cells, a cell line of epithelial origin [100, 101]. Although we have not presently determined whether the chromosome 11p deletions in primary breast tumors are associated with the activation of a cellular gene expression or the loss of a suppressor gene, or both, this should represent a focus for future work.

4. Conclusions and caveats

Table 1 summarizes the genetic alterations that have been detected in primary breast tumors and the clinical parameters with which they are associated. The most frequent mutations (10–34% of the tumor DNAs) involve c-*myc*, *int*-2, and c-*erb* B-2 proto-oncogenes and c-H-*ras*-1 and other genes on chromosome 11p. In addition, mutations have been identified less frequently at the c-*erb* A-1 (5%), c-*erb* B (2%), and c-*myb* (<1%) loci. No mutations have been detected at the c-K-*ras*-2, N-*ras*, N-*myc*, c-*sis*, c-*mos*, and *met* loci. Some of

Table 1. Frequent mutations associated with primary breast tumors

Gene or chromosome	Mutation	Frequency of mutation (%)	Association with clinical parameters	*P* values
c-*myc*	amplification/ rearrangement	34	patients >51 years of age	<0.02
int-2	amplification	16	distal metastasis	<0.001
c-*erb* B-2	amplification	28[a]	>3 affected lymph nodes	<0.002
			ER-negative tumors	<0.05
			PR-negative tumors	<0.06
			survival	<0.0011
			relapse	<0.0001
		16[b]	none	—
		10[c]	none	—
chromosome 11p	deletion	20[c]	histopathological grade III tumors	<0.006
			PR-negative tumors	<0.002
			distal metastasis	<0.05
c-*erb* A-1		6	none	—
c-*erb* B		2	none	—
c-*myb*		<1	none	—

[a] Slamon *et al.* [56].
[b] van de Vijver *et al.* [60].
[c] Ali *et al.* [58, 59].

these alterations seem to have significant association with one or more clinical parameters and therefore could be of potential prognostic value. There are some limitations, however. One problem is the variation in frequency of specific mutations observed by different laboratories (Table 1). The variable amount of adjacent normal stroma in the surgical specimen and the cellular heterogeneity of the breast tumors could partly be responsible for these discrepancies and could have profound effects on: (1) the apparent frequency of mutations, (2) the apparent copy number of an amplified gene, and (3) statistical correlations between the frequency of the mutation and clinical parameters. Thus, to accurately compare tumor samples within a study or studies undertaken by different laboratories, it may be necessary to develop criteria to quantitatively assess the tumor cellularity in the biopsy material used for DNA or RNA extraction.

Another major inconsistency between the results of Slamon *et al.* [56] and those of ours [58, 59] is the lack of association between the copy number of c-*erb* B-2 and the number of positive lymph nodes or the disease-free and overall survival periods. Further studies on an expanded number of tumors by several laboratories are warranted to determine the effects of various factors, including inadvertant sampling errors due to differences in geographical location, genetic background, and dietary factors, and to more firmly establish the clinical usefulness of these markers.

The search for molecular genetic alterations in breast carcinoma has just begun. The number of the genetic abnormalities in breast tumors would very likely increase as more proto-oncogenes and other genetic lesions are analyzed. In view of the extensive genetic heterogeneity of human breast neoplasia, this is not surprising. In fact, many human diseases are probably caused by one of several genes under the given circumstances. The extent of genetic diversity in breast carcinoma is reminiscent of that observed in other inherited disorders, such as xeroderma pigmentosum (nine loci) [102], ataxia telangiectasia (five loci) [103], and cockayne syndrome (three loci) [104]. Conversely, there is also evidence for the existence of shared chromosomal lesions for a variety of related cancers. The carriers of the retinoblastoma locus have several hundred-fold increased potential for the development of osteosarcoma [105, 106]. Similarly, a common loss of heterozygosity of genes on band p13 on chromosome 11 in several embryonal malignancies, including Wilms' tumor, rhabdomyosarcoma, hepatoblastoma, and adrenal carcinoma [80], suggests that these genes might be closely linked. About 5–10% of breast cancers seem to have a genetic element in their etiology [107]. Genetic epidemiologic studies have indicated a high incidence of breast cancer in some families. In members of these high risk families, endometrial carcinomas and other soft tissue sarcomas and brain tumors are also found quite frequently [108, 109]. It has also been reported that mothers of children with osteosarcoma and chondrosarcoma are at 3-fold excess risk of developing breast cancer [110].

The idea of shared chromosomal lesions for related cancers implies the

presence of one locus playing a pleiotropic role in developing various malignancies. This could happen, for example, by controlled stage-specific and/or tissue-specific expression of a particular gene. One could also envision a large multigene complex being responsible for many cancers. It is then conceivable that different overlapping deletions on that chromosome involving different genes may give rise to one or the other type of cancer.

In breast carcinoma, approximately 20% of tumors were found to have lost alleles of several genes on the short arm of chromosome 11. At a molecular level, how might deletions of chromosome 11p contribute to the evolution of the tumor? One possibility is that a deletion can bring the regulation of a cellular gene under new *cis*-acting regulatory elements in a manner similar to that observed with translocations or insertions of foreign genetic material. Such a phenomenon has been observed with c-*myb* in chromosome 6q-leukemias and lymphomas [111]. An alternative possibility is the unmasking of the pre-existing germinal or somatic recessive mutations, thus contributing to the origin of breast tumors.

The amplification of one of the three proto-oncogenes (c-*myc*, c-*erb* B-2, and *int*-2) and deletions on chromsome 11 occur usually in different subsets of breast tumors. However, there were a few tumors (approximately 5%) that had multiple genetic lesions (i.e., amplification of more than one proto-oncogene or deletion of sequences on chromosome 11p together with amplification of one or more proto-oncogene). The clinicopathological parameters of such tumors were not very different from other tumors with single mutations. Therefore, the significance of multiple genetic lesions occuring simultaneously in a single tumor is not clear at present.

FUTURE PROSPECTS

The focus of future studies will be on the characterization of these recently discovered molecular alterations (listed in Table 1) in breast tumors. Some of these areas include structural and functional characterization of the amplified and rearranged oncogenes in primary breast tumors. The transforming potential of c-*erb* B-2, like c-H-*ras*, was attributed to point mutations that cause substitution of a single amino acid in the transmembrane domain of the 185-kDa protein that displays tyrosine protein kinase activity [112]. The cloning and nucleotide analysis of the amplified c-*erb* B-2 sequence from primary breast tumors, with and without lymph node involvement, might shed some light on the role of this proto-oncogene in tumors that apparently have different biological behaviors.

Only a fraction of the known proto-oncogenes have been examined in primary breast tumor DNA. In addition, due to the complexity of factors contributing to breast tumor growth and development, it seems probable that other cellular genes (i.e., growth factors and hormone receptors) will be found to be frequently altered in tumor DNA. In this regard, analysis of the

c-*erb* A family of proto-oncogenes may prove informative. This family is composed of at least five cellular genes, located on chromosomes 3 and 17, which are related to the avian v-*erb* A oncogene [60, 113, 114]. Recently, it has been reported that the thyroid hormone receptor is the gene product of one of the members of the c-*erb* A gene family [114, 115]. Similarly, the vitamin D3 receptor has been found to have homology with the members of this super gene family [116]. All of these receptors are located in the cell nucleus and are believed to modulate gene expression at the level of transcription [117]. The effect of the estrogen and progesterone receptors on breast tumor growth and their association with patient prognosis is well established. Several studies have suggested that thyroid hormone may also profoundly affect the course of breast tumor growth in rodents and perhaps humans [118]. Based on these considerations, it seems reasonable to speculate that genetically altered members of the c-*erb* A family may be found in primary breast tumor DNA.

The current studies have focused primarily on advanced, moderately to poorly differentiated ductal carcinomas. These tumors represent a late stage in the development of the disease. Although this histiotype is the most common, others are recognized [1]. In addition, we do not know whether the mutations that have been detected are associated with the initial stages of malignant transformation or are positively selected during later stages in the evolution of the disease. The recent development of techniques that lead to the recovery of high molecular weight DNA from formalin-fixed tissue specimens embedded in paraffin [119] should provide an additional resource to obtain adequate samples of rare histiotypes, early tumors, and tumors from patients with inheritable breast cancer. Moreover, the vast archives of tumor specimens in pathology departments throughout the world could be used in retrospective studies to determine the prognostic significance of specific genetic mutations.

Approximately 5–10% of breast cancers have a genetic etiology and seem to cluster in families [107–109]. Linkage analysis of large pedigrees by use of polymorphic genetic markers on chromosome 11 might provide useful information and help following the segregation of the disease with reference to a particular gene in families. Also, the significance of the rare c-H-*ras*-1 allele can be determined by studies of breast cancer families.

The deletion of sequences on the short arm of chromosome 11 suggests that cryptic recessive mutations in the genes with possible regulatory functions might underlie the development of at least a subset of breast tumors. Even if the importance of the region between the globin and PTH loci is born out by analysis of a large number of tumors, identification of the putative gene(s) will not be an easy task because of the substantial genetic distance involved. The recently developed techniques of pulsed field gel electrophoresis [120–122] in combination with chromosome 'jumping' [122–125] may make our search for these abstract diseased genes over long stretches of human genomic DNA a little more practical. Other limitations in the study of breast

neoplasms and many other human diseases have been their unknown biochemical nature and a lack of suitable functional assays. The approach of 'reverse genetics' [126] may provide a better understanding of the diseased genes once they are identified. In this regard, it would be interesting to see if the introduction of the recently cloned retinoblastoma gene [127, 128] would revert the malignant phenotype of the tumor cells in culture. In case of the breast neoplasm, given the diversity of the disease, it may not be that simple.

In summary, abnormalities of at least four and maybe more proto-oncogenes exist in breast tumors. There is also suggestive evidence for the presence of recessive oncogenes. A molecular understanding of human breast neoplasia, a disease with very diversified biological traits and genetic heterogeneity, represents a challenge and also provides a possible model system to understand the involvement of dominant proto-oncogenes and tumor suppressor genes and their possible interaction in this human cancer.

References

1. Harris JR, Hellman S, Canellos GP, Fisher B: Cancer of the breast. In Cancer principles and practice of oncology, DeVita VT, Hellman S, Rosenberg SA (eds), Philadelphia, JB Lippincott Company, 1982, pp 1119–1178.
2. Gompel G, Van Kerkem C: The breast. In: Principles of Surgical Pathology, Vol. I, Silverberg S (ed), New York, Wiley Medical Publication, 1983, pp 245–255.
3. Barker PE, Hsu TC: Double minutes with special reference to breast carcinomas. J Natl Cancer Inst 62: 257–262, 1979.
4. Kovacs G: Homogeneously staining regions on marker chromosomes in malignancy. Int J Cancer 23: 299–301, 1979.
5. Quinn LA, Moore GE, Morgan RT, Woods LK: Cell lines from human colon carcinoma with unique cell products, double minutes and homogeneously staining regions. Cancer Res 39: 4914–4924, 1979.
6. Barker PE, Lau YF, Hsu TC: A heterochromatic homogeneously staining region (HSR) in the karyotype of a human breast carcinoma cell line. Cancer Genet Cytogenet 1: 311–319, 1980.
7. Rodgers CS, Hill SM, Hulten MA: Cytogenetic analysis in human breast carcinoma. I. Nine cases in the diploid range investigated using direct preparations. Cancer Genet Cytogenet 13: 95–119, 1984.
8. Kovacs G, Preferential involvement of chromosome 1q in a primary breast carcinoma. Cancer Genet Cytogenet 3: 115–129, 1981.
9. Trent JM: Cytogenetic and molecular biologic alterations in human breast cancer: A review. Breast Cancer Res Treat 5: 221–229, 1985.
10. Satya-Prakash KL, Podhak S, Hsu TC, Olive M, Cailleau R: Cytogenetic analysis on eight human breast tumor cell lines: High frequencies of 1q, 11q, and HeLa-like marker chromosomes. Cancer Genet Cytogenet 3: 61–73, 1981.
11. Kraus MH, Yuasa Y, Aaronson SA: A position 12-activated H-*ras* oncogene in all HS578T mammary carcinoma cells but not normal mammary cells of the same patient. Proc Natl Acad Sci USA 81: 5384–5388, 1984.
12. Fasano O, Birnbaum D, Edlund L, Fogh J, Wigler M: New human transforming genes detected by a tumorigenicity assay. Mol Cell Biol 4: 1695–1705, 1984.
13. Groner B, Kozma S, Hynes NE, Redmond S, Jaggi R, Gunzburg W, Slamons B, Ball R, Buser K, Reichmann E, Anders AC: The search for oncogenes in breast cancer. In: Breast

cancer: origins, detection and treatment, Rich MA, Hager JC, Taylor-Papadimitriou J (eds), Boston, Martinus Nijhoff, 1985. pp 195–204.
14. Manolagas SC, Provvedini DM, Murray EJ, Murray SS, Tsonis PA, Spandialos DA: Association between the expression of the c-*myc* oncogene mRNA and the expression of the receptor protein for 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci USA 84: 856–860, 1987.
15. Kasid A, Lippman ME, Papageorge AG, Lowy DR, Gelmann EP: Transfection of v-*ras*^{H} DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorigenicity. Science 228: 725–728, 1985.
16. Graham KA, Richardson CL, Minden MD, Trent JM, Buick, RN: Varying degrees of amplification of the N-*ras* oncogene in the human breast cancer cell line MCF7. Cancer Res 45: 2201–2205, 1985.
17. Kozbor D, Croce C: Amplification of the c-*myc* oncogene in one of five human breast carcinoma cell lines: Cancer Res 44: 438–441, 1984.
18. Modjtahedi N, Lavielle C, Poupon M-F, Landin RM, Cassingena R, Monier R, Brison O: Increased level of amplification of the c-*myc* oncogene in tumors induced in nude mice by a human breast carcinoma cell line. Cancer Res 45: 4372–4379, 1985.
19. King CR, Kraus MH, Aaronson SA: Amplification of a novel v-*erb* B related gene in a human mammary carcinoma. Science 229: 974–976, 1985 .
20. Aaronson SA, Tronick SR: The role of oncogenes in human neoplasia. In: Important Advances in Oncology 1985, DeVita VT, Hellman S, Rosenberg SA (eds), Philadelphia, J.B. Lippincott Company, 1985, pp. 3–15.
21. Bishop JM: The molecular genetics of cancer. Science 235: 305–311, 1987.
22. Alitalo K: Amplification of cellular oncogene in cancer cell. Med Biol 62: 304–317, 1984.
23. Rabbits TH: The c-*myc* proto-oncogene: Involvement in chromosomal abnormalities. Trends Genet 1: 327–331, 1985.
24. Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM: Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80: 1707–1711, 1983.
25. Dalla Favera R, Wong-Staal F, Gallo RC: One gene amplification in promyelocytic leukemia cell line HL-60 and primary leukemic cells of the same patient. Nature 299: 61–63, 1982.
26. Schwab M, Alitalo K, Klempnauer K-H, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J: Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305: 245–248, 1983.
27. Little CD, Nau MM, Calney DN, Gazdar AF, Minna JD: Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306: 194–196, 1983.
28. Nau MM, Brooks BJ, Battery J, Sausville E, Gazdar AF, Kirsh JR, McBride DW, Beetness V, Hollis GF, Minna JD: L-*myc*, a new *myc*-related gene amplified in human small cell lung cancer. Nature 318: 69–73, 1985.
29. Stewart T, Pattengale P, Leder P: Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/*myc* fusion genes. Cell 38: 627–637, 1984.
30. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme MH, Gest J, Callahan R: Genetic alteration of the c-*myc* proto-oncogene in human primary breast carcinoma. Proc Natl Acad Sci USA 83: 4834–4838, 1986.
31. Lidereau R, Mathieu-Mahul D, Theillet C, Renaud M, Manchauffe M, Gest J, Larsen CJ: Presence of allelic *Eco*RI restriction fragment of the c-*mos* locus in leukocyte and tumor cell DNAs of breast cancer patients. Proc Natl Acad Sci USA 82: 7068–7070, 1985.
32. Yokota J, Tsunetsugu-Yokota Y, Battifora H, LeFevre C, Cline MJ: Alterations of *myc*, *myb* and *ras*^{Ha} proto-oncogenes in cancers are frequent and show clinical correlation. Science 231: 261–265, 1986.
33. Slamon DJ, de Kernion JB, Verma IM, Cline MJ: Expression of cellular oncogenes in

human malignancies. Science 224: 256–262, 1984.
34. Erisman MD, Rothberg PG, Diehl RE, Morse CC, Spandorfer JM, Actrin SM: Deregulation of c-*myc* gene expression in human colon carcinoma is not accompanied by amplification or rearrangement of the gene. Mol Cell Biol 5: 1969–1976, 1985.
35. Mariani-Costantini R, Escot C, Theillet C, Gentile A, Merlo G, Lidereau R, Callahan R: *In situ* c-*myc* expression and genomic status of the c-*myc* locus in infiltrating ductal carcinomas of the breast. Cancer Res 48:199–205, 1988.
36. Callahan R: Retrovirus and proto-oncogene involvement in the etiology of breast neoplasia. In: The mammary gland: development, regulation, and function, Neville P, Daniel C (eds), New York, Plenum Press, in press, 1987.
37. Nusse R: this volume.
38. Varmus HE: Recent evidence for oncogenesis by insertion mutagenesis and gene activation. Cancer Surv 1: 309–319, 1982.
39. Nusse R, Varmus H: Mammary tumor induced by the mouse mammary tumor virus: Evidence for a common region for provirus integration in the same region of the host genome. Cell 31: 99–109, 1982.
40. Peters G, Brookes S, Smith R, Dickson C: Tumorigenesis by mouse mammary tumor virus: Evidence for a common region for provirus integration in mammary tumors. Cell 33: 369–377, 1983.
41. Gallahan D, Callahan R: Mammary tumorigenesis in feral mice: Identification of a new *int* locus in MMTV (Czech II)-induced mammary tumors. J Virol 61: 66–76, 1986.
42. Nusse R, van Ooyen A, Cox D, Fung Y-K, Varmus H: Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature 307: 131–136, 1984.
43. Dickson C, Smith R, Brookes S, Peters G: Tumorigenesis by mouse mammary tumor virus: Proviral activation of a cellular gene in the common integration region *int*-2. Cell 37: 529–536, 1984.
44. Jakobovits A, Shackleford GM, Varmus HE, Martin GR, Two proto-oncogenes implicated in mammary carcinogenesis, *int*-1 and *int*-2, are independently regulated during mouse development. Proc Natl Acad Sci USA 83: 7806–7810, 1986.
45. Peters G, Lee AE, Dickson C: Activation of cellular gene by mouse mammary tumor virus may occur early in mammary tumor development. Nature 309: 273–275, 1986.
46. Brown AMC, Wildin RS, Prendergast TJ, Varmus HE, A retrovirus vector expressing the putative mammary oncogene *int*-1 causes partial transformation of a mammary epithelial cell line. Cell 46: 1001–1009, 1987.
47. Rijsewijk R, Van Deemter L, Wagenaar E, Sonnenberg A, Nusse R: Transfection of the *int*-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J 6: 127–131, 1987.
48. Lidereau R, Callahan R, Escot C, Dickson C, Peters G, Ali IU: Amplification of MMTV-related *int*-2 locus in primary human breast tumors, Oncogene Res 2, 285–291, 1988.
49. Casey G, Smith R, McGillivray D, Peters G, Dickson, C: Characterization and chromosome assignments of the human homolog of *int*-2, a potential proto-oncogene. Mol Cell Biol 6: 502–510, 1986.
50. Shih C, Padney L, Murray M, Weinberg RA: Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature 290, 261–264, 1981.
51. Schechter AL, Hung MC, Valdyanathan L, Weinberg RA, Yang-Feng TL, Francke U, Ullrich A, Coussens L: The *neu* gene: An *erb* B homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 229: 976–978, 1985.
52. Schechter AL, Stern DF, Valdyanathan L, Decker SJ, Drebin JA, Greene ME, Weinberg RA, The *neu* oncogene: an *erb* B related gene encoding a 185,000-M tumor antigen. Nature 312: 513–516, 1984.
53. Coussens L, Yang-Feng TL, Liao Y-C, Chen E., Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A, and Ullirch A (1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science* 230, 1132–1139.

54. Semba K, Kamata N, Toyoshima F, Yamamoto T: A v-*erb* B related protooncogene, c-*erb* B-2, is distinct from the c-*erb* B-1/EGF receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA 82: 6497–6501, 1985.
55. Fukushige SI, Mastsubara KI, Yoshida M, Sasaki M, Suzuki T, Semba K, Toyoshima K, Yamamoto T, Localization of a novel v-*erb* B related gene, c-*erb* B-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol Cell Biol 6: 955–958, 1986.
56. Slamon DJ, Clark GM, Wong SG, Levin WS, Ullrich A, McGuire WL, Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235: 177–182, 1987.
57. Brodeur GM, Seager RC, Schwab M, Varmus HE, Bishop JM: Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. Science 224: 1121–1124, 1984.
58. Ali IU, Campbell G, Lidereau R, and Callahan R: Is there evidence for an association between c-*erb* B_2 amplification and aggressiveness of human breast carcinoma? *Science* in press.
59. Ali IU, Campbell G, Lidereau R, and Callahan R: Lack of evidence for the prognostic significance of c-*erb*B-2 in human breast carcinoma. *Oncogene Research* in press.
60. van de Vijver M, van de Bersselaar R, Devilee P, Cornelisse C, Peterse J, Nusse R: Amplification of *neu* (c-*erb* B-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-*erb* A oncogene. Mol Cell Biol. In press, 1987.
61. Spurr NK, Solomon B, Jansson M, Sheer D, Goodfellow PN, Bodmer WF, Venstrom B: Chromosomal location of the human homologues to the oncogenes *erb* A and B. EMBO J 3: 159–163, 1984.
62. Mathieu-Mahul D, Xu DO, Lidereau R, Galibelt F, Berger R, Mauchauffe M, Larsen CJ: An *Eco*RI restriction fragment length polymorphism (RFLP) in the human c-*erb* A locus: Hum Genet 71: 41–44, 1985.
63. Isobe M, Emmanuel BS, Givol D, Oren M, Croce CM: Localization of the gene for human p53 tumor antigen to band 17p13. Nature 320: 84–85, 1986.
64. Capon DJ, Chen EY, Levinson AD, Seeburg PH, Goeddel DB, Complete nucleotide sequences of the T24 human bladder carinoma oncogene and its normal homologue. Nature 302: 33–37, 1983.
65. Krontiris TG, DiMartino NA, Colb M, Parkinson DR: Unique allelic restriction fragments of the human Ha-*ras* locus in leukocyte and tumor DNAs of cancer patients. Nature 313: 369–374, 1985.
66. Lidereau R, Escot C, Theillet C, Champeme MH, Brunet M, Gest J, Callahan R: High frequency of rare alleles of the human c-Ha-*ras*-1 p-rotooncogene in breast cancer patients. J Natl Cancer Inst 77: 697–701, 1986.
67. Horan Hand P, Thor A, Wunderlich D, Muraro R, Caruso A, Schlom J: Monoclonal antibodies of predefined specificity detect activated *ras* gene expression in human mammary and colon carcinomas. Proc Natl Acad Sci USA 81: 5227–5231, 1984.
68. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R, Frequent loss of a H-*ras*-1 allele correlates with aggressive human primary breast carcinomas. Cancer Res 46: 4476–4781, 1986.
69. Spandidos DA, Agnantis NJ: Human malignant tumors of the breast, as compared to their respective normal tissue, have elevated expression of the Harvey-*ras* oncogene. Anticancer Res 4: 269–279, 1984.
70. Lidereau R, Simpson J, Thor A, Ohuchi N, Gentile A, Schlom J, Callahan R: A correlation between c-H-*ras*-1 genotype and *ras* p21 expression in primary human breast tumors. Manuscript in preparation, 1988.
71. Ohuchi N, Thor A, Page DL, Horan Hand P, Halter SA, Schlom J: Expression of the 21,000 molecular weight *ras* protein in a spectrum of benign and malignant human mammary tumors. Cancer Res 46: 2511–2519, 1986.
72. Theillet C, Mariani-Costantini R, Hutzell P, Merlo G, French D, Schlom J, Callahan R,

The c-*myc* and c-H-*ras*-1 proto-oncogenes in primary human colon adenocarcinomas: Gene structure and expression. Submitted to J Histochem Cytochem, 1988.
73. Heighway J, Thatcher N, Cerny T, Hasleton PS: Genetic predisposition to human lung cancer. Br J Cancer 53: 453–457, 1986.
74. Thein SL, Oscier DG, Flint J, Wainscoat JS: Ha-*ras* hypervariable alleles in myelodysplasia. Nature 321: 84–85, 1986.
75. Gerhard DS, Dracopoli NC, Bale SJ, Houghton AN, Watkins P, Payne CE, Greene MH, Housman DE: Evidence against Ha-*ras*-1 involvement in sporadic and familial melanoma. Nature 325: 73–75, 1987.
76. Knudson AG: Mutation and cancer: Statistical study of retinoblastoma. Proc Natl Acad Sci USA 68: 820–823, 1971.
77. Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Godbort R, Gallie BL, Murphee AL, Strong LC, White RL: Expression of recessive alleles by chromosomal mechanism in retinoblastoma. Nature 305: 779–784, 1983.
78. Benedict WF, Murphee AL, Banerjee A, Spina CA, Sparkes MC, Sparkes RS: Patient with 13 chromosome deletion: Evidence that the retinoblastoma gene is a recessive cancer gene. Science 219: 973–975, 1983.
79. Dryja TP, Cavanee W, White R, Rapaport JM, Peterson R, Albert DM, Burns GAP: Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. N Engl J Med 310: 550–553, 1984.
80. Koufos A, Hansen MF, Copeland NG, Jenkins NA, Lampkin BC, Cavanee WK: Loss of heterozygosity in three embryonal tumors suggests a common pathogenetic mechanism. Nature 316: 330–334, 1985.
81. Koufos A, Hansen MF, Lampkin BC, Wakman ML, Copeland NG, Jenkins NA, Cavanee WK: Loss of allele at loci on human chromosome 11 during genesis of Wilms' tumor. Nature 309: 170–172, 1984.
82. Orkin SH, Goldman DS, Sallan SE: Development of homozygosity for chromosome 11p markers in Wilms' tumor. Nature 309: 172–178, 1984.
83. Reeve AE, Harsiaux PJ, Gardner RJM, Chewings WE, Grindley RM, Millow LJ: Loss of Harvey *ras* allele in sporadic Wilms' tumour. Nature 309: 174–176, 1984.
84. Fearon ER, Vogelstein B, Feinberg AP: Somatic deletion and duplication of genes on chromosome 11 in Wilms' tumors. Nature 309: 176–178, 1984.
85. Fearon ER, Feinberg AP, Hamilton SH, Vogelstein B: Loss of genes on the short arm of chromosome 11 in bladder cancer. Nature 318: 377–380, 1985.
86. Seizinger BR, Martuza RL, Gusella JF: Loss of genes on chromosome 22 in tumorigenesis of human acoustic neuroma. Nature 322: 644–647, 1986.
87. Mukai S, Dryja TP: Loss of alleles at polymorphic loci on chromosome 2 in uveal mela noma. Cancer Genet Cytogenet 22: 45–53, 1986.
88. Knudson AG: Model hereditary cancers of man. Prog Nucleic Acid Res Mol Biol 29: 17–25, 1983.
89. Ali IU, Lidereau R, Thiellet C, Callahan R: Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science 238:185–188, 1987.
90. Turleau C, de Grouchey J, Nihoul-Fekete C, Dufier JL, Chavin-Colin F, Junien C: Del 11p13/nephroblastoma without aniridia, Hum Genet 67: 455–456, 1984.
91. Michalopoulos EE, Bevilacqua PJ, Stokoe N, Powers VE, Willard HF, Lewis WH: Molecular analysis of gene deletion in aniridia-Wilms' tumor association. Hum Genet 70: 157–162, 1985.
92. Van Heyningen V, Boyd PA, Seawright A, Fletcher JM, Fantes JA, Buckton KE, Spowart G, Porteous DJ, Hill RE, Newton MS, Hastie ND: Molecular analysis of chromosome 11 deletions in aniridia-Wilms' tumor syndrome. Proc Natl Acad Sci USA 82: 8592–8596, 1985.
93. Glaser T, Lewis WH, Bruns GAP, Watkins PC, Rogler CE, Shows TB, Powers VE, Willand HF, Goguen JM, Simola KOJ, Housman DE: The subunit of follicle-stimulating hormone is deleted in patients with aniridia and Wilms' tumor, allowing a further definition

of the WGAR locus. Nature 321: 882–887, 1986.
94. Clark GM, McGuire WL, Hubag CA, Pearson OH, Marshall JS: Progresterone receptors as a prognostic factor in stage II breast cancer. N Engl J Med 309: 1343–1347, 1983.
95. Murphee AL, Benedict WF: Retinoblastoma: Clues to human oncogenesis. Science 223: 1028–1033, 1984.
96. Cavanee WK, Hansen MF, Nordenskjold M, Kock E, Maumenee I, Squire JA, Phillips RA, Gallie BL: Genetic origin of mutations predisposing to retinoblastoma. Science 228: 501–503, 1985.
97. Dracapoli NC, Houghton AN, Lloyd JO: Loss of polymorphic restriction fragments in malignant melanoma: Implication for tumor heterogeneity. Proc Natl Acad Sci USA 82: 1470–1474, 1985.
98. Stanbridge EJ: A case for human tumor-suppressor genes. BioEssays 3: 252–255, 1985.
99. Sager R: Genetic suppression of tumor formation: A new frontier in cancer research. Cancer Res 46: 1573–1580, 1986.
100. Stanbridge EJ, Flandermeyer RR, Daniels DW, Nelson-Rees WA, Specific chromosome loss associated with the expression of tumorigenicity in human cell hybrids. Somatic Cell Genet 7: 699–712, 1981.
101. Kaelbling M, Klinger HP: Suppression of tumorigenicity in somatic cell hybrids. III. Cosegregation of human chromosome 11 of a normal cell and suppression of tumorigenicity in intraspecies hybrids of normal diploid X malignant cells. Cytogenet Cell Genet 41: 65–70, 1985.
102. Keijzer W, Jaspers NGJ, Abrahams PJ, Taylor AMR, Arlett CF, Zelle B, Takebe H, Kinmont PDS, Bootsma D: A seventh complementation group in xeroderma pigmentosum. Mutat Res 62: 183–190, 1979.
103. Jaspers NGJ, Painter RB, Paterson MC, Kidson C, Inoue T. In: Ataxia-Telangiectasia, Gatti RA, Swift M, (eds), New York, Liss, 1985, pp 147–162.
104. Lehman AR: Three complementation groups in cockayne's syndrome. Mutat Res 106: 347–356, 1982.
105. Hansen MF, Koufos A, Gallie BL, Phillips RA, Fodstad O, Brogger A, Gedde-Dahl T, Cavanee WK: Osteosarcoma and retinoblastoma: A shared chromosomal mechanism revealing recessive predisposition. Proc Natl Acad Sci USA 82: 6216–6220, 1985.
106. Dryja TP, Rapaport JM, Epstein J, Goorin AM, Weichsebaum R, Koujos A, Cavanee WK: Chromosome 13 homozygosity in osteosarcoma without retinoblastoma. Am J Hum Genet 38: 59–66, 1986.
107. Lynch HT, Albano WA, Danes S, Layton MA, Kimberling WJ, Lynch JF, Cheng SC, Costello KA, Mulcahy GM, Wagner CA, Tindall SL: Genetic predisposition to breast cancer. Cancer 53: 612–622, 1984.
108. Go RCP, King MC, Bailey-Wilson J, Elston RC, Lynch HT, Genetic epidemiology of breast cancer and associated cancers in high risk families. I. Segregation analysis. J Natl Cancer Inst 71: 455–461, 1983.
109. King MC, Go RCP, Lynch HT, Elston RC, Terasaki PI, Petrakis NL, Rodgers GC, Lattanzio D, Bailey-Wilson J: Genetic epidemiology of breast cancer and associated cancers in high risk families. II. Linkage analysis. J Natl Cancer Inst 71: 463–467, 1983.
110. Hartley AL, Birch JM, Marsden HB, Harris M: Breast cancer risk in mothers of children with osteosarcoma and chondrosarcoma. Br J Cancer 54: 819–823, 1986.
111. Barletta C, Pelicci PG, Kenyon LC, Smith SD, Dalla-Favera R: Relationship between the c-*myb* locus and the 6q- chromosomal aberration in leukemias and lymphomas. Science 235: 1064–1067, 1987.
112. Bargmann CI, Hung MC, Weinberg RA: Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. Cell 45: 649–657, 1986.
113. Jansson M, Philipson L, Venstrom B: Isolation and characterization of multiple human genes homologous to the oncogenes of avian erythroblastosis virus. EMBO J 2: 561–565, 1983.

114. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruot DJ, Evans RM: The c-*erb* A gene encodes a thyroid hormone receptor. Nature 324: 641–646, 1986.
115. Sap J, Munoz A, Damm K, Goldberg Y, Ghysdad J, Leutz A, Berg H, Venstrom B: The c-*erb* A protein is a high-affinity receptor for thyroid hormone. Nature 324: 635–640, 1986.
116. McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR, O'Malley BW: Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235: 1214–1217, 1987.
117. Yamamoto KR: Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet 19: 209–252, 1985.
118. Vonderhaar BK: Effect of thyroid hormones on mammary tumor induction and growth. In: Hormonal Regulation of Mammary Tumors Leung BS, (ed), Montreal, Canada, Eden Press, pp 138–154, 1982.
119. Dubeau L, Chandler LA, Gralow JR, Nichols PW, Jones PA: Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. Cancer Res 46: 2964–2969, 1986.
120. Carle GF, Frank M, Olson MV: Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. Science 232: 65–68, 1986.
121. Smith CL, Lawrence SK, Gillespie GA, Cantor CR, Weissman SM, Collins FS: Strategies for mapping and cloning macro-regions of mammalian genomes. Methods Enzymol: 151:461–489, 1987.
122. Chu G, Vollrath D, Davis RW: Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science 234: 1582–1585, 1986.
123. Collins FS, Wesisman SM: Directional cloning of DNA fragments at a large distance from an initial probe: A circularization method. Proc Natl Acad Sci USA 81: 6812–6816, 1984.
124. Poustka A, Lehrach H: Jumping libraries and linking libraries: The next generation of molecular tools in mammalian genetics. Trends Genet 2: 174–179, 1986.
125. Collins FS, Drumm ML, Cole JL, Lockwood WK, Van de Woude GF, Iannuzzi MC: Construction of a general human chromosome jumping library, with application to cystic fibrosis. Science 235: 1046–1049, 1987.
126. Orkin S: Reverse genetics and human disease. Cell 47: 845–850, 1987.
127. Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP: A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323: 643–646, 1986.
128. Lee W-H, Bookstein R, Hong F, Young L-H, Shew J-Y, Lee EY-UP: Human retinoblastoma susceptibility gene: Cloning, identification and sequence. Science 235: 1394–1399, 1987.
129. Grzeschik A, Kazazian HH: Report of the committee on the genetic constitution of chromosome 10, 11, and 12. Cytogenet Cell Genet 40: 179–205, 1985.

3. Different mechanisms are responsible for oncogene activation in human mammary neoplasia

Matthias H. Kraus, Pier Paolo Di Fiore, Jacalyn H. Pierce, and Stuart A. Aaronson

1. Introduction

Investigations of genetic alterations associated with neoplasia have identified a limited set of cellular genes, termed proto-oncogenes, that are highly conserved in vertebrate evolution. Acute transforming retroviruses have substituted viral genes essential for replication with these discrete segments of host genetic information. When incorporated within the retroviral genome, such transduced sequences acquire the ability to induce neoplastic transformation as viral oncogenes, an observation that initially linked these genes to the neoplastic process [1, 2].

The analysis of their encoded transforming protein products has provided important insights into the physiological role of oncogenes and proto-oncogenes in malignant and normal cells. Certain oncogenes share such properties as enzymatic activities, subcellular location, or sequence homologies and thus can be classified according to biological functions of their gene products [3–5]. The first class is represented by the v-*sis* oncogene, whose gene product is closely related to a growth factor polypeptide. A second class comprises genes encoding protein kinases. Their gene products phosphorylate tyrosine, serine, or threonine residues in proteins, the majority possessing the first activity. All of these transforming protein share significant amino acid sequence similarities and are located in the cytoplasm or plasma membrane. The v-*erb*B and v-*fms* oncogenes are related to growth factor receptors. Receptors for a number of other growth factors and hormones appear to be protein kinases, suggesting that additional relationships between known oncogenes and receptor molecules are likely to be discovered. The homology of certain proto-oncogenes with effector molecules of growth regulation has established the importance of proto-oncogene products for normal cellular growth.

Another major class comprises the *ras* genes, which encode closely related proteins with molecular weights of 21 kd. *Ras* p21 proteins bind guanine nucleotides, and catalyze the hydrolysis of GTP. Although the functions of *ras* proto-oncogenes remain to be elucidated, there is evidence that normal *ras*-encoded p21 may be part of a second messenger system for signal trans-

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

duction in the cytoplasm of the cell by virtue of the associated GTP binding and GTPase activity. A fourth class of viral oncogenes encode proteins located in the cell nucleus. Some of these nuclear oncogene products have been found to bind double-stranded DNA and are thus suspected of influencing transcriptional regulation.

In human malignancies and experimentally-induced animal tumors, evidence has emerged that some of the very same cellular genes which have given rise to retroviral *onc* genes (v-*onc*) can be activated as cellular transforming genes (c-*onc*), completely independent of retroviral transduction. Mechanisms by which proto-oncogenes become activated in human tumors include point mutations, DNA rearrangement, promoter insertion, and gene amplification. These alterations commonly result in elevated mRNA and protein levels or expression of a structurally altered proto-oncogene product [6].

This chapter focuses on different mechanisms by which proto-oncogenes can become activated in human mammary tumor cells including somatic activation of a cellular *ras* gene by point mutation and overexpression of a novel *erb*B-related growth factor receptor-like gene, *erb*B-2, in the presence or absence of gene amplification. Expression of such activated oncogene products in cultured assay cells demonstrated that each mechanism independently suffices to confer the neoplastic phenotype to NIH/3T3 fibroblasts.

2. Cellular transforming genes in human tumors

The development of DNA-mediated gene transfer techniques has provided an approach for the detection of cellular transforming genes. DNA, from a variety of human tumors and tumor cell lines subjected to transfection analysis in NIH/3T3 cells, induces focal transformation of this contact-inhibited murine cell line. A number of oncogenes have been detected by this approach. Those most often detected have been members of the *ras* gene family comprised of three highly conserved genes, designated H-*ras*, K-*ras* and N-*ras*. The H-*ras* and K-*ras* genes have been initially identified as viral oncogenes, while N-*ras* thus far has eluded capture by a retrovirus. The availability of molecular clones of the normal and activated alleles of human *ras* proto-oncogenes has made it possible to determine the molecular mechanisms responsible for the malignant conversion of such genes. The genetic lesions responsible for activation of cellular *ras* genes in tumors have been localized primarily to single base changes at codons 12 and 61 [1, 6].

The frequency at which cellular *ras* oncogenes have been observed varies but appears at least in part to be dependent on the type of tumor analyzed (Table 1). As many as 30–50% of colon and lung carcinomas have been reported to contain activated K-*ras* oncogenes, whereas activated N-*ras* alleles have been observed at highest frequency in a wide variety of hematopoietic malignancies. N-*ras* oncogenes have been detected in >50 of acute myelog-

Table 1. Detection of *ras* oncogenes in human tumors

Tumor source	Percent positive	ras oncogene activated		
		H-*ras*	K-*ras*	N-*ras*
Carcinoma (lung, gastrointestinal, genitourinary)	10–30	4/12	6/12	2/12
Sarcoma (fibrosarcoma, rhabdomyosarcoma)	~10	0/2	0/2	2/2
Hematopoietic (AML, CML, ALL, CLL)	10–50	0/9	1/9	8/9

enous leukemias analyzed and at lower frequencies in other human tumors of hematopoietic origin [7–10].

3. Ras oncogenes in human mammary neoplasia

To investigate the role of such genes in human mammary neoplasia, we initially analyzed DNAs of mammary tumor cell lines and tissues for transforming activity upon transfection onto NIH/3T3 cells. Analysis of a large series of human mammary tumors and tumor cell lines revealed that among 21 tumors analyzed, only DNA of a carcinosarcoma cell line, HS578T, scored positive in the NIH/3T3 focus-forming assay [11]. These findings established that *ras* oncogenes are detectable in the NIH/3T3 transfection assay at only relatively low frequency in human mammary tumors. However, the detection and molecular cloning of an H-*ras*-related oncogene from the HS578T cell line, derived from a rare human mammary tumor possessing histopathologic features of both carcinoma and sarcoma, has initially demonstrated that *ras* gene activation can occur in human mammary tumor cells.

4. Activated H-ras oncogene in HS578T mammary carcinosarcoma cells

The oncogene activated in HS578T carcinosarcoma cells was identified by Southern blot analysis as a homolog of the human H-*ras* proto-oncogene [11]. To directly establish its mechanism of activation, we cloned the HS578T transforming DNA sequence from a primary transfectant. To map the position of the genetic lesion in the HS578T oncogene leading to its malignant activation, recombinant molecules of the HS578T oncogene and the normal human H-*ras* proto-oncogene were constructed and assayed in transfection. By this analysis, replacement of first coding exon sequences in the H-*ras*

proto-oncogene with homologous sequences of the HS578T oncogene resulted in transforming activity of 3T3 cells, mapping the activating lesion of the HS578T oncogene to its first coding exon. Nucleotide sequence analysis in this region revealed that the HS578T oncogene and the human H-*ras* proto-oncogene differed at a single base within codon 12. The change of a G to an A resulted in a substitution of aspartic acid for glycine at position 12 in the coding sequence (Figure 1).

5. Somatic activation and selection of the HS578T oncogene

It is of critical importance to establish the relevance of an oncogene activated in a human tumor to the neoplastic process. Indirect evidence in support of this relationship derives from the close homology of human *ras* oncogenes to *ras* oncogenes present in retroviruses that induce a variety of malignancies *in vivo*. The G → A base substitution at codon 12 conferred a restriction polymorphism for Msp I/Hpa II on the activated H-*ras* oncogene in HS578T. By

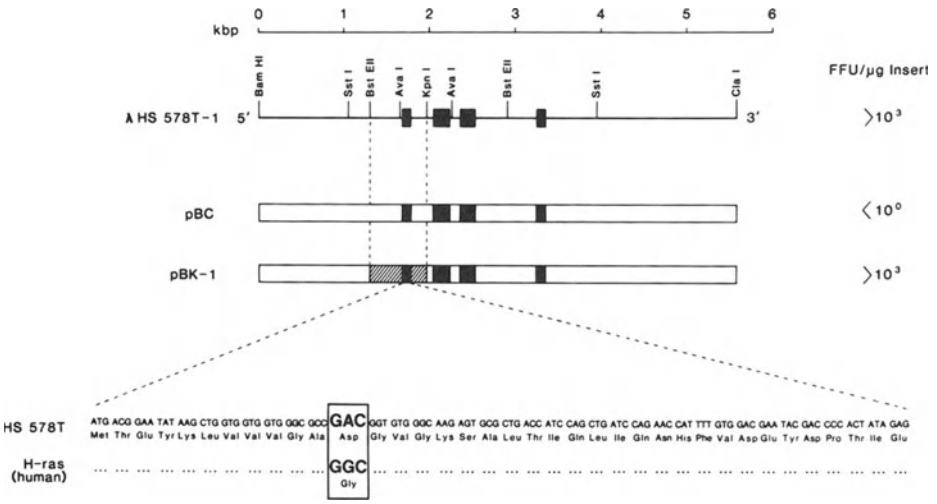


Figure 1. Identification of the lesion that led to activation of the HS578T oncogene. Restriction maps of the λ HS578T oncogene and the H-*ras* proto-oncogene show homologous cleavage sites for various enzymes. The hybrid plasmid pBK-1 was constructed by replacing the 0.69-kbp BstE II/Kpn I fragment in pBC, a plasmid contaning the 5.6-kbp BamH I/Cla I fragment of the human H-*ras* proto-oncogene, by the corresponding domain of the HS578T oncogene (hatched box). After removing the 1.6-kbp BstE II fragment of pBC, this plasmid was ligated with 0.69-kbp BstE II/Kpn I fragment of λ HS578T and the 0.88-kbp Kpn I/BstE II fragment of the H-*ras* proto-oncogene. Transfection analysis was carried out by addition of 0.001-1.0 mg of cloned DNA per plate. The nucleotide and predicted amino acid sequences of the first exon are compared for the HS578T oncogene and the human H-*ras* proto-oncogene. A single base change (guanine to adenine) and the consequent amino acid change (glycine to aspartic acid) are boxed. ffu, Focus forming unit.

molecular genetic analysis we demonstrated the lack of this substitution in alleles present in normal cells from the same patient (Figure 2). These results established that the mutation activating HS578T oncogene was the result of a somatic event selected for within the tumor [11].

To ascertain the importance of this selection process to the tumor itself, we biologically cloned individual tumor cells from a mass population of HS578T breast carcinosarcoma cells. All tumor cell clones analyzed contained the activated allele as demonstrated by the presence of a 411 bp restriction fragment following Msp I digestion and Southern blot analysis using a probe specific for this region of human H-*ras* gene locus. Some clones possessed, whereas others appeared to lack, the faster migrating 355 bp DNA fragment characteristic of the normal H-*ras* allele (Figure 2). Taken together, these findings suggest a powerful selective advantage for tumor cells possessing

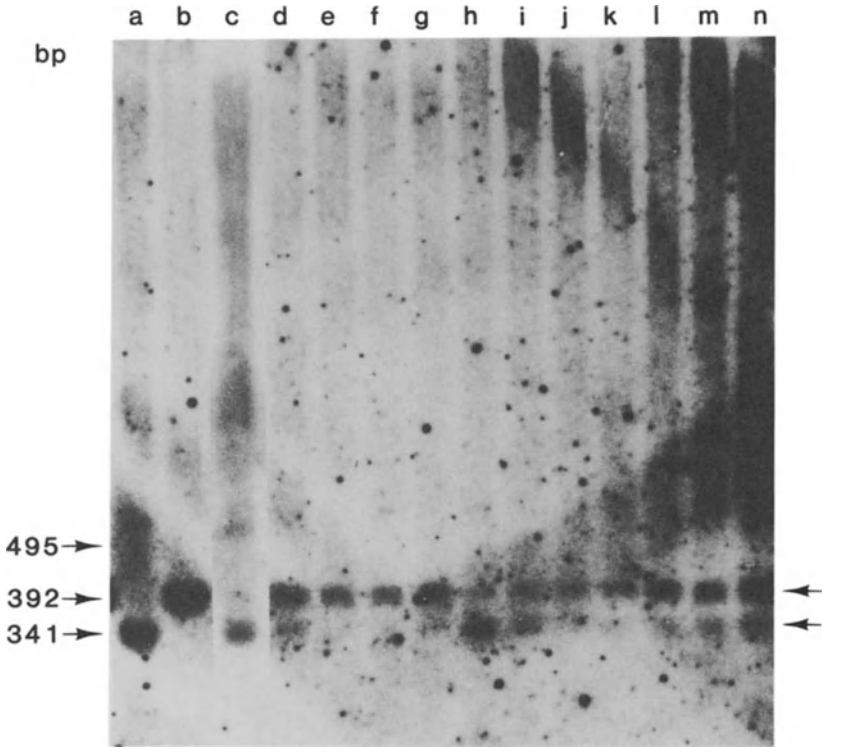


Figure 2. Somatic activation and selection of the HS578T oncogene. The Msp I/Hpa II restriction sensitivity at position 12 of H-*ras* was analyzed in HS578T mammary carcinosarcoma cells (lane d), HS578 Bst normal mammary cells derived from the same patient (lane c), and 10 single-cell-derived cell clones of HS578T (lanes e-n). Human placenta (lane a) and T24 bladder carcinoma DNA (lane b) represent controls for normal position 12 and position 12-mutated human H-*ras* alleles, respectively. $\phi \times 174$ replicative form DNA digested with Hinc II was coelectrophoresed as molecular size standards (labeled in bp).

the H-*ras* oncogene. As such, these findings implied that activation of the oncogene very likely contributed to, rather than represented a consequence of the neoplastic process [11].

6. Increased proto-oncogene expression in human tumors

Other mechanisms including gene amplification, mutations in regulatory sequences, and DNA rearrangement, can result in the altered expression of a proto-oncogene product [6]. Evidence for the differential expression of oncogenes in human tumors was initially obtained by probing mRNAs from human cell lines and tumor tissues with cloned retroviral oncogene sequences [12]. High levels of transcripts related to the v-*myc* oncogene were observed in several tumor cell lines. In the cell line HL 60, a high level of *myc* transcript was shown to be associated with gene amplification of the human c-*myc* proto-oncogene [13]. Similarly, c-*myc* gene amplification results in abundance of c-*myc* mRNA in a colon carcinoma [14]. More recently, a high percentage of human lung carcinoma cell lines have been shown to contain amplified c-*myc* DNA [15]. Many tumor cells containing amplified genes often display abnormal karyotypic markers such as homogeneously staining regions (HSRs), double minutes (DMs) and abnormally banded regions (ABRs). In several instances, amplified *myc* DNA sequences have been found to be associated with such cytogenetic abnormalities [14, 16].

Additional members of the *myc* gene family have been identified in the human genome by finding their related sequences sufficiently amplified in particular tumors to allow detection. This approach facilitated the detection of N-*myc* gene amplification in human neuroblastomas [17, 18] and L-*myc* in human lung tumors [19]. In the case of human neuroblastomas the frequently observed N-*myc* gene amplification is associated with a progressed clinical disease course [20].

7. Identification of a novel EGF receptor-related gene amplified in a human mammary adenocarcinoma

In a further approach to identify activated oncogenes in human mammary neoplasia, we searched for oncogene-related sequences amplified in such tumors. We subjected DNAs of mammary tumor cell lines and tissues to Southern blot analysis utilizing v-*erb*B as a probe [21]. We did so due to the homology of *erb*B to the EGF receptor [22] and the observation that certain mammary tumor cell lines exhibit high EGF binding levels, comparable to those observed in the epidermoid carcinoma cell line A431, which displays EGF receptor gene amplification and rearrangement [23, 24]. In an effort to identify genes that might be candidates for new receptor coding sequences of this gene family, we employed hybridization conditions of moderate stringency

under which proto-oncogenes related to other viral oncogenes of the tyrosine kinase family did not hybridize (data not shown). Thus any gene detected might be expected to have a closer relationship to v-*erb*B than to other members of the typrosine kinase family.

DNA prepared from tissue of a human mammary carcinoma, MAC117, showed a pattern of hybridization (Figure 3A) differing both from that observed with DNA of normal human placenta and the A431 squamous-cell carcinoma line. In A431 DNA, we observed four EcoR I fragments that had increased signal intensities compared to those of corresponding fragments in placenta DNA (Figure 3A). In contrast, MAC117 DNA contained a 6-kilobase pair (kbp) fragment, which appeared to be amplified compared to corresponding fragments observed in both A431 and placenta DNAs (Figure 3A). These findings were consistent with the possibility that the MAC117 tumor contained an amplified DNA sequence related to, but distinct from, the cellular *erb*B proto-oncogene [21].

To define its structure, we undertook the molecular cloning of the 6-kbp EcoR I fragment and we determined its nucleotide sequence in the region

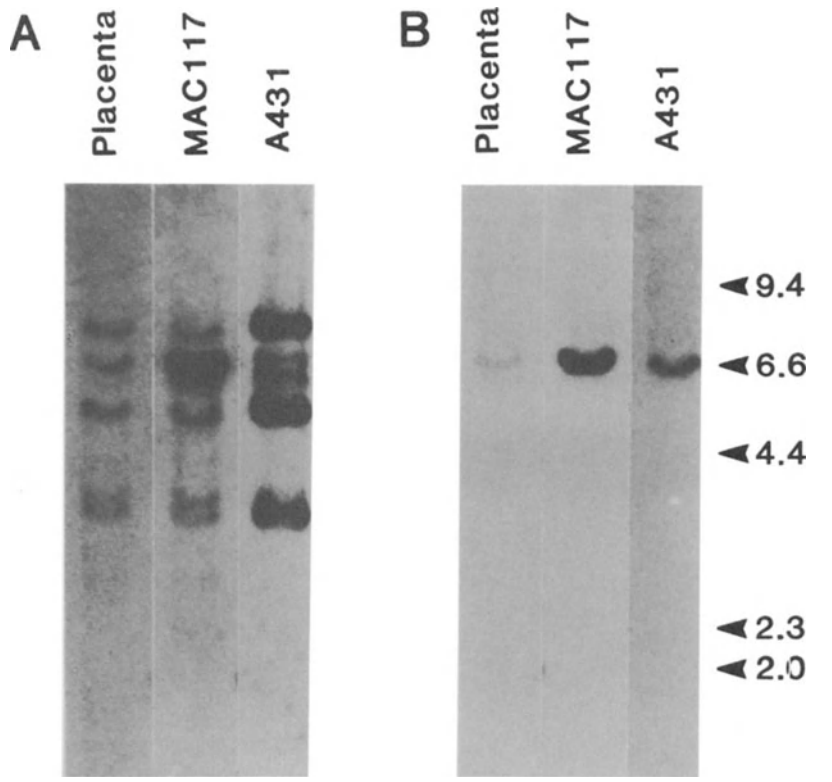


Figure 3. Detection of v-*erb* (panel A) and pMAC117-specific (panel B) gene fragments in normal human placenta, A431 cells, or human mammary carcinoma MAC117.

most homologous to v-*erb*B. This sequence contained two regions of nucleotide sequence homology to v-*erb*B separated by 122 nucleotides. These regions shared 69% nucleotide sequence identity with both the v-*erb*B and the human EGF receptor gene. The predicted amino acid sequence (Figure 4) was 85% homologous to two regions that are contiguous in the EGF receptor sequence [23]. Furthermore, the two putative coding regions were each flanked by the AG and GT dinucleotides which are known to border the exons of eukaryotic genes. All of these findings argue strongly that the sequence represents two exons, separated by an intron, of a gene related to but distinct from the v-*erb*B/EGF receptor gene.

By comparison of the predicted amino acid sequence of the clones designated pMAC117 with corresponding sequences of several members of the tyrosine kinase family, the most striking homology was observed with the human EGF receptor and v-*erb*B. However, we observed 42% to 52% homology with the predicted amino acid sequences of other tyrosine kinase encoding genes as well. In fact, at 25% of the positions, there was identity among each of the sequences analyzed. All of these findings placed the pMAC117 coding sequence within the tyrosine kinase gene family (Figure 4).

The availability of cloned probes of the gene made it possible to investigate its expression in a variety of cell types. The probe detected a single 5-kb transcript in A431 cells (Figure 4). Under the stringent conditions of hybridization utilized, this probe did not detect any of the three RNA species recognized by EGF receptor complementary DNA. Thus, the gene, designated

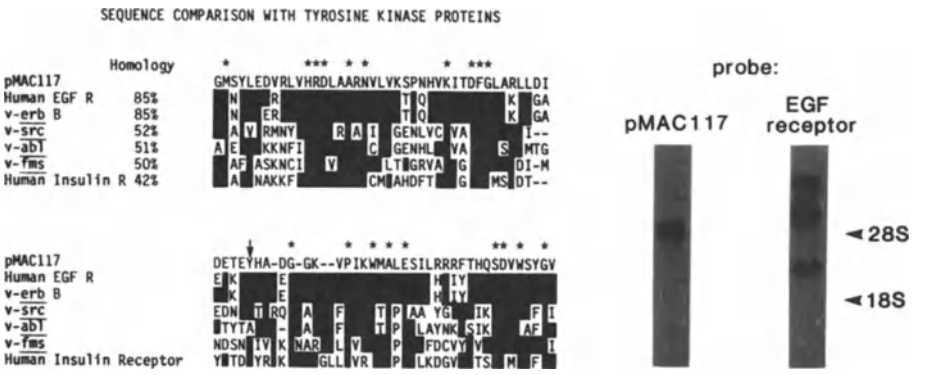


Figure 4. (Left) Comparison of the putative encoded amino acid sequence in pMAC117 with known tyrosine kinase sequence. Black regions represent homologous amino acids. Differing amino acid residues are shown in one letter code. Amino acid positions conserved in all sequences are denoted by (*). the tyrosine homologous to that autophosphorylated by the v-*src* protein is shown by an arrow. The v-*abl* sequence contains a tyrosine residue in this region displaced by two positions. The amino acid sequences of human EGF receptor, v-*erb*B, v-*src*, v-*abl*, v-*fms*, and human insulin receptor were aligned by the computer program described [50]. The homology observed with the predicted amino acid sequences of v-*yes* and v-*fes* was 51% and 48%, respectively. (Right) Detection of distinct messenger RNA species derived from the pMAC117 gene and the human EGF receptor gene.

*erb*B-2, represented a new functional gene within the tyrosine kinase family, closely related to, but distinct from the gene encoding the EGF receptor (21).

8. Overexpression of the *erb*B-2 gene in human mammary tumor cell lines by different molecular mechanisms

The initial identification of *erb*B-2 gene amplification in tissue from a primary mammary adenocarcinoma (MAC 117) suggested the possibility that *erb*B-2 overexpression might contribute to neoplastic growth in this tumor type. To assess the role of *erb*B-2 in human mammary neoplasia we compared mRNAs of 16 mammary tumor cell lines to normal human fibroblasts, M413, and a human mammary epithelial cell line, HBL100 [25]. Increased expression of an apparently normal size 5-kb transcript was detected in 8 of 16 tumor cell lines, when total cellular RNA was subjected to Northern blot analysis (Figure 5). An aberrantly sized *erb*B-2 mRNA was not detected in any of the cell lines analyzed [25].

To quantitate more precisely the amount of *erb*B-2 transcript in eight mammary tumor cell lines which overexpress *erb*B-2, serial 2-fold dilutions of total cellular RNA were subjected to dot blot analysis using human β action as a control for the amount of RNA applied to the nitrocellulose filters [25]. The highest levels of *erb*B-2 mRNA, which ranged from 64- to 128-fold over that of our controls, were observed in the cell lines MDA-MB453, SK-BR-3, MDA-MB361 and BT474. Moreover, *erb*B-2 mRNA levels were increased 4- to 8-fold in four cell lines including BT483, MDA-MB175, ZR-75-30 and ZR-75-1 (Table 2).

To investigate alterations of the *erb*B-2 gene associated with its overexpression, we examined the *erb*B-2 gene locus by Southern blot analysis, in these cell lines. The normal restriction pattern was detected in all DNA samples tested, indicating that gross rearrangements in the proximity of the *erb*B-2 coding region did not occur in these cell lines. When compared with normal human fibroblast DNA, the *erb*B-2 specific restriction fragments appeared amplified in several cell lines including SK-BR-3, BT474 and MDA-MB361 [25].

Quantitation of *erb*B-2 gene copy number was accomplished using DNA dot-blot analysis. These studies revealed a 4- to 8-fold *erb*B-2 gene amplification in SK-BR-3 and BT474 relative to normal human DNA and a 2- to 4-fold *erb*B-2 gene amplification in the MDA-MB453 and MDA-MB361 cell lines. Thus, gene amplification was associated with overexpression in the four tumor cell lines with the highest levels of *erb*B-2 mRNA (Table 2). In contrast, gene amplification could not be detected by Southern blot analysis or DNA dot-blot analysis in four tumor cell lines in which the *erb*B-2 transcript was increased to intermediate levels [25].

In chemically induced rat neuroblastomas, a point mutation within the transmembrane domain activates *neu*, the rat homologue of *erb*B-2 to ac-

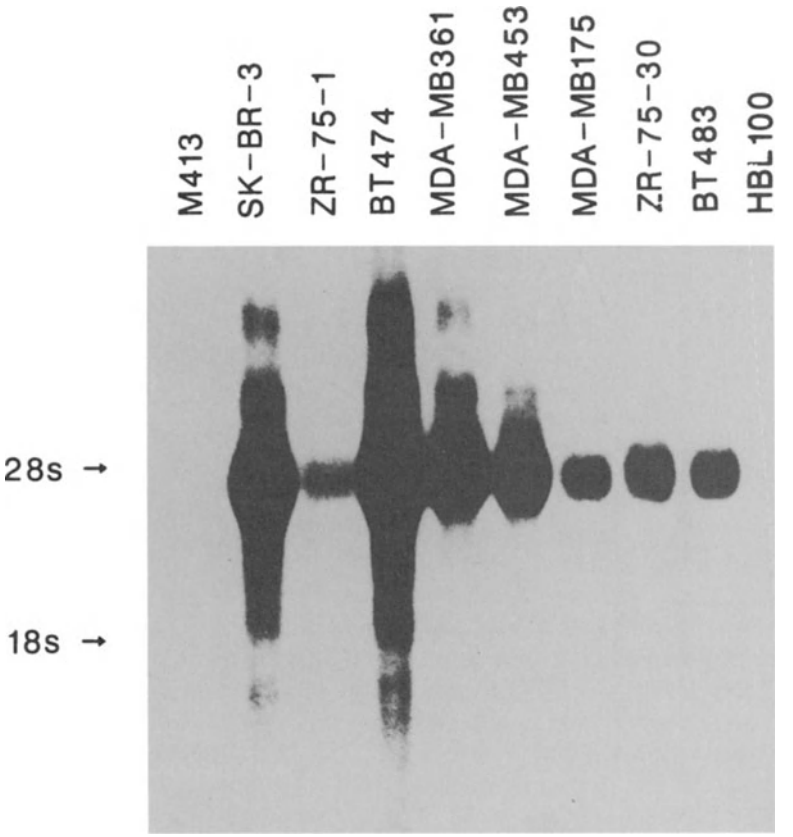


Figure 5. Overexpression of *erb*B-2 in human mammary tumor cell lines. Northern blot analysis. Total cellular RNA (10 μg) of mammary tumor cell lines, normal human fibroblasts M413 and HBL100 was hybridized with a human *erb*B-2 cDNA probe. M413 and HBL100 cells contain *erb*B-2 specific mRNA detectable after longer autoradiographic exposures. RNA amounts were standardized by rehybridization of the same filter with a human β actin probe.

quire transforming activity in the NIH/3T3 transfection assay [26]. The lack of transforming activity in the NIH/3T3 focus forming assay of a large group of mammary tumors and tumor cell lines [11], including those which exhibited *erb*B-2 amplification and/or overexpression in the absence of aberrant transcript size suggested that a structurally normal *erb*B-2 coding sequence was overexpressed in some human mammary tumor cell lines [25].

9. The *erb*B-2 gene is a potent oncogene when overexpressed in NIH/3T3 cells

To directly assess the effects of *erb*B-2 overexpression on cell growth properties, we undertook the isolation of cDNA clones comprising the entire human *erb*B-2 coding sequence. A series of overlapping cDNA clones were

Table 2. Overexpression and gene amplification of *erbB*-2 in human mammary neoplasia

Source	Overexpression of mRNA[a]	Gene amplification
M413	1	1
HBL100	1	1
MCF-7	1	1
SK-BR-3	128	4–8
BT474	128	4–8
MDA-MB361	64	2–4
MDA-MB453	64	2
ZR-75-1	8	1
ZR-75-30	4	<1
MDA-MB175	8	1
BT483	8	<1

[a] Overexpression above normal fibroblast and HBL100.

isolated from normal human fibroblast and MCF-7 cDNA libraries utilizing fragments from the previously described human genomic *erb*B-2 clones as probes [25]. A full-length normal human *erb*B-2 clone was assembled from overlapping clones [27]. By sequence analysis the coding sequence was found to be identical to previously published normal *erb*B-2 coding sequences except for a few conservative substitutions [27–29].

Expression vectors based on the transcriptional initiation sequences of either the Moloney murine leukemia virus long terminal repeat (MuLV LTR) or the SV40 early promoter were constructed in an attempt to express the *erb*B-2 cDNA at different levels in NIH/3T3 cells (Figure 6) [30]. Because of the presence of the MuLV donor splice site close to the 5′ LTR [31], we engineered one of the LTR-based vector (LTR-1/*erb*B-2 to contain an acceptor splice site immediately upstream of the translation initiation codon of the *erb*B-2 coding sequence (Figure 6). This vector was constructed in order to ensure correct splicing of the message even if a cryptic splice acceptor site were present within the *erb*B-2 open reading frame. In the SV40-based expression vector (SV40/*erb*B-2) the *erb*B-2 coding sequence replaced the neomycin-resistance gene of pSV2/neo [32] (Figure 6).

To assess the biologic activity of our human *erb*B-2 vectors, we transfected NIH/3T3 cells with serial dilutions of each DNA. As shown in Table 3, both LTR-1/*erb*B-2 and LTR-2/*erb*B-2 DNAs induced transformed foci at high efficiencies of 4.1×10^4 and 2.0×10^4 focus-forming units per picomole of DNA (ffu/pM), respectively. In striking contrast, the SV40/*erb*B-2 construct failed to induce any detectable morphological alteration of NIH/3T3 cells transfected under identical assay conditions (Table 3). Since the SV40/*erb*B-2 construct lacked transforming activity, these results demonstrated that the higher levels of *erb*B-2 expression under LTR influence correlated with its ability to exert transforming activity.

To compare the growth properties of NIH/3T3 cells transfected by these

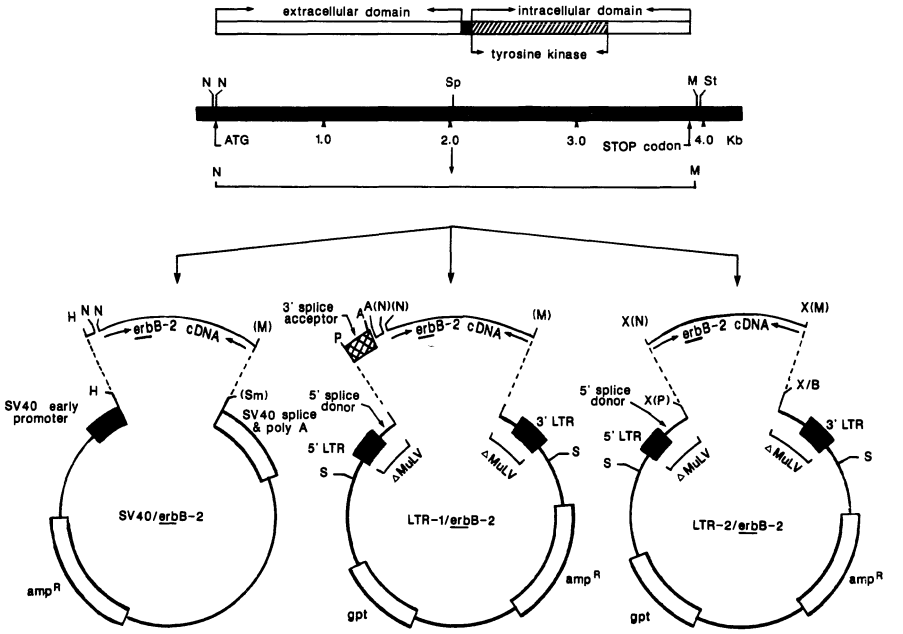


Figure 6. Construction of expression vectors for the human *erb*B-2 cDNA. A Nco I-Mst II fragment encompassing the entire *erb*B-2 open reading frame was cloned under the transcriptional control of either the SV40 early promoter or MuLV LTR. Symbols: ▨, *erb*A-*erb*B intergeneic region of pAEV11 containing the 3′ splice acceptor site; N, Nco I; Sp, SphI; M, Mst II; St, Stu I; H, Hind III, Sm, Sma I; P, Pst I; B, Bam HI; X, Xho I. Sites indicated in parentheses were not reconstituted after the cloning procedures.

Table 3. Transformed phenotype of *erb*B-2 transfectants

DNA transfectant[a]	Specific transforming activity[b] (ffu/pM)	Colony-forming efficiency in agar (%)[c]	Cell number required for 50% tumor incidence[d]
LTR-1/*erb*B-2	4.1×10^4	45	10^3
SV40/*erb*B-2	$<10^0$	<0.01	$>10^6$
LTR/*erb*B	5.0×10^2	20	5×10^4
LTR/*ras*	3.6×10^4	35	10^3
pSV2/gpt	$<10^0$	<0.01	$>10^6$

[a] All transfectants were isolated from plates which received 1 μg cloned DNA and were selected by their ability to grow in the presence of killer HAT medium [52].

[b] Focus-forming units were adjusted to ffu/pM of cloned DNA added based on the relative molecular weights of the respective plasmids.

[c] Cells were plated at 10-fold serial dilutions in 0.33% soft agar medium containing 10% calf serum. Visible colonies comprising >100 cells were scored at 14 days.

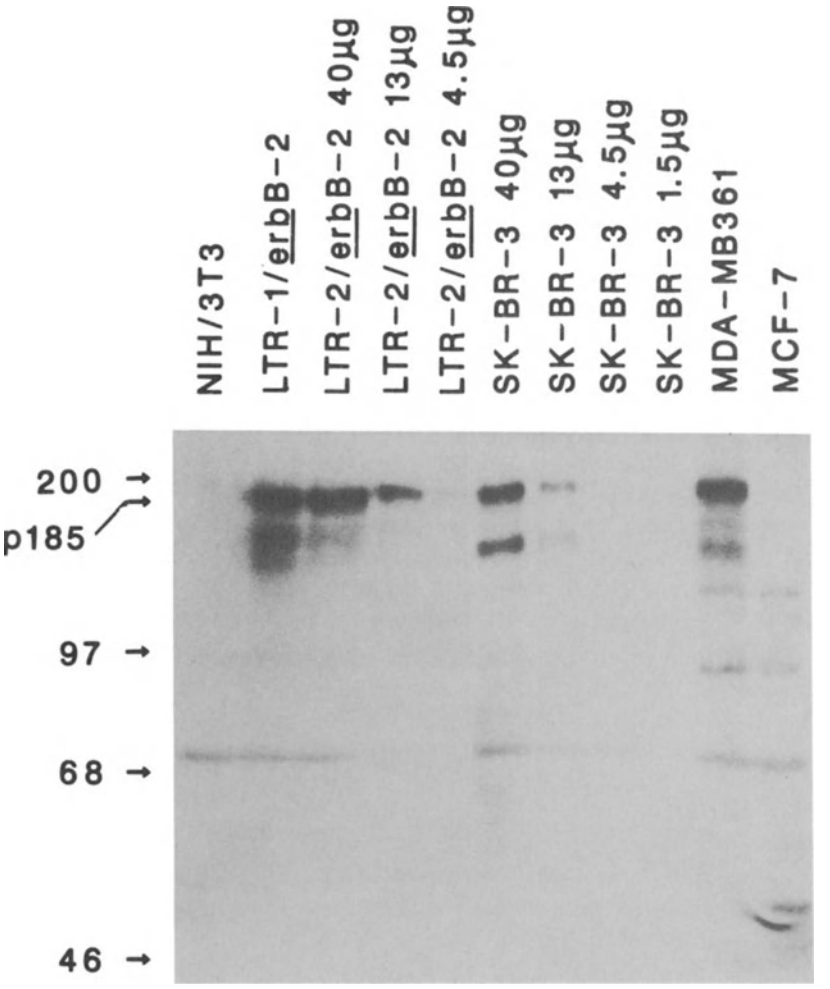
[d] NFR nude mice were inoculated subcutaneously with each cell line. Ten mice were tested at cell concentrations ranging from 10^6 to 10^3 cells/mouse. Tumor formation was monitored at least twice weekly for up to 30 days.

genes, we analyzed the transfectants for anchorage-independent growth in culture, a property of many transformed cells. The colony-forming efficiency of a LTR-1/*erb*B-2 transformant was very high and comparable to that of cells transformed by LTR-driven v-H-*ras* and v-*erb*B (Table 3). Moreover, the LTR-1/*erb*B-2 transfectants were as malignant *in vivo* as cells transformed by the highly potent v-H-*ras* oncogene and 50-fold more tumorigenic than cells transfected with v-*erb*B. In contrast, SV40/*erb*B-2 transfectants failed to display anchorage-independent growth in vitro and did not grow as tumors in nude mice even when 10^6 cells were injected (Table 3).

While the predicted *erb*B-2 protein bears structural similarity to the EGF receptor, there is evidence that EGF is not the ligand for the *erb*B-2 product [33, 34]. In fact, the normal ligand for this receptor-like protein has yet to be identified. If present in serum, this ligand might be responsible for stimulating the overexpressed *erb*B-2 product and triggering its transforming ability. To address this possibility, we investigated whether *erb*B-2 transformed cells maintained their altered phenotype when cultured in medium lacking serum [27]. NIH/3T3 cells grow as a contact-inhibited monolayer in a chemically defined medium which contains EGF, fibroblast growth factor (FGF), and insulin (W. Taylor, O. Segatto, S.A. Aaronson, unpublished). These growth factors have been excluded as possible exogenous ligand for the *erb*B-2 gene product [33, 34]. In this medium, LTR-1/*erb*B-2-transfected cells continued to exhibit a stable transformed phenotype by growing as foci of densely packed cells. These findings demonstrate that neither EGF nor any factors present in serum are required for maintaining the transformed phenotype of NIH/3T3 cells overexpressing *erb*B-2.

10. Human mammary tumors with amplified *erb*B-2 genes express the *erb*B-2 protein at high levels comparable to LTR/*erb*B-2 NIH/3T3 transformants

In order to assess the relevance of *erb*B-2 protein levels inducing *in vitro* transformation for *erb*B-2 overexpression in mammary neoplasia, we sought to compare the level of overexpression of the *erb*B-2-encoded 185 kd protein in human mammary tumor cell lines possessing amplified *erb*B-2 genes with that of NIH/3T3 cells transformed by the *erb*B-2 coding sequence [27]. An anti-*erb*B-2 peptide serum detected several discrete protein species ranging in size from 150 to 185 kD in extracts of MDA-MB361 and SK-BR-3 mammary tumor cell lines, as well as LTR/*erb*B-2 NIH/3T3 transformants (Figure 7). The relative levels of the 185-kD *erb*B-2 product were similar in each of the cell lines and markedly elevated over that expressed by MCF-7 cells, where the 185 kD *erb*B-2 protein was not detectable under these assay conditions (Figure 7). Thus, human mammary tumor cells which overexpressed the *erb*B-2 gene demonstrated levels of the *erb*B-2 gene product capable of inducing malignant transformation in a model system.



11. Implications

While knowledge about the chain of events leading a normal cell along the pathway to malignancy is incomplete, there is considerable evidence that activated oncogenes often subvert the pathways by which growth factors commonly stimulate cell proliferation. The continued search for activated oncogenes in mammary tumors will hopefully help to identify genes within these pathways and as in the case of *erb*B-2 provide important targets for the development of better diagnostic and therapeutic approaches to this malignancy.

Oncogenes activated by structural alteration of their coding sequence are detectable in a wide variety of human malignancies [1, 6]. Although these mechanisms can account for proto-oncogene activation in human mammary cancer thus far only at low frequency, the observation of a somatically activated H-*ras* allele selected for in every HS578T tumor cell suggests a causative role of an activated H-*ras* gene in the development of this tumor.

There is considerable evidence that genes encoding growth factor receptors can be activated as transforming genes by structural alterations in their coding sequences [23, 26, 35–42]. For example, the *neu* gene, chemically activated by a point mutation in the coding sequence for its transmembrane domain [26], was initially detected by its ability to induce foci upon DNA transfection of NIH/3T3 cells [43]. To date, transforming genes encoding growth factor receptors have not been detected in human tumors by this approach. Instead, abnormalities involving growth factor receptors in human malignancies appear most commonly to involve their overexpression as reported for EGF receptor or *erb*B-2 genes in a significant fraction of human epithelial malignancies [21, 25, 44–50].

Our studies show that the human *erb*B-2 gene can be activated as an oncogene by its overexpression in NIH/3T3 cells. The level of the *erb*B-2 product was shown to be critical in determining its transforming ability. Our results further indicate that a ligand in serum is not required for transforming activity of the overexpressed *erb*B-2 product. In fact, transfected NIH/3T3 cells overexpressing the *erb*B-2 product were still capable of altered growth in chemically defined medium supplemented with EGF, FGF and insulin, which have been excluded as exogenous ligands for this receptor-like protein. As the ligand for the *erb*B-2 protein has yet to be identified, it is not possible to exclude the possibility that *erb*B-2 transformed cells themselves might produce the ligand for the *erb*B-2 receptor protein. Alternatively, an increased number of receptors may cause transformation either by raising the level of constitutive tyrosine kinase activity to a threshhold required for growth stimulation or by facilitating receptor-receptor interactions that may be a prerequisite for their activation.

Our *in vitro* observations are paralleled by *in vivo* findings that a number of human mammary tumors and tumor cell lines display overexpression of the *erb*B-2 gene. In addition, we demonstrated that the levels at which the *erb*B-2 protein is capable of inducing an *in vitro* transformation are comparable to those detected in naturally occurring tumors exhibiting *erb*B-2 overexpression. Recently, an inverse correlation of *erb*B-2 gene amplification and disease-free survival has been reported in breast cancer patients, suggesting a predictive role of *erb*B-2 gene amplification in an aggressive disease course in human mammary neoplasia [51]. Taken together, all these observations establish a mechanistic basis for growth factor receptor gene amplification and over-expression as representing a causal driving force in the clonal evolution of a tumor cell rather than being an incidental consequence of tumorigenesis.

References

1. Weiss RA, Teich N, Varmus H, Coffin J: Molecular Biology of Tumor Viruses. 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984.
2. Bishop JM: Cellular oncogenes and retroviruses. Ann Rev Biochem 52: 301–354, 1983.
3. Bishop JM: Viral oncogenes. Cell 42: 23–38, 1985.
4. Weinberg RA: The action of oncogenes in the cytoplasm and nucleus. Science 230: 770–776, 1985.
5. Hunter T, Cooper JA: Protein-tyrosine kinases. Ann Rev Biochem 54: 897–930, 1985.
6. Varmus HE: The molecular genetics of cellular oncogenes. Ann Rev Genet 18: 553–612, 1984.
7. Pulciani S, Santos E, Lauver AV, Long KL, Aaronson SA, Barbacid M: Oncogenes in solid human tumors. Nature 300: 539–542, 1982.
8. Eva A, Tronick SR, Gol RA, Pierce JH, Aaronson SA: Transforming genes of human hematopoietic tumors: Frequent detection of *ras*-related oncogenes whose activation appears to be independent of tumor phenotype. Proc Natl Acad Sci USA 80: 4926–4930, 1983.
9. Bos JL, Toksoz D, Marshall CJ, Verlaan-de Vries M, Veeneman GH, van der Ed AJ, van Boom JH, Janssen JWG, Steenvoorden CM: Amino-acid substitutions at codon 13 of the N-*ras* oncogene in human acute myeloid leukemia. Nature 315: 726–730, 1985.
10. Needleman SW, Kraus MH, Srivastava SK, Levine PH, Aaronson SA: High frequency of N-*ras* activation in acute myelogenous leukemia. Blood 67: 753–757, 1986.
11. Kraus MH, Yuasa Y, Aaronson SA: A position 12-activated H-*ras* oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proc Natl Acad Sci USA 81: 5384–5388, 1984.
12. Eva A, Robbins KC, Andersen PR, Srinivasan A, Tronick SR, Reddy P, Ellmore NW, Galen AT, Lautenberger JA, Papas TS, Westin EH, Wong-Staal F, Gallo RC, Aaronson SA: Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells. Nature 295: 116–119, 1982.
13. Dalla Favera R, Wong-Staal F, Gallo RC: *Onc* gene amplification in promyelocytic leukemia cell line HL-60 and primary leukemic cells of the same patient. Nature 299: 61–63, 1982.
14. Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM: Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80: 1707–1711, 1983.
15. Little CP, Nau MM, Carney DN, Gazdar AF, Minna JD: Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306: 194–196, 1983.
16. Schwab M, Varmus HE, Bishop JM, Grzeschnick KH, Naylor SL, Sakaguchi AY, Brodeur G, Trent J: Chromosome localization in normal human cells and neuroblastomas of a gene related to c-*myc*. Nature 300: 788–791, 1984.
17. Schwab M, Alitalo K, Klempnower KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J: Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305: 245–248, 1983.
18. Kohl NE, Kanda N, Schreck RR, Bruns G, Latt SA, Gilbert F, Alt FW: Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell 35: 359–367, 1983.
19. Nau MM, Brooks BJ, Battey J, Sausville E, Gazdar AF, Kirsch IR, McBride OW, Bertness V, Hollis GF, Minna JD: L-*myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. Nature 318, 69–73, 1985.
20. Schwab M, Ellison J, Busch M, Rosenau W, Varmus HE, Bishop JM: Enhanced expression of the human gene N-*myc* consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc Natl Acad Sci USA 81: 4940–4944, 1984.
21. King CR, Kraus MH, Aaronson SA: Amplification of a novel v-*erb*B-related gene in a

human mammary carcinoma. Science 229: 974–976, 1985.
22. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. Nature 307: 521–527, 1984.
23. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH: Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309: 418–425, 1984.
24. Xu YH, Ishii S, Clark AJL, Sullivan M, Wilson RK, Ma DP, Roe BA, Merlino GT, Pastan I: Human epidermal growth factor receptor cDNA is homologous to a variety of RNAs overproduced in A431 carcinoma cells. Nature 309: 806–810, 1984.
25. Kraus MH, Popescu NC, Amsbaugh SC, King CR: Overexpression of the EGF receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 6: 605–610, 1987.
26. Bargmann CI, Hung MC, Weinberg RA: Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. Cell 45: 649–657, 1986.
27. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA: *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237: 178–182, 1987.
28. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, Saito T, Toyoshima K: Similarity of protein encoded by the human c-*erb*-B-2 gene to epidermal growth factor receptor. Nature (London) 319: 230–234, 1986.
29. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke V, Levinson A, Ullrich A: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230: 1132–1139, 1985.
30. Gorman CM, Merlino GT, Willingham MC, Pastan I, Howard BH: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc Natl Acad Sci USA 79: 6777–6781, 1982.
31. Shinnick TM, Lerner RA, Sutcliff JG: Nucleotide sequence of Moloney murine leukemia virus. Nature 293: 543–548, 1981.
32. Southern PJ, Berg P: Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter, J Mol Appl Genet 1: 327–341, 1982.
33. Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T: The product of the human c-*erb*B-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232: 1644–1646, 1986.
34. Stern DF, Heffernan PA, Weinberg RA: p185, a product of the *neu* proto-oncogene, is a receptor-like protein associated with tyrosine kinase activity. Mol Cell Biol 5: 1729–1740, 1986.
35. Yamamoto T, Nishida T, Miyajima N, Kawai S, Ooi T, Toyoshima K: The *erb*B gene of avian erythroblastosis virus is a member of the *src* gene family. Cell 35: 71–78, 1985.
36. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT Stanley ER: The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41: 665–676, 1985.
37. Weber W, Gill GN, Spiess J: Production of an epidermal growth factor receptor-related protein. Science 224: 294–297, 1984.
38. Merlino GT, Xu YH, Ishii S, Clark AJ, Semba K, Toyoshima K Yamamoto T, Pastan I: Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. Science 224: 417–419, 1984.
39. Hampe A, Gobet M, Sherr CJ, Galibert F: Nucleotide sequence of the feline retroviral oncogene v-*fms* shows unexpected homology with oncogenes encoding tyrosine-specific protein kinases. Proc Natl Acad Sci USA 81: 85–89, 1984.
40. Lax I, Kris R, Sasson I, Ullrich A, Hayman MJ, Beug H, Schlessinger J: Activation of c-*erb*B

in avian leukosis virusinduced erythroblastosis leads to the expression of a truncated EGF receptor kinase. EMBO J 4: 3179–3182, 1985.

41. Nilsen TW, Maroney PA, Goodwin RG, Rottman FM, Crittenden LB, Raines MA, Kung HJ: c-*erb*B activation in ALV-induced erythroblastosis: novel RNA processing and promoter insertion result in expression of an amino-truncated EGF receptor. Cell 41: 719–726, 1985.
42. Coussens L, Van Beveren C, Smith D, Chen E, Mitchell RL, Isacke CM, Verma IM, Ullrich A: Structural alteration of viral homologue of receptor proto-oncogene *fms* at carboxyl terminus. Nature 320: 277–280, 1986.
43. Shih C, Padhy LC, Murray M, Weinberg RA: Transforming genes of cacinomas and neuroblastomas introduced into mouse fibroblasts. Nature 290, 261–264, 1981.
44. Liberman TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J: Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature 313: 144–147, 1985.
45. Xu YH, Richert, N, Ito S, Merlino GT, Pastan I: Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. Proc Natl Acad Sci USA 81: 7308–7312, 1984.
46. King CR, Kraus MH, Williams LT, Merlino GT, Pastan IH, Aaronson SA: Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNAs. Nucleic Acids Res 13: 8477–8486, 1985.
47. Fukushige S, Matsubara K, Yoshida M, Sasaki M, Suzuki T, Semba K, Toyoshima K, Yamamoto T: Localization of a novel v-*erb*B-related gene, c-*erb*B-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol Cell Biol 6: 955–958, 1986.
48. Semba K, Kamata N, Toyoshima K, Yamamoto T: A v-*erb*B-related proto-oncogene, c-*erb*B-2, is distinct from the c-*erb*B-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA 82: 6497–6501, 1985.
49. Yokota J, Yamamoto T, Toyoshima K, Terada M, Sugimura T, Battifora H, Cline MJ: Amplification of c-*erb*B-2 oncogene in human adenocarcinomas in vivo. Lancet 1: 765–767, 1986.
50. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M *et al*: Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature 313: 756–761, 1985.
51. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235: 177–182, 1987.
52. Mulligan RC, Berg P: Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyl-transferase. Proc Natl Acad Sci USA 78: 2072–2076, 1981.

4. Identification of oncogenes in breast tumors and their effects on growth and differentiation

B. Groner, N.E. Hynes, S. Kozma, S. Redmond, S. Saurer, M. Schmitt-Ney, R. Ball, E. Reichmann, C. Shöenberger, and A.C. Andres

1. Introduction

The mammary gland is a complex structure of parenchymal and stromal cells. It consists of a system of branching ducts of epithelial and myoepithelial cells embedded in adipose stroma. The epithelial cells of the mammary ducts proliferate and differentiate under the hormonal influence of pregnancy [1, 2]. They form lobuloalveolar structures in which centrally located secretory cells synthesize the constituents of milk during lactation. Morphological, topographical and functional criteria can be used to classify the parenchymal cells [3, 4]. Myoepithelial cells are myofilament-containing, contractile cells located along the basement membrane. Epithelial cells are constituted by ductal and alveolar luminal cells. The epithelial cells are in close proximity to fibroblasts and adipocytes, and it has been proposed that the interaction of different cell types is important for hormonal responsiveness of growth and differentiation [5, 6]. Multiple steroid and polypeptide hormones cooperate in lobuloalveolar differentiation and induction of milk component synthesis. Estrogen and progesterone are required, together with prolactin, placental lactogen and possibly chorionic gonadotropin, for alveolar development [1, 3]. Thyroid hormone and adrenal steroids enhance the formation of lobules. Insulin, hydrocortisone and prolactin induce the expression of casein synthesis [7]. The complexity of cell-cell interactions is compounded by the diverse hormonal stimuli and only a concerted view will allow a description of cell growth, differentiation and neoplastic disease of breast tissue [8].

We have used the methods of molecular and cellular biology and genetics to gain insights into the hormonal regulation of mammary epithelial development and aberrations induced by activated oncogenes. We also investigated the possibility that malignant transformation of epithelial cells *in vivo* is caused by the activation of oncogenes. For these purposes we used two experimental strategies: 1) DNA from breast tumor cells was transfected into sensitive indicator cells which signalled the transfer of an activated oncogene and allowed a molecular characterization, and 2) cloned oncogenes were introduced into mammary epithelial cells *in vitro* or tissue specifically expressed in transgenic mice. The effects on transformation and differentia-

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

tion markers were monitored. We observed that activated, dominantly acting oncogenes are present in established mammary carcinoma cells as well as in primary tumor cells. At least three oncogenes could be associated with the human tumor cells. Introduction of oncogenes into mammary epithelial cells *in vitro* causes severe interference with the cellular physiology. Growth and differentiation parameters are affected and the hormonal responsiveness is impaired. These processes are concentration dependent with respect to the oncogene product p21-*ras*. Corresponding effects could be observed *in vivo* when oncogene expression was targeted by promoter recombination in transgenic mice. Interference with normal cell differentiation and predisposition to tumor development are the consequences of the activated oncogene products. We can conclude that activated oncogenes are found in mammary carcinoma cells and very likely contribute to the process of tumorigenesis. The introduction of oncogenes and expression in mammary epithelial cells, however, suggests that single oncogene activation is not a sufficient prerequisite for transformation and that a concerted effect of several genetic alterations is responsible for breast cancer *in vivo*.

2. Oncogene identification in human breast tumor cells

Three lines of evidence are being used to correlate the etiology of human tumors with the occurrence of activated oncogenes: (1) Human tumor cell DNA can be transfected into non-transformed mouse cells and the transfer of a dominantly acting human oncogene can be monitored by the emergence of transformed cell clones [9, 10, 11, 12]; (2) Amplified proto-oncogene sequences are found in a large percentage of certain human tumors [13]; and (3) Consistent chromosomal translocations are observed in certain leukemias and proto-oncogene sequences have been identified at the chromosomal breakpoints [14]. All three points might indicate that slightly altered gene products or the deregulated expression of proto-oncogene products are instrumental in the transformation process [15, 16]. We have applied the first two principles (genomic transfection procedures and screening of tumor DNA with cloned oncogene probes) to the study of human breast cancer cells. We could identify at least three distinct oncogenes in DNA of primary and established carcinoma cells.

In order to detect the presence of a dominantly acting oncogene in cells of primary tumor tissue, genomic DNA was extracted from a metastasis of a breast cancer patient (HM347). Genomic DNA was cotransfected with the selectable marker plasmid, pSV2-*neo*, into NIH 3T3 mouse fibroblasts. G418 selection yielded several thousand individually transfected cell clones. These transfected cells were pooled and expanded into mass culture. 10^6 G418-resistant cells were injected into nude mice and tumor growth at the site of injection was monitored [17]. Tumors were obtained 2 to 5 weeks after injection of NIH 3T3 cells transfected with HM347 DNA. Second and third

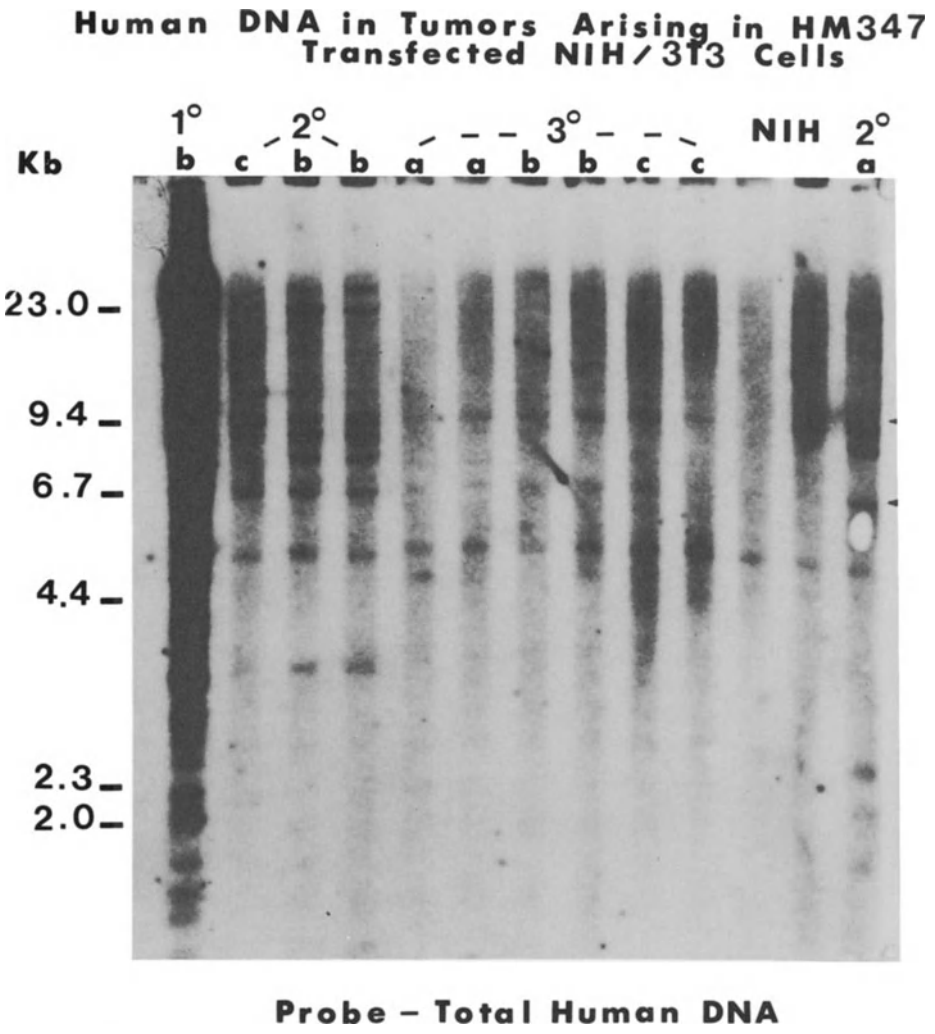


Figure 1. Human DNA sequences present in transformed NIH 3T3 cells. DNA from the human breast cancer metastasis HM 347 was extracted and cotransfected with the pSV2*neo* resistance plasmid into NIH 3T3 cells. The G418 resistant cells were selected, pooled and grown into mass culture. The transfected cells were injected into nude mice and tumors appeared at the site of injection after about two weeks. The primary (1°) tumor DNA was extracted and cotransfected into NIH 3T3 cells to yield secondary tumors (2°) after injection into nude mice. The procedure was repeated with secondary tumor DNA to yield tertiary (3°) tumors. DNA of 1°, 2° or 3° tumors or of NIH 3T3 cells was digested with BamHI, electrophoresed and blotted on a nitrocellulose filter. The blots were hybridized to nick-translated total human DNA to visualize highly repetitive human sequences. The pattern of human sequences becomes simpler going from 1° to 2° and 3° tumors, indicating the transfer of a few, distinct restriction fragments carrying a dominantly acting oncogene. a, b and c denote individually derived tumor DNAs.

rounds of transfection into NIH 3T3 cells were carried out using the genomic DNA of the nude mouse tumors. The presence of human DNA sequences in the transfected NIH 3T3 cells can be visualized by hybridization of filter bound nude mouse tumor DNA to radioactive human DNA. If total human DNA is used as a probe the repetitive sequences present in transfected NIH 3T3 cells become apparent in the DNA blotting procedure. Figure 1 shows the presence of human DNA sequences in primary, secondary and tertiary nude mouse tumor cells. The primary nude mouse tumor cells represent the clonal outgrowth of the single NIH 3T3 cell which has acquired a dominantly acting human oncogene in the transfection of the HM347 DNA. Because individual, transfected NIH 3T3 cells acquire a few thousand kb of exogenous donor DNA, a rather complex pattern of human DNA sequences is represented by the restriction fragments identified by human repetitive DNA in lane 1. The complexity of the pattern is markedly reduced in secondary (lanes 2–4) and tertiary (lanes 5–10) nude mouse tumors. The majority of the human sequences are lost. The addition of the hybridizing fragments in tertiary tumors shows that about 25 kb of DNA remain associated with tumorigenic cells. The tertiary tumor DNA was cloned into a λ gene library and three Bam H1 restriction fragments (6, 9 and 13 kb) were molecularly isolated using a probe of repetitive human DNA. The molecular clones were analyzed for sequence homologies with 10 cellular and 14 viral oncogenes and none were found. This experimental strategy thus led to the isolation of a new oncogene, potentially involved in the etiology of human breast tumors or their metastasis.

Using a similar experimental approach, a dominantly acting oncogene was detected in the genomic DNA of the human breast carcinoma cell line MDA-MB231. The presence of the human *K-ras* gene was detected in the tumorigenic NIH 3T3 cells transfected with the genomic DNA of MDA-MB231. Hybridisation of synthetic oligonucleotides to *in vitro* amplified *K-ras* sequences of MDA-MB231 DNA showed a specific mutation in codon 13. A guanosine to adenosine transition at the second position of codon 13 results in the code for aspartic acid instead of glycine. Codon 13 codes for glycine in all members of the human proto-*ras* gene family and transforming mutations have been found in position 13 of the N-*ras* gene (glycine to aspartic acid) and the H-*ras* gene (glycine to serine). It is therefore reasonable to assume that the glycine to aspartic acid mutation of the K-*ras* gene is responsible for the transformation of the MDA-MB231 cell line [18]. An activated *H-ras* oncogene was previously detected in the carcinosarcoma line HS578T [19].

We have also used cloned oncogene probes to detect gene amplifications in primary human breast cancer cell DNA. The c-*mos* gene and the EGF receptor gene probes did not yield evidence for amplification in about 50 DNA samples which were analyzed. Our studies showed, however, that 23% (12/51) of human breast tumor DNAs contain multiple copies of the c-*erb*B-2 gene. Normal breast DNA from the same cancer patients was tested in some cases and the amplification was shown to be specific for tumor cells. This gene

shares sequence homology with members of the tyrosine kinase gene family. Its protein structure is similar to the EGF receptor and it has been suggested that it might function as a growth factor receptor [20]. The correlation between c-*erb*B2 gene amplification and protein expression was investigated. Paraffin sections of tumor material from the same patients were screened for c-*erb*B-2 protein using an anti-peptide antibody [21]. Our results suggest that with the exception of one, the tumors exhibiting amplified DNA sequences all stained positively with the c-*erb*B-2 antibody. No staining was detected in normal breast tissue. In addition, other tumors which do not contain extra gene copies also reacted positively with the antibody. Altogether, of the 38 tumors evaluated for c-*erb*B-2 expression, 24 expressed the c-*erb*B-2 protein. The high expression of c-*erb*B-2 seems to correlate with an unfavorable prognosis for early relapse. These results suggest that over-expression of the c-erbB-2 gene may be involved in human breast cancer development [22, 23, 24, 25].

3. The H-ras oncogene product affects primary and established mouse mammary epithelial cells in a concentration dependent manner

Three members of the *ras* gene family have been discovered in the mammalian genome (H-*ras*-1, K-*ras*-2 and N-*ras*) [26]. These genes code for highly related proteins of 21 kd (p 21). The proteins bind guanine nucleotides, exhibit GTPase activity and are associated with the plasma membrane. Their sequence analysis revealed homologies to G proteins and they may participate in the transduction of signals across the cellular membrane. The ras genes have been called proto-oncogenes, since mutations in their coding sequences result in their activation, i.e., confer transforming potential to the p21 gene products. Skin tumors [26] and mammary carcinomas [27] induced by carcinogens often show activation of the H-*ras* proto-oncogene. Over a third of the human colorectal cancers exhibit *ras* gene mutations [28, 29]. To investigate the role of the H-*ras* oncogene in mammary carcinogenesis we introduced it experimentally into primary and established mammary epithelial cells. We observed the phenotypic effects of p21 v-*ras* expression on various transformation parameters.

The advantages offered by retroviral gene transfer were exploited. Recombination of the v-*ras* oncogene into the pZIPneoSV(X) vector and transfection into Ψ2 cells results in a retrovirus (pZSR) which can simultaneously transduce a selectable marker gene (neo = G418 resistance) and an activated oncogene [30]. At the same time gene transfer efficiency is much higher than can be achieved by calcium phosphate DNA coprecipitation. Primary cultures of mouse mammary epithelial cells were infected with pZSR virus. The infected cells were initially selected for G418 resistance. A very small fraction of the cells (about 0.01%) yielded proliferating colonies. No colonies were detected in cells infected with the pZIPneoSV(X) virus or in uninfected

cultures. The cell clones exhibited an epithelial morphology and expressed cytokeratins. These cells are highly tumorigenic and grow anchorage independently (Table 1). Very high levels of the viral transcript encoding p21 virus were found in the transformed primary cells. We assume that the very high expression of p21 virus is required for the transformation and might partly explain the low frequency of cell clones observed after G418 selection. In addition to the very high expression levels, additional genetic changes might be required for the establishment and transformation of the primary epithelial cells 31. Primary mammary epithelial cells have been infected by SV40 virus [32, 33, 34] and multiple phenotypes have been observed.

The observation of very high levels of p21 v-*ras* in primary cell transformation caused us to investigate concentration effects on transformation parameters in an established epithelial cell line. A single cell clone (NOG8) was selected by limiting dilution from the NMuMG line. This clone has a normal cuboidal epithelial morphology, does not grow in soft agar and is non-tumorigenic. Its karyotype analysis shows that these cells are almost diploid, containing 42 chromosomes (trisomies in chromosomes 8 and 11). NOG8 cells were infected with pZSR virus and independent cell clones resistant to G418 were selected. Clones with different cellular morphologies were detected and three clones (NOG8/SR3, NOG8/SR1 and NOG8/SR2) were analyzed in detail (Table 1). Clone NOG8/SR3 has a normal morphology, not distinguishable from NOG8 cells or cells infected with the pZIPneoSV(X) virus. Increasing morphological transformation, i.e., growth to higher cell density, refractile appearance and cell shape, were observed in the cell clones NOG8/SR1 and NOG8/SR2. These morphological parameters were paralleled by the extent of p21 v-*ras* expression. The highly transformed clone NOG8/SR2 expresses 30-fold more viral RNA than the normal-looking clone NOG8/SR3. The clone NOG8/SR1 with a moderately transformed morphology expresses 8 times more pZSR RNA than NOG8/SR3. Similar ratios of expression were found for the p21 v-*ras* gene product (Table 1).

Other indicators of the transformed phenotype, such as anchorage independent growth and tumorigenicity, were shown to be proportional to the extent of p21 v-*ras* expression. The correlation between tumorigenicity and the p21 v-*ras* dosage is particularly well documented in clone NOG8/SR1. This clone is tumorigenic with a latency of 6 to 7 weeks. The resulting tumor cells probably reflect a selection of clonal variants. Their expression of p21 v-*ras* is increased four-fold over the parental NOG8/SR1 cells (Table 1). The high p21 v-ras expression level might be a prerequisite for tumor formation. We have used the levels of mRNA expression and enzyme activity of ornithine decarboxylase (ODC) as an additional transformation parameter. ODC is expressed in the G1 phase of the cell cycle and is a sensitive indicator of the cellular growth state [35]. NOG8 cells were found to drastically reduce their ODC activity when they reach confluence (Table 1). ODC mRNA and enzyme activity was measured in pZSR virus infected cells to examine the effects of p21 v-*ras* dosage. Measurements were carried out at different cell

Table 1. Dosage effects of V-H *ras* expression

Cells	PZSR RNA level	p21 protein level	Transformed morphology	Soft agar growth	Tumorigenicity	Latency in weeks	ODC mRNA levels	ODC activity change at high cell density
NOG8	0	1	−	<0.02%	0/4	−	1	15.8
NOG8/SVX	0	N.D.	−	<0.02%	N.D.	N.D.	N.D.	17.6
NOG8/SR3	1	1.6	−	0.05%	1/3	20	3	16.5
NOG8/SR1	8	6	+	4%	2/2	6, 7	11	9.7
NOG8/SR1 T.O.	30	24	++		−	−	N.D.	2.3
NOG8/SR2	240	53	++	40%	2/2	2, 2	20	1.9
Primary Cells/pZSR			++	20%	2/2	2, 2	N.D.	N.D.
Clone 1/Passage 8	0							
NIH			−	1%	0/2	−		14.5
NIH/SR6			++	85%	N.D.	N.D.		3.1
NIH/SR11			++	53%	2/2	3, 5		2.4

NOG cells are derived from a single cell clone of established mammary epithelial cells, NOG8/SVX are cells infected with pZIP SVX virus; NOG8/SR3, NOG8/SR1 and NOG8/SR2 are independent cell clones infected with pZSR virus; NOG8/SR1 T.O. is derived from a tumor induced by NOG8/SR1 and re-established in culture: − denotes normal morphology; +, ++ denote moderately and strongly transformed morphology. ODC activity change shows the drop in ODC activity when comparing parallel cultures at high (>95% confluent) and low (10%–20%) cell density.

densities. ODC mRNA levels were elevated 3-, 11- and 20-fold in NOG8/SR3, NOG8/SR1 and NOG8/SR2 cells when compared to uninfected cells at low cell density. The enzyme activity of ODC was determined at low and high cell densities. NOG8 and NOG8/SR3 cells with a normal morphology show a 15- to 18-fold decrease in ODC activity when they reach high density. NOG8/SR1 cells, moderately transformed, show a 9- to 10-fold decrease in dense culture. The highly transformed NOG8/SR2 cells only decrease their ODC activity two-fold upon reaching confluence. Similar observations were made with pZSR virus transformed NIH 3T3 cells (Table 1). These experiments show that an additional level can be identified which is crucial in the transformation of epithelial cells when compared to fibroblasts. In all NIH 3T3 cells which have acquired a *ras* oncogene and which express it, the fully transformed phenotype was observed [31]. Epithelial cells seem to exhibit a threshold, i.e., a certain concentration of oncogene product has to be present before a phenotype is observed and gradual changes occur with increasing oncogene expression. This dosage requirement might buffer the effects of oncogene activation *in vivo*.

4. Oncogene expression interferes with in vitro differentiation and hormonal responsiveness of cultured mouse mammary epithelial cells

Oncogene transfer studies, in which different classes of oncogenes and their influence on differentiation parameters are evaluated, provide insights into their role in the transformation process. In the context of breast tumor studies it is most desirable to test oncogenes which have been detected in tumors by introduction into normal mammary epithelial cells. We have studied three classes of oncogenes and have assayed their biological effects upon growth and differentiation *in vitro*. In addition to the ras oncogene, which has been detected in two breast cancer cell lines [18, 19], we studied the *myc* oncogene which has been shown to be amplified in a mammary carcinoma cell line [36] and in about one-third of primary breast cancer cells [37]. Targeted c-*myc* expression predisposes transgenic mice to mammary cancer [38, 39]. The *fgr* oncogene which represents the most prevalent family of oncogenes, those related to tyrosine specific protein kinases [40]. Two cell lines (COMMA-1D and IM-2) were chosen as representative cell lines in these studies.

The COMMA-1D cell line [41, 42] is a spontaneously immortalized cell line derived from the BALB/c mouse mammary epithelium. It was non-tumorigenic, had a normal karyotype, expressed cytokeratins, repopulated the cleared mammary fat pad with ductal epithelium and could be induced by lactogenic hormones to produce the beta-casein milk protein. Since the COMMA-1D cell line was heterogeneous and rapidly progressed to tumorigenicity during culture [42], the cell line was cloned by transfection with the hygromycin B resistance plasmid, pY3 [42]. Clone 14 (HC14) was chosen for further study [44]. HC14 was non-tumorigenic in nude mice, did not show

anchorage independent growth, and expressed cytokeratins detected by the monoclonal antibody LE61 [45]. HC14 could also be induced to differentiate further by lactogenic hormones to produce beta-casein. Clone HC14 appeared to be the most suitable clone for studying the effect of the introduction of oncogenes upon the growth and differentiation of mammary epithelial cells.

V-*fgr* was introduced into HC14 by transfection of a proviral copy of the Gardner-Rasheed feline sarcoma virus [40] and cells selected by tumorigenicity in nude mice. The viral H-*ras* coding sequence [46] was cloned into the pZIP*neo*SV(X) plasmid [30] to generate pZIP-she-*ras* [31]. pMMCV-*neo* [47, 48] contained the v-*myc* oncogene from the avian retrovirus OK10 inserted upstream of a bacterial *neo* gene within a MLV based retrovirus vector pMV4-*neo*. Both pZIP-she-*ras* and pMMCV-*neo* were used to transfect Ψ-2 cells [49] to produce recombinant murine retroviruses free of helper virus. Pools of HC14 cells infected with the v-H-*ras* or v-*myc* virus were produced.

The presence of mutant proviral copies of the three oncogenes within HC14 cells was demonstrated by DNA blot analysis. Expression of the oncogenes was confirmed. The p21 *ras* protein was detected by protein blot analysis. The 4.8 kb spliced v-*myc* transcript was detected in a RNA blot analysis. The $p70^{gag\text{-}actin\text{-}fgr}$ fusion protein was revealed by a kinase reaction after immuno-precipitation with anti-gag antibodies. The fusion protein possessed mainly phosphotyrosine residues and produced a five-fold increase in total cellular phosphotyrosine.

The effects of the oncogenes upon the growth and differentiation were studied. Only v-*fgr* produced a change in morphology of the HC14 cells in culture. All oncogene-containing HC14 cells retained density inhibition of growth. No change in growth factor requirements could be detected. The oncogenes induced anchorage independent growth in soft agar with low efficiency (Table 2) compared to transformation of fibroblasts with the same constructs. Tumorigenicity of the oncogene-containing HC14 cells was tested by subcutaneous injection into nude mice. V-*fgr* or v-H-*ras* expressing cells gave rise to tumors with short latencies. Introduction of v-*myc* did not result in tumorigenicity (Table 2).

The effect of the oncogenes upon parameters specific to mammary epithelial cells was assayed. Unlike the parent cell line, COMMA-1D, no clone was able to repopulate the cleared mammary fat pad [41]. No *in vivo* assays could be performed. Instead, the branching growth within rat tail collagen was used as an *in vitro* analogue of the three-dimensional growth of mammary epithelium [50, 51]. Clone HC14 cells show 3-D branching growth in hydrated collagen gel, as does v-H-*ras* transformed HC14. However, v-*myc* or v-*fgr* transformed HC14 cells grew as tight clusters of cells in collagen (Table 2). Thus, v-*myc* and v-*fgr* disturbed the growth behavior within an artificial basement membrane matrix.

The effects of oncogenes upon tissue specific gene expression and differentiation were examined. The 33A10 antigen [52] is expressed in highly dif-

Table 2. Effects of oncogenes on growth and differentiation of HC14

	HC14 containing:—				
	No construct	SVX control	v-H-*ras*	v-*myc*	v-*fgr*
a) Growth in soft agar	0	0	0.43	0.16	0.69
b) Tumorigenicity	0/7	0/2	1/2 (3 w)	0/2	5/5 (1 w)
c) Branching growth in collagen	+	+	+	−	−
d) % LE61$^+$ cells	90%	80%	80%	20%	0%
e) Beta-casein induction	15	0	0	196	0

a) % cells forming colonies in 0.378% agar at 3 weeks.
b) No. of tumours/no. of mice injected (tumor latency in weeks).
c) Protocol according to Yang *et al.*, 1980.
d) % cells staining with LE61 monoclonal anti-keratin antibody.
e) No. of cells staining with anti-casein antibodies per 2,000 screened after 5 d induction with lactogenic hormones.

ferentiated mammary epithelial cells and its expression in HC14 cells was unaffected by the oncogenes. Cytokeratins were detected in HC14 by indirect immunoflourescence using the LE61 monoclonal antibody (Table 2). The number of stained cells was partially reduced by v-*myc* and no cytokeratin staining was detected after introduction of v-*fgr*. Detergent insoluble cytoskeletal proteins were analyzed by two-dimensional gel electrophoresis [53]. Most of them were no longer observed after transformation by v-*fgr*. One remaining protein, most likely vimentin, was detected with increased intensity. Alteration of expression of these epithelium specific intermediate filaments after transformation has been previously reported [54, 55].

Terminal differentiation could be induced in HC14 by culturing on plastic dishes with the combination of lactogenic hormones, dexamethasone, insulin and prolactin for five days. We used polyclonal rabbit anti-mouse casein antisera capable of detecting alpha, beta and gamma caseins in mouse milk [56]. Only beta-casein was observed in a protein blot assay of hormone induced HC14 cells. The β casein remained intracellular and no secretion was observed. The absence of secretion has been reported for cells grown on a plastic substrate [57]. Unlike primary cultures of mammary epithelial cells, HC14 did not show increased induction of beta-casein upon co-culture with converted 3T3-L1 adipocytes when compared to culture on plastic [6].

The effect of the introduced oncogenes upon the induction of beta-casein was examined (Table 2). One percent of HC14 could be stained with anti-casein antibodies after lactogenic hormone stimulation. This frequency was elevated ten-fold in HC14 cells containing v-*myc*. No stained cells were detected in v-*fgr* or v-H-*ras* transformed HC14. However, the control virus vectors ZIP*neo*SV(X) or MV4-*neo* also abolished the low level of casein induction. It appears that the recombinant retroviral vectors had an inhibitory

effect of their own. RNA blot analysis with a probe from a mouse beta-casein cDNA clone [58] was carried out. Transformation of HC14 by v-*myc* was shown to increase the lactogenic hormone induction of beta casein mRNA. Protein blot analysis demonstrated that approximately 50-fold more beta-casein protein was induced in the v-*myc* transformed cells than in HC14. V-*myc* could be altering the hormonal dependence of beta-casein induction. All possible combinations of the three lactogenic hormones were tested and the synergistic action of all three was found to be required for the induction of beta-casein in HC14 and v-*myc* transformed HC14 (Table 3). The response of mammary epithelial cells *in vitro* is known to be dependent upon the matrix used in culture. It is possible that v-*myc* could be acting by substituting for some tissue organization required specifically for milk protein expression.

These results suggest that the expression of v-*myc* somehow promotes the differentiation of HC14. Constitutive *myc* expression was previously found to inhibit differentiation [59, 60, 61] and endogenous c-*myc* expression is usually inhibited during induced cell differentiation [62, 63]. However, *myc* expression does not always preclude differentiation [64, 65].

Although targets of action of the *myc* oncogene are not known, there are many examples in which *myc* alters the cellular response to hormones [66, 67, 68, 69]. In addition, v-*mos* and v-H-*ras* have been shown to interfere with glucocorticoid hormone dependent gene transcription [70].

We have shown that the introduction of three different oncogenes, v-*myc*, v-H-*ras* and v-*fgr*, into a single cloned mouse mammary epithelial cell line, HC14, produces three different sets of alterations of growth and differentiation of these cells. *myc* is known to be involved in the control of growth and differentiation. In the case of mammary epithelial cells, this appears to involve at least a change in responsiveness to lactogenic hormones.

A second cell line was used for the studies of oncogene effects on mammary epithelial differentiation. We established a new, spontaneously-immortalized mammary epithelial cell line which is derived from normal mammary epithelium of mid-pregnant BALB/c mice. This cell line, designa-

Table 3. Lactogenic hormone dependence of beta-casein induction in HC14 is unaltered by v-*myc* transformation

	Hormone addition[a)]							
Cells	None	D	I	P	DI	DP	IP	DIP
HC14	0[b)]	0	0	0	0	0	0	5
HC14 with v-*myc*	0	0	0	0	0	0	3	54
COMMA-1D cell line	0	0	0	0	0	0	4	265

[a)] D = 10^{-6}M dexamethasone, I = 5 μg/ml bovine insulin, P = 5 μg/ml ovine prolactin. 5 days of induction.

[b)] Casein$^+$ cells/2,000, detected by indirect immunofluorescence with rabbit anti-casein antisera.

ted IM-2, and its subline, LU-1, form three-dimensional duct- and endbud-like structures in monolayer cultures, and show extensive dome formation at high density (Figure 2). When parental IM-2 or LU-1 cells were exposed to the lactogenic hormones hydrocortisone, insulin and prolactin, they synthesize large amounts of β-casein, a marker for functional differentiation.

Immunocytochemical methods revealed that casein-synthesizing cells were strictly confined to the three-dimensional ductal structures. The monoclonal antibody, LE61 [45], specifically reacts with cytokeratins of the luminal, secretory type of mammary epithelial cells. We showed that cells within the duct-like structures stained positively in immunofluorescence experiments with this particular type of cytokeratin antibody. The pattern of cytokeratin polypeptides resolved by two-dimensional gel electrophoresis was essentially identical in IM-2 cells and in freshly cultured primary mammary epithelial cells. The typical cytokeratins usually expressed in the mammary epithelium of the mouse could be detected in the detergent high salt insoluble fraction of the IM-2 cell lysate. Laminin, a component of the basal lamina, was synthesized and secreted only upon formation of the ductal structures.

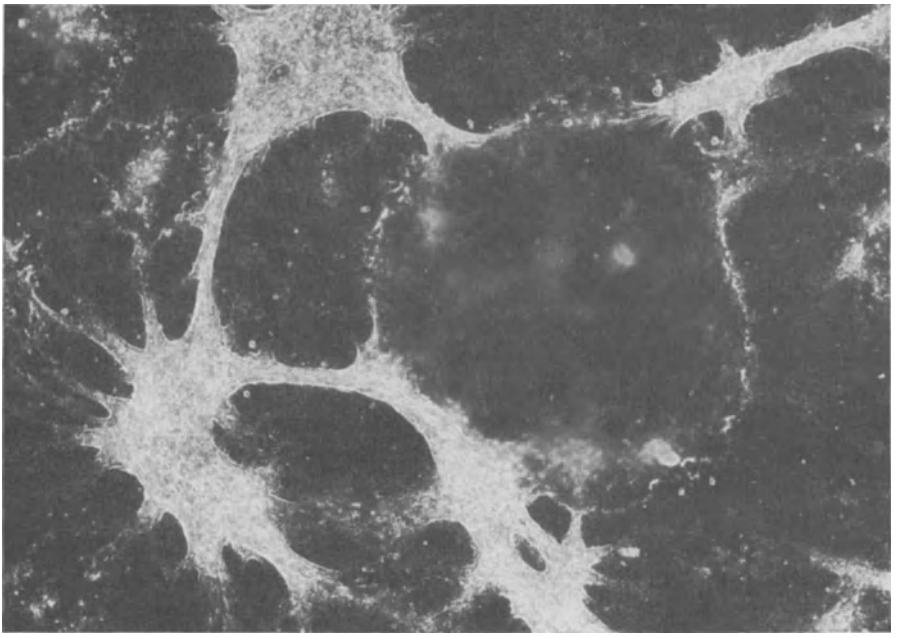


Figure 2. Morphogenic potential of an established mammary epithelial cell line derived from mid-pregnant Balb/c mice. LU-1 cells were cultured in DMEM supplemented with 10% FCS, 5 μg/ml insulin and 10 ng/ml EGF. By 3 days after plating the cells reached confluency and by 8 days duct-like structures and domes were fully developed. The photograph illustrates duct-like structures originating from a dome. At least 2 different cell types are required in order to cause the branching network of duct-like structures which when exposed to hydrocortisone (5 μg/ml) within three days shows maximal levels of β casein expression.

In contrast to cultures of primary mammary epithelial cells described previously, IM-2 cells are not dependent upon a collageneous substratum for inducibility of casein synthesis. The β-casein protein, as was shown by protein blot analysis, is strongly expressed 24 hours after lactogenic hormone induction. A maximal level of expression is reached after three days and remains constant for at least seven days. The β-casein mRNA could be shown three hours after hormone addition by RNA blot analysis.

The IM-2 line is a heterogeneous cell population consisting of at least two different cell types. We have derived single cell clones from the IM-2 line. No single cell clone was inducible by lactogenic hormones to synthesize β-casein. Two morphologically different cell clones were co-cultured to reconstitute a cell population in which lactogenic hormone dependent casein synthesis could be demonstrated. These cells organized into duct-like structures at high cell density. The casein-synthesizing cells were strictly confined to the duct-like structures. We suggest that the cell-cell contact of the two distinct cell types may be a prerequisite for the synthesis and secretion of basement membrane components allowing the luminal secretory epithelial cells to become responsive to lactogenic hormones. This may explain why IM-2 cells may be induced to differentiate to produce milk proteins in the absence of exogenously added matrix components, which were previously considered essential [71, 72, 73].

We also investigated the influence of viral oncogenes (v-*H-ras* and v-*myc*) on the hormonally induced differentiation of IM-2 cells. We introduced the oncogenes into IM-2 cells via retroviral vectors containing the neomycin resistance gene. Our experiments with single cell clones derived from the IM-2 cell line had indicated that they are unresponsive to lactogenic hormones. For this reason we pooled the virally infected G418-resistant cell colonies. Populations of the G418-resistant infected cells were tested for their response to lactogenic hormones. Cells infected with a retrovirus expressing only the neomycin resistance gene without an oncogene are inducible for β-casein synthesis. They show the typical pattern of histodifferentiation and dome formation following lactogenic hormone treatment. The amount of casein made is comparable to that detected in the original IM-2 line. The introduction of the *myc* oncogene did not change this phenotypic pattern. Populations infected with a retroviral construct encoding both neomycin resistance and the *ras* oncogene could be shown to express the p21 oncogene product. These cells become highly tumorigenic in both BALB/c and nude mice. They exhibited neither casein synthesis nor morphological histodifferentiation and dome formation following hormone treatment. It could be demonstrated that transformed populations expressing the *ras* oncogene synthesized two additional cytokeratin proteins not detected in the normal counterparts.

From this data we conclude that the IM-2 line, although immortalized, still exhibits relatively "normal" differentiation properties *in vitro*. IM-2 represents a cell culture system suitable for the investigation of the influence of hormones, basement membrane components and oncogene products on the growth and differentiation of mouse mammary epithelial cells and might be

useful in the study of the mechanism of synergistic action of lactogenic hormones at the gene level.

The introduction and expression of the v-H-*ras* oncogene rendered IM-2 cells highly tumorigenic and clearly interfered with the functional and morphological differentiation of these cells, while v-*myc* expression had no obvious effects on the differentiation of IM-2 cells.

5. The effect of the ras and the myc genes in vivo: oncogene expression directed by a milk protein gene promoter in transgenic mice

In vivo and *in vitro* transformation studies indicate that tumorigenesis is a multistep process. The establishment of cell lines and their malignant transformation are two processes that can be distinguished in *in vitro* studies. *In vivo* different cell types interact and genes and mutations may be crucial for tumorigenesis which are difficult to detect *in vitro*. During the past few years the method of establishing transgenic mice has been developed [74]. Foreign genes are injected into the male pronucleus of fertilized mouse eggs. The injected eggs are reimplanted into pseudo-pregnant foster mothers to develop. Usually the DNA integrates randomly into the host genome before the first cell division and is equally segregated to all daughter cells. Therefore, the foreign gene will be inherited to the progeny in a Mendelian fashion.

The information responsible for cell type-specific gene expression is often located in the surroundings of the gene promoter. Recombination of these regulatory sequences has generally been sufficient to target gene expression in transgenic mice [75]. This raises the possibility of analyzing the effect of an experimentally introduced oncogene in a particular cell type *in vivo* by coupling the oncogene to a tissue-specific promoter. Our interest in transformation of mammary epithelial cells led us to study the effect of the activated H-*ras* and the *myc* oncogenes on this particular cell type *in vivo* [39, 76]. To target the expression to the mammary epithelial cells, the oncogenes were subjected to the control of the murine they acidic protein (Wap) gene promoter. The Wap is one of the major whey proteins in the milk of rodents and it is synthesized under the influence of lactogenic hormones in mammary epithelial cells during late pregnancy and lactation [7]. The expression of the *ras* or the *myc* oncogene directed by the Wap promoter in transgenic mice offers an opportunity to compare the effect of two different oncogenes on the same cell type *in vivo*.

To direct the expression of *ras* or *myc* oncogenes to the mammary epithelial cells in transgenic mice, we isolated the control region of the Wap gene as a 2.5 kb restriction fragment, including the RNA cap site, the promoter region and putative 5' regulatory sequences [77]. The chimeric Wap-*ras* oncogene was constructed by linking this 2.5 kb Wap control region to the coding part of the human activated H-*ras* oncogene which was originally isolated from a human bladder carcinoma cell line [78].

The hybrid Wap-*ras* gene was purified from vector sequences and the linear 7.4 kb fragment was introduced into the germ line of C57 B1/6 × SJL mice by microinjection into the male pronucleus of fertilized eggs. 560 eggs were injected with approximately 200 copies each of the Wap-*ras* hybrid gene and subsequently reimplanted into pseudo-pregnant recipient mice. Seventy-one mice were born and analyzed for the presence of the Wap-*ras* DNA.

A corresponding approach was chosen to obtain transgenic mice expressing the WAP-*myc* hybrid oncogene. We recombined a 4.8 kb DNA fragment containing the second and third exons [79] of the mouse cellular *myc* gene with the Wap control region. The resulting 7.4 kb hybrid construct was used for microinjection. From 297 reimplanted eggs 45 mice were born.

In the offspring resulting from injected eggs the presence of the transgene was demonstrated in five Wap-*ras* and four Wap-*myc* mice (Table 4). These transgenic mice were backcrossed to wild type mice and the transmission of the transgene to the offspring in a Mendelian fashion typical for autosomal genes was confirmed in all but one of the lines. For line 69 we obtained only male transgenic mice despite a normal ratio of male and female progeny. This inheritance suggests that the hybrid gene is integrated into the Y chromosome.

The transgenic lines which have stably integrated either the Wap-*ras* or the Wap-*myc* gene were analyzed for chimeric gene expression. The expression patterns of all transgenic lines are summarized in Table 4. The hormone-dependent transgene expression was found in two lines of the Wap-*ras* strain and in three lines of the Wap-*myc* strain. In these lines, transgene expression in mammary glands was restricted to late pregnancy and lactation and parallels the expression of the endogenous Wap gene. This indicates that the 2.5 kb 5′ Wap sequence conferred hormone-dependence on either transgenic *myc* or *ras* expression. Analysis of RNA extracted from different tissues other than the mammary gland during lactation revealed that hormonally controlled expression of the Wap-*myc* gene is strictly confined to the mammary gland. In Wap-*ras* expressing females from lines 3 and 58 hormone dependent transgene expression was also found in the brain. In three lines the gene was silent.

The transgenic line with the WAP-*ras* gene integrated in the Y chromosome (line 69) expressed the chimeric gene constitutively in the parotid gland. This expression is peculiar to line 69. It has been shown that tissue-specific expression of introduced genes can be influenced by the integration site [80]. Thus we presume a correlation between integration and parotid expression of the transgene in line 69.

The tissue-specific expression of the transgenes allow us to investigate the phenotypic consequences of the presence of oncogene products in these animals. Initially all animals were morphologically normal and healthy. Only after a latency of 7–11 months tumor formation was observed in part of the animals (Table 4). There is a remarkable difference in tumor incidence depending on whether the *ras* or the *myc* oncogene is expressed in the mammary epithelial cells. From the *Wap-ras* expressing females only founder

Table 4. Transgene inheritance, expression pattern and tumor formation in the different transgenic strains

Transgenic strain	Line	Inheritance	Expression			
			Hormone-dependent (lactation)	Constitutive		
	11	autosomal	—	—	*Tumor formation*	*Latency*
	40	autosomal	—	—		
WAP-*ras*	3	autosomal	mgl, brain	—	0/100	
	58	autosomal	mgl, brain	—	1/20	11 mos
	69	Y-linked	—	parotid gl	19/24	7–11 mos
	33	autosomal	mgl	—	5/6	9 mos
WAP-*myc*	19	autosomal	mgl	—	2/4	11 mos
	21	autosomal	mgl	—	(1)	
	20	autosomal	—	—		(5 mos)

female 58 developed bilateral tumors in the second pair of mammery glands. As summarized in Table 4, Wap-*myc* expressing females from all three transgenic lines develop mammary adenocarcinomas with an incidence of about 80%. Females of line 21 are now approaching the age to develop tumors.

The low tumor incidence in the females of lines 3 and 58 may be a consequence of the defined expression period of the Wap-*ras* gene. Histological examination of mammary glands from these females under different hormonal conditions revealed a reduced differentiation of the mammary epithelium and sometimes neoplastic lesions during pregnancy and lactation. After lactation the mammary gland in these lines shows a completely normal morphology. The *ras* oncogene expression has effects on the differentiation and transformation of the epithelial cells but is not able to overcome the developmental control. The cells undergo their normal fate of involution after lactation. In line 69, where the Wap-*ras* integrated into the Y chromosome and is expressed constitutively in the parotid gland, most of the males develop adenocarcinomas of the parotids. This shows that also *ras* expression predisposes epithelial cells to transformation *in vivo*. The histological examination revealed that all tumors, in the Wap-*ras* or in the Wap-*myc* transgenic mice, were surrounded by normal tissue also expressing the transgenes. The clonal origin of the tumors and the long latency suggests that the expression of one oncogene is not sufficient to transform epithelial cells *in vivo*. Additional genetic or epigenetic events have to be postulated for tumor formation. However, in nontransgenic control animals no tumors were observed and in the transgenic mice tumors arose only in those tissues where the transgene was already expressed. This suggests that the oncogene expression is a predisposing factor for the tumor formation *in vivo*.

Provided that the expression of the transgene was involved in the transformation, its expression should be detected in the tumors. In Figure 3, the expression of the Wap-*ras* gene in the parotid adenocarcinomas and in normal parotis of males of line 69 is shown in a RNase protection assay. In this assay, isolated cytoplasmatic RNA is hybridized to an *in vitro* transcribed, radiolabelled anti-sense RNA probe of a defined size. Specific RNA will form double-stranded hybrids with the probe which are protected from digestion during the subsequent RNase treatment [81]. After gel separation, autoradiography reveals a band corresponding to the size of the protected fragment indicative of the specific RNA. For Wap-*ras* RNA we expect a protected fragment of 143 nucleotides. Compared to the abundancy in normal parotids (lane 2), Wap-*ras* transcripts are highly increased in the tumors (lane 1 & 3). This increase in abundancy was consistently found in all parotid tumors and was also observed in the two mammary tumors of founder female 58. This increase of Wap-*ras* RNA could be due to a more homogenous population of Wap-*ras* expressing cells in the tumors compared to normal tissue. Alternatively, the higher abundancy directly reflect an increased expression of the transgene in the transformed cells.

To distinguish between these two possibilities we performed *in situ* hybrid-

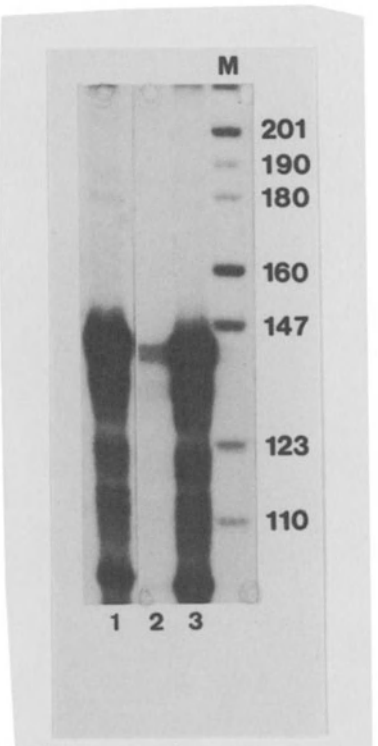


Figure 3. Expression of the Wap-*ras* gene in tumors.
RNA (10 g) was prepared from parotid adenocarcinomas (lanes 1 & 3) and from normal parotis (lane 2) of males from line 69 and analyzed in a RNase protection assay. The RNA was hybridized to a ^{32} P-labelled single-stranded antisense probe specific for exon 1 of the transgene. A protected fragment of 143 nucleotides is indicative for Wap-*ras* transcripts.
M: HpaII digested, ^{32}P-end-labelled pBR 322 DNA was used as size marker (indicated in nucleotides).

izations. This method allows the localization and quantification of specific transcripts in tissue sections. Essentially, we followed the method described by [82]. Thin sections of 6 μM were cut from tissues frozen in liquid nitrogen and were fixed in 4% paraformaldehyde. To make the cells more accessible to the probe, the sections were subjected to a limited proteinase K treatment. Subsequently, they were hybridized to a ^{32}P-labelled probe specific for the human H-*ras* gene. Since this probe does not crossreact with endogenous murine *ras* RNA, we expect to detect only transcripts derived from the Wap-*ras* transgene. After autoradiography the hybridized probe is visualized as black silver grains over the tissue sections which are counter-stained with hematoxilin-eosin.

Figure 4a shows the histology of normal parotids. The secretory alveoli are embedded in stromal tissue and can clearly be distinguished from the ducts. The histology of the parotid adenocarcinomas (Figure 4b) shows the epithelial tumor cells surrounded by stromal tissue. Hybridization of the *ras* probe to

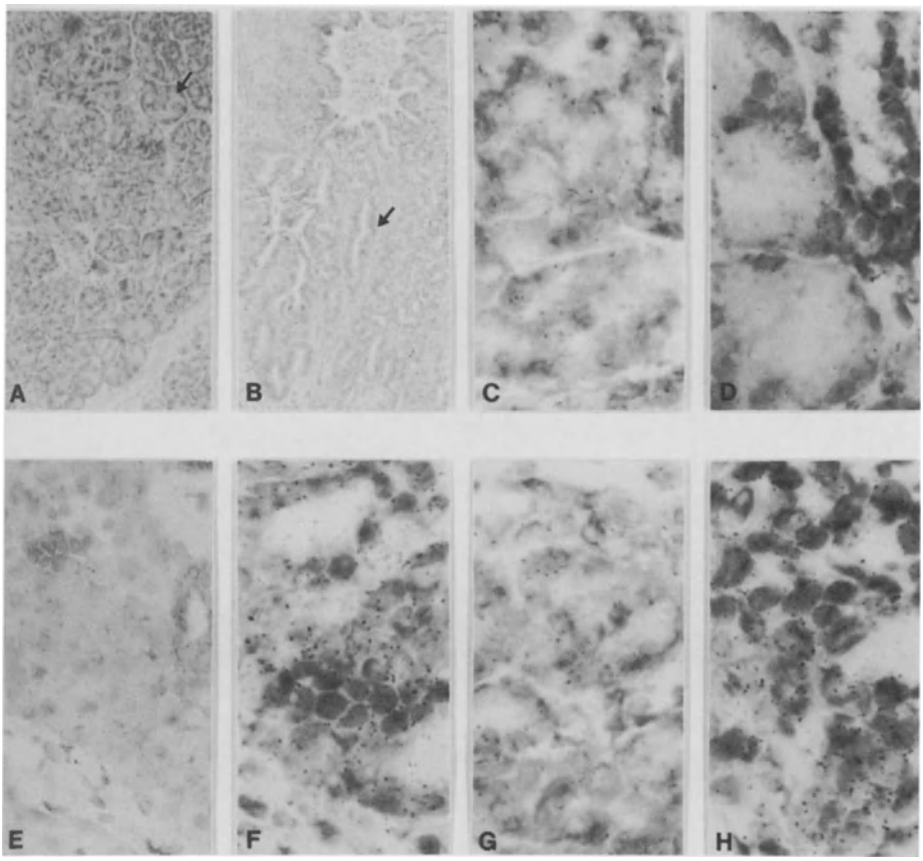


Figure 4. In situ detection of WAP-*ras* RNA in normal and tumor tissue of males of line 69. A: Paraffin section of normal parotids. The secretory alveoli are indicated by an arrow. B: Paraffin section of a parotid adenocarcinoma. Epithelial structures are indicated by an arrow. (Magnification 400×).
The frozen sections (C-H) were hybridized to a ^{32}P-labelled probe specific for exon 1 of the activated human H-*ras* gene (600 nucleotides). C: Normal parotis of a transgenic male of line 69. D: Submaxillary gland from the same animal. E: Parotids of a nontransgenic control animal. F: Tumor tissue from a male of line 69. G: Normal parotis of a male from line 69. H: Parotid tumor of the same individual. (Magnification 1,600×).

normal parotis (Figure 4c) revealed a weak signal over the acinar cells of the gland. No silver grains were detected over the stromal or ductal structures. Neither in the submaxillary gland of a male from line 69 nor in parotid glands of nontransgenic control animals a specific reaction of the *ras* probe was found (Figure 4d & e). These results are consistent with the results obtained in RNase protection assay. The transgene is expressed in the secretory alveolar cells of the parotis in males of line 69. The analysis of tumor tissue (Figure 4f) reveals hybridization of the *ras* probe to the epithelial cells and not

to the stromal cells of the tumor. Compared to the normal parotis of the same individual (Figure 4g), the silver grains are much more abundant on the epithelial cells of the tumor (Figure 4h). We counted on the average 0.83 (± 0.5) grains per acinar cell of the parotids and 4.85 (± 0.7) gains per epithelial tumor cell. The higher density of silver grains over the tumor cells indicates that these cells contain higher levels of of Wap-*ras* RNA than the untransformed acinar cells in the normal parotids of the same individual.

The result contained by *in situ* hybridization revealed that the transformation involves the secretory epithelial cells already expressing the transgene and is accompanied by its increased expression. We argued above that expression of the Wap-*ras* gene is not sufficient for transformation *in vivo*. In this experimental system an additional event increasing the level of Wap-*ras* expression might have been the critical factor for transformation.

Although the expression of the *ras* and the *myc* oncogene in transgenic females is regulated identically, the *ras* oncogene is not able to cause tumor formation in the mammary epithelium whereas the *myc* oncogene is highly efficient in the transformation of these cells. The high incidence of mammary gland tumors in the Wap-*myc* expressing line indicates a different mechanism of oncogene action. Analysis of the expression of the Wap-*myc* gene revealed its constitutive expression in the tumors. In addition to the Wap-*myc* gene, the expression of th endogenous Wap and β-casein gene was found to be independent from lactogenic hormones in the tumors. This constitutive expression was found in all mammary adenocarcinomas of females from all three Wap-*myc* lines and was maintained even after transplanting the tumors into virgin nude mice. This suggests that Wap-*myc* induced transformation results in the escape of milk protein genes from their regulation physiologically exerted by the lactogenic hormones. The abrogation of the milk protein genes from the hormonal control was not observed in the tumors of the Wap-*ras* bearing female 58 and supports the different action of *ras* and *myc* in mammary epithelial cells. In contrast to *ras*, Wap-*myc* expression may be able to overcome the hormonal control of the mammary epithelial cells. Targeting *myc* expression to the lymphatic compartement of transgenic mice [83] revealed that the transgene expression favored proliferation over maturation of the pre-B cells [84]. From this population lymphomas of monoclonal origin arose. Similarly, *myc* expression in differentiating mammary epithelial cells may, at least in some cells, result in a block of differentiation endowing the cells with proliferative capacities and the ability to produce differentiation specific products. The inhibition of terminal differentiation uncouples them from the normal control ultimately leading to their involution. The tumors might arise from the cells persisting after lactation.

The main difference between the effects of the *ras* and the *myc* oncogene observed in transgenic mice are emphasized by the different ability to interfere with the strict hormonal control of the mammary epithelium. By crossing Wap-*ras* and Wap-*myc* mice we established a supertransgenic line bearing both, the *ras* and *the* myc oncogene. This line will allow us to investigate if the

two different mechanisms of oncogene action are able to cooperate in transformation of mammary epithelial cells *in vivo*. The tumor incidence will reveal if after abrogated from the hormonal control by *myc*, the *ras* oncogene exerts its transforming potential on this particular cell type.

6. Conclusions

The activation of oncogenes as a trigger for tumorigenesis has been a fruitful concept and has contributed important new experimental procedures to the study of cancer [16]. A number of fundamental questions are under investigation in many laboratories: 1) Are there consistently involved oncogenes which are responsible for a particular type of tumor; 2) Are there cooperative modes of action of mutations in different genes; 3) Are oncogenes interfering with growth and differentiation in ways other than transformation; 4) Can oncogenes or their functions be utilized in diagnosis and treatment of cancer? Although many of these questions are far from being answered, interesting new leads are being pursued. The study of breast cancer has provided insights into the complexity of the oncogene interactions and a few conclusions can be drawn.

Oncogene expression studies in breast tumor tissues or functional oncogene assays using genomic DNA have shown that different oncogenes can be involved in the disease. Members of the *ras* gene family, the c-*myc* oncogene, the EGF receptor gene [85] and the c-*erb*B-2 oncogene have been found. Considering the different cellular localizations of the oncoproteins encoded by these genes and the different subcellular localizations, it is likely that different mechanisms of transformation must be involved. Not only the activation of the oncogenes but also their expression levels contribute to the transformed phenotype. Transformation parameters can be observed *in vitro* and they are proportional to the concentration of certain oncoproteins. We could furthermore observe that oncogenes can cause effects short of transformation. They can influence the growth parameters and the hormonal responsiveness of mammary epithelial cells *in vitro* and *in vivo*. The effects are specific and different for individual oncogenes.

The different effects of oncogenes on different cell types brings us back to the complexity of the mammary epithelium. Cell-cell, cell-substratum and cell-hormone interactions all contribute to the function of the gland. It is conceivable that these networks of interactions are influenced at very different points. Oncogene transformation could interfere with, for instance, the synthesis of growth factors [86], the synthesis of the basement membrane or the hormonal responsiveness of epithelial cells. Cells might be blocked at different stages of differentiation, or subpopulations of proliferating cells might be expanded and become susceptible to transformation. The usefulness for diagnostic and therapeutical purposes will very much depend on insights gained into the function of the oncoproteins. Important leads can be expec-

ted, especially from proteins located on the cell surface and accessible to externally administered drugs [87].

References

1. Banerjee MR: Responses of mammary cells to hormones. Int Rev Cytol 47:1–97 1976.
2. Kano-Sueoka T: Factors affecting mammary cells in culture. In: Biochem Actions of Hormones, Vol. X, Litwack G (ed), New York, Academic Press, pp 163–185.
3. Topper YJ, Freeman CS: Multiple hormone interactions in the developmental biology of the mammary gland. Physiol Rev 60:1049–1106, 1980.
4. Russo J, Tay LK, Russo IH: Differentiation of the mammary gland susceptibility to carcinogenesis. Breast Cancer Res Treat 2:5–73, 1982.
5. Kratochwil K: Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. Rev Biol 20:46–71, 1969.
6. Levine JF, Stockdale FE: Cell-cell interactions promote mammary epithelial cell differentiation. J Cell Biol 100:1415–1422, 1985.
7. Hobbs AA, Richards DA, Kessler DJ, Rosen JM: Complex hormonal regulation of rat casein gene expression. J Biol Chem. 257:3598–3605, 1982.
8. Nandi S, Yang J, Richards J, Guzman R: 1981 Role of hormones in mammogenesis and carcinogenesis. In: Banbury Report, Vol. 8, Hormones and Breast Cancer, Pike MC, Siiteri PK, Welsch CW (eds), New York, Cold Spring Harbor Laboratory, pp 445–453, 1981.
9. Krontiris TG, Cooper GM: Transforming activity of human tumor DNAs. Proc Natl Acad Sci USA 78:1181–1184, 1981.
10. Murray JJ, Shilo BZ, Shih C, Cowing D, Hsu HW, Weinberg RA: Three different human tumor cell lines contain different oncogenes. Cell 25:355–361, 1981.
11. Perucho M, Goldfarb M, Shimizu K, Lama C, Fogh J, Wigler M: Human tumor-derived cell lines contain common and different transforming genes. Cell 27:467–476, 1981.
12. Pulciani S, Santos E, Lauver AV, Long LK, Aaronson SA, Barbacid M: Oncogenes in solid human tumours. Nature 300:539–542, 1982.
13. Alitalo K, Schwab, M. Oncogene amplification in tumor cells: Advances in Cancer Research 47, 235–281, 1986.
14. Croce CM: Chromosome translocations and human cancer. Cancer Res 46:6019–6023, 1986.
15. Varmus HE: The molecular genetics of cellular oncogenes. Ann Rev Genet. 18:533–612, 1984.
16. Bishop JM: The molecular genetics of cancer. Science 235:305–311, 1987.
17. Hynes NE, Jaggi R, Kozma SC, Ball R Muellener D, Wethereall NT, Davis BW, Groner B: New acceptor cell for transfected genomic DNA: Oncogene transfer into a mouse mammary epithelial cell line. Mol Cell Biol 5:268–272, 1985.
18. Kozma SC, Bogaard ME, Buser K, Saurer SM, Bos JC, Groner B, Hynes NE: The human c-kirsten *ras* gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231. Nucleic Acids Res 15:5963–5971, 1987.
19. Kraus MH, Yuasa Y, Aaronson SA: A position 12-activated H-*ras* oncogene in all HS 578 T mammary carcinoma cells but not normal mammary cells of the same patient. Proc Natl Acad Sci USA 81:5384–5388, 1984.
20. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, Saito T, and Toyoshima K: Similarity of protein encoded by the human c-*erb*B-2 gene to epidermal growth factor receptor. Nature 319:230–234, 1986.
21. Gullick WJ, Berger MS, Bennett PLP, Rothbard JB, Waterfield AD: Expression of the c-*erb*B-2 protein in normal and transformed cells. Int J Cancer 40:246–254, 1987.
22. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–182, 1987.

23. Venter DJ, Tuzi NL, Kumar S, Gullick WJ: Overexpression of the c-*erb*B-2 onco-protein in human breast carcinomas: immunohistological assessment correlates with gene amplification. Lancet ii:69–72, 1987.
24. Van de Vijver M, van de Bersselaar R, Devilee P, Cornelisse C, Peterse J, Nusse R: Amplification of the neu (c-*erb*B-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-*erb*A oncogene. Mol Cell Biol 7:2019–2023, 1987.
25. Berger M, Locher G, Saurer S, Gullick W, Waterfield M, Groner B, Hynes NE: c-*erb*B-2 gene amplification and protein expression in human breast carcinoma correlate with nodal status and nuclear grading. Cancer Res 48; March, 1988.
26. Barbacid M: *ras* genes. Annual Rev Biochem 56:779–827, 1987.
26. Balmain A, Ramsdem M Bowden GT, Smith J. Activation of the mouse cellular Harvey-*ras* gene in chemically induced benign skin papillomas. Nature 307: 658–660, 1984.
27. Zarbl H, Sukumar S, Arthur AV, Martin-Zanca D, Barbacid M: Direct mutagenesis of H-*ras*-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315:382–385, 1985.
28. Bos JL, Fearon ER, Hamilton SR, Verlaan de Vries M, van Boom JH, van der Eb AJ, Vogelstein B: Prevalence of *ras* gene mutations in human colorectal cancers. Nature *327*, 293–297, 1987.
29. Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M: Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. Nature 327:298–303, 1987.
30. Cepko CL, Roberts BE, Mulligan RC: Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell 37:1053–1062, 1984.
31. Redmond SMS, Reichmann E, Müller RG, Friis R, Groner B, Hynes NE: The transformation of primary and established mouse mammary epithelial cells by p21-*ras* is concentration dependent. Oncogene 2, 1988.
32. Butel JS, Wong C, Median D: Transformation of mouse mammary epithelial cells by papovavirus SV40. Exper Mol Pathol 40:79–108, 1984.
33. Chang SE, Kun J, Lane EB, Taylor-Papadimitriou J: Establishment and characterization of SV40 transformed human breast epithelial cell lines. Cancer Res 42:2040–2053, 1982.
34. Garcia I, Sordat B, Rauccio-Farinon E, Dunand M, Krähenbühl JP, Diggelmann H: Establishment of two rabbit mammary epithelial cell lines with distinct oncogenic potential and differentiated phenotype after microinjection of transforming genes. Mol Cell Biol 6:1974–1982, 1986.
35. McConlogue L, Gupta M, Wu L, Coffino P: Molecular cloning and expression of the mouse ornithine decarboxylase gene. Proc Natl Acad Sci USA 81:540–544, 1984.
36. Kozbor D, Croce CM: Amplification of the c-*myc* oncogene in one of five human breast carcinoma cell lines. Cancer Res 44:438–441, 1984.
37. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme MH, Gest J, Callahan R: Genetic alterations of the c-*myc* proto oncogene in human primary breast carcinomas. Proc Natl Acad Sci USA 83:4834–4838, 1986.
38. Leder A, Pattengale PK, Kuo A, Stewart TA, Leder P: Consequences of widespread deregulation of the c-*myc* gene in transgenic mice: multiple neoplasms and normal development. Cell 45:485–495, 1986.
39. Schönenberger CA, Andres AC, Groner B, van der Valk M, Le Meur M, Gerlinger P: Targeted c-*myc* gene expression in mammary glands of transgenic mice induces mammary tumors with constitutive milk protein gene transcription. EMBO J 7:169–175, 1988.
40. Naharro G, Robbins KC, Reddy EP: Gene product of v-fgr onc: hybrid protein containing a protion of actin and a tyrosine specific protein kinase. Science 223:63–66, 1984.
41. Danielson KG, Oborn CJ, Durban EM, Butel JS, Medina D: Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc. Natl. Acad. Sci. USA *81*, 3756–3760.
42. Medina D, Oborn CJ, Kittrell FS, Ullrich RL: Properties of mouse mammary epithelial cell lines characterized by *in vivo* transplantation and in vitro immunocytochemical methods. J

Natl Cancer Inst 76:1143–1156, 1986.
43. Blochlinger K, Diggelmann H: Hygromycin β phosphotransferase as a selectable marker for DNA transfer experiments in higher eukaryotic cells. Mol Cell Biol 4:2929–2931, 1984.
44. Ball RK, Ziemiecki A, Schoenenberger CA, Reichman E, Redmond SMS, Groner B: v-*myc* alters the response of a cloned mouse mammary epithelial cell line to lactogenic hormones. Mol Endocrinol. (Submitted.) 1987.
45. Lane EB: Monoclonal antibodies provide specific intramolecular markers for the study of epithelial tonofilament organization. J Cell Biol 92:605–673, 1982.
46. Ellis RW, De Feo D, Maryak JM, Young HA, Shih TY, Chang EH, Lowy DR, Scolnick EM: Dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. J Virol 36:408–420, 1980.
47. Vennström B, Kahn P, Adkins B, Enrietto P, Hayman MJ, Graf T, Luciw P: Transformation of mammalian fibroblast and macrophages *in vitro* by a retrovirus encoding an avian v-*myc* oncogene. EMBO J 3:3223–3229, 1984.
48. Wagner EF, Vanek M, Vennström B: Transfer of genes into embryonal carcinoma cells by retrovirus infection: efficient expression from an internal promoter. EMBO J 4:663–666, 1985.
49. Mann R, Mulligan RC, Baltimore D: Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 33:153–159, 1983.
50. Yang J Richards J, Bowman P, Guzman R, Enami J, McCormick K, Hamamoto S, Pitelka D, Nandi S: Sustained growth and three dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. Proc Natl Acad Sci USA 76:3401–3405, 1979.
51. Daniel CW, Berger JJ, Strickland P, Garcia R: Similar growth patterns of mouse mammary epithelium cultivated in collagen matrix in vivo and in vitro. Dev Biol 104:57–64, 1984.
52. Sonnenberg A, Daams H, van der Valk MA, Hilkens J, Hilgers J: Development of mouse mammary gland, identification of stages in the differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent anti-serum against keratin. J Histochem Cytochem 34:1037–1046, 1986.
53. Asch HL, Asch BB: Expression of keratins and other cytoskeletal proteins in mouse mammary epithelium during the normal development cycle and primary culture. Dev Biol 107:470–482, 1985.
54. Banks-Schlegel SP, Howley PM: Differentiation of human epidermal cells transformed by SV40. J Cell Biol 96:330–337, 1983.
55. Mervis A, Steinberg ML, Defendi V: Keratin gene expression in simian virus 40 transformed human keratinocytes. Proc Natl Acad Sci USA 82:8498–8502, 1985.
56. Durban EM, Median D, Butel JS: Comparative analysis of casein synthesis during mammary cell differentiation in collagen and mammary gland development *in vivo*. Dev Biol 109:288–298, 1985.
57. Lee EY, Lee HP, Kaetzel CS, Parry G, Bissell MJ: Interaction of mouse mammary epithelial cells with collagen substrates: regulation of casein gene expression and secretion. Proc. Natl Acad Sci USA 82:1419–1423, 1985.
58. Hennighausen LG, Sippel AE: Characterization and cloning of the mRNAs specific for the lactating mouse mammary gland. Eur J Biochem 125:131–141, 1982.
59. Falcone G, Tato F, Alema S: Distinctive effects of the viral oncogenes *myc*, *erb*, *fps* and *src* on the differentiation propan of quail myogenic cells. Proc Natl Acad Sci USA 82:426–430, 1985.
60. Coppola JA, Cole MD: Constitutive c-*myc* oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. Nature 320:760–763, 1986.
61. Prochownik EV, Kukowska J: Deregulated expression of c-*myc* by murine erythroleukemia cells prevents differentiation. Nature 322:848–850, 1986.
62. Dony C, Kessel M, Gruss P: Post-transcriptional control of *myc* and p53 expression during differentiation of the embryonal carcinoma cell line F9. Nature 317:636–639, 1985.
63. Müller R: Protooncogenes and differentiation. Trends Biochem Sci 11:129–132, 1986.

64. Alema S, Tato F, Boettiger D: *myc* and *src* oncogenes have complementary effects on cell proliferation and expression of specific extracellular matrix components in definitive chondroblasts. Mol Cell Biol 5:538–544, 1985.
65. Noda M, Ko M, Ogura A, Liu DG, Anano T, Takano T, Ikawa Y: Sarcoma viruses carrying *ras* oncogenes induce differentiation associated properties in a neuronal cell line. Nature 318:73–75, 1985.
66. Balk SD, Riley TM, Gunther HS, Morisi A: Heparin treated, v-*myc* transformed chicken heart mesenchymal cells assume a normal morphology but are hypersensitive to epidermal growth factor (EGF) and brain fibroblast growth factor (bFGF); cells transformed by the v-Ha-*ras* oncogene are refractory to EGF and FGF but are hypersenstive to insulin-like growth factors. Proc Natl Acad Sci USA 82:5781–5785, 1985.
67. Sorrentino V, Drozdoff V, McKinney MD, Zeitz L, Fleissner E: Potentiation of growth factor activity by exogenous c-*myc* expression. Proc Natl Acad Sci USA 83:8167–8171, 1986.
68. Stern DF, Roberts AB, Roche NS, Sporn MB, Weinberg RA: Differential responsiveness of *myc* and *ras* transfected cells to growth factors: selective stimulation of *myc* transfected cells by epidermal growth factor. Mol Cell Biol 6:870–877, 1986.
69. Rapp UR, Cleveland JL, Brightmare K, Scott A, Ihle JN: Abrogation of IL-3 and IL-2 dependence by recombinant murine retroviruses expressing v-*myc* oncogenes. Nature 317:434–348, 1985.
70. Jaggi R, Salmons B, Muellener D, Groner B: The v-*mos* and H-*ras* oncogene expression represses glucocorticoid hormone dependent transcription from the mouse mammary tumor virus LTR. EMBO J 5:2609–2616, 1986.
71. Yang J, Richards J, Guzman R, Imagawa W, Nandi S: Sustained growth in primary culture of normal mammary epithelial cells embedded in collagen gels. Proc Natl Acad Sci USA 77:2088–2092, 1980.
72. Richards J, Guzman R, Konrad M, Yang J, Nandi S: Growth of mouse mammary gland end buds cultured in a collagen gel matrix. Exp Cell Res 141:433–443, 1982.
73. Bissell MJ: The differentiated state of normal and malignant cells or how to define a normal cell in culture. Int Rev Cytol 70:27–100, 1981.
74. Palmiter RD, Chen HY, Brinster RL: Differential regulation of metallothionein thymidine kinase fusion genes in transgenic mice and their offspring. Cell 29:701–710, 1982.
75. Palmiter RD, Brinster RC: Germ line transformation of mice. Ann Rev Genet 20:465–499, 1986.
76. Andres AC, Schönenberger CA, Groner B, Henninghausen L, LeMeur M, and Gerlinger, P: Ha-*ras* oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation, and tumor induction in transgenic mice. Proc Natl Acad Sci USA 84:1299–1303, 1987.
77. Campbell SM, Rosen JM, Henninghausen LG, Strech-Jurk U, Sippel AE: Comparison of the whey acidic protein genes of the rat and mouse. Nucleic Acids Res 12:8685–8697, 1984.
78. Capon DJ, Chen EY, Levinson AD, Seeburg PH, Goeddel PV: Complete nucleotide sequence of the T24 human bladder carcinoma oncogene and its normal homologue. Nature 302:33–37, 1983.
79. Land H, Parada LF, Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596–601, 1983.
80. Kollias G, Wrighton N, Hurst J, and Grosveld F: Regulated expression of human γ-, β- and hybrid γ-β globin genes in transgenic mice: manipulation of the developmental expression patterns. Cell 46:89–94, 1986.
81. Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR: Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res 12, 7035–7056, 1984.
82. Hafen E, Levine M, Garber RL, Gehring WJ: An improved *in situ* hybridization method for the detection of cellular RNAs in Drosophila tissue sections and its application for localizing transcripts of the homeotic antennapedia gene complex. EMBO J 2:617–623, 1983.
83. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD,

Brinster RL: The c-*myc* oncogene, driven by immunoglobulin enhancers, induces lymphoid malignancies in transgenic mice. Nature 318:533–538, 1985.

84. Langdon WY, Harris AW, Cory S, Adams JM: The c-*myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. Cell 47:11–18, 1986.
85. Filmus J, Trent JM, Pollack MN, Buick RN: Epidermal growth factor receptor gene—amplified MDA 468 breast cancer cell line and its nonamplified variants. Mol Cell Biol 7: 251–257, 1987.
86. Dickson RB, Lippman ME: Hormonal control of human breast cancer cell lines. In: Cancer Surveys, Vol. 5, Bulbrook RD, (ed) Oxford University Press, pp 617–624.
87. Drebin JA, Link VC, Stern D, Weinberg RA, Greene MT: Down modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell 41:695–706, 1985.

5. Epidermal growth factor receptors in human breast cancer

Adrian L. Harris, and Stewart Nicholson

1. Isolation of Epidermal Growth Factor (EGF)

Epidermal Growth Factor (EGF) is a small (6045 Dalton) protein that stimulates cell proliferation in both cell culture systems and in intact animals. EGF was first isolated from the submaxillary gland of the mouse by Cohen in 1962 [1]. Extracts of the gland when injected into newborn animals induced precocious eyelid opening and incisor eruption. At the time, Cohen was involved in research on Nerve Growth Factor but realized the potential importance of a substance with such profound biological effects. The biological response to injection of extracts of the submaxillary gland was due to a stimulation of epidermal growth and keratinization. The factor responsible for these effects was named Epidermal Growth Factor. The mouse EGF molecule contains 53 amino acid residues [2] and has disulphide bonds between cysteine residues 5 and 20, 14 and 31, 33 and 42, producing three disulphide loops in the secondary structure [3]. Mouse EGF is produced from a much larger precursor molecular of 130,000 Daltons [4]. In the mid 1970s, EGF was detected [5] and isolated from human urine [6]. In 1975 Gregory demonstrated that human EGF was equivalent to urogastrone, a hormone capable of inhibiting gastric acid secretion. Mouse EGF and urogastrone (Uro-EGF) share a 70% homology but are antigenically distinct as shown by radioimmunoassay [7]. They are, however, interchangeable in radioligand assays and growth stimulatory experiments. Several reviews are available which detail the various biological and chemical aspects of EGF [8, 9].

2. Distribution of EGF in human tissues

EGF is found in human tissues, including Brunner's glands of the duodenum, the anterior pituitary, certain cells in the bone marrow, the skin and its appendages, the kidney and male genital tract and it is secreted in milk [10–13]. Large quantities of EGF are found in human urine and are probably the result of secretion by renal tubular cells. A 4,700 nucleotide sequence

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

messenger RNA has been found in kidney which codes for a large molecular weight precursor of EGF—prepro-EGF [14]. Prepro-EGF consists of around 1,200 amino acids and part of it is similar to the EGF receptor. It is possible that prepro-EGF is the source of urinary EGF that is released by a processing enzyme on the plasma membrane of the renal tubular cell [15].

Recent studies have also shown a high concentration of EGF in human prostatic secretion (up to 280 ng/ml) [16]. However, EGF was not identified in prostatic tissue and the authors proposed that the prostate—like the breast—concentrates EGF from serum. Significantly lower concentrations of EGF were found in the prostatic secretion of patients with benign hyperplasia than in controls. The concentration of EGF in urine is about 30 ng/ml and between 50 μg to 60 μg of EGF are excreted in the urine daily [17]. The concentration in milk is between 20 ng/ml–90 ng/ml and in saliva between 2 ng/ml to 17 ng/ml. Serum concentrations of EGF are between 130 pg/ml and 160 pg/ml but it is not clear if these increase during pregnancy or lactation [18, 19].

3. Effects of EGF

The first demonstration of the effect of EGF on epithelial cell proliferation was made using fragments of skin derived from chick embryos in an organ culture system [20]. Subsequently, the mitogenic effects of EGF have been demonstrated in a wide variety of cell types, both in culture and *in vivo*. EGF enhances a cascade of cellular events including: stimulation of amino acid transport, enhanced turnover of phosphatidyl inositol, extensive ruffling of the plasma membrane, enhanced bulk endocytosis, activation of glycolysis, induction of ornithine decarboxylase, activation of RNA and protein synthesis, activation of synthesis of extracellular macromolecules and initiation of DNA synthesis and cell replication [9, 21].

The physiological role of EGF has not yet been clarified. In addition to its effects on the newborn mouse, it induces maturation of the foetal lung [22] and may play a role in the normal regeneration of the epithelial surfaces of the gut and urinary tract. For instance, EGF given intravenously to an infant with microvillous atrophy was recently reported to result in increaed crypt cell proliferation [23].

4. EGF receptors

The biological effects of EGF are mediated through high affinity binding to specific cell membrane receptors. The receptor is an integral membrane protein of 170,000 daltons [24]. It is a glycoprotein having approximately 130,000 daltons of protein and a considerable amount of N-linked carbohydrate. Re-

cently a high degree of homology has been shown to sequences of the V-erb B transforming protein of the avian erythroblastosis virus [25]. Comparison with v-erb B structure suggests that the EGF receptor has a cytoplasmic domain of approximately 60,000 daltons (542 amino acids), a short transmembrane sequence, and a large external domain of 100,000 daltons (621 amino acids) that is not present in the v-erb B protein. The EGF receptor has no subunit structure and the binding site for EGF on the extracellular portion and an EGF-activated tyrosine kinase activity on the intracellular (cytoplasmic) portion are located in separate domains of a single polypeptide chain [24, 26].

The EGF receptor gene has been mapped to human chromosome 7 in the p13-p11 region [27]. Chromosome analysis of A431 epidermoid carcinoma cells, which possess a very high number of EGF receptors, and are hence an extremely useful tool in EGF receptor research, has shown that these cells contain two copies of chromosome 7 [28].

Another oncogene (neu) has been identified in rat glioblastoma and a gastric carcinoma cell line which is closely related in amino acid sequence to c-erb-B and the EGF receptor but which does not cross-react with antibodies raised against the EGF receptor [29]. It has been proposed that this oncogene protein is the receptor for an as yet unidentified growth factor. It has been shown to be amplified in approximately 40% of patients with breast cancer and positive lymph nodes [30].

5. Tyrosine kinases

Binding of EGF to the external domain of the receptor initiates the tyrosinase specific protein kinase activity intrinsic to the cytoplasmic domain. This reaction requires energy in the form of ATP [21]. Several lines of evidence point to the protein kinase activity being intrinsic to the receptor [26]. EGF stimulated protein phorphorylation at tyrosine residues leads to autophosphorylation of the receptor as well as acting on exogenous substrates [31]. The EGF receptor, and in particular its internal domain, is related to certain transforming proteins both in structure and function. The cytoplasmic domain shares 95% homology with the erb-B transforming protein of avian erythroblastosis virus [25, 32]. Of all transforming proteins known, 30% have tyrosine kinase activity (src, yes, fps, fes, fos, abl, fgs) [33, 34]. Phosphotyrosine represents usually less than 0.03% of the total phosphoaminoacids in the non-transformed cell but increases approximately 30-fold in transformed cells [35]. This property is also shared with other receptors for growth promoting factors such as PDGF, insulin and somatomedin C [33].

The EGF receptor and pp60 src, the oncogene of the Rous sarcoma virus, phosphorylate a Mr 36,000 normal cellular protein at the same site [36]. They may stimulate cell growth through the same pathway. One substrate has recently been described for the EGF receptor. Cohen has described in A431

cells a 35,000d soluble protein which, in the presence of calcium, is phosphorylated on tyrosine residues when EGF binds to its receptor. Large amounts of this protein have been found in lung and placenta and it is homologous to lipocortin [37–39].

6. Receptor clustering and second messengers within the cell

Following the interaction of EGF and its receptor, growth factor-receptor complexes are formed and internalized (down-regulation) [40]. This is a complex process beginning with the clustering of growth factor-receptor complexes in indentations of the plasma membrane followed by endocytic internalisation, followed by rapid degradation within lysosomes. EGF has a marked effect on the rate of degradation of its receptor. With diploid human fibroblasts, the half life of the receptor has been shown to be approximately 10 hours in the absence of EGF but when EGF is added to the medium, the half life was reduced to about one hour [41]. Similar experiments using A431 cells revealed a reduction in half life from 20 to 7 hours [42]. The internalised EGFr remains capable of tyrosine phosphorylation and may be able to interact at sites on the nuclear scaffold [43] and cytoskeletal elements [44].

Binding of PDGF has been found to activate phosphoinositidase C which hydrolyzes phosphoinositol-4,5-biphosphate to D-inositol-1,4,5-tris-phosphate and 1,2-diacylglycerol [45]. The former leads to release of calcium ions stored in the endoplasmic reticulum—a process which also occurs in cells expressing activated oncogenes [46]. The latter activates protein kinase C. Increases in intracellular calcium occurring after exposure to other growth factors may not be mediated by this mechanism, however. It has been shown recently that, after exposure to EGF, the increase in intracellular calcium is due to opening of a voltage-independent calcium channel in the cell membrane [47]. EGF can increase phosphoinositol triphosphate levels in A431 cells, but this does not occur in most cell types stimulated by EGF.

Protein kinase C (PKC) phosphorylates proteins mainly on serine and threonine residues. The stimulation of PKC by phorbol esters results in the phosphorylation of the EGF receptor on serine and threonine residues and decreases the affinity for EGF [48, 49]. This provides a mechanism by which one growth factor can modulate the activity of another. These modulations of EGFr number or affinity show the problem of trying to interpret the significance of a given level of expression in any particular tumour.

7. Transforming growth factors

Transforming growth factors are polypeptide hormones capable of inducing anchorage-independent growth of nontransformed cells *in vitro* [50]. There are two major subgroups of transforming growth factor, the TGF-alpha (or

TGF-1) and B TGF [51]. TGF-α binds to the EGFr. Mary transformed cells produce TGF-alpha and this has been shown to interact with EGF receptors in radioreceptor assays and bears considerable sequence homology of EGF [52]. The TGF alpha polypeptide contains 50 amino acid residues compared with the 53 of EGF, with homologies to either mouse or human EGF at 19 positions (approx. 40%). Homologies are most preserved in the relative positioning of the 6 cysteine residues that form disulphide bonds and within the first disulphide loop, resides 6–20 of the mouse EGF structure, where homology is approximately 60%, TGF-alpha is more homologous to EGF than any of the several EGF-like peptides in the large EGF gene [53, 54]. TGF-alpha mimics most, if not all, of the actions of EGF including receptor autophosphorylation, receptor down-regulation, stimulation of cell growth, and promotion of precocious tooth eruption and eyelid opening [55–61].

8. EGF effects in normal breast

In recent years, there has been much interest in the possible role of EGF in breast cancer. This interest dates back to some observations in animals. EGF has been shown to promote both normal and malignant mammary growth in rodents [62]. EGF stimulates proliferation of mouse mammary epithelial cells [63–66], and is necessary for lobulo-alveolar development of mouse mammary gland in organ culture [64]. Recent studies also indicate that EGF plays a physiological role in the development of the mouse mammary gland during pregnancy [67]. Certain strains of mice, such as C3H, have a high incidence of mammary tumors and development of these is related to a virus which is transmitted to the suckling infants in the milk [68, 69]. In the mouse, the only important site of EGF production is in the submandibular salivary gland as shown by Cohen's early work. Surgical removal of this gland in these C3H mice before the age of 22 weeks almost abolished spontaneous tumorigenesis, but if the sialoadenectomized animals were subsequently given EGF, spontaneous tumorigenesis was restored [70].

9. EGF and EGF receptors in human breast cancer cell lines

EGF is known to stimulate proliferation of human breast cancer cells in culture [71–73]. In human breast cancer cell lines there is a reciprocal relationship of EGFr to oestrogen receptors (ER). The ER-positive cell lines have lower EGFr concentrations than ER-negative lines. However, EGF stimulates the growth of those with the lowest number of EGFr [74]. The affinity of the receptors for EGF is higher in the ER-positive cell lines [75]. It has therefore been suggested that there is an interaction between protein kinase C and the EGFr in the lines expressing the highest amounts of receptor, such that there is a decrease in affinity for EGF. It is possible that

another growth factor is interacting with protein kinase C, modulating EGFr [76].

This demonstrates the problem of interpreting EGFr on tumors *in vivo*, since expression of the receptor does not necessarily correlate with response to EGF or growth factor-α.

Recent work by Lippman's group [77] has shown that oestrogen-stimulated growth is mediated via autocrine secretion of TGF-α and points to the importance of EGFr for response to steroid hormone therapy. Progestins can also regulate EGFr expression and increase EGFr 2–3-fold in the T47D cell lines [78]. Conversely, phosphorylation of the progesterone receptor may occur as a result of binding of EGF [78, 79]. EGF caused a decrease in amount of progesterone binding to its receptor in the breast cancer cell line T47D. *In vivo*, oestrogens stimulate an increase in EGFr on uterine membranes in rats [80]. There are therefore complex interactions between steroids and EGFr *in vitro* which are relevant to interpretation of EGFr measurements in human tumors.

10. EGF receptor in human breast cancer

The study of the EGF receptor in human breast cancer has been of major interest. The most widely used method of receptor identification has been a radioligand assay in which ^{125}I-labelled EGF is incubated with membranes prepared from both primary and metastatic tumors alone and in the presence of a large excess of unlabelled EGF, the difference in binding under these two conditions representing specific binding [81]. Minor differences in methodology exist between the groups who have published in this field but some important and consistent observations can be made. High affinity binding specific for EGF is present on between 30–50% of primary breast tumors [82–84], with levels of between 1–120 fmol/mg receptor protein deduced usually by Scatchard analysis of binding data.

However, there is no general agreement of a clinically significant level. The three original publications all analyzed oestrogen receptor levels by the dextran-coated charcoal method, and one group also analysed progesterone receptor levels [84]. Two groups arbitrarily chose a cut-off point of 1 fmol/mg membrane protein [83, 84] but in our department a higher level was used [82].

11. Measurement of EGF receptor in human breast cancer

Freshly collected tumor is stored in blocks of 0.5 × 0.5 × 1 cm in cold sucrose/glycerol buffer medium and stored at −20°C [85]. The tumor membranes are prepared by homogenization in 10 mM Tris HCl/50 mM NaCl, pH 7.4, and then centrifugation at 800 g. The supernatant is then centrifuged at 100,000 g for 35 minutes and the membrane pellet can be stored at −70°C.

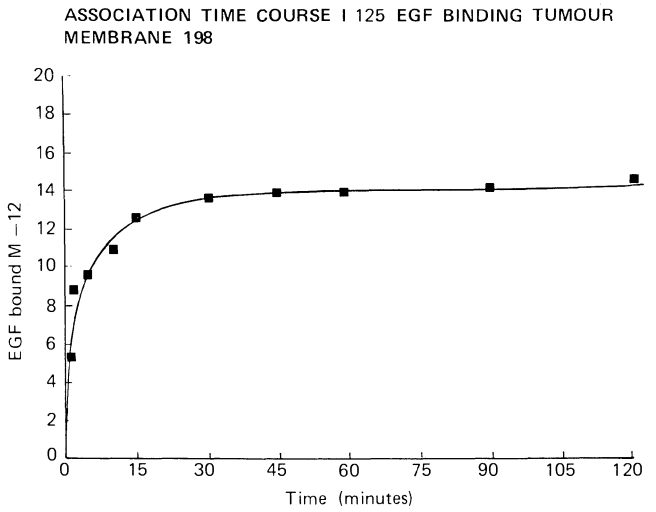


Figure 1. Association time course for I^{125} EGF binding to primary breast cancer membranes.

EGF is labelled by the iodogen method to a specific activity of approx. 80–130 μCi/ug.

In ligand binding assays, 100-fold excess EGF is used to determine non-specific binding. Time-course studies showed equilibration with binding sites by 60 minutes at 26°C (Figure 1). Scatchard analyses were carried out in 2 ways. Firstly, a fixed concentration of 0.3 nM EGF was used and increasing concentrations of unlabelled EGF. Alternatively, increasing concentrations of labelled EGF can be used. A direct comparison of the methods on the same tumors shows that the second method is better for defining more precisely the affinity of high affinity sites, but both give similar results for receptor density of the high affinity sites (Figures 2, 3).

In contrast to other studies, we find the majority of tumors exhibiting high affinity sites have curvilinear rather than straight line Scatchard plots. Occasionally some tumors show only one site, but the biological significance of this is not clear (Figure 4).

In our earlier series, we used the method with increasing unlabelled EGF, but now use the second method. There is no difference in distribution of total amount of specifically bound EGF but the Kd range is lower (0.17–1.0 nM v. 0.6–2.9 nM). The displacement method can yield a direct estimate of Kd by the concentration of unlabelled EGF required to displace the labelled EGF by 50%. However, this will be a combination of both high and low affinity sites, leading to a slightly lower estimate of affinity. We have used the amount of receptor for comparative purposes and this does not differ with the 2 methods.

In an initial screen of a tumor, we carry out a 2 point assay in triplicate. Initially this used 0.6 nM labelled EGF, in order to identify particularly the

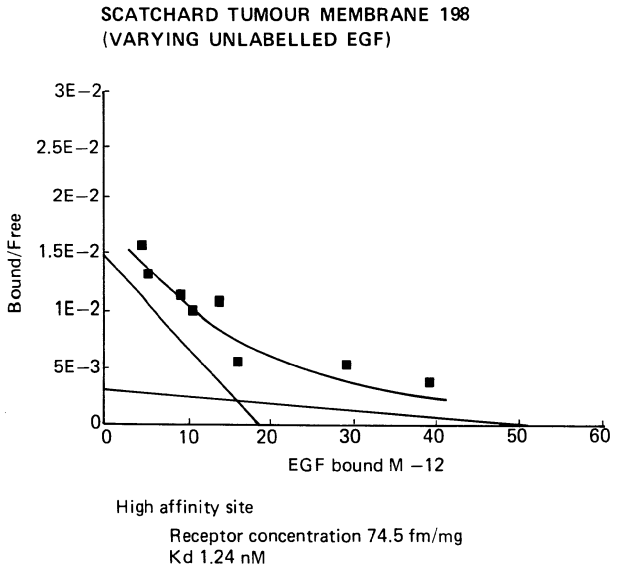


Figure 2. Scatchard analysis of EGF binding to primary breast cancer membranes using increasing concentrations of unlabelled EGF.

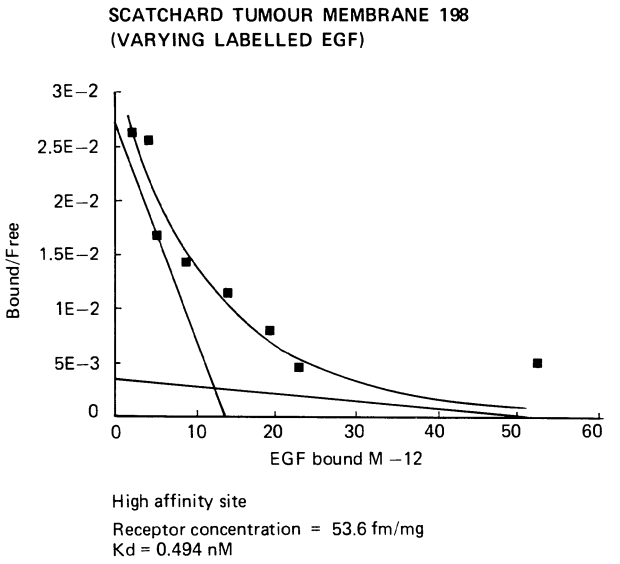


Figure 3. Scatchard analysis of EGF binding to primary breast cancer membrances using increasing concentrations of labelled EGF.

high affinity site. More recently, this has been increased to 1 nM to increase the sensitivity of the assay.

The criteria used for the interpretation of these assays is crucial. At a ^{125}I EGF concentration of 1 nM in a volume of 400 µl and a protein concentra-

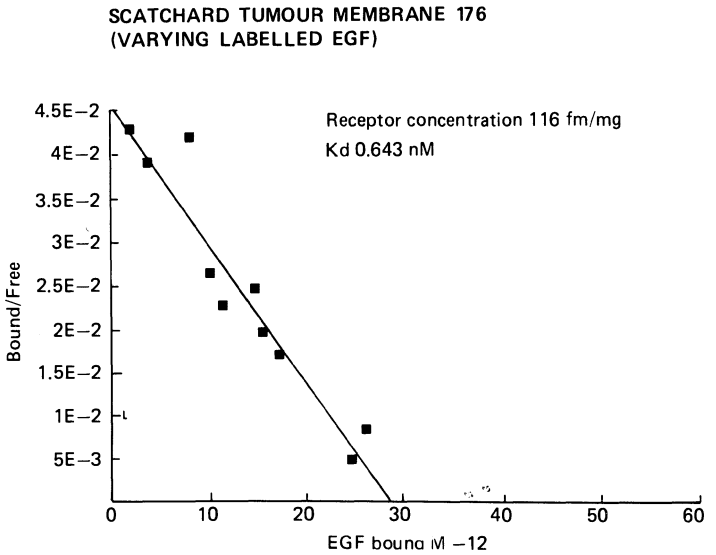


Figure 4. Scatchard analysis of EGF binding to primary breast cancer membranes: only one class of binding site is detectable.

tion of 100 μg, specific binding of 0.25% represents a receptor concentration of 10 fm/mg membrane protein. In addition to this criterion of positivity, we apply a simple statistical test to the result. An unpaired T test between the three figures for total binding and the three for nonspecific binding is performed and if the difference reaches the 95% confidence limit ($p < 0.05$), and specific binding is $> 0.25\%$ (equivalent to 10 fm/mg), then the tumor is considered positive for EGFr. This is based on the reliability of counting with the specific activity of EGF we use routinely.

12. Normal human tissue distribution of EGF receptor

Using immunohistological techniques and a monoclonal antibody to the EGFr, Gusterson *et al.* [86] surveyed the localization of EGFr. It was present in a wide range of tissues. EGFr were detected on the undifferentiated basal cells of stratified epithelia and other proliferating cells of adnexial structures. Levels were also detectable on non-proliferating cells and the ducts of bronchial glands, hepatocytes, bile ducts, and breast ducts. Therefore EGFr in normal tissues are not necessarily a marker for growth and they may have many other physiological effects in differentiated cells. Real *et al.* [87] also found wide distribution of EGFr with different antibodies, including epithelium and glands in the oesophagus, stomach, small bowel and large bowel. Alveoli, smooth muscle, endothelium and nerve fibres were positive for EGFr. If any selective targeting to tumors via EGFr is planned, it is essential that the tumor express EGFr to a much higher level than these normal tissues.

13. Relationship of EGFr to estrogen receptor

In our studies, we measured "nuclear" and "cytoplasmic" receptor in all primary tumors [82]. Although it is clear that ER is nuclear in location, there is a more easily extractable component, the "cytoplasmic receptor". In analyses we included tumors with either type of ER positivity as ER positive.

EGF receptor was detected in the range of 10–187 fmol/mg membrane protein. Two binding sites were identified by Scatchard analysis, the higher affinity having a Kd range of 0.17–2.9 nM. Steady state binding was reached in 1 hour at 26°C and was similar to that observed at 37°C. In 228 primary tumors, there was a striking inverse correlation of EGFr with ER (Table 1) ($p < 0.0001$). For the purposes of statistical analysis, EGF binding < 10 fmol/mg membrane proteins was considered to be EGFr negative and ER binding < 5 fmol/mg cytosol protein was ER negative. The ER negative tumors can thus be split into two groups, those which are EGFr +ve and those which are EGFr −ve.

The ligand binding results were compared with semiquantitative grading of EGFr by immunohistochemistry and there was a significant correlation (Table 2) [82]. EGFr measured by ligand binding is more sensitive than immunochemistry, accounting for EGFr +ve tumors that are negative in histochemistry. These results suggest that endogenous ligands are not interfering with the EGFr assay, since EGFR[1], antibody reacts with a peptide external domain of the EGFr and does not interfere with the binding site for EGF [88].

Failure to demonstrate EGFr on the majority of ER positive tumors contrasts with *in vitro* work on the MCF7 (ER positive) human breast cancer cell line. They express a low level of EGFr, are stimulated to grow by EGF and produce TGF alpha [89]. It is possible that low levels of EGFr *in vivo* in ER positive tumors are further down-regulated by TGF alpha and hence not detectable by ligand binding studies. There is, however, a good correlation of immunohistochemical demonstration of EGFr with ligand binding so high surface levels of EGFr occupied by locally secreted TGF alpha would not account for the EGFr negative tumors.

Autophosphorylation of EGFr and immunoprecipitation of labelled receptor appears to be more sensitive than ligand binding (results in preparation)

Table 1. EGFr in primary breast carcinoma

	EGFr		
	+	−	
ER +	12	86	98
−	68	62	130
	80	148	228

Table 2. Relation between immunohistochemical and radioligand epidermal growth factor receptor assays

Radioligand assay	Immunohistochemical assay EGFr +	−	Total
EGFr			
+	21	5	26
−	2	20	22
	23	25	48

Specificity of immunochemistry 91%.
Sensitivity of immunohistochemistry 81%.

and receptor detected by this method may therefore be present in ER positive tumors. Nevertheless, quantitative ligand binding does separate a group of patients with poor prognosis (see later).

14. Correlation of EGF receptors with differentiation, size of tumour and lymph nodes

To correlate EGFr with other prognostic variables, Bloom and Richardson grading was carried out on 108 primary tumors (Table 3) [90]. EGFr were correlated with poorly differentiated tumors. The inverse correlation of EGFr with ER also occurred in regional lymph node metastases, 3 ER +ve metastases were EGFr −ve and 10 EGFr +ve metastases were ER −ve ($p < 0.02$). No EGFr +ve metastases arose from primary tumors that were EGFr −ve.

There was also a correlation of EGFr with increasing tumor size (Table 4). There were more EGFr +ve tumors in patients with involved regional lymph nodes (Table 5). To assess serial changes in EGFr status, we are now using fluorescence activated cell sorting of needle aspirates stained with EGFR[1] antibody.

Table 3. Bloom and Richardson grading v. EGF receptor

	I	II	III	
EGFr +	2	11	32	45
−	10	25	28	63
	12	36	60	108

$p < 0.002$.

Table 4. Relation between size of tumor and epidermal growth factor receptor state

	Size (cm)				
	<2	2.1–3.5	3.6–5	>5	Total
Epidermal growth factor receptors:					
Positive	8	22	7	8	45
Negative	21	28	9	5	63
Total	29	50	16	13	108
Positive for epidermal growth factor receptors (%)	28	44	44	62	

λ^2 for logit-linear trend in proportions = 3.97 with 1 df; p = 0.046.

Table 5. Lymph node status and EGFr status

	EGFr+	EGFr−
Nodes clear	8	18
Nodes involved	25	20

15. Are these abnormal EGF receptors?

Both the monoclonal antibody and EGF ligand studies only detect the external domain of the receptor, but do not give information on the activity of the receptor. Therefore, immunoprecipitation studies were carried out to detect autophosphorylation of the EGFr. Membranes were incubated with or without 100 nM unlabelled EGF and ^{32}ATP and the labelled receptor precipitated with EGFR[1] antibody after solubilization. Autoradiography showed that EGFr were functional and that in some cases EGFr undetectable by ligand binding or immunochemistry could be detected by enhancement of autophosphorylation with EGF. However, for prognostic purposes and correlation with other variables, ligand binding results have been used.

None of these methods would detect EGFr analogous to erb-B, which lacks the external domain and shows much less autophosphorylation than EGFr. We therefore used a polyclonal antibody raised to a synthetic peptide homologous to a region of the internal EGFr domain (gift of Dr W. Gullick). 42 primary tumors were studied and the particular immunochemical pattern that would show an erb-B-type protein would be negative staining with EGFR[1] but positive staining with the internal antibody. The control for the internal antibody consisted of preincubation with the synthetic peptide to which it was raised. Only 2 tumors showed this pattern and they were also EGFr negative by ligand binding. Unfortunately, there was insufficient material, for molecular biological studies—so this remains to be confirmed. How-

ever, it is clear that this is not a common finding in breast cancer (<5% of cases).

16. Proportion of EGF receptor positive tumors

Two other groups have confirmed the reciprocal relationship of EGFr to ER, although in the case of Fitzpatrick *et al.* [84] the results did not reach significance. Their EGFr values ranged from 1–121 fmol/mg membrane protein in 137 tumors. Perez *et al.* [83] found a range of 1–64 fmol/mg membrane protein and a significant inverse relationship in 95 human breast cancers. The Kd mean was 3.7×10^{-9} M, and in pooled samples Fitzpatrick *et al.* found a value of 2 nM. Thus a total of 460 breast tumors have been described with good agreement of Kd and binding capacity. However, the cut-off point for correlating EGFr with other variables is different in each series—for example, Fitzpatrick *et al.* [84] had a background binding of 54% to membrane filters used to separate bound from free EGF. They considered specific binding greater than 15% of total binding minus filter binding to be EGFr +ve. They probably estimated tumors to be EGFr +ve which we would not have done on statistical grounds. Thus, they found 48% of tumors were positive and we found 35% positive. Perez *et al.* [83] found 42% of tumors positive but did not describe how they decided on EGFr +ve status, or what their background non-specific binding was.

Since we have shown a highly significant association of EGFr status with ER −ve and poorly differentiated tumors, it would appear that our definition of EGFr +ve can be justified on the usefulness of the clinical correlation as well as the grounds of analysis of counting reliability.

17. Prognostic significancce of EGF receptors

The prognostic significance of EGFr status as defined above has now been analyzed on the first 135 patients followed prospectively [91]. As expected, there is a slower relapse initially with ER +ve tumours but by three years from first diagnosis, the relapse-free survival (RFS) curves come together again. Nevertheless, the curves for RFS and overall survival (OS) are both significantly different for ER +ve and ER −ve tumors.

Of particular interest is the result for ER −ve tumours stratified by EGFr status (Figure 5). There is a highly significant difference in OS as well as RFS, with EGFr +ve patients having a much higher mortality. RFS is 76% vs. 39% at 3 years and OS is 82% vs. 41% (Figure 6). The difference between ER +ve tumors and ER −ve EGFr −ve tumors is not significant. Thus the early separation of survival curves for ER −ve and ER +ve tumors can be accounted for by the rapid demise of ER −ve EGFr +ve patients, and the coming together of the survival curves is related to the better prognosis of ER −ve

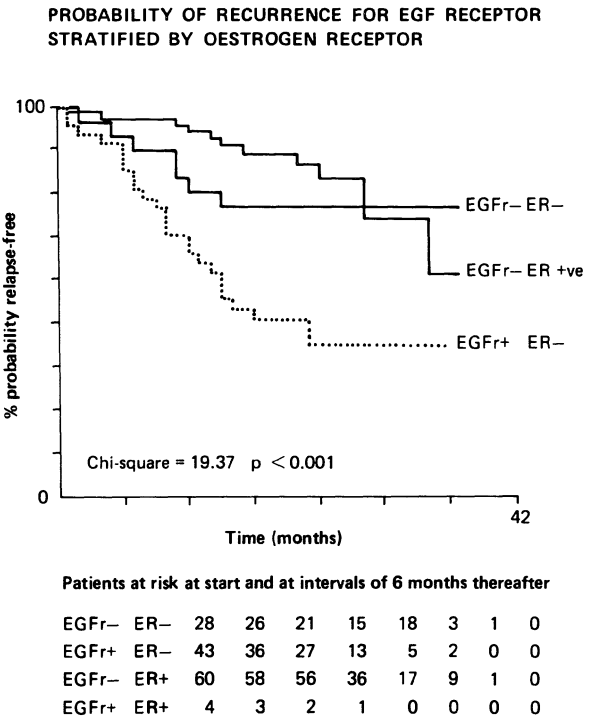


Figure 5. Recurrence-free survival for EGFr positive and negative tumors stratified by ER status.

EGFr −ve patients. These results provide an explanation for a major controversy in the literature on the effect of ER status on RFS. Studies following patients for over 5 years generally do not show a significant effect on RFS, but those analyzing at 1–2 years do. An early relapsing, poor prognosis subgroup would account for these results. We have defined such a subgroup by EGFr status.

There are other known prognostic factors such as tumor size and Bloom and Richardson grading, so we analyzed these factors in our patients. There was no significant separation of survival (OS or RFS) using these criteria. Stratifying for these variables, EGFr +ve tumours always had the worst survival. Positive lymph node status and ER −ve tumors were associated with worse survival, but the EGFr +ve tumors within a subgroup always had worse survival than the EGFr −ve tumors.

The separation of a poor prognostic subgroup in a relatively low number of patients followed up for 3 years suggests that EGFr status is a powerful discriminator and could be used for prospective trials of adjuvant therapy. The expression of EGFr and poor prognosis suggests that the initial hypothesis that tumors expressing higher EGFr could be more responsive to endogenous growth factors is correct. This therefore provides new therapeutic options for this group of patients.

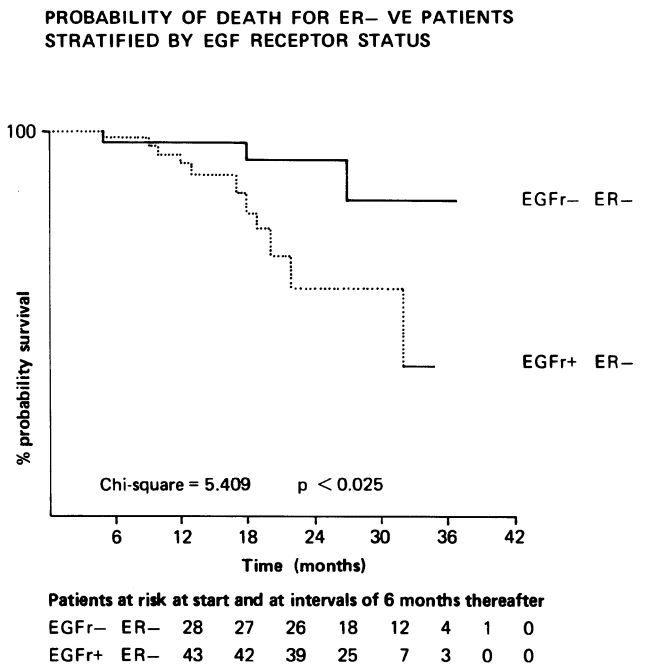


Figure 6. Overall survival for ER negative patients stratified by EGFr status.

18. The association of EGF receptor and estrogen receptor with membrane-bound tissue plasminogen activator and urokinase

To try and relate the expression of EGFr to other biological variables associated with aggressive tumor behavior, we investigated plasminogen activators in the primary tumor membranes. Plasminogen activators (PA) are serine protease enzymes which initiate extracellular proteolysis by cleaving plasminogen to yield the active protease plasmin. The PAs are involved in numerous physiological processes in which proteolysis is featured, such as tissue remodelling, regression of breast. There is a large amount of evidence that the PAs are also involved in tumor invasion and metastasis [92].

Two types of PA are described, namely tissue plasminogen activator (PA) and urokinase (uPA). They differ not only in molecular weight and immunologically but also in their function and it is known that they are products of separate genes [93]. Tissue plasminogen activator depends upon the presence of fibrin to achieve efficient activation of plasminogen and so it is considered to be the main PA involved in fibrinolysis. Urokinase has no fibrin dependence and it is associated with the regulation of extracellular proteolysis [92]. Both types of PA have been identified in cancers.

The regulation of PA secretion is influenced by hormones in several cancer cell lines. Oestradiol stimulates PA secretion in MCF–7 [94], ZR75–1 [95] and UCT Br–1 [96] breast cancer cell lines and epidermal growth factor

(EGF) stimulates PA secretion in HeLa [97] and A431 [98] cells. An association between PA activity and ER concentration has been identified in human breast tumors [99] but no work so far exists concerning the relationship between EGF receptor and PA activity in human tumors.

Plasminogen activators are strongly bound to cells, although they are also found in solution in many body fluids. The membrane bound form may be of more significance than the soluble, since it would block enzyme activity around the tumor. Inhibitors do not block bound urokinase. A membrane receptor for uPA was described recently which binds uPA to the external surface of the cell in active form [100]. We have demonstrated that this receptor is present in breast cancers [101].

Because of *in vitro* evidence of hormonal regulation of different types of PA, and because membrane bound PA may be of most biological relevance, we have investigated the occurrence of membrane associated PA in 43 breast cancers and have studied its relationship to both ER and EGF receptor status.

The PA assay used fibrin as substrate to ensure that tPA activity was not underestimated and antibodies to uPA and tPA were used to quench the activity of each PA type so that they could be measured separately.

Plasminogen activators were measured by the fibrin plate method and the two types were distinguished using antibodies to UK and tPA to quench the reaction. Membranes were prepared for EGFr assay and PA assays as described earlier. The majority of PA activity resided in the membrane fraction, the membrane: cytosol PA activity ranging from 10–300:1. The total PA activity did not differ significantly between ER +ve and ER −ve tumors (Figure 7). However, the tPA levels were significantly higher in ER positive tumours (Figure 7) and the % of total PA that was UK was lower in ER +ve tumors.

Tumors were divided further into ER+EGFr−, ER−EGFr+ and ER−EGFr− groups. While there was no significant difference in total PA and UK between any group and another, tPA was significantly lower in ER−EGFr+ tumors (Table 6). The ER−EGFr− tumors did not differ significantly from ER+EGFr− tumors. Therefore ER status alone was not the major determinant of tPA but lack of ER plus the presence of EGFr was associated with low tPA levels.

The membrane bound form of PA appears to be important. Jones *et al.* demonstrated that cell associated PA but not secreted PA was correlated to tumorigenicity in osteosarcoma cells. Cell bound PA is not neutralized by naturally occurring inhibitors [103] and plasmin production may thereby be localized to the vicinity of the cell.

Urokinase is secreted as a single chain proenzyme which binds to specific UK membrane receptors upon release [104]. It can subsequently be activated at the receptor. We have demonstrated the presence of uPA receptors in breast cancers which link active uPA to the cell membrane through the inactive chain [101]. Excess pro-uPA remains in the extracellular fluid in solution where it may be activated and neutralized. There is little data available

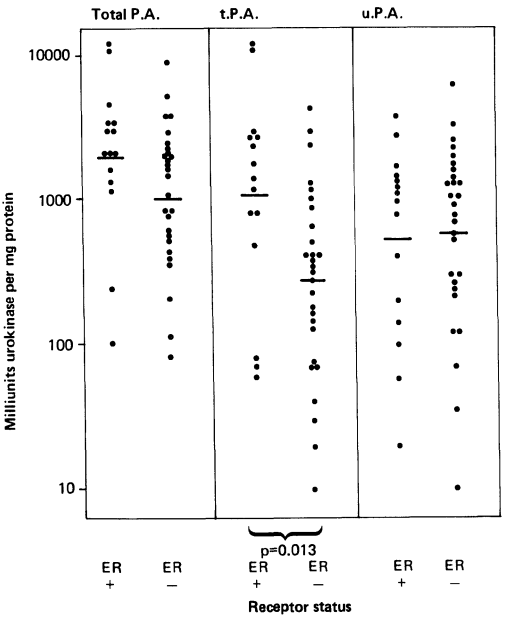


Figure 7. Total plasminogen activator, tissue plasminogen activator and urokinase in primary breast cancer membranes stratified by ER status.

Table 6.

n	ER	EGFr	Total PA	tPA	uPA	% uPA
3	+	+	1948	777	255	7
12	+	−	2005	1132*	625	49†
10	−	+	1034	140*	826	87†
18	−	−	1030	384	475	55

Log mean PA activity min uPA mg protein^{-1}.
*p = 0.003.
†p = 0.0004.

concerning the release of tPA from cancer cells and its binding to cell components, although tPA is strongly bound to cells and its molecule has homologous regions to those of uPA which mediate binding to its receptor [104, 105].

Although many studies have shown PA to be associated with the plasma membrane in cell cultures [92], none have so far examined membrane associated PA in human tumors. Ng *et al.* [106] used a rat mammary adenocarcinoma model to show that membrane from lung metastases contained twice as much PA activity as membrane from primary growths, although the respective cytosols contained similar activities. They suggested that membrane bound PA was greater in cells possessing a tendency to metastasize.

Our present results are consistent with the findings of Duffy *et al.* [99] who demonstrated a correlation between tPA and ER. ER negative tumors possessed lower tPA activity than ER positive tumors. Higher tPA levels in ER positive tumors might be expected also, since oestrogens are known to stimulate tPA but not uPA secretion in the cell line MCF-7 [107]. The lack of tPA appears to be associated with an aggressive group of tumors and it is possible that EGFr have a role in suppressing tPA secretion. Alternatively, they may be independent features in the phenotype of these tumors. Thorsen postulated that tumor cells arrested in the circulation by microthrombi might free themselves if they were able to secrete PA [108]. Since tPA is mostly concerned with fibrinolysis, this is one mechanism by which lack of tPA may contribute to a more aggressive phenotype.

19. Relevance of EGF receptor to Malignant Behavior and Gene amplification

The role of EGFr in malignant behavior almost certainly depends on which tumor type is considered and the mechanisms associated with EGFr expression.

In tumors where genomic rearrangements or gene amplification for EGFr can be demonstrated, it is plausible that EGFr expression is primarily involved in the malignant transformation—i.e. a genetic event. Such tumors include several types of squamous carcinoma (e.g. head and neck, lung, vulva) [109, 110, 111, 112] and gliomas. These tumors may have greatly increased EGFr expression and also abnormal mRNA transcripts in some cases. Human breast cancer EGFr amplification is unusual [30].

However, functional expression of the full length cDNA clone for EGFr in rodent cell lines does not lead to transformation [113] although this may be because of insufficiently elevated expression. It may also be essential for cells to secrete TGF-α in an autocrine manner and receptor expression per se may not lead to enhanced proliferation. However, in the presence of EGF, the human EGF receptor proto-oncogene does transform NIH3T3 cells.

20. Cooperation between oncogenes and growth factors

The tumorigenic conversion of primary embryo fibroblasts requires at least 2 cooperating oncogenes. Transformation of cells by viruses such as Kirsten and Moloney murine sarcoma viruses (which carry *ras* and *mos* oncogenes) causes secretion of growth factors. Stern *et al.* [115] and Balk *et al.* [116] investigated whether certain oncogenes would alter the responsiveness of cells to exogenously supplied growth factors. Cells transfected with the *myc* gene become much more sensitive to the effects of EGF on stimulating anchorage independent growth. *Myc* transfected cells did not produce more TGF-α-like factors than control cells.

In contrast, *ras* transfected cells secreted growth factors but did not respond to exogenous EGF. EGF receptors were barely detectable in the *ras* transfected cells. These results were interpreted as showing downregulation of receptors due to autocrine stimulation by the secreted growth factors. There was no increase in EGFr in the *myc* transfected cells, suggesting that the *myc* may amplify the effects of autocrine stimulation. Cooperation between *myc* and *ras* may therefore occur from growth factor production induced by *ras* and increased responsiveness due to *myc*. This hypothesis provides a key role for normally expressed EGFr in the action of these cytoplasmic and nuclear oncogenes.

21. Relation to tumor stage and differentiation

Although EGFr is homologous to the *erb*-B oncogene, we have shown in breast cancer that the receptor is essentially normal in ligand binding and functional properties, in contrast to *erb*-B which lacks the external ligand binding domain. Also, *erb*-B leads to erthroleukemia in chickens, whereas the EGFr is expressed in epithelial malignancies.

EGFr was related to tumor stage and differentiation, suggesting that it is not a directly acting transforming gene. Our interpretation is therefore that concomitant increased expression of EGFr modifies the biological behavior but is not per se related to transformation. It is possible that phenotypic selection occurred so that cells showing marginally more EGFr than others had a survival advantage and eventually dominated the tumor, but we did not see foci of EGFr positive cells immunochemically.

Since basal epithelial cells express EGFr (although not to the extent in the positive tumors), EGFr expression may just correlate with the stage of differentiation at which malignant cells are arrested. However, although there is a correlation of EGFr with poor differentiation, well differentiated tumors can also show high EGFr expression (see Table 3).

In the very early stages of carcinoma growth, if EGF receptor expression was increased as part of an abnormally activated genetic program, in addition to the direct transforming events, then the results we observed may occur. As an early event in the life of the carcinoma, there would be relatively little heterogeneity in tumor staining. Also EGFr could be expressed at any stage of differentiation but it may be more likely to be further activated at stages where it was already expressed (i.e. early basal layers of epithelia, less well differentiated cells). Interaction with other oncogenes are particularly likely to modify behavior.

22. Amplified EGF receptors

The differential expression of EGFr on malignant compared with normal cells, or the amplification of sensitivity to TGF-α are potential therapeutic

targets. Gross increases in EGFr as in squamous cancers and gliomas provide targets for monoclonal antibodies and hence the very high number of receptors will be critical for differential toxicity. The internalization of EGFr on stimulation by growth factors still occurs in cells with amplified EGFr (e.g. A431), hence drugs covalently linked to EGF or TGF-α could be selectively concentrated and released intracellularly.

However, cell lines with very high EGFr expression are not usually stimulated by EGF and can be inhibited by EGF (see earlier section). These types of cancer may not be suitable for EGF or TGF-α antagonists, since there are much high levels of tyrosine kinase activity independent of growth factor stimulation.

23. Normal EGF receptors

Although apparently normal EGFr are overexpressed relative to the surrounding normal tissues in some common epithelial cancers, the basal epithelial layers also express EGFr. There is still a larger amount of EGFr in a tumor compared with an equal volume of normal epithelial tissue, so selectivity could be obtained with antibodies to EGFr or drugs linked to EGF.

A more attractive approach may be to use peptide antagonists as described by Todaro's group [117]. This is particularly so if the tumor has been "sensitized" to EGFr stimulation, as discussed above for the *myc* oncogene. Then a highly selective antiproliferative effect may be obtained. Another approach could be to immunize a patient against his own TGF-α if these factors are not essential growth factors for normal adult tissue growth. The Na^+K^+ ATPase stimulated by EGF is another potential target for inhibition by analogues of amiloride [118]. The EGFr is occupied by dioxin, which provides a possible lead compound for simple non-peptide growth factor receptor antagonists [119].

In normal cell lines, high levels of EGF can be growth inhibitory and in sheep EGF inhibits hair growth, allowing wool to be sheared by the hand [120]. Therefore high levels of EGF by infusion or subcutaneous release may be inhibitory to tumour growth. Alternatively, low doses of EGF to stimulate tumor proliferation prior to chemotherapy or radiotherapy could be tried, similar to oestrogen priming in breast cancer.

24. Conclusion

These results show the importance of applying the fundamental knowledge on oncogenes developed from molecular biology to the clinical situation. The oncogenes may interact very differently in common human epithelial malignancies—as shown for EGFr. EGFr are of prognostic significance in some tumor types and it may therefore be desirable to measure EGFr on primary

human tumors routinely. An approach to this would be to use monoclonal antibodies that reacted with EGFr in paraffin fixed sections. Since EGFr also provide a therapeutic target, measurement of EGFr may become important in selecting patients for appropriate therapy.

References

1. Cohen S: Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. J Biol Chem 237:1555–1562, 1962.
2. Savage CR Jr, Inagami T, Cohen S: The primary structure of epidermal growth factor. J Biol Chem 247:7612–7621, 1972.
3. Savage CR Jr, Hash JH, Cohen S: Epidermal growth factor: location of disulphide bonds. J Biol Chem 248:7669–7672, 1973.
4. Scott J, Patterson S, Rall L, Bell GI, Crawford R, Penschow J, Coghlan N and Coghlan J: The structure and biosynthesis of epidermal growth factor precursor. J Cell Sci Suppl 3:19–28, 1985.
5. Starkey RH, Cohen S, Orth DN: Epidermal growth factor: identification of a new hormone in human urine. Science 189:800–802, 1975.
6. Cohen S, Carpenter G: Human epidermal growth factor: isolation and chemical and biological properties. Proc Natl Acad Sci USA 72:1317–1321, 1975.
7. Gregory H: Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature 257:325–327, 1975.
8. Carpenter G, Cohen S: Epidermal growth factor. Ann Rev Biochem 48:193–216, 1979.
9. Carpenter G, Cohen S: EGF: Receptor interactions and the stimulation of cell growth. In: Receptors and Recognition, Series B, Lefkowitz RJ (ed). London: Chapman and Hall 13: 43–66, 1981.
10. Elder JB, Williams G, Lacey E, Gregory H: Cellular localization of human urogastrone-epidermal growth factor. Nature 271:466–467, 1978.
11. Nanney LB, Magid M, Stoscheck CM, King LE: Comparison of epidermal growth factor binding and receptor distribution in normal human epidermis and epidermal appendages. J Invest Dermatol 83:385–393, 1984.
12. Elson SD, Brown CA, Thorburn GD: Identification of epidermal growth factor-like activity in human male reproductive tissues and fluids. J Clin Endocrinol Metabol 58:589–594, 1984.
13. Kasselberg AG, Orth DN, Gray ME, Stahlman MT: Immunocytochemical localization of human epidermal growth factor/urogastrone in several human tissues. J Histochem Cytochem 33:315–322, 1985.
14. Rall LB, Scott J, Bell GI: Mouse prepro-epidermal growth factor snthesis by the kidney and other tissues. Nature 313:228–231, 1985.
15. Pfeffer S and Ullrich A: EGF—is the precursor a receptor? Nature 313:184, 1985.
16. Gregory H, Willshire IR, Kavanagh JP, Blacklock NJ, Chowdury S, Richards RC Urogastrone—epidermal growth factor concentrations in prostatic fluid of normal individuals and patients with benign prostatic hypertrophy. Clin. Sci. 70:359–363, 1986.
17. Dailey GE, Kraus JW and Orth DN: Homologous radioimmunoassay for human epidermal growth factor (urogastrone). J Clin Endocrinol Metabol 46:929–936, 1978.
18. Ances IG: Serum concentrations of epidermal growth factor in human pregnancy. Am J Obstet Gynecol 115:357–362, 1973.
19. Hirata Y, Moore GW, Bertagna C, Orth DN: Plasma concentrations of immunoreactive human epidermal growth factor (urogastrone) in man. J Clin Endocrinol Metabol 50:440–444, 1980.
20. Cohen S: The stimulation of epidermal proliferation by a specific protein (EGF). Dev Biol 12:394–403, 1965.

21. Carpenter G, King L, Cohen S: Rapid enhancement of protein phosphorylation in A431 cell membrane preparations by epidermal growth factor. J Biol Chem 254:4884–4891, 1979.
22. Goldin GV, Operman LA: Induction of supernumerary tracheal buds and the stimulation of DNA synthesis in the embryonic chick lung and trachea by epidermal growth factor. J Embryol Exp Morphol 60:235–243, 1980.
23. Walker-Smith JA, Phillips AD, Walford N, Gregory H, Fitzgerald JD, MacCollagh K and Wright NA. Intravenous epidermal growth factor/urogastrone increases small intestinal cell proliferation in congenital microvillous atrophy. Lancet ii:1239–1240, 1985.
24. Cohen S, Ushiro H, Stoscheck C, Chinkers M: A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J Biol Chem 257:1523–1531, 1982.
25. Downward J, Yarden Y, Scrace G, Torry N, Stockwell P. Ullrich A, Schlessinger Waterfield MD: Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. Nature 307:521–527, 1984.
26. Buhrow SA, Cohen S and Stavos JV: Affinity labelling of the protein kinase associated with the epidermal growth factor receptor in membrane vesicles from A431 cells. J Biol Chem 251:4019–4022, 1982.
27. Konda I, Shimizu N: Mapping of the human gene for epidermal growth factor receptor (EGFr) on the p13-q22 region of chromosome 7. Cytogenet Cell Genet 35:9–14, 1983.
28. Shimizu N, Kondo I, Gamou S, Behzadian MA, Shimizu Y: Genetic analysis of hyperproduction of epidermal growth factor receptors in human epidermoid carcinoma A-431 cells. Somat Cell Mol Genet 10:45–53, 1984.
29. Bargmann CI, Hung M–C, Weinberg RA: The *neu* oncogene encodes an epidermal growth factor receptor related protein. Nature 319:226–230, 1986.
30. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–181, 1987.
31. Ushiro H, Cohen S: Identification of phosphotyrosine as a product of epidermal growth factor-associated protein kinase in A-431 cell membranes. J Biol Chem 255:8363–8365, 1980.
32. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlesinger J, Downward J, Mayers ELV, Whittle N, Waterfield MD & Seeburg PH. Human epidermal growth factor receptor cDNA sequences and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (London) 309: 418–425, 1984.
33. Heldin CH, Westermark B: Growth factors: mechanism of action and relation to oncogenes. Cell 37: 9–20, 1984.
34. Chinkers M, Cohen S: Purified EGF receptor kinase interacts specifically with antibodies to Rous sarcoma virus transforming protein. Nature 290:516–519, 1981.
35. Sefton BM, Hunter T, Beeman K, Eckhart W: Evidence that the phosphorylation of tyrosine is essential for transformation by Rous sarcoma virus. Cell 20:807–816, 1980.
36. Erikson E, Shealy DJ, Erikson RL: Evidence that viral transforming gene products and epidermal growth factor stimulate phosphorylation with similar specificity. J Biol Chem 256:11381–11384, 1981.
37. Cohen S: Internalization of functional epidermal growth factor receptor/kinase vesicles and the isolation of a calcium binding 35 kDa substrate. J Cell Biochem Suppl 10C:99, 1986.
38. Huang K–S, Wallner BP, Mattaliano RJ, Tizard R, Burne C, Frey A, Hession C, McGray P, Sinclair LK, Chow EP, Browning JL, Ramachandran KL, Tang J, Smart JE, Pepinsky RB: Two human 35 kd inhibitors of phospholipase A^2 are related to substrates of $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase. Cell 46:191–199, 1986.
39. Saris CJM, Tack BF, Kristensen T, Glenney JR, Hunter T: The cDNA sequence for the protein-tyrosine kinase substrate p36 (Calpactin I heavy chain) reveals a multidomain protein with internal repeats. Cell 46:201–212, 1986.
40. Carpenter G, Cohen S: ^{125}I-labelled human epidermal growth factor (hEGF): binding,

internalization, and degradation in human fibroblasts. J Cell Biol 71:159–171, 1976.
41. Stoscheck CM, Carpenter G: Down-regulation of epidermal growth factor receptors: direct demonstration of receptor degradation in human fibroblasts. J Cell Biol 98:1048–1053, 1984a.
42. Stoscheck CM, Carpenter G: Characterization of the metabolic turnover of epidermal growth factor receptor protein in A-431 cells. J Cell Physiol 120:296–302, 1984b.
43. Murthy U, Basu M, Sen-Majumdar A, Das M: Perinuclear location and recycling of epidermal growth factor receptor kinase: immunofluorescent visualization using antibodies directed to kinase and extracellular domains. J Cell Biol 103:333–342, 1986.
44. Wiegant FAC, Blok FJ, Defize LHK, Linnemanns WAM, Verkley AJ, Boonstra J: Epidermal growth factor receptors associated to cytoskeletal elements of epidermoid carcinoma (A431) cells. J Cell Biol 103:87–94, 1986.
45. Newmark P: Events at the surface of the cell. Nature 317:380, 1985.
46. May WS, Sahyoun N, Wolf M, Cuatrecasas P: Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. Nature 317:549–561, 1985.
47. Moolenaar WH, Rob JA, Tertoolen LGJ, de Laat SW: The epidermal growth factor induced signal in A431 cells. J Biol Chem 261:279–284, 1986.
48. Cochet C, Gill GN, Meisenhelder J, Cooper JA, Hunter T: C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor stimulated tyrosine kinase activity. J Biol Chem 259:2553–2558, 1984.
49. Iwashita S, Fox CF: Epidermal growth factor and potent phorbol tumor promoters induce epidermal growth factor receptor phosphorylation in a similar but distinctively different manner in human epidermoid carcinoma A431 cells. J. Biol. Chem., 259:2559–2567, 1984.
50. Delarco JE, Todaro GJ: Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci USA 75:4001–4005, 1978.
51. Roberts AB, Anzano MA, Lamb LC, Smith LM Frolik CA, Marquardt H, Todaro GJ and Spom MB: Isolation from murine sarcoma cells of novel transforming growth factors potentiated by EGF. Nature (London) 295:417–419, 1982.
52. Marquardt H, Hunkapiller MW, Hood LE, Todaro GJ: Rat transforming growth factor type 1: structure and relationship to epidermal growth factor. Science 223:1079–1082, 1984.
53. Scott J, Urdea M, Quiroga M, Sanchez-Prescadar R, Fong N, Selby M, Rutter WJ and Bell GI: Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins. Science 221:236–240, 1983.
54. Gray A, Dull TJ, Ullrich A: Nucleotide sequence of epidermal growth factor c-DNA predicts a 128,000 molecular weight protein precursor. Nature 303:722–725, 1983.
55. Todaro GJ, Fryling C, DeLarco JE: Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. Proc Natl Acad Sci USA 77:5258–5262, 1980.
56. Anzano MA, Roberts AB, Meyers CA, Komoriya A *et al*: Synergistic interaction of two clones of transforming growth factors from murine sarcoma cells. Cancer Res 42:4776–4778, 1982.
57. Massague J: Epidermal growth factor-like transforming growth factor. I. Isolation, chemical characterization and potentiation by other transforming factors from feline sarcoma virus-transformed rat cells. J Biol Chem 258:13606–13613, 1983a.
58. Massague J: Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J Biol Chem 258:13614–13620, 1983b.
59. Reynolds FH Jr, Todaro GJ, Fryling C, Stephenson JR: Human transforming growth factors induce tyrosine phosphorylation of EGF receptors. Nature (London) 292:259–262, 1981.
60. Twardzik DR, Todaro GJ, Reynolds FH, Stephenson JR: Similar transforming growth factors (TGFs) produced by cells transformed by different isolates of feline sarcoma virus. Virology 124:201–207, 1983.

61. Tam JP: Physiological effects of transforming growth factor in the newborn mouse. Science 229:673–675, 1985.
62. Turkington RW: Stimulation of mammary carcinoma cell proliferation by epithelial growth factor *in vitro*. Cancer Res 29:1457–1458, 1969.
63. Yang J, Guzman R, Richards J, Imagawa W, McCormick K, Nandi S: 1980 Growth factor- and cyclic nucleotide-induced proliferation of normal and malignant mammary epithelial cells in primary culture. Endocrinology 107:35–41, 1980.
64. Tonelli QJ, Sorof S: Epidermal growth factor requirement for development of cultured mammary gland. Nature 285:250–252, 1980.
65. Taketami Y, Oka T: Biological action of epidermal growth factor and its functional receptors in normal mammary cells. Proc Natl Acad Sci USA 80:2647–2650, 1983.
66. Taketami Y, Oka T: Epidermal growth factor stimulates cell proliferation and inhibits functional differentiation of mouse mammary epithelial cells in culture. Endocrinology 113: 871–877, 1983.
67. Okamoto S, Oka T: Evidence for physiological function of epidermal growth factors: Pregestational sialoadenectomy in mice decreases milk production and increases offspring mortality during lactation period. Proc Natl Acad Sci USA 81:6059–6063, 1984.
68. Nandi S, McGrath CM: Mammary neoplasia in mice. Adv Cancer Res 17: 353–414, 1973.
69. Medina D: In: The Mouse in Biomedical Research, Vol. 4, Foster HL, Small JD and Fox JG (eds). New York, Academic Press, 373–396.
70. Kurachi H, Okamoto S, Oka T: Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorigenesis. Proc Natl Acad Sci USA 82:5940–5943, 1985.
71. Osborne CK, Hamilton B, Titus G, Livingston RB: Epidermal growth factor stimulation of human breast cancer cells in culture. Cancer Res 40:2362–2366, 1980.
72. Fitzpatrick SL, LaChance MP, Schultz GS: Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res 44:3442–3447, 1984.
73. Imai Y, Leung CKH, Friesen HG, Shiu RPC: Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture. Cancer Res 42:4394–4398, 1982.
74. Costa SD, Fabbro D, Regazzi R, Kung W and Eppenberger U: The cytosolic phorboid receptor correlates with hormone dependency in six mammary carcinoma cell lines. Biochem Biophys Res Commun 133:814–822, 1985.
75. Fabbro D, Regazzi R, Costa SD, Borner C and Eppenberger U: Protein kinase C desensitization by phorbol esters and its impact on growth of human breast cancer cells. Biochem Biophys Res Commun 135:65–73, 1986.
76. Roos W, Fabbro D, Kung W, Costa SD, Eppenberger U: Correlation between hormone dependency and the regulation of epidermal growth factor receptor by tumor promoters in human mammary carcinoma cells. Proc Natl Acad Sci USA 83:991–995, 1986.
77. Lippman ME, Dickson RB, Bates S, Knabbe C, Huff K, Swain S, McManaway M, Bronzert D, Kasid A, Gelmann EP: Autocrine and paracrine growth regulation of human breast cancer. Breast Cancer Res Treat 7:59–70, 1986.
78. Murphy LJ, Sutherland RL, Stead B, Murphy LC, Lazarus L: Progestin regulation of epidermal growth factor receptors in human mammary carcinoma cells. Cancer Res 46:728–734, 1986.
79. Fox CF: Actions of tyrosine protein kinases on steroid hormone receptor properties. J Cell Biochem Suppl 10C:107, 1986.
80. Mukku VR, Stancel GM: Regulation of epidermal growth factor receptor by estrogen. J Biol Chem 260:9820–9824, 1985.
81. Carpenter G: Binding assays for epidermal growth factor. Methods in Enzymology 109: 101–110, 1985.
82. Sainsbury JRC, Farndon JR, Sherbet GV and Harris AL: Epidermal growth factor receptors and oestrogen receptors in human breast cancers. Lancet i:364–366, 1985.

83. Perez R, Pascual M, Macias A, Lage A: Epidermal growth factor receptors in human breast cancer. Breast Cancer Res Treat 4:189–193, 1984.
84. Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS: Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen and progestin receptor levels. Cancer Res 44:3448–3453, 1984.
85. Crawford D: New storage procedure for human tumor biopsies prior to oestrogen receptor measurement. Cancer Res 44:2348–2351, 1984.
86. Gusterson B, Cowley G, Smith JA, Ozanne B: Cellular localization of human epidermal growth factor. Cell Biol Internat Rep 8:649–657, 1984.
87. Real FX, Rettig WJ, Chesa PG, Melamed MR, Old LJ, Mendelsohn J: Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. Cancer Res 46:4726–4731, 1986.
88. Waterfield MD, Mayers ELV, Stroobant P, Bennet PLP, Young S, Goodfellow PN, Banting GS, Ozanne B: A Monoclonal Antibody to The human epidermal growth factor receptor. J Cell Biochem 20:149–161, 1982.
89. Dickson RB, Rates SE, McManaway ME and Lippman ME: Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res 46:1707–1713, 1986.
90. Sainsbury JRC, Malcolm AJ, Appleton DR, Farndon JR, Harris AL: Presence of epidermal growth factor receptor as an indicator of poor prognosis in patients with breast cancer. J Clin Pathol 38:1225–1228, 1985.
91. Sainsburg JRC, Farndon JR, Needham GK, Malcohn AJ and Hamis AC:Epidemal growth factor receptor status as predictor of early recurrence of and death from breart Cancer. Lancet 1:1398–1402, 1987.
92. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Neilsen LS, Skriver L: Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 44:139–226, 1985.
93. Tripputi P, Blasi F, Ny T, Emanuel BS, Lefofsky J, Croce CM: Tissue type plasminogen activator gene is on chromosome 8. Cytogenet Cell Genet 42:24–28, 1986.
94. Butler WB, Kirkland WL, Jorgensen TL: Induction of plasminogen activator by estrogen in a human breast cancer cell line (MCF–7). Biochem Biophys Res Commun 90:1328–1334, 1979.
95. Huff K, Lippmann ME: Hormonal control of plasminogen activator secretion in ZR-75-1 human breast cancer cells in culture. Endocrinology 114:1702–1710, 1984.
96. Wilson EL, Dutlow C, Dowdle EB: Effect of hormones on the secretion of plasminogen activator by a new line of human breast carcinoma cells, UCT-Brl. In: *Growth of Cells in Hormonally Defined Media*, New York, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1982, pp 849–854.
97. Lee LS, Weinstein IB: Epidermal growth factor-like phorbol esters induce plasminogen activator in HeLa cells. Nature 274–696–697, 1978.
98. Gross JL, Krupp MN, Rifkin DB, Lane DM: Down-regulation of epidermal growth factor receptor correlates with plasminogen activator activity in human A431 epidermoid carcinoma cells. Proc Natl Acad Sci USA 80:2276–2280, 1983.
99. Duffy MJ, O'Grady P, Simon J, Rose M, Lijnen HR: Tissue type plasminogen activator in breast cancer: relationship with estradiol and progesterone receptors. J Natl Cancer Inst 77: 621–623, 1986.
100. Vassalli JD, Baccino D, Belin D: A cellular binding site for the Mr 55,000 form of the human plasminogen activator urokinase. J Cell Biol 100:86–92, 1985.
101. Needham GK, Sherbet GV, Farndon JR, Harris AL: Binding of urokinase to specific receptor sites on human breast cancer membranes. Br J Cancer 55:13–16, 1987.
102. Jones PA, Rhim JS, Isaacs H, McAllister RM: The relationship between tumorigenicity, growth in agar and fibrin lytic activity in a line of human osteosarcoma cells. Int J Cancer 16:616–621, 1975.
103. Chapman HA, Varsin Z and Hibbs JB: Macrophage fibrinolytic activity: Identification of two pathways of plasmin formation by intact cells and of a plasminogen activator inhibitor.

Cell 28:653–662, 1982.
104. Blasi F, Stoppelli MP, Cubellis MV: The receptor for urokinase, plasminogen activator. J Cell Biochem 32:179–186, 1986.
105. Bachman F, Kruithof EKO: Tissue plasminogen activator. Chemical and physiological aspects. Sem Thromb & Hemostas 10:6–17, 1984.
106. Ng R, Wong A, Kellen JA: Localization of plasminogen activator(s) in primary and secondary rat adenocarcinoma cells. Clin Exptl Metastasis 3:73, 1985.
107. Ryan TJ, Seeger JI, Kumar SA, Dickerman HW: Estradiol preferentially enhances extracellular tissue plasminogen activators of MCF-7 breast cancer cells. J Biol Chem 259:14324–14327, 1984.
108. Thorsen T: Association of plasminogen activator activity and steroid receptors in human breast cancers. Eur J Cancer Clin Oncol 18:129–132, 1982.
109. Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A and Schlessinger J: Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumors of glial origin. Nature 313:144–147, 1985.
110. Ozanne B, Schum A, Richards CS *et al*: Evidence for increased EGF receptor in epidermal malignancies. In: Cancer Cell Vol. III: Growth Factors and Transformation. New York, 1985, Cold Spring Harbor Labs, pp 41–49.
111. King CR, Kraus MH, Williams LT, Merlino GT, Pastan IH, Aaronson SA: Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNAs. Nucl Acids Res 13:8477–8486, 1985.
112. Yamamoto T, Kamata N, Kawano H, Shimizu S, Kuroki T, Toyoshima K, Rikimaru K, Nomura N, Ishizaki R, Pastan I, Gamou S, Shimizu N: High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. Cancer Res 46:414–416, 1986.
113. Lin CR, Chen WS, Lazar CS, Carpenter CD, Gill GN, Evans RM, Rosenfeld MG: Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. Cell 44:839–848, 1986.
114. Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I. and Lowy, DR. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. Science, 238, 1408–1410, 1987.
115. Stern DF, Roberts AB, Roche NS, Sporn MB, Weinberg RA: Differential responsiveness of *myc*- and *ras*-transfected cells to growth factors: selective stimulation of *myc*-transfected cells by epidermal growth factor. Mol Cell Biol 6:870–877, 1986.
116. Balk SD, Riley TM, Gunther HS and Morisi M: Heparin-treated, v-*myc*-transformed chicken heart mesenchymal cells assume a normal morphology but are hypersensitive to epidermal growth factor (EGF) and brain fibroblast growth factor (bFGF); cells transformed by the v-Ha-*ras* oncogene are refractory to EGF and bFGF but are hypersensitive to insulin-like growth factors. Proc Natl Acad Sci USA 82:5781–5785, 1985.
117. Nestor JJ Jr, Newman SR, DeLustro B, Todaro GJ, Schreiber AB: A synthetic fragment of rat transforming growth factor a with receptor binding and antigenic properties. Biochem Biophys Res Commun 129:226–232, 1985.
118. Davis RJ, Czech MP: Amiloride directly inhibits growth factor receptor tyrosine kinase activity. J Biol Chem 260:2543–2551, 1985.
119. Madhukar BV, Brewster DW, Matsumura F: Effects of *in vivo*-administered 2,3,7,8-tetrachlorodibenzo-p-dioxin on receptor binding of epidermal growth factor in the hepatic plasma membrane of rat, guinea-pig, mouse and hamster. Proc Natl Acad Sci USA 81: 7407–7411, 1984.
120. Moore GPM, Panaretto BA, Robertson D: Inhibition of wool growth in merino sheep following administration of mouse epidermal growth factor and a derivative. Aust J Biol Sci 35:163–172, 1982.

6. Control of human breast cancer by estrogen, growth factors, and oncogenes

Robert B. Dickson and Marc E. Lippman

1. Introduction: the central role of estrogen in endocrine control of breast cancer

Cellular mechanisms of control of cancer proliferation have received considerable attention. In particular, recent studies have provided unifying links among growth factors [1], their receptors, and oncogene products. Neoplastic growth of leukemia, prostate carcinoma, endometrial carcinoma, and breast carcinoma has long been recognized to be under endocrine control by steroid hormones. Of particular note are the observations that receptors for estrogens, glucocorticoids, vitamin D metabolites, and progestins contain amino acid sequences that are highly homologous to the erb A oncogene (Chapter by G. Stack, *et al.*, this volume).

Breast cancer has been known for many years to be growth stimulated by estrogenic hormones and growth inhibited by antiestrogenic compounds in about 1/3 of clinical cases of metastatic disease [2]. The proportion of primary (nonmetastatic) breast cancers that are hormone dependent is unknown, but almost certainly larger. Breast cancer occurs in women who have never had functional ovaries with only 1% of the frequency of that of women with intact ovaries. In addition, normal mammary tissue responds to increases in estrogens at puberty. Thus, estrogens appear to have a stimulatory role in both normal and neoplastic breast epithelium. Estrogenic hormones may play multiple roles in neoplastic progression of breast cancer as carcinogens, permissive, promotional and tumor growth inducing agents. Though still controversial, several recent studies have shown that cyclical estrogens plus progestins in the form of oral contraceptives do not significantly enhance the risk of breast cancer when taken by adults [3]. Thus, it would appear likely that any effect of estrogen on early stages of breast tumorigenesis would occur shortly after or prior to puberty. Alternatively, such artificial ovulatory cycles may have different consequences from cycles in which hormones are secreted. An estrogenic component of neoplastic growth control would appear to be a remnant of a normal mechanism of mammary epithelial proliferation and differentiation during puberty (and possibly fetal development). While estrogens are mitogens for both normal and malignant breast epithelium, the

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

hypothalamus-pituitary axis is indirectly in control of ovarian estrogen secretion by virtue of GnRH and gonadotropin stimulation [4]. In addition, the pituitary gland (or other organs) may also secrete other direct or indirect acting mitogens [5]. Elsewhere in this volume, R.P. Shiu, *et al.* summarize some recent literature concerning identification of these mitogens. As pointed out in the chapters (this volume) on murine model systems, estogen can control breast tumor growth by inducing pituitary synthesis and secretion of prolactin. Sirbasku has employed the term 'estromedin' for other analogous, but still hypothetical, estrogen induced, endocrine acting mitogens [6]. It has also been proposed by other investigators that estrogen acts by allowing breast cancers to overcome growth inhibitory agents in their environment [7, 8]. These inhibitory compounds could be serum derived, produced by the cancer itself, or produced by nearby tissues. This review will, however, concentrate primarily on the estrogen-induced biochemical events which are associated with direct stimulation of proliferation of human breast cancer cell lines *in vitro* and *in vivo*. This type of approach in studies of hormonal control of breast cancer has proceeded over the past few years in number of laboratories using cell lines usually derived from pleural or ascites fluids of patients (Table 1). Several such estrogen responsive lines exist including MCF-7, T47D, MDA-MB-134, ZR-75-1, PMC42 and CAMA-1 [9–24], the best characterized of these being MCF-7 [9]. MCF-7 has an absolute requirement for estrogenic stimulation to form tumors in the athymic (nude) mouse model *in vivo* [14]. Experimental findings obtained using these cell lines must be regarded with some circumspection. After years in laboratory culture, subclonings, and assorted selective pressures, one can only hope that data derived from these cell lines will prove relevant to understanding of tumorigenesis *in vivo*.

Initial studies on *in vitro* hormone responsivity of MCF-7 cells produced disparate reports concerning the growth responses to estrogen. We and others

Table 1. Breast cancer cell lines in common usage

Cell line	Estrogen receptor	Histology/oncogenes
PMC42	+	very well differentiated adenocarcinoma
MCF-7	+	adenocarcinoma
ZR-75-1	+	adenocarcinoma
T47D	+	adenocarcinoma
CAMA-1	+	adenocarcinoma
MDA-MB-134	+	adenocarcinoma
Hs578T	–	carcinosarcoma, activated c-H-ras oncogene
MDA-MB-231	–	adenocarcinoma, activated c-K-ras
MDA-MB-468	–	adenocarcinoma, EGF receptor gene amplified (c-*erb*B)
SKBR-3	–	adenocarcinoma, c-*myc* and c-*erb*B2 genes amplified

succeeded in demonstrating receptors for [9; 10] and direct proliferative responses to physiologic doses of 17β-estradiol (E_2) *in vitro* [10–13, 18, 21–24] and *in vivo* in the nude mouse [14, 17]. However, a number of groups failed to observe such responses [7, 8, 19, 20]. These discrepancies have now been largely resolved with a more complete understanding of relevant variables in culture conditions. Serum is a rich source of estrogenic compounds, including sulfate conjugates, which must be removed to observe effects of exogenous estrogen *in vitro* [11, 12, 19]. Furthermore, it is now known that phenol red, commonly present in culture medium as a pH indicator can produce estrogenic effects [24]. Finally, growth factors in the cellular environment, particularly of the insulin family, can critically govern estrogen responses [19].

A well differentiated estrogen responsive breast cancer cell line has been recently described: PMC42. Monoclonal antibodies prepared against surface antigens of this line cross reacted with intraductal (early stage) breast cancer biopsies [25, 26]. At the other end of the spectrum, numerous estrogen independent breast cancer lines exist [17] such as the adenocarcinoma MDA-MB-231 or the carcinosarcoma Hs578T. It should be pointed out that while existing cell lines can be rank ordered according to their estrogen receptor states, nearly all were derived from metastatic sites in patients and are fully malignant in that sense. Thus, controls on metastatic behavior have been difficult to address. We will return to considerations of metastasis later in this article.

Throughout this review we will attempt to summarize the literature addressing the hypothesis that estrogens can directly interact with receptor-containing breast cancer cells to modulate gene expression and phenotypic properties. In addition, we will propose that polypeptide growth factors may be common mediators of growth control for both estrogen regulated and autonomous breast cancer. By stressing direct effects of estrogens on cancer cells *in vitro* we in no way imply that growth control of tumors *in vivo* might not be a much more complex phenomenon resulting from many more interactions among other cell types, hormones, proteases, and basement membrane components.

2. The estrogen receptor

In the late 1950's work by Jensen focused attention on high affinity estrogen binding components in estrogen target tissues [27]. Initial cell localization studies utilizing radiolabeled estrogen demonstrated long term retention of estrogen by the rodent uterus. The principal binding component, the estrogen receptor, has been partially characterized. Many studies have also localized the estrogen receptor to neural and many other non-reproductive organs of both male and female mammals [reviewed in 28]. The estrogen receptor appears to be a necessary mediator of estrogen action, initiating diverse developmental and physiological roles in many tissues [29].

Based on subcellular fractionation results, early studies proposed that the unoccupied estrogen receptor was located in the cellular cytoplasm. Following ligand binding, the receptor affinity for chromatin increased (a process called activation or transformation) and a 'translocation' to the nucleus was proposed to occur [30]. However, Zava and McGuire observed 'unoccupied' nuclear receptors in MCF-7 breast cancer cells, a finding inconsistent with the translocation hypothesis [31]. Although work by Edwards and co-workers [32] called into question the existence of unoccupied nuclear receptors in the intact cells, this receptor form is now generally accepted based on two other lines of evidence. Following characterization of monoclonal antiestrogen receptor antibodies, King and Greene reported nuclear immunolocalization of the unoccupied estrogen receptor, further suggesting that the nuclear translocation model was incorrect [33]. Similar results were obtained by Gorski's group using a cell enucleation procedure [34]. Though still controversial, both unoccupied and occupied estrogen receptors are now believed to reside largely in the nucleus. The precise nature of the estrogen receptor-nuclear interaction is unknown. Presumably the receptor interacts both with DNA and chromosomal proteins. Nuclear 'acceptor' binding proteins have been isolated for the uterine estrogen receptor [35] and other steroid receptors [36]. In addition, the estrogen receptor forms a complex with the nuclear matrix [37], a chromatin scaffolding structure which may be involved in regulation of transcription and replication of DNA [38, 39]. Toft and co-workers [40] have also recently shown that receptors for estrogen and other steroids associate (at least *in vitro*) with a 90 kDa heat shock protein. This heat shock protein also associates with the Rous sarcoma virus transforming protein $pp60^{v\text{-}src}$, a plasma membrane protein. The function of the 90 kDa protein in receptor function and hormone action is not yet known.

Chambon and colleagues (see chapter by Stack *et al.*, this volume) have recently obtained cDNA clones of the estrogen receptor from MCF-7 cells [41]. The mRNA codes for a 66 kDa protein which contains a long 3′ untranslated region (like the glucocorticoid receptor) [42]. The DNA binding domain of both the estrogen receptor and the glucocorticoid receptor share a strong homology with one of the transforming proteins of avian erythroblastosis virus (v-*erb* A) [41–44]. Recent studies have shown that the cellular homologue of v-*erb* A, known as c-*erb* A, is a receptor for thyroid hormones [45, 46]. The complete functional significance of the homology between these oncogenes is not yet known; though all are DNA-binding proteins [47]. Following expression in transfected cells or after *in vitro* translation, the protein product of c-*erb* A cDNA is able to bind E_2 with high affinity [41, 48]. The *in vitro* translation experiments, coupled with detailed sequence analysis, strongly suggest that the estrogen receptor is not a protein kinase and does not require post translational modifications, such as phosphorylation, for binding activity.

A possible role of phosphorylation in the action of the estrogen receptor remains to be fully evaluated. Auricchio and co-workers [49, 50] have purified

tyrosine kinase and phosphatase activities from calf and rodent uteri and demonstrated that the purified estrogen receptor is a substrate. The state of tyrosine phosphorylation is associated with the ability of the receptor to bind E_2 in *in vitro* assays [51]. These investigators have proposed that a phosphorylation-dephosphorylation cycle might exist in intact cells to regulate receptor binding and nuclear localization. Another study has reported that cAMP decreased and cGMP increased estrogen binding in cytosol fractions of endometrial cancer cells [52]. However, this study did not directly evaluate receptor phosphorylation. Recent work in our laboratory using intact MCF-7 cells and estrogen receptor immunoprecipitation following metabolic labeling with radioactive phosphate have failed to detect either phosphotyrosine or changes in receptor binding following treatment of cells with activators of adenylate cyclase. However, phorbol ester treatment of MCF-7 cells resulted in a reduction of estrogen receptor binding activity and cell proliferation and was associated with a loss of E_2-inducibility of proliferation and progesterone receptor [53, 54]. Phosphorylation of the estrogen receptor or an associated protein may play a negative modulatory role analogous to phosphorylation of receptors for some growth factors [40]. Reported differences among investigations concerning identity, inducibility and function of amino acid phosphorylation in the estrogen receptor remain to be resolved.

3. Biological and biochemical effects of estrogen on *in vitro* model human breast cancer systems

Estrogen induces a large number of enzymes involved in nucleic acid synthesis including DNA polymerase, thymidine and uridine kinases, thymidylate synthetase, carbamyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase, and dihydrofolate reductase [20, 56, 57]. Physiologic concentrations of E_2 stimulate DNA synthesis by both scavenger and *de novo* biosynthetic pathways. For example, estrogen regulates thymidine kinase and dihydrofolate reductase at the mRNA level [58, 59]. Regulation of thymidine kinase mRNA also occurs at the transcriptional level [59]. Though increases in global transcription appear to be tightly coupled to estrogen action [60] no study has yet identified the most critically regulated gene(s). The existence of 'second message' regulatory systems in the growth induction process is also possible but has not yet been proven. In MCF-7 cells, E_2-induced stimulation of phosphotidyl inositol turnover occurs with an exceptionally long lag time [61]. In contrast, in a variety of other model systems, this metabolic effect is quite rapid (within minutes as opposed to hours for estrogenic effects) and tightly coupled to growth control by proteases and hormones, particularly the polypeptide growth factors [62, 63]. Thus, phosphatidyl inositol turnover, with its associated stimulation of protein kinase C and Ca^{++} fluxes [63] could serve as a metabolic mediator of mitogenic effects of E_2-induced growth factors.

The progesterone receptor is also induced by estrogen [64]. However,

progestins are growth inhibitory for human breast cancer while inducing specific protein of 48 kDa [65]. The presence of the progesterone receptor is generally coupled to functional growth regulation by estrogens *in vivo* and *in vitro*. Thus, progesterone receptor content of breast tumors is used in addition to the estrogen receptor as a marker for estrogen and antiestrogen responsiveness of tumors in clinical therapy [2]. Widespread exceptions do, however, exist *in vitro* in the coexpression of these two receptors; for example, in cell lines T47D and MD-MB-134 [16, 64] and *in vivo* in some patients' tumors [2].

Both estrogens and antiestrogens alter the cellular synthesis and/or secretion of several other proteins, but their role in growth control is unclear. These proteins include various plasminogen activators and collagenolytic enzymes [71]. Plasminogen activator (along with other proteases) is thought to contribute to tumor progression and growth by allowing the tumor to digest and traverse encapsulating basement membrane [66, 67]. While this is likely, it is conceivable that proteases may serve additional roles such as facilitating release of mitogenic growth factors like IGF-I (somatomedin C) from carrier proteins, processing inactive precursor growth factors and proteases to active species [68] or interacting directly with cellular receptors [69, 70]. In addition, several relatively abundant breast cancer cells secrete proteins of 24 kDa [72], 52 and 160 kDa [73], 37–39 kDa, 32 kDa [74, 75] and 7 kDa (initially identified by detection of an estrogen induced mRNA species termed pS2) [76]. Four other mRNA species termed pNR 1–4 [77], and the cytoplasmic enzyme LDH [78] are also under estrogen regulation. The 52 kDa glycoprotein, one of the major secreted proteins, has cathepsin D-like activity in purified form; it is also mitogenic for MCF-7 cells *in vitro* [79, 80] (see chapter by H. Rochefort, this volume). The nature of the 160, 37–39, 32, 24, and 7 kDa proteins are unknown at present, but the 160, 52, and 7 kDa secreted proteins may be disassociated from estrogen and antiestrogen modulation of MCF-7 cells growth using two MCF-7 clonal variants [81–83]. These three protein species are decreased by antiestrogen to the same extent in both MCF-7 and LY2, the latter being a stable antiestrogen resistant variant of MCF-7. In I-13, an MCF-7 clonal variant which is growth arrested by physiologic concentrations of estrogen, the same three proteins are induced to the same extent as in MCF-7. These observations suggest that a significant reduction in secretion of these proteins has no impact on growth in the case of LY2, and their induction does not affect I-13.

Finally, it has been recently demonstrated that estrogen induces the cell surface 'receptor' or binding protein for laminin in MCF-7 cells [84]. The laminin receptor is thought to mediate attachment of cells to basement membrane laminin (66, 67; see chapters by L. Liotta *et al.* and R. Thompson *et al.*, this volume) to contribute to invasiveness by tumor cells, and to promote colonization of new host tissues. Estrogen treatment of MCF-7 cells increases I^{125}-laminin binding, cell attachment to artificial, laminin-coated membranes, and the migration of the same cells across an artificial membrane toward a diffusable source of laminin [84]. E_2 treatment of MCF-7 cells also

induces marked rearrangements of cytoskeletal and adhesion structures [85] and alterations in the plasma membrane microvilli as observed by scanning electron microscopy [86].

In summary, estrogens exert a considerable number of influences *in vivo* which may indirectly alter breast cancer progression [87]. Direct effects of estrogens on isolated breast cancer cells are also well established. These effects include growth regulation as well as modulation of enzymes and other activities thought to mediate mitogenic, metastatic and differentiated status. Some of these activities are secreted and can be detected as products of the normal gland, such as in milk [88, 89; see also chapter by D. Salomon and W. Kidwell].

4. Antiestrogens

Among the first antiestrogen compounds utilized were MER 25 and clomiphene. The triphenylethylene antiestrogen tamoxifen has subsequently become a mainstay of therapy both in advanced disease and stage II disease in postmenopausal women. In contrast to cytotoxic chemotherapy agents, antiestrogens appear to be cytostatic rather than cytocidal and have a low incidence of significant side effects. Many investigators have noted the close correlation between the initial clinical response to antiestrogens and the presence of the estrogen receptor (and its induced product—the progesterone receptor) [90]. Since antiestrogens and their active metabolites have a high affinity for the estrogen receptor, the most likely mechanism of antiestrogen action appears to be simple antagonism of the growth promoting effects of estrogen [91]. However, alternate views involving other microsomal binding sites for antiestrogen have been presented [92]. In addition, it has been proposed that antiestrogen can have direct antimitogenic effects mediated through the estrogen receptor but independent of estrogen occupancy [93]. It is possible that an alternative receptor confirmation and/or chromosomal localization may mediate such effects. In addition, high doses of antiestrogen inhibit both calmodulin and protein kinase C [91, 94] but the physiological relevance of these observations is not yet certain. Antiestrogen treatment of estrogen dependent breast cancer leads to cell cycle blockade (early G_1) of most of the cells *in vitro* and to reduction in tumor growth *in vivo* [91, 95–97]. It had been initially observed that MCF-7 cells responded *in vitro* to both estrogens and antiestrogens under normal cell culture conditions [10]. These experiments were initially interpreted to suggest that antiestrogens could act to arrest growth independently of an occupied estrogen receptor complex. However, as previously mentioned recent work by Katzenellenbogen and co-workers has clearly shown that high concentrations of phenol red present in the culture medium of the cells in these studies produced estrogenic effects [18]. Removal of phenol red, whose structure resembles that of certain nonsteroidal estrogens, abrogated antiestrogen action on MCF-7 cells and dramatically

enhanced the responsiveness of the cells to estrogen induction of cell growth and progesterone receptor. At the present time it appears that antiestrogens act at physiologic doses primarily by direct antagonism of the initiation of signals generated by an agonist occupied receptor.

The principle clinical limitation to the utility of antiestrogens is the gradual resistance which develops in tumors treated with these agents. While in some cases, antiestrogen resistant tumors lack the estrogen receptor, it is unlikely that loss of the estrogen receptors fully explains the loss of antiestrogen sensitivity during clinical treatment [2, 3]. At least 40% of all breast cancers contain the estrogen receptor. Furthermore, a stable clone of MCF-7 cells selected stepwise *in vitro* for antiestrogen resistance (as well as other less stable resistant clones—R3 and R27), still contains high levels of the estrogen receptor [82].

Another limitation to the antineoplastic efficacy of antiestrogens has been the partial estrogenic (or agonist) activity characteristic of the compounds in clinical use. For instance, studies in the nude mouse model system with MCF-7 cells have only achieved modest inhibition of tumor growth with tamoxifen [96]. Tamoxifen and some other antiestrogens are weakly stimulatory for uterine growth in rodent model systems [91]. However, novel 7α-alkyl amide derivatives of E_2 have been reported as pure antiestrogens and are devoid of uterine growth promoting potential *in vitro*. Such compounds might have greater clinical utility than tamoxifen in the suppression of breast tumor growth [98, 99].

5. Non-estrogenic growth regulatory hormones and breast cancer: antocrine, paracrine and endocrine actions

Many groups have shown growth regulation of MCF-7 cells in monolayer culture by a variety of lipid soluble trophic hormones in addition to E_2. These include glucocorticoids, iodothyronines, androgens and retinoids [reviewed in 100]. MCF-7 cells have receptors but are not growth stimulated by progesterone or vitamin D [29, 65, 100, 101]. Progesterone induces a specific protein [65] and can be growth inhibitory *in vitro* [reviewed in 93]. Additional studies have demonstrated receptors for and responses to the polypeptides insulin, epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) [29, 101–103] and transforming growth factors α and β (TGFα, and TGFβ) [Reviewed in 104]. Somatostatin is growth inhibitory for breast cancer [105]. Finally, receptors and metabolic effects, but little cellular growth response has been demonstrated for other hormones, such as prolactin, growth hormone, glucagon and calcitonin [100] (see also chapter by R.P. Shiu, *et al.*, this volume). The multiplicity of growth modulatory hormones for *in vitro* breast cancer systems suggests the possibility that many serum borne modulators of growth may play important regulatory roles *in vivo* [106]. Alternatively, or additionally, growth factors with a similar spectrum of activities could

be elaborated by the breast cancer cells themselves. In the nude (athymic) mouse model system E_2 is an absolute tumor growth requirement for two human cell lines, MCF-7 and T47D, and a growth stimulator for a third cell line, ZR-75-1. [14, 96, 107]. McGrath and his colleagues have further defined this system by showing that E_2 need not enter the systemic circulation in nude mice to promote MCF-7 tumorigenesis; elevation of local E_2 concentration was sufficient to promote local but not distant tumor growth [108]. This suggests that although estrogens might be required to induce a host of regulatory factors required by the tumor, the production and action of such regulated factors is probably restricted to the local area of the tumor.

What are these local, E_2 induced events that contribute to tumor growth? (as opposed to the 'estromedin' hypothesis noted above) [6]. The mammary stroma is likely to provide as yet unidentified contributory factor(s) *in vivo* for full mitogenicity of estrogen [109]. In culture, normal mouse mammary epithelial cells require exposure to mammary fibroblasts for estrogen effects [110]. Estrogen induction of progesterone receptor requires the presence of either glutaraldehyde killed fibroblasts, type I (stromal) collagen, or conditioned medium from fibroblasts. This effect may be mediated by a basement membrane or substratum effect of fibroblasts. In contrast, estrogen dependent DNA synthesis in normal mammary epithelium occurs only when live fibroblasts were in coculture [106]. These observations imply that a labile secreted fibroblast material was permissive for estrogen induced growth. Furthermore, fibroblasts proliferate in response to combinations of E_2 and mammary epithelial cells but not to estrogen alone. Taken together, these data provide evidence for intimate or paracrine communication between stromal and epithelial cells, each requiring the presence of the other for growth in response to E_2. In embryonic development of the male, androgen treatment of the receptor-containing mesenchyme results in necrosis of the epithelium [111]. Thus, the stromal cells may contribute both negative and positive growth modulation of epithelium. A wide range of possible mechanisms exists for such communication including secretion of soluble mediators, or basement membrane or even exchange of cell surface components. It is possible that cancer may represent a partial escape from dependence on a stromal requirement or an abnormally strong response to a stromal component (see chapter by Salomon and Kidwell, this volume).

How might mammary epithelium become autonomous from stromal controls? Several groups have directed their attention to the possible involvement of tumor epithelial derived, secreted growth factors in growth control of breast cancer. It has been observed that initial growth rate of MCF-7 cells *in vitro* is proportional to number of cells plated [112]. While multiple interpretations of these data are possible, one possibility is the production of autostimulatory or autocrine growth factors which would stimulate the growth of MCF-7 cells. More direct studies using estrogen-free extracts of conditioned medium harvested from MCF-7 cells treated with E_2 (CME_2) observed that CME_2 are capable of stimulating increases in both thymidine incorporation

and cell proliferation [6, 113, 86]. Continuous subcutaneous infusion of proteins obtained from CME_2 into nude mouse also stimulated MCF-7 tumorigenesis in the absence of E_2 [114]. Thus, adenocarcinomas of up to 0.5 cm diameter appeared at MCF-7 implantation sites within 2 weeks. Tumors in CME_2 infused animals appeared with 2- to 3-fold greater frequency than in CM infused animals. While CME_2-supported tumors reached maximum size within 2-3 weeks of treatment, but subsequently usually declined in size thereafter, E_2-pellet implanted animals had continuously growing tumors. In this study, uterine weight was unaffected by CME_2 and CME_2 activity was decreased by treatment with either trypsin, a reducing reagent, or heating to 56°C for 1 hour. This suggests that tumor growth promoting substance(s) in CME_2 are likely to be proteins. Thus, following estrogenic stimulation, cultured human breast cancer cells release an autocrine, tumor-promoting factor(s) which can act both *in vitro* and *in vivo* after release of sufficient quantities into the general circulation of the athymic mouse. Furthermore, autocrine growth factors might be able to act in an endocrine fashion if they achieve high enough concentrations in the blood.

6. Growth factors and transformed phenotype in fibroblast model systems

What kinds of hormones might act in an autocrine or paracrine fashion? A well established system for the study of mediators of growth control has been provided by rodent fibroblasts *in vitro*. Studies were initially carried out in cell monolayers on plastic surfaces. Smith, Scher and Todaro, among others, identified 'restriction points' in the cell cycle of 'normal' (but immortalized) fibroblasts. Various polypeptide growth factors abrogate these restriction points, allowing the cell cycle to progress [115]. Platelet derived growth factor (PDGF), a 'competence' growth factor, allows cells to pass a restriction point in early G_1. As PDGF acts to initiate the cell cycle, several genes known as protooncogenes are sequentially induced. Among these are c-*fos*, c-*myc*, and c-*ras* [reviewed in 116]. EGF (or the related transforming growth factor α, TGFα) acts later, while IGF-I (also known as somatomedin C) and other hormones still later in G_1 [Reviewed in 116]. EGF and IGF-I are termed 'progession growth factors'. Human diploid fibroblasts treated with PDGF, EGF, or growth hormone secrete their own IGF-I. Secreted IGF-I is capable of self-stimulation to promote mitogenesis. Anti IGF-I antibodies blocked growth hormone stimulation of DNA synthesis [117–118].

A number of investigators have noted that when fibroblasts and other cells are transformed with various tumor viruses, oncogenes, chemicals, or radiation, they lose some requirements for exogenous growth factors [116, 119–121]. Thus, malignant transformation was proposed to result from ectopic production of growth factors, abolishing both competence and progression restriction points in a cell's own cycle. This may reflect the decreased serum requirement of some cancer cells [122, 123].

In an attempt to generate a more cancer-specific growth assay for growth factor effects, an 'anchorage independent growth' assay has been developed. It had been observed that the ability of some cells to grow in colonies under anchorage independent conditions (growth suspended in agar or agarose) was correlated with their tumorigenicity or state of malignant 'transformation' [124]. At least four growth factor activities have been identified which together can reversibly induce this transformed phenotype of murine fibroblasts: PDGF, EGF (or TGFα), IGF-I (or IGF-II, a different somatomedin activity) and an additional growth factor, transforming growth factor β (TGFβ) (122, 125, 126). Results from this assay suggest that these growth factors are likely to be involved in cancer growth control, but little direct evidence for an *in vivo* role in tumor growth has yet emerged. It should be emphasized that conclusions drawn from the murine fibroblast model system may not apply to cancers of other tissues or species of origin.

The principle restriction point(s), if any, for epithelial cell cycles are unknown. However, it is now clear that normal human mammary epithelial cells require hydrocortisone insulin, EGF, PGE, transferrin, and an undefined pituitary component to proliferate in serum free medium [127] (see also chapter by M. Stampfer, this volume). In contrast to the fibroblast model, TGFβ is a growth inhibitor for many types of primary and malignant epithelial cells [128, 129]. Therefore, while some of the same growth factors may facilitate traverse of the cell cycle in fibroblasts and epithelial cells, control of anchorage independent growth may involve another less well defined growth factor(s). A candidate for such a growth factor is provided by the work of Halper and Moses [130]. They have identified an adrenal carcinoma cell line (SW13) which is extremely sensitive for anchorage independent cloning to a mitogen (TGFe) found in epithelial cancers or cell lines. Basic pituitary FGF can also subserve such a function in cloning of SW13 but the epithelial cancer derived growth factor does not appear to be chemically related to FGF. Notwithstanding the unknown features of cell cycle control in breast cancer, a number of studies have begun the analysis of its secreted growth factors with representatives of all 5 above mentioned activity classes under investigation: PDGF, TGFα, IGF-I, TGFβ, and an epithelial transformation factor. A summary of growth factors commonly found in tumors is presented in Table 1.

7. Transforming growth factor α—an estrogen induced growth stimulator

Conditioned medium from MCF-7 cells, other breast cancer cell lines, and extracts of tumors have been fractionated to facilitate identification of the growth factors present. The cell lines secrete stimulatory activity for MCF-7 and murine 3T3 fibroblast monolayer cultures as well as 'transforming growth activity' (TGF) (Table 2) as assessed by stimulation of anchorage independent growth of rodent NRK and AKR-2B fibroblasts in soft agar culture [107,

Table 2. Tumor growth factors

	Protein size	Structure	RNA size	Source	Receptor
TGFα	6 KDa + Higher MW Forms	EGF Homology	4.8 Kb (Others?)	Many cancers, fetal tissues	170 KDa EGF Receptor
PDGF	28 KDa	Heterodimer and Homodimers	A (1) Chain 2.5, 2.9 Kb B (2) Chian 4.1 Kb	Sarcomas glioma, some carcinomas, platelets	185 KDa Receptor
TGFβ	25 KDa	Disulfide-linked Homodimer	2.5 Kb	Many normal and cancer cells, platelets	280 KDa Receptor Homodimeric and lower MW forms
IGF-I	7.6 KDa	Disulfide-linked Preporinsulin Homology	Multiple	Carcinomas, fetal tissues, many adult tissues including liver, fibroblasts	135 KDa, 90 KDa Heterotetramer, Related to Insulin Receptor

112, 131–136]. Breast cancer cells produce a 30 KDa apparent molecular weight peak of transforming activity for NRK fibroblasts which has been identified. This species also coincides with a peak of MCF-7 autostimulatory activity and is the principle species of EGF receptor competing activity [107–112]. Antisera specific to TGFα reacts with this species [136]. Thus, this activity may be related to TGFα, but it appears to be significantly larger than the cloned and sequenced 6 KDa species from transformed rodent fibroblasts [137]. It is not yet certain if this protein is related to the 17–19 KDa TGFα precursor protein observed in fibroblasts [138], whether it is modified by glycosylation, palmitoylation [139], or if it is the product of alternative mRNA splicing. The precursor species is thought to be membrane bound in cell lines which express it [138–140]. It cannot be ruled out at present that the breast cancer derived TGFα might be the product of a novel TGFα-related gene. The 30 KDa TGFα-like species is induced by estrogen treatment of MCF-7, T47D, and ZR-75-1 cells ranging from 2–14 fold depending on cell type and culture conditions [94, 99]. The expected 4.8 Kb TGFα mRNA species has been detected by Derynck and co-workers in MCF-7 and some other human breast cancer cell lines and tumors [137, 141, 142] and by ourselves in a series of human tumors ranging from low to high estrogen receptor levels [135]. No correlation of TGFα mRNA expression was observed with estrogen receptor status; 70% of the adenocarcinomas contained TGFα mRNA. One breast cancer cell line, the estrogen receptor negative, carcinosarcinomas Hs578T (143) has been reported to not contain detectable levels of TGFα protein [107] or its mRNA (144). When MCF-7 cells were treated with estradiol *in vitro* TGFα mRNA was induced in 6 hours (131, 139). Estrogen withdrawal of MCF-7 cells grown as estrogen dependent tumors in nude mice led to decreased TGFα mRNA [144]. Similar results have been reported in murine breast tumor models (see chapter by Salomon and Kidwell, this volume). Recent studies using antibodies directed against either TGFα or its receptor (the EGF receptor, discussed in a later section) have reported growth suppression of MCF-7 cells grown as anchorage independent colonies or as estrogen stimulated, high density monolayer cultures [144]. TGFα is currently one of the most likely growth factors to exert a positive autocrine effect in breast cancer. It has also been detected as a tumor burden marker in the urine of patients and nude mice bearing breast and other tumor [145–148]. Thus it may be provide a marker for tumor mass, disease progression, or risk of malignancy. Detection of urinary TGFα has been complicated by the presence of very high levels of EGF-related growth factors present even in normal control urine. Preliminary evidence has been obtained in our laboratory for synthesis of EGF-related activities in some breast cancer cell lines. EGF is also found in milk [88, 89] but has not yet been identified in breast tumors. One other cell line, a human salivary adenocarcinoma cell line has been reported to secrete EGF [149].

Is TGFα a breast tumor-specific growth factor? A number of studies have failed to detect TGFα in untransformed fibroblasts [1, 115, 122, 123] or in

normal epithelial tissue biopsies [142], although it is found in embryonic tissue [116]. The hypothesis that TGFα is an onco-fetal marker has been recently disproven with the discovery that normal bovine anterior pituitary cells proliferating in culture produce TGFα [150]. Further, it is also now known that normal human mammary epithelial cells rapidly proliferating in culture also secrete high quantities of TGFα and produce its expected mRNA species (134). Thus TGFα may contribute to growth control processes of normal as well as malignant breast tissue. Alternatively, TGFα could act to facilitate adaption of cells to growth in tissue culture. It is possible that the response of breast tissue to TGFα or EGF rather than the absolute levels of its production might distinguish the cancer from normal *in vivo*. Indeed, MCF-7 cells grown as tumors in nude mice respond to E_2 or EGF infusions with tumor formation; normal rodent breast tissue *in situ* does not form a tumor [113], though it does proliferate *in vitro* (see chapter by Oka *et al.*, this volume). Derynck and co-workers have recently found that the TGFα gene expressed under an SV 40 promoter in murine fibroblasts can act as an oncogene to induce anchorage independent growth *in vitro* and tumor formation in nude mice [151]. Furthermore, EGF under promotion by a MoMULV promoter can also act as an oncogene in fibroblasts to induce their tumor formation in nude mice [152].

A large body of literature also exists demonstrating that EGF has tumor promotional activity [reviewed in 153]. In studies investigating mouse mammary carcingenesis, Oka and coworkers have recently demonstrated a likely role of EGF in both mammary tumor onset and subsequent growth support [154]. Using a mouse strain highly susceptible to spontaneous mammary tumors, removal of the submandibular glands (sialoadenectomy) dramatically reduced the incidence of tumor formation and/or the rate of growth of the breast tumors allowed to form. The submandibular gland is a source of EGF in mammals and reinfusion of EGF into such sialoadenectomized mice returned tumor incidence and growth rate of tumors to their normally high level. Thus, TGFα- and EGF-like activities may have endocrine functions in tumor onset and support [154]. TGFα and EGF are also thought to be produced locally by murine mammary epithelium during ductal development (see chapter by Vanderhaar). As the data with MCF-7 cells show, one mechanism of tumor progression might involve local production of TGFα. Clearly, TGFα- or EGF-like growth factors are likely to be important regulators of mammary tumor progression by a variety of possible mechanisms.

Both EGF and TGFα can act via the EGF receptor, a ligand inducible tyrosine kinase, on both normal and cancerous cell lines [1]. The EGF receptor has been detected in human and rodent mammary tumor biopsies and malignant cell lines [155–158]. In one breast cancer cell line (MDA-MB-468) Kudlow and co-workers have observed that EGF induces the biosynthesis of its own receptor [158]. The receptor observed in breast cancer cells appears to be very similar to the cloned and sequenced human EGF receptor. The apparent molecular size is 170 kDa, and the kinase domain is unaltered as

determined by S1 ribonuclease analysis [156]. However, the state of phosphorylation or the tyrosine kinase activity of the receptor and the physiologic substrates of the receptor kinase in breast cancer are unknown. A recent study has shown that EGF induces phosphorylation of lipocortin (a phospholipase inhibitor) in A431 carcinoma cells [159]. The relevance of this effect to other EGF/TGFα actions remains to be determined. EGF also is known to induce trans-acting nuclear factors with appear to regulate transcription of responsive genes [160]. Finally, self-association (dimerization or oligomerization) of the EGF receptor can itself activate tyrosine kinase. Self-association is triggered by EGF/TGFα ligand occupancy and might also be the consequence of receptor overexpression on cell surfaces [reviewed in 161].

Does TGFα have roles other than for proliferation or transformation in cells other than breast epithelium? After secretion by the normal gland into milk, TGFα and EGF act on neonatal development; the best known example is in eyelid opening [162]. TGFα may also have roles in epithelial wound healing [163], and angiogenesis [164]. However, such processes are quite complex, involving a variety of hormones and other components in addition to the TGFα which stimulate chemotaxis and mitogenesis of several cell types [165–172]. In breast cancer, TGFα/EGF may also contribute to desmoplasia, the fibrotic, stromal proliferative response surrounding the tumor [115, 173] (see also section V) and to hypercalcemia and bone resorption [174]. Finally, it is also possible that growth factors, such as EGF/TGFα [143], can be immunosuppressive, acting to counter host immune rejection of cancer cells. Some of these complexities are outlined in Figure 1.

8. Insulin-like growth factors

As mentioned in section 6, somatomedins are required both for anchorage dependent and independent proliferation of fibroblast model systems. Do they also play a role in breast cancer? IGF-I is mitogenic for some breast cancer cells in culture [102, 175]. Using radioimmunoassay, we and others have also noted that an IGF-I (somatomedin C)-related species is secreted by all human breast cancer cells examined to date [175, 176]. After partial purification from MCF-7 cell conditioned medium this growth factor comigrates on gel exclusion chromatography with authentic human serum-derived IGF-I. Acid ethanol extraction is required to disrupt a high molecular weight form of the growth factor. A complex series of IGF-I-related mRNA species are also detected with northern blot analysis using a cDNA probe to authentic IGF-I [177]. Similar complex species of IGF-I cross hybridizing mRNA's have been previously described for the human fetus [178]. No E_2 induction of secreted IGF-I-like growth factor is observed in standard culture conditions using phenol red-containing medium with MCF-7 cells. Subsequent studies, utilizing the more substantially estrogen depleted phenol red-free medium have

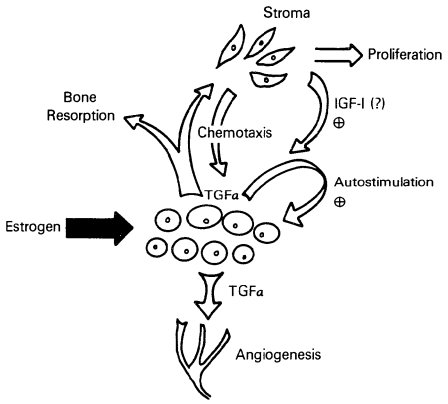


Figure 1. TGF α is induced by estrogen in hormone responsive breast cancer. It may have autostimulatory roles as well as paracrine effects on blood vessels (angiogenesis), surrounding stroma (proliferation, chemotaxis and IGF-I release), and some resorption.

revealed a 3–6-fold induction of the IGF-I-like growth factor with E_2, TGFα, EGF, or insulin treatment [179, 180]. IGF-I-like growth factor secretion is inhibited by growth inhibitory antiestrogens (in phenol red-containing medium) TGFβ, and glucocorticoids. While growth hormone is a strong stimulus for IGF-I production by liver, fibroblasts, and other normal tissues it is without effect on IGF-I-like growth factor production by MCF-7 breast cancer cells [116, 117, 181]. Breast cancer may have a unique hormonal specificity for its regulation of IGF-I-like growth factor secretion. The production and regulation of IGF-I-like growth factor by normal human breast epithelium has not yet been examined. As previously mentioned IGF-I related polypeptides are secreted by fibroblasts and smooth muscle and contribute to autocrine growth control in these cell types [116, 117]. It remains to be seen whether IGF-I produced by breast cancer acts primarily on breast cancer itself in an autostimulatory mode or on surrounding stroma to promote chemotaxis and growth [173] (Figure 2).

IGF-I mitogenesis is thought to be mediated by its receptor, a close homologue of the insulin receptor, though it also weakly binds to the insulin receptor. The IGF-I receptor in a variety of cell types consists of a 450 kDa complex (2α chains of 130 kDa and 2β chains of 85 kDa) [1]. The receptor has been purified, cloned and sequenced; it is strongly homologous to the insulin receptor and it posesses tyrosine kinase activity [1, 182, 183]. Its mechanism of action is unclear but IGF-I binding appears to stimulate growth by a post transcriptional mechanism [184]. IGF-I also stimulates transcription from the promoter of the ribosomal RNA gene in mouse fibroblasts [185], but the relevance of this observation for mitogenesis is not yet known.

The results of site directed mutagenesis studies with the highly homologous insulin receptor [186] suggest that IGF-I receptor function might also be mediated by activation of the tyrosine kinase activity. IGF-I receptors of the

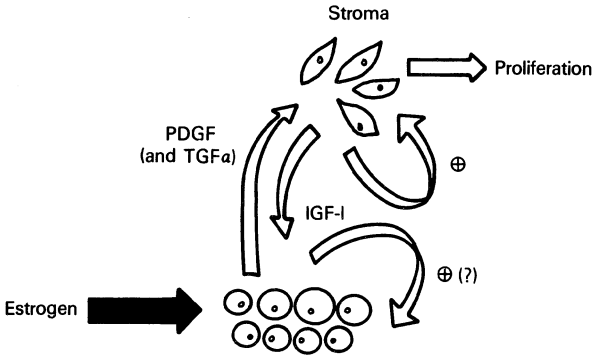


Figure 2. IGF-I is induced by estrogen in hormone responsive breast cancer. It may be autostimulatory or have paracrine effects on stroma. Stromal cells in the vincinity of the tumor probably also make their own IGF-I under stimulation of other tumor derived hormones.

expected size have been detected by cross-linking studies on human breast cancer cell lines [102]. Research on IGF-I receptors has been somewhat hampered by the presence of a secreted, non-receptor binding protein which interferes in ligand binding assays [187–189]. At the present time insufficient information is available to fully evaluate the structure, regulation, and function of breast cancer-derived IGF-I-related proteins and compare them to those produced by embryonic and adult tissues [190, 191]. The mechanisms of IGF-I induction and its possible biological role(s) in breast cancer are not yet known. However, two strongly tumorigenic, estrogen receptor negative breast cancer cell lines (MDA-MB-231 and Hs578T) secrete high levels of IGF-I and (presumably on this basis) have blunted responsiveness to exogenous IGF-I [175].

It is possible that an IGF-II-related gene product(s) are produced by normal and malignant breast tissue. IGF-I mRNA has been recently reported in other human tumors: lung, colon, and liposarcoma [192, 193], IGF-II has been observed to be overproduced in Wilms tumor [194]. Somatomedins appear to be among the most ubiquitous growth factors, produced by nearly all normal tissues [115, 181] and found in the blood [195].

9. Transforming growth factor β

Transforming growth factor β is a 25 kDa polypeptide (see Table 1) which is required (along with other growth factors) for full induction of the transformed phenotype in fibroblasts (section 6). It is also produced autonomously in fibroblasts transformed by oncogenes [196]. In contrast to TGFα and many other growth factors TGFβ is growth inhibitory for most epithelial cells [128, 129]. We wished, therefore, to ask whether TGFβ might be an autocrine inhibitory (chalone [197])-type substance in breast cancer.

Breast cancer cells have been shown to contain a TGFβ-related activity [129, 132, 198]. MCF-7 conditioned medium has been fractionated by gel exclusion chromatography. A major peak of activity binds to TGFβ receptors, transforms AKR-2B and NRK fibroblasts, and comigrates with platelet-derived TGFβ [132]. SDS gel electrophoresis of metabolically labeled, immunoprecipitated material confirmed its close similarity. All breast cancer cells examined expressed a 2.5 Kb mRNA species, expected based on other studies [132, 141, 198]. TGFβ secretion is inhibited by treatment of MCF-7 cells with mitogens, for example E_2 and insulin [132], but growth inhibitory antiestrogens and glucocorticoids strongly stimulate its secretion. Intracellular TGFβ does not appear to change in concentration following treatment with mitogens or growth inhibitors [132]. TGFβ from antiestrogen induced MCF-7 cells strongly inhibits the growth of an estrogen receptor negative cell line MDA-MB-231. This growth inhibitor was reversed in the presence of a polyclonal antibody directed against native TGFβ [132]. In addition to a possible role as an antiestrogen-induced chalone, it also might be possible that antiestrogen induced TGFβ in hormone responsive breast cancer might act to expand the growth inhibitory potential of antiestrogens in clinical therapy. Breast cancers exist as mixtures of estrogen receptor positive and negative tumor cells [199, 200]. Since breast cancers do not necessarily become TGFβ unresponsive as they become antiestrogen unresponsive, TGFβ may act in such mixed cell populations to make antiestrogen more effective than might otherwise be expected based only upon blockade of estrogen action [132]. We have observed that in LY2, an MCF-7 variant stepwise selected for antiestrogen resistance, TGFβ is no longer induced by antiestrogen, but the cells still retain the TGFβ response and receptor [132]. The mechanism of TGFβ induction in MCF-7 cells is not yet fully defined, but it is not at the steady state mRNA level. Among other possibilities it may involve both synthesis of protein and conversion of a latent form to an active form of TGFβ [132]. In contrast in other systems, [196, 201] there is significant active TGFβ present in breast cancer conditioned medium. The biochemical details of the conversion of a secreted inactive to active TGFβ remain to be elucidated. Inactive TGFβ may be activated by acidification. A 62 kDa precursor can be cleaved by plasmin to yield the 25 kDa mature species [202].

TGFβ is proposed to act through a high molecular weight (615 kDa) receptor complex, the receptor subunits being two 280 kDa species. In addition, 65 kDa and 85 kDa binding components have been reported [203]. The TGFβ receptor does not appear to 'down regulate' from the cell surface following ligand occupancy. It appears to rapidly recycle and not to follow a lysosomal degradation pathway taken by tyrosine kinase-encoding receptors such as IGF-I, EGF, and PDGF. Presumably, it follows an endocytosis-recycling pathway (like transferrin) or is rapidly replenished, following endocytosis from a large intracellular store [204, 205]. This receptor has not yet been purified, cloned or sequenced, but is reported not to have tyrosine kinase activity [1]. In the cow and pig, an alternate gene for TGFβ (known as

TGFβ_2) exists. The gene product forms either a homodimeric complex or heterodimeric complex with TGFβ, appears to bind to the same 280 kDa receptor species as TGFβ but has lower affinity for the 65 kDa and 85 kDa receptor species [203]. TGFβ has been reported to induce production of c-sis mRNA and its encoded PDGF activity [206], fibronectin, and collagen [207] in fibroblasts. Since anchorage independent growth of fibroblasts is also induced by fibronectin (in the absence of TGFβ) and anti-fibronectin antibodies interrupted TGFβ-induced anchorage independent growth of fibroblasts, it has been proposed that fibronectin mediates at least some of the actions of TGFβ [207]. Thus, a model has been proposed by Massague whereby a critical growth factor for anchorage independent growth may act by stimulating production of basement membrane. It is conceivable that in anchorage independent states, basement membrane synthesis, accumulation, or assembly might be rate limiting for cell growth. TGFβ has also been reported to modulate the levels of EGF receptor on fibroblast cell surfaces [208] and to induce a secreted activity which somehow prolongs the cellular ruffling response of fibroblasts to TGFβ [209].

TGFβ influences the differentiated state of many cell types, possibly as a result of induced basement membrane synthesis. For example, it inhibits myogenic differentiation [210, 211], it inhibits hepatocyte growth, and it prevents dedifferentiation of other epithelial cell types [212]. TGFβ is apparently part of a larger gene family, including Müllerian inhibiting substance and inhibin [213]; members of the gene family have pervasive actions on development and metabolism. It is not yet known what effects on differentiation and metabolism TGFβ has in breast cancer. High affinity binding sites for TGFβ have been reported on responsive (growth inhibited) human breast cancer cell lines [129, 132].

Finally, like TGFα and IGF-I, TGFβ produced by tumor cells might have marked paracrine effects on surrounding tissue (Figure 3). TGFβ may contribute to the marked stromal proliferation and basement membrane deposition commonly observed in breast tumors [173]. It also has been reported that TGFβ can induce bone resorption [174]. In the context of endothelial regeneration, TGFβ inhibits replication of endothelial cells *in vitro* [214–215]. However, Roberts and co-workers have proposed that TGFβ acts *in vivo* to promote fibrosis and angiogenesis [216]. TGFβ might indirectly induce angiogenesis by stimulating macrophage chemotaxis at the site of the tumor and degranulation [165, 196].

10. Platelet derived growth factor

PDGF-like growth factors (Table 1) are produced by a variety of model transformed murine fibroblast lines and by some human tumors of diverse origins. Some human tumors were derived from PDGF-responsive cell types (sarcomas and gliobastomas) while others were derived from PDGF-

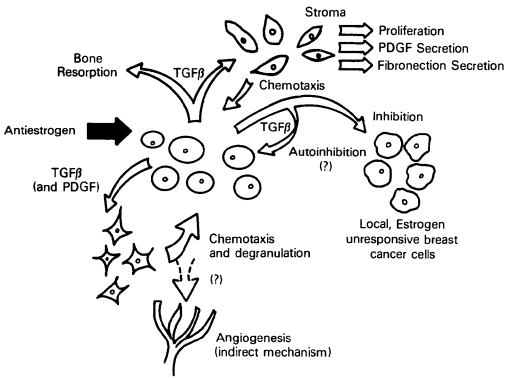


Figure 3. TGF β is induced by antiestrogen in hormone responsive breast cancer. It may be autoinhibitory and inhibit other antiestrogen unresponsive tumor cells in the vicinity of the tumor. Other paracrine effects may include indirect mediation of angiogenesis through chemotaxis and degranulation of macrophages, bone resorption, and multiple effects on stromal cells.

unresponsive epithelial, or white blood cell hematopoeitic cell types (hepatoma, T Cell leukemia, bladder carcinoma, erythroleukemia). The v-*sis* oncogene is related to PDGF B chain homodimer and can transform PDGF-receptor-containing cell types. Consequently, PDGF could subserve an autocrine role in such tumors. However, this has not been directly proven and the PDGF receptor is no longer present (presumably 'downregulated') in such tumors. In tumors derived from cell types initially lacking the PDGF receptor, PDGF presumably functions in stromal proliferation (desmoplasia) and chemotaxis and degranulation of monocytes and neutrophils [Figure 4].

Transformation of fibroblastic cells with simian sarcoma virus (SSV) provides a model system for the function of PDGF in initially responsive cell types. In such a system, the PDGF-B chain related protein encoded by the virus forms a homodimer and is sometimes secreted by the cell. In such cases, antibodies directed against PDGF have been reported to exert antiproliferative and antitransforming activity [reviewed in 217]. However, in many instances the PDGF is largely cell associated and presumably already bound to its receptor. Thus, anti-PDGF antisera have been only partially effective as anti-proliproferative reagents [218]. The subcellular origin or fate of PDGF in such instances remains to be fully characterized, however, immunoreactive PDGF has been observed in the cell nucleus [219] in SSV transformed cells.

Many breast cancer cell lines which we have examined to date secrete a PDGF-related activity detected by anchorage dependent growth stimulation of mouse 3T3 fibroblasts in the presence of platelet poor plasma. This is known as a 'competency' assay for early mitogenic signals [220]. 28 kDa and 16 kDa species were observed by immunoprecipitation of metabolically labeled MCF-7 and MDA-MB-231 breast cancer cell extracts and medium. The 28 kDa species (the unreduced form) was biologically active after elution

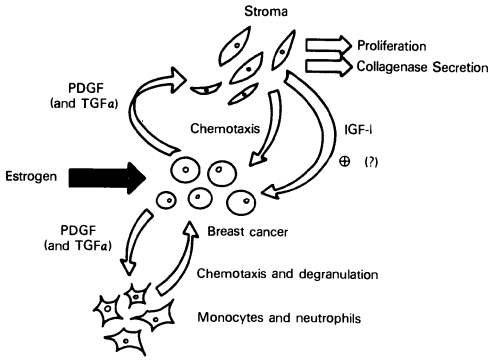


Figure 4. PDGF is induced by estrogen in hormone responsive breast cancer. Its effects may be entirely paracrine to effect chemotaxis and degranulation of monocytes and neutrophils, and multiple effects (including IGF-I release) on stromal cells.

from non-reducing SDS-polyacrilamide gels, and its activity was blocked with anti-PDGF antiserum. Upon examination of poly A selected mRNA from either cell line, transcripts of both PDGF A [1] and B [2] chains are observed. Estrogen treatment of the estrogen responsive MCF-7 induces mRNA encoding both A and B chain [221]. A and B chain have been recently reported to be widely expressed in breast cancer and other cell lines [222–224]. While the B chain is homologous to the v-*sis* oncogene, the A chain is not known to have a retroviral oncogene homologue. The A and B chain share substantial sequence homology to each other [222]. It is not yet known how A and B chains assemble in breast cancer cells.

PDGF acts through a 185 kDa receptor on a variety of mesenchymal cell types which encode a ligand inducable tyrosine kinase, similar to that of EGF, IGF-I, and insulin [1]. The receptor has been purified, sequenced, and cloned but does not appear to be expressed in any human mammary carcinoma cell lines [225].

PDGF is known to mediate proliferation of stromal cells such as fibroblasts *in vitro* and possibly in such physiological and pathological conditions as wound healing, vasoconstriction, atherosclerosis, embryonic development, myeloproliferative diseases, and desmoplasia [reviewed in 217]. PDGF circulates bound to at least one carrier protein, α_2 macroglobulin [217]. In model fibroblast systems, PDGF is known to act (similar to fibroblast growth factor) as a 'competency' growth factor [1, 217]. That is, it acts to allow density arrested fibroblasts in platelet poor plasma to respond to 'progression' factors such as EGF or IGF-I. The presence of both competency and progression factors allows fibroblasts to fully traverse G1 and enter S phase of the cell cycle. PDGF is known to rapidly induce both the turnover of phosphotidylinositol and the release of prostaglandins PGI_2 and PGE_2 [217]. Prostaglandins mediate vasodilatory and bone resorption functions [217]. In fibroblasts, PDGF induces proliferation, collagenase and collagen secretion

[217, 220] and induces IGF-I production [117]. IGF-I is autocrine-acting in such a system. PDGF induces mitogenic stimulation of mitogenesis in cultured human fibroblasts and porcine aortic smooth muscle cells, being largely abolished in the presence of anti IGF-I antibodies [117].

Such observations make it likely that in breast cancer, estrogen induced PDGF (along with other growth factors such as TGFα) act in a paracrine manner on fibroblasts and possibly other surrounding tissue. This could result in the proliferation of fibroblasts and further enhanced tumor growth by released fibroblast mediators such as IGF-I. It is possible that fibroblast-derived IGF-I might be one of the stromal factors (see section 5) which are required *in vivo* to initiate all the estrogenic effects observed on epithelial proliferation (Figure 4). One report already exists which suggests a correlation between PDGF mRNA expression and degree of stromal desmoplasia in primary breast cancer [226].

11. Other growth factors

This review has so far presented evidence for the production of autocrine and paracrine growth factors by breast cancer. The situation is undoubtedly more complex *in vivo*: for example, it is possible that *in vivo* growth factors and/or E_2 act in concert with other systemic mitogens to promote tumor growth. Shiu and coworkers have isolated a pituitary derived activity which potentiates the mitogenic effects of E_2 on MCF-7 cells [227]. One pituitary factor has already been identified as IGF-II (see chapter by R.P. Shiu, *et al.*, this volume) [228]. In addition, pituitary derived GnRH may also directly interact with breast cancer to inhibit its proliferation [5]. The *in vivo* role of either of these two hormones remains to be determined.

As mentioned in section 1 of this article, there has been a search for 'estromedins', or systemic mediators of estrogen action in breast cancer [6]. However, work by McGrath and others [108–110] has ruled out a major importance of non-tumor tissue other than in the immediate vicinity of the tumor. Thus, estrogen action in normal and malignant breast is probably restricted to direct effects on epithelial cells as well as to complex paracrine interactions with the stroma and possibly other local tissue types. However, it cannot be ruled out that pituitary-derived or other factors might play important permissive or modulatory roles in the tumor environment *in vivo*. In keeping with this possibility, evidence has been presented that growth factors (and/or E_2) could act to neutralize inhibitory, blood derived or endogenous inhibitors of tumor growth [7, 8]. Gaffney and co-workers have found a 68 KDa growth inhibitor for MCF-7 cells in bovine serum [229]. In addition, other investigators have identified but not yet isolated inhibitory activities for human breast cancer in fetal bovine and fetal human serum [7, 8]. It is possible that while direct effects of breast cancer derived growth stimulators (E_2, 52 KDa protein, TGFα, IGF-I) and growth inhibitors (TGFβ, other serum derived inhibitor(s)) can be demonstrated *in vitro*, *in vivo* growth control

may be influenced by a more complex interplay of growth stimulatory and inhibitory substances.

Other workers have focussed on approaches which identify secreted breast cancer growth factors which promote deposition of basement membrane collagen by stromal cells. Such differentiation-maintaining growth factors could be important in growth control of early stage *in situ* breast cancer (see chapter by Salomon and Kidwell, this volume). Two growth factors identified so far which subserve this function are TGFα (initially called MDGF-II) and a novel, 62 KDa (pI 4.8) growth factor known as MDGF-I [230]. These growth factors are found predominantly in more differentiated murine mammary carcinomas but have also been identified in human milk and human breast tumors.

Currently unidentified secreted growth factor activities also stimulate neoplastic growth. Halper and Moses [231] have established a model system with human SW-13 adrenal carcinoma cells in soft agar culture. These cells clone poorly unless basic fibroblast growth factor (FGF) or conditioned medium from certain epithelial cancers is applied. No other growth factors are known to be active. This activity has been only partially characterized from kidney but appears to be 40–42 kDa in size [231]. A recent study has begun the characterization of a similar activity from human breast cancer cells [232]. The estrogen independent, poorly differentiated and tumorigenic lines MDA-MB-231 and Hs578T produce high levels of the activity, while estrogen receptor containing lines (MCF-7, ZR-75-1, T47D) produce much lower levels. The activity from MDA-MB-231 cells has an acidic isoelectric point, and is approximately 60 kDa in size by gel filtration and gel electrophoresis. It has been purified to near homogeneity by an acid-ethanol extraction, isoelectric focussing, and HPLC sizing. It is not yet clear whether this activity is related to MDGF-I [230], or to basic FGF [233]. Future studies may utilize milk as a source of activities for anchorage independent growth assays of epithelial cells since it is such a rich source of many epithelial mitogens (see chapter by Salomon and Kidwell, this volume).

Finally, we turn to growth factor involvement in breast tumor metastasis. A very late event in tumor progression is the metastatic spread of the tumor out of its site of initial development to colonize other host areas. As previously mentioned, proteases and the laminin receptor might contribute to this process (see chapters by Liotta and Thompson *et al.*, this volume). Liotta and coworkers have also isolated a 55 kDa 'tumor cell autocrine motility factor' from a variety of cancer types; this activity may play an additional role in tumor invasion of basement membrane and metastasis [234]. Future studies using *in vivo* application of this agent should evaluate this hypothesis.

12. Role of oncogenes in malignant progression of breast cancer

Recent studies carried out in rodent systems (see chapters by Ali *et al.*, Groner *et al.*, Hager, Darbre *et al.*, and Nusse, this volume) have impli-

cated specific genetic alterations leading to malignant transformation and tumor progression. In the murine model (see Table 3), mouse mammary tumor virus (MMTV) inserts itself into the genome of susceptible mouse strains at specific sites and induces expression of at least 2 cellular genes [235]. One of these genes, int-1 (in a retroviral vector) can itself cause partial transformation of a mouse mammary epithelial cell line [236]. Another of these cellular genes, int-2 is highly homologous to basic FGF [237]. Thus MMTV appears to cause transformation by insertional activation (overexpression) of cellular protooncogenes.

In the NMU carcinogen treated rat model system, activation of the oncogene known as Harvey *ras* (c-H-*ras*) occurs by a different mechanism: point mutation [238]. However, prior estrogen exposure of the mammary glands to estrogen at puberty is required for subsequent tumor formation, the carcinogen induced tumors being generally estrogen responsive [238]. This model points to multiple roles for estrogenic activities beyond direct carcinogenic effects (see also chapter by M. Stampfer *et al.*). Thus, estrogens may be permissive (required at puberty), promotional (required to indirectly initiate tumors along with a direct acting carcinogen), and growth stimulating for tumors once they have formed. In the transgenic mouse model, an activated c-H-*ras* oncogene under promotion of a mammary specific DNA sequence (the whey acidic protein promoter) was expressed in mammary glands after a long latency involving multiple pregnancies, mammary adenocarcinomas in a female expressing the Wap-*ras* construction [239]. Transfection of an activated c-H-*ras* into a non-tumorigenic but established (immortal) mouse mammary line led to the ability to form tumors in nude mice in the absence of estrogen supplementation [241]. Transfection of an activated c-H-*ras* into a tumorigenic but non-metastatic mouse mammary carcinoma line led to increased metastasis capacity in syngeneic mice. Increased metastasis was measured by both lung colinization ability following intreavenous injection or spontaneous metastasis from subcutaneous tumors [240]. Thus, expression of *ras* mutations can allow malignant progression of tumors to estrogen dependent as well as independent and metastatic states. Overexpression of unmutated c-H-

Table 3. Detection of oncogene expression in mammary carcinoma

Species	Oncogene	System
Human	c-H-*ras*	tumor biopsies and cell lines
	c-*erb*B (EGF receptor)	ER negative tumor biopsies and cell lines
	c-*erb*B-2 (neu)	tumor biopsies and cell lines
	c-*myc*	tumor biopsies and cell lines
	c-*src*	tumor biopsies, ER positive cell lines
Rat	activated c-H-*ras*	carcinogen treated rats
Mouse	int 1, 2, etc.	MMTV insertional activation of cellular genes

ras in transfected murine fibroblasts can also lead to malignant transformation [242]. In human diploid fibroblasts it has been shown that nuclear microinjection of c-H-*ras* DNA directly induces DNA synthesis [243]. Inducible promoter systems have shown that expression of c-N-*ras* 20-50-fold over normal is required for complete transformation; somewhat higher than required for *ras* oncogenes activated by point mutation [243]. Transformation of fibroblasts by v-H-*ras* oncogene induces growth factor secretion in association with mitogenesis and transformation [1]. *Ras* oncogene expression in fibroblasts is also associated with increased metastatic behavior of the cells [245, 246]. In addition, cells transformed with an activated c-H-*ras* have been shown to be more prone to chromosomal rearrangements, suggesting that *ras* itself might be mutagenic and predispose cancer cells to mutations leading to more aggressive states of malignancy [247]. The mechanisms of these multiple effects of the *ras* oncogene is beyond the scope of the current article (see chapters by Ali, Groner *et al.* and Liotta *et al.*, this volume); the subject has been recently reviewed [116, 248].

To summarize, in mouse and rat model systems a case can be made for the underlying importance of steroidal hormones, particularly estrogen to allow carcinogen induction of tumors and to support the growth of tumors once they form. Probably more than one genetic event plus estrogen are minimal requirements for tumorigenesis. *Ras* is the most commonly activated oncogene in these rodent systems; activation or overexpression can lead to tumors, invasive-metastatic behavior, and chromosomal abberations.

At the present time, no such unifying statements can be made about human breast cancer. Rather, diverse observations of oncogene expression [249, 250] suggest a plethora of mechanisms at work in malignant progression (Table 3). In only one human breast cancer cell line, Hs578T, has an activated c-H-*ras* oncogene has been observed [251]. In another line, MCF-7, c-N-*ras* is gene amplified [252]. However this potential mechanism appears far from universal. NIH-3T3 fibroblasts have been utilized as recipients for DNA mediated transfection studies to identify activated oncogenes in human breast cancer [reviewed in 248]. This assay has been used extensively to identify activated *ras* oncogenes in other human tumors. Such studies with human breast cancer have been largely negative. In fact, one group found no codon 12 mutations of the c-H-*ras* or c-K-*ras* genes respectively in 32 and 64 breast tumors [253]. In transfection studies using MCF-7 cells, the only transforming activities definitively identified have been unmutated, amplified c-N-*ras* and two oncogenes rearranged to activate as an artifact of the transfection strategy [254–257]. In contrast, the most productive lines of investigation, outlined below, have concentrated on overexpression of protocellular oncogenes as an alternative strategy in understanding states of breast tumor malignancy. Perhaps future studies using mammary epithelial cells as transfection recipients for breast tumor DNA will uncover oncogenes which are not recognized by the 3T3 fibroblast assay.

C-*myc* codes for one of several related nuclear proteins which have been

implicated in neoplasia. The c-*myc* gene consistently has been shown to be altered in Burkitt's lymphoma [258]. C-*myc* is also expressed normally during PDGF induced transitions in the fibroblast cell cycle from G_0 to G_1 [259–261]. Recent studies using anti c-*myc* antibodies have suggested that this protooncogene is directly involved in DNA synthesis [262]. The gene for glutamate-pyruvate transaminase (GPT) was shown to be linked to a familial predisposition to human breast cancer and was known to be located on chromosome 8, the same chromosome as c-*myc* [263]. Kozbor and Croce examined the extent of c-*myc* amplification in five human breast cancer cell lines: SKBR-5, SKBR-3, Cama-1, MCF-7, and BT-20. They found that SKBR-3 had a c-*myc* gene amplified 4–8 fold and three of the other cell lines had a somewhat elevated level of c-*myc* expression. The significance of this was not established [264].

Another human breast cancer cell line, SW 613-S, was found to have amplified copies of the c-*myc* gene both on double minute chromosomes and integrated into chromosomes [265]. Passage of the cells as tumors in nude mice results in an increase in-*myc* copy number in the cell line. Since c-*myc* amplification appears to be co-selected with the capacity for the cells to grow as tumors *in vivo*, this experiment suggests a relationship between c-*myc* expression and tumorigenic capacity. A similar inference was made by the experiments with transgenic mice carrying a normal mouse c-*myc* gene under the control of the glucocorticoid-responsive murine mammary tumor virus (MMTV) long terminal repeat [266]. In these strains of mice carrying additional copies of the hybrid MMTV/*myc* gene in every cell and expressing high levels of MTV/*myc* mRNA in a number of tissues, the selective formation of mammary adenocarcinomas occurrs in females after more than one pregnancy.

A comprehensive study of the c-*myc* gene was conducted in 121 primary breast cancers [267]. This survey of c-*myc* revealed that the gene was amplified 2–15 fold in 32% of the samples tested. In five cases there was a nongermline restriction enzyme fragment, which in one case, was found to be due to a rearrangement distal to the third c-*myc* exon. Alterations of the c-*myc* gene correlated with invasive ductal histology and age of the patient. Ninety-five of the 121 specimens were from invasive ductal carcinomas and 40 of those had alterations of c-*myc*; benign fibroadenomas did not have amplified c-*myc*. The actual function of c-*myc* expression in breast cancer remains to be determined. One study has shown that a reduction in the growth rate of MCF-7 cells leads to a reduced c-*myc* expression. While expression of c-*myc* is not stimulated, c-*fos*, another nuclear oncogene, is induced by TGFα [268]. Another study has reported that c-*myc* is induced by estrogen treatment of MCF-7 or T47D cells through a posttranscriptional mechanism [269]. Finally, in neuroblastoma cells, N-*myc* amplification following gene transfer is associated with down modulation of MHC class I antigen expression [270]. C-*myc* expression may be associated with proliferation invasion and possibly other functions in early stage, invasive ductal carcinoma.

There are at least three closely related human ras genes, c-H-*ras* (on chromosome 11), c-K-*ras* (on chromosome 12), and c-N-*ras* (on chromosome 1) [271–272]. A number of studies have found c-H-*ras* expression to be elevated in invasive mammary carcinoma. In hyperplastic lesions or normal mammary tissue *ras* expression is lower than in malignant lesions [273–278]. Expression in cancers is heterogenous among primary and metastatic lesions. Although there is a trend toward higher expression in postmenopausal patients than in premenopausal patients, there is no correlation of expression with estrogen receptor status. However, one report has noted a trend toward higher TGFα content of tumors expressing the highest levels of *ras* protein [136].

The state of the c-H-*ras* gene in breast cancers has been examined with respect to genomic structure and allelic exclusion (see also chapter by Ali, *et al.*, this volume). Southern analysis of DNA from 104 breast cancer samples failed to uncover any evidence of rearrangement or amplification of c-H-*ras* [253]. The c-H-*ras* gene does have frequent BamHI restriction fragment length polymorphisms (RFLP). Among 51 patients who were heterozygous for these polymorphisms, 14 exhibited allelic exclusion in the tumor tissue. Although loss of the allele did not alter c-H-*ras* protein expression, it did correlate significantly with advanced histologic grade, lack of hormone receptors, and subsequent occurrence of distal metastases. Such a correlation, coupled with negative protein expression data, may reflect more general chromosomal instability rather than oncogenic activation or overexpression [see also reference 247]. Moreover, rare c-H-*ras* genotypes as determined by RFLP analysis were found much more frequently and common alleles were markedly diminished among the 104 breast cancer patients in this survey [279]. In summary, *ras* oncogene expression rearrangement appears to correlate with increased invasive/metastatic behavior of the tumor. Its expression appears to be independent of estrogen receptor expression in tumors.

The epidermal growth factor receptor is coded by the c-*erb*B oncogene (discussed below; see also chapters by Harris, *et al.* and Aaronson *et al.*) [280]. A gene closely related in structure to the EGF receptor, but probably distinct in function is c-*erb*B-2 (chromosome 17). From structural studies of the cloned gene it appears that c-*erb*B-2 is a transmembrane protein with an extracellular ligand binding domain and an intracellular tyrosine kinase site [281]. In carcinogen-induced murine neural tumors c-*erb*B-2 is activated by point mutation in the hydrophobic transmembrane domain of the molecule [282].

Amplification of c-*erb*B-2 appears to be relatively frequent in human breast cancer cell lines and tumor biopsies [283–287]. Amplification of this oncogene has been found only sporadically in other human tumors such as stomach, kidney, and salivary gland. In an extensive study by Slamon and co-workers [287], c-*erb*B-2 amplification was found to be a significant prognostic indicator of overall patient survival and time to relapse in breast cancer patients. While no association is observed between amplification and estrogen receptor status, amplification is significantly correlated with the number of positive lymph

nodes (an indication of metastatic progression). This study did not address either primary lesions versus metastatic disease lesion or expression of c-*erb*B-2. In common with c-*myc* and c-H-*ras* expression, c-*erb*B-2 expression appears to be related to increased invasive/metastatic behavior of the tumor rather than estrogen receptor status. c-*erb*B-2 appears to be significantly correlated with especially poor prognosis breast cancer [287].

Many of the known protooncogenes (like c-*erb*B-2) code for proteins with tyrosine kinase activity. The most extensively studied of these is c-*src* which codes for a 60 kDa phosphoprotein, $pp60^{c\text{-}src}$. This protein autophosphorylates its own tyrosine residues and those of a number of cellular proteins. Studies with the viral transforming gene, v-*src*, have shown that the kinase activity is required for the transforming potential of the protein [288]. Jacobs and Rubsamen assayed c-*src* activity in 21 human mammary carcinoma specimens [289]. To assay protein kinase activity they exploited the ability of the c-*src* molecule to phosphorylate the immunoglobulin used to carry out the immunoprecipitation. Nearly half the breast carcinomas tested (10/21) had elevated levels of c-*src* activity in comparison to one normal specimen. When Rosen and co-workers [290] utilized assays of both c-*src* autophosphorylation and phosphorylation of casein they found that three hormone-dependent breast cancer cell lines but not two hormone-independent lines, had elevated activity. Western blot analysis of the cellular proteins demonstrated that the amount of c-*src* protein did not differ between the cells with high and low kinase activity. Moreover, analysis of a breast tumor and adjacent normal tissue showed that both normal and neoplastic tissues had the same levels of c-*src* protein but the tumors had markedly elevated kinase activity. Future studies with more tumors of defined estrogen receptor status are required to evaluate the hypothesis that c-*src* is expressed in more differentiated, estrogen receptor positive breast cancer.

In contrast to c-*src*, c-*erb*B (the EGF receptor) is expressed to a greater extent in estrogen receptor negative cell lines and tumor biopsies (see chapter by A. Harris *et al.* this volume) [156, 291–298]. It appears to be a marker for differentiation as well as invasion/metastatis in breast cancer. Results from recent studies using cells grown *in vitro* suggest that the mechanism for the high level of EGF receptor expression may be either transcriptional activation [156] or (more rarely) gene amplification [297].

In breast tumor biopsies, overexpression of the EGF receptor protein is more common than amplification of the gene [287]. However, MDA-MB-468 cells (see Table 3) have an amplified c-*erb*B and a subclone analysis has determined how c-*erb*B amplification and expression relate to tumorigenic status. In these cells, loss of c-*erb*B gene amplification and expression are inversely related to tumor growth rate in the nude mouse (though tumors formed in all cases) [298]. In a vulvar carcinoma line (A431) subclone differences in c-*erb*B receptor expression parallel tumorigenic status of cells [299]. In bladder cancer c-*erb*B correlates with tumor invasiveness [300], but in melanoma, overexpression seems to be a marker for dedifferentiation [301].

In the most complete study of tumor specimens to date, relapse free survival and overall survival were worse for c-*erb*B positive than estrogen receptor negative tumors. There was a significant inverse relationship between c-*erb*B and estrogen receptor expression [296]. It is not yet known whether overexpression of the c-*erb*B protein in cancer directly contributes to the transformed phenotype or indirectly mediates the effects of EGF (or TGFα) produced in an autocrine loop. In summary, c-*erb*B appears to be an important indicator of dedifferentiation and poor prognosis in breast cancer. Its overexpression may be the functional equivalent of the activation present in v-*erb*B.

12. *Ras* oncogene and hormonal autonomy: model systems

The diverse observations concerning oncogene activation and cellular protooncogene overexpression suggest that many mechanisms may exist in the malignant progression of breast cancer. Alternatively, observations of cellular protooncogene expression could simply reflect the malignant state rather than be the cause of its induction. Oncogene activity in breast cancer could be investigated by directly inserting the oncogene of interest into a breast epithelial cell test system. Thus, normal diploid human mammary epithelium first immortalized with brief treatment with the carcinogen benzo[a]pyrene and then transfected with oncogenes [302]. Stampfer (see chapter, this volume) has observed that treatment of normal mammary epithelial cells in culture with benzo[a]pyrene conferred immortalization but not tumorigenicity in the nude mouse are well-differentiated and karyotypically stable [303], but the event induced by the carcinogen to confer immortality remains unknown. Using retroviral vectors, Clarke has inserted various oncogenes into one of these lines to determine the phenotype effects. Insertion of either v-H-*ras*, v-*mos*, or simian virus 40 T antigen (SV40 T) renders the cells capable of growth in high concentrations of serum, but does not confer tumorigenicity in the nude mouse. Transfectants containing v-H-*ras* plus either SV40 T or v-*mos* are strongly tumorigenic in nude mice.

Primary rabbit mammary epithelial cells have also been immortalized and established in culture, by microinjection with SV 40 DNA. The rabbit cells were not tumorigenic in nude mice unless an activated human c-H-*ras* gene was coinjected with the SV40 DNA [304]. Thus, it appears that normal or nonmalignant mammary epithelium of several species [238, 302–304] can be converted to an estrogen independent-tumorigenic state with at least two genetic manipulations. In each of the three cases cited, transfection of an activated *ras* oncogene into the system allowed expression of the malignant phenotype.

In many cases, breast cancer patients present with tumors which are initially responsive to hormonal therapy such as antiestrogens. Following endocrine therapy, the tumors generally become hormone unresponsive although some

will respond to second and even third hormone treatments. We wished to develop a model system to study the conversion of a cell line from hormone responsive to independent. For this purpose we chose to transfect DNA from the tumor-causing Harvey sarcoma retrovirus to MCF-7 cells. The tumor-inducing portion of this viral DNA (v-H-*ras*) is closely related to the most commonly detected protooncogene in some highly malignant human cancers (but not breast cancer—see previous section). MCF-7 cells do not initially contain an activated c-H-*ras* oncogene. The v-H-*ras* oncogene was incorporated in MCF-7 cells by the calcium phosphate method [305].

MCF-7 cells containing stably integrated v-H-*ras* genes in their DNA ($MCF\text{-}7_{ras}$) express 5–8 times the level of mRNA present in control cells and have detectable phosphorylated v-H-*ras* protein. The ability of $MCF\text{-}7_{ras}$ cells to respond to stimulation estrogen and antiestrogens *in vitro* was blunted. While non-transfected cells failed to produce tumors in nude mice in the absence of E_2 supplementation, the transfected cells were tumorigenic in the absence of estrogen in 85% of inoculated, oophorectomized females [305]. $MCF\text{-}7_{ras}$ cells also expressed increased levels of the laminin receptor on their surfaces [84] and several unique intracellular proteins [306]. They also exhibit increased rates of turnover of phosphatidyl inositol, which occurs following E_2 treatment of MCF-7 cells [61]. Increased phosphatidyl inositol turnover is known to generate diacylglycerol, an inducer of protein kinase C and inositol triphoshate, a stimulator of Ca^{++} flux in the cell [307]. Ca^{++} flux probably has numerous physiological consequences including contributing to activation of protein kinase C and calcium-calmodulin protein kinase [307]. It is not yet known what the physiological relevance of these two signalling pathways might be in breast cancer. Protein kinase C has now been cloned and sequenced; it is a family of related enzymes whose detailed function is the subject of intensive, current investigation [308, 309]. One study has proposed that the *ras* oncogenes functionally couple growth factor receptors to phosphatidyl inositol turnover, though it is not yet known if the coupling is direct such as for adenylate cyclase-G protein interaction [310].

Growth factor secretion was also studied in $MCF\text{-}7_{ras}$ cells [311]. $MCF\text{-}7_{ras}$ cells were cocultured with MCF-7 cells which lacked the active oncogene. $MCF\text{-}7_{ras}$ cells growing at high density secrete growth promoting activity for MCF-7 cells seeded at clonal density. However, $MCF\text{-}7_{ras}$ cells are substituted by the parental MCF-7 cells, at high density the colony formation of the indicator MCF-7 cells at clonal is significantly lower. Conditioned medium prepared from $MCF\text{-}7_{ras}$ cultures contains 3–4 fold evlevated concentrations of radioreceptor assayable and bioactive TGFα in comparison with conditioned medium from a control transfectant cell line. On gel exclusion chromatography of $MCF\text{-}7_{ras}$ conditioned medium, a single peak of TGFα-like activity elutes with an apparent molecular weight of 30 kDa. Furthermore, secretion of immunoreactive IGF-I and TGFβ were elevated 3–4 fold in $MCF\text{-}7_{ras}$ cells but PDGF secretion was unaltered. A similar induction of TGFα was observed in mouse mammary cells induced to hormone independent tumori-

genesis with the *ras* oncogene [312]. It should be noted, however, that *ras* activation is not always associated with TGFα secretion in breast cancer. For instance, the human breast carcinosarcoma line Hs578T expresses the activated c-H-*ras* protein but does not synthesize detectable TGFα mRNA or protein [107, 135, 144, 251].

The published data on expression of an activated *ras* oncogene in MCF-7 cells suggests that its activation can bring about phenotypic and tumorigenic changes in human breast cancer cells. Some of these changes are also induced by estrogens. However, the cells retain the capacity to bind estrogen and respond to estrogens as indicated by estrogenic induction of the progesterone receptor [305]. Thus, *ras* gene transfection may partially bypass estrogen activation of the transformed phenotype but by a pathway which appears to be similar but not identical to the E_2 induction pathway. Future studies are required to more clearly define the similarities and differences between estrogenic and v-H-*ras* induced malignant progression of MCF-7 cells.

14. Summary and future prospects

We have emphasized some of the literature pointing to the role of estrogen, polypeptide growth factors and their receptors as regulators of breast cancer growth and malignant progression. We have used estrogen/antiestrogen regulated breast cancer cell lines growing *in vitro* as a model system to determine which growth factor modulations were consistent with a mediating or modulating role in cellular proliferation. At present, data from a number of laboratories suggests that TGFα might be an important autostimulatory and TGFβ an autoinhibitory component of growth regulation. The roles of IGF-I, PDGF, and a variety of other secreted growth factors are less certain. We also summarized the importance *in vivo* of paracrine endothelial and stromal interactions with the tumor to support its growth. New breast cancer therapies could be designed on the basis of interrupting the growth stimulatory TGFα, enhancing growth inhibitory effects of TGFβ, or blocking paracrine interactions.

While paracrine acting hormones are more difficult to identify, they might be promising targets of antineoplastic therapy. For example, in colon cancer, a new angiogenic factor known as angiogenin has just been discovered [168]. It does not appear to be related to systems of angiogenesis in normal tissues [166] and may be tumor specific. A major obstacle in utilizing such antigrowth factor strategies is the lack of information on the role of growth factors in the proliferation and function of normal tissues. However, such approaches have succeeded in other experimental tumor systems utilizing antigrowth factor and antireceptor antibodies [313, 314].

We have also presented evidence implicating protooncogenes expression in some steps in the malignant progression of normal tissue to cancer. Thus, c-*myc*, c-H-*ras*, and c-*erb*B-2 appear to relate to the invasive state of the tumor

without regard to estrogen receptor status. In *ras* gene transfection experiments it has been possible to induce MCF-7 and other breast epithelial cells to secrete increased amounts of growth factors and to become partially autonomous from estrogen controls. The c-*src* kinase is expressed in estrogen receptor-containing breast cancer cell lines and high levels of c-*erb*B (EGF receptor) in estrogen receptor negative cell lines and tumor biopsies. The overexpression of both receptors and growth factors might be mechanisms of malignant progression towards estrogen autonomy. Future chemotherapeutic approaches could utilize anti-receptor molecules conjugated with toxins to produce tumor-specific agents. These approaches have produced encouraging preliminary results in animal model systems [315, 316].

References

1. Goustin AS, Leof EB, Shipley GD, Moses HL: Growth factors and cancer. Cancer Res 46:1015–1029, 1986.
2. Lippman ME: Endocrine responsive cancers of man. In: Textbook of Endocrinology, Williams RH (ed), Philadelphia, W.B. Saunders Co. 1985, pp 1309–1326.
3. The cancer and steroid hormone study of the centers for disease control and the National Institute of Child Health and Human Development. Oral contraceptive use and the risk of breast cancer. New Eng J Med 315:405–411, 1986.
4. Ross GT, Vande Wiele RL, Frantz AG: The ovaries and the Breasts. In: Textbook of Endocrinology, Williams, RH (ed) W.B. Saunders Co., Philadelphia, 1981, pp 355–411.
5. Eidne KA, Flanagan CA, Millar RP: Gonadotropin—releasing hormone binding sites in human breast carcinoma. Science 229:989–991, 1985.
6. Ikeda T, Danielpour D, Sirbasku BA: Isolation and properties of endocrine and autocrine type mammary tumor cell growth factors (estromedins). In: Bresciani F, King RJB, Lippman ME, Namer M, Raynaud JP (eds). Progress in Cancer Research and Therapy, Vol. 31, Raven Press, New York, 1983, pp 171–186.
7. Soto A, Sonnenschein, C: Cell proliferation of estrogen-sensitive cells: the case for negative control. Endocrine Reviews, 8:44–52, 1987.
8. Lykkesfeldt AE, Briand, P: Indirect mechanism of estradiol stimulation of cell proliferation of human breast cancer cell lines. Br J Cancer 53:29–35, 1986.
9. Brooks SC, Locke ER, Soule HD: Estrogen receptor in a human breast cell line (MCF-7) from breast carcinoma. J Biol Chem 248:6251–6261, 1973.
10. Lippman ME, Bolan G, Huff K: The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long term culture. Cancer Res 36:4595–4601, 1976.
11. Page MJ, Field JK, Everett NP, Green CD: Serumregulation of the estrogen responsiveness of the human breast cancer cell line MCF-7. Cancer Res 43:1244–1249, 1983.
12. Darbe P, Yates J, Curtis S, King RJB: Effect of estradiol on human breast cancer cells in culture. Cancer Res 43:349–354, 1983.
13. Chablos D, Vignon F, Keydar I, Rochefort H: Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). J Clin Endocrinol Metab 55:276–283, 1982.
14. Soule HD, McGrath CM: Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. Cancer Lett 10:177–189, 1980.
15. Leung BS, Qureshi S, Leung JS: Response to estrogen by the human mammary carcinoma cell line CAMA-1. Cancer Res 42:5060–5066, 1982.
16. Reiner GCA, Katzenellenbogen BS: Characterization of estrogen and progesterone re-

ceptors in MDA-MB-134 human breast cancer cells. Cancer Res 46:1124–1131, 1986.
17. Engle LW, Young NW: Human breast carcinoma cells in continuous culture: a review. Cancer Res 38:4327–4339, 1978.
18. Katzenellenbogen B, Norman MJ, Eckert RL, Peltz SW, Mangel WF: Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxy-tamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res 44:112–119, 1983.
19. Butler WB, Kelsey WH, Goran N: Effects of serum and insulin on the sensitivity of the human breast cancer cell line MCF-7 to estrogens and antiestrogens. Cancer Res 41:82–88, 1981.
20. Edwards DP, Murphy SR, McGuire WI: Effect of estrogen and antiestrogen on DNA polymerase in human breast cancer. Cancer Res 40: 1722–1726, 1980.
21. Natoli C, Sica G, Natoli V, Serra A, Iacobelli S: Two new estrogen-supersensitive variants of the MCF-7 human breast cancer cell line. Breast Cancer Res Treat 3:23–32, 1983.
22. Weichselbaum RW, Hellman S, Piro A, *et al.* Proliferation kinetics of a human breast cancer cell line *in vitro* following treatment with 17B-estradiol and 1-B-o-arabinofuran-asylcytosine. Cancer Res 38:2339–2342, 1978.
23. Simon WE, Albrecht M, Trams G, *et al. In vitro* growth promotion of human mammary carcinoma cells by steroid hormones, tamoxifen, and prolactin. J Natl Cancer Inst 73: 313–321, 1984.
24. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS: Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500, 1986.
25. Whitehead RH, Quirk SJ, Vitali AA, Funder JW, Sutherland RL, and Murphy LC. A new human breast carcinoma cell line (PMC42) with stem cell characteristics. III. Hormone receptor status and responsiveness. J Natl Cancer Inst 73:643–648, 1984.
26. Dempsey PJ, Feleppa FP, Brown RW, deKretser TA, Whitehead, RH, Jose DG: Development of monoclonal antibodies to the human breast carcinoma cell line PMC42. J Natl Cancer Inst 77:1–15, 1986.
27. Jensen EV: Studies of growth phenomenon using tritium-labeled steroids. Proc. 4th Int. Congress of Biochem, Pergamon Press, Vienna, Vol. 15, 1958, p 119.
28. Dickson RB, Clark CR: Estrogen receptors in the male. Arch Androl 7:205–217, 1981.
29. Strobl JS, Thompson EB: Mechanisms of steroid hormone action. In: Sex Steroid Receptors, Auricchio F (ed). Field Educational Halia Acta Medica, Rome, 1985, pp 9–36.
30. Jensen EV, Desombre ER: Mechanism of action of the female sex hormones. Ann Rev Biochem 41:203–230, 1972.
31. Zava DT, McGuire WI: Estrogen receptor: unoccupied sites in nuclei of a breast tumor cell line. J Biol Chem 252:2703–3708, 1977.
32. Edwards PP, Martin PM, Horwitz KB, Chamness GC, McGuire WL: Subcellular compartmentalization of estrogen receptors in human breast cancer cells. Exp Cell Res 127:197–213, 1980.
33. King WJ, Greene GL: Monoclonal antibodies localize estrogen receptor in the nuclei of target cells. Nature 307:745–747, 1984.
34. Welshons WV, Lieberman ME, Gorski J: Nuclear localization of unoccupied estrogen receptors. Nature 307:747–749, 1984.
35. Puca GA, Sica V, Nola E: Identification of a high affinity nuclear acceptor site for estrogen receptor of calf uterus. Proc Natl Acad USA 171:979–83, 1974.
36. Spelsberg TC, Webster RA, Pikler GM: Chromosomal proteins regulate steroid binding to chromatin. Nature 262:65–67, 1976.
37. Barrack ER, Coffey DS: The specific binding of estrogen and androgens to the nuclear matrix of sex hormone responsive tissues. J Biol Chem, 255:7265–75, 1980.
38. Pardoll DM, Vogelstein B, Coffey DS: A fixed site of DNA replication in eucaryotic cells. Cell 19:527–36, 1980.
39. Robinson SI, Nelkin BD, Vogelstein B: The ovalbumin gene is associated with the nuclear

matrix of chicken oviduct cells. Cell, 28: 99–106, 1985.
40. Schuh S, Yamemoto W, Brugge J, Bauer VJ, Riehl RM, Sullivan WP, Toft DO: A 90,000 dalton binding protein common to both steroid receptors and the rous sarcoma virus transforming protein $pp60^{v\text{-}src}$. J Biol Chem 260:14292–14296, 1985.
41. Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch, J-M, Straub A, Jensen E, Scrace G, Waterfield M, Chambon P: Cloning of the human estrogen receptor cDNA. Proc Natl Acad Sci USA, 82:889–893, 1985.
42. Giguere SM, Hollenberg SM, Rosenfeld MG, Evans RM, Functional domains of the human glucocorticoid receptor. Cell 46: 645–652, 1986.
43. Green S, Walter P, Kumar V, Krust A, Bornertt J-M, Argos P, Chambon P: Human oestrogen receptor cDNA: Sequence, expression and homology to v-*erb* A. Nature 320: 134–139, 1986.
44. Greene GL, Gilna P, Waterfield M, Baker H, Hort Y, Shine J: Sequence and expression of human estrogen receptor complementary DNA. Science 231:1150–1154, 1986.
45. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM: The c-*erb* A gene encodes a thyroid hormone receptor. Nature 234:641–646, 1986.
46. Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B: The c-*erb* A protein is a high-affinity receptor for thyroid hormone. Nature 234:635–640, 1986.
47. Lumar V, Green S, Stark A, Chambon P: Localization of the oestrodial-binding and putative DNA-binding domains of the human oestrogen receptor. EMBO 5:2231–2236, 1986.
48. Druege PM, Klein-Hitpass L, Green S, Stock G, Chambon P, Ryffel GU: Introduction of estrogen-responsiveness into mammalian cell lines. Nucleic Acids Res 14:9329–9337, 1986.
49. Migliaccio A, Rotondi A, Auricchio F: Calmodulin-stimulated phosphorylation of 17β-estradiol receptor on tyrosine. Proc Natl Acad Sci USA, 81:5921–5925, 1985.
50. Auricchio F, Migliaccio A, Castoria G, Rotondi A: Regulation of hormone binding of 17β-estradiol receptor by phosphorylation-dephosphorylation of receptor on tyrosine. In: Sex. Steroid Receptors, Auricchio F (ed), Field Educational Italia Acta Medica, Rome, 1985, pp 98–107.
51. Migliaccio A, Rotondi A, Auricchio F: Estradiol receptor: phosphorylation on tyrosine in uterus and interaction with anti-phosphotyrosine antibody. EMBO 5:2867–2872, 1986.
52. Fleming H, Blumenthal R, Gurpide E: Rapid change in specific estrogen binding elicited by cGMP or cAMP in cytosol from human endometrial cells. Proc Natl Acad Sci USA, 80:2486–2490, 1983.
53. Knabbe CK, Lippman ME, Greene GL, Dickson RB: Phorbol ester induced phosphorylation of the estrogen receptor in intact MCF-7 human breast cancer cells. Fed Proc 45: 1899 Washington DC, 1986 (Abstract #2437).
54. Darbon J-M, Valette A, Bayard F: Phorbol esters inhibit the proliferation of MCF-7 cells. Biochem Pharmacol 35:2683–2686, 1986.
55. Hanover JA, Dickson RB: The possible link between receptor phosphorylation and internalization. Trends Phar Sci 6:164–167, 1985.
56. Aitken SC, Lippman ME: Hormonal regulation of *de novo* pyrimidine synthesis and utilization in human breast cancer cells in tissue culture. Cancer Res 43: 4681–4690, 1983.
57. Aitken SC, Lippman ME: Effect of estrogens and antiestrogens on growth-regulatory enzymes in human breast cancer cells in tissue culture. Cancer Res 45:1611–1620, 1985.
58. Cowan K, Levine R, Aitken S, Goldsmith M, Douglass E, Clendeninn N, Nienhuis A, Lippman ME. Dihydrofolate reductase gene amplification and possible rearrangement in estrogen-responsive methotrexate resistant human breast cancer cells. J Biol Chem 257: 15079–15086, 1982.
59. Kasid A, Davidson N, Gelmann E, Lippman ME: Transcriptional control of thymidine kinase gene expression by estrogens and antiestrogens in MCF-7 human breast cancer cells. J Biol Chem 261:5562–5567, 1986.
60. Aitken SC, Lippman ME, Kasid A, Schoenberg DR: Relationship between the expression of estrogen regulated genes and estrogen-stimulated proliferation of MCF-7 mammary

tumor cells. Cancer Res 45:2608–2615, 1985.
61. Freter CE, Lippman ME, Cheville A, Zinn S and Gelmann EP: Alterations in phosphoinositide metabolism associated with 17-β estradiol and growth factor treatment of MCF-7 breast cancer cells. Mol Encocrin, in press, 1988.
62. Carney DH, Scott DL, Gordon EA, LaBelle EF: Phosphoinositides in mitogenesis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. Cell 42:479–488, 1985.
63. Nishizuka Y: Protein kinases in a signal transduction. Trends Biochem Sci 9:163–171, 1984.
64. Horwitz KB, McGuire WL: Estrogen control of progesterone receptor in human breast cancer. J Biol Chem 253:2223–2228, 1978.
65. Chablos D, Rochefort H: Dual effects of progestin R5020 on proteins released by the T47D human breast cancer cells. J Biol Chem 259:1231–1238, 1984.
66. Liotta LA, Rao CN, Weiner UM: Biochemical interactions of tumor cells with the basement membrane. Ann Rev Biochem 55:1037–1057, 1986.
67. Terranova VP, Hujanen ES, Martin GR: Basement membrane and the invasive activity of metastatic tumor cells. J Natl Cancer Inst 177:311–316, 1986.
68. Kaufman U, Zapf J, Torretti B, Froesch ER: Demonstration of a specific serum carrier protein of nonsuppressible insulin-like activity *in vivo*. J Clin Endocrinol Metab 44:160–166, 1977.
69. Neufeld EF, Ashwell G: In: The Biochemistry of Glycoproteins and Proteoglycans, Lennarz WJ (ed) Plenum Press, New York, 1980, pp 241–266.
70. Stoppelli MP, Tacchetti C, Cubellis MV, Corti A, Hearing VJ, Cassani G, Appella E, Blasi F: Autocrine saturation of prourokinase receptors on human A431 cells. Cell 45:675–684, 1986.
71. Butler WB, Kirkland WL, Jorgensen TL: Induction of plasminogen activator by estrogen in a human breast cancer cell line (MCF-7). Biochem Biophys Res Comm 90:1328–1334, 1979.
72. Ciocca DR, Adams DJ, Edwards DP, Bjerke RJ, McGuire WL: Distribution of an estrogen induced protein with a molecular weight of 24,000 in normal and malignant human tissues and cells. Cancer Res 43:1204–1210, 1983.
73. Westley B, Rochefort H: A secreted glycoprotein induced by estrogen in human breast cancer cell lines. Cell 20:353–362, 1980.
74. Bronzert DA, Silverman S, Lippman ME: Estrogen inhibtion of a Mr 39,000 glycoprotein secreted by human breast cancer cells. Cancer Res 47:1234–1238, 1987.
75. Sheen YY Katzenellenbogen BS: Antiestrogen stimulation of the production of a 37,000 molecular weight secreted protein and estrogen stimulation of the production of a 32,000 molecular weight secreted protein in MCF-7 human breast cancer cells. Endocrinology 120: 1140–1151, 1987.
76. Jakolew SB, Breathnack R, Jeltsch J, Chambon P: Sequence of the pS2 mRNA induced estrogen in the human breast cancer cell line MCF-7. Nucleic Acids Res 12:2861–2874, 1984.
77. May FEB, Westley BR: Cloning of estrogen-regulated messenger RNA sequences from human breast cancer cells. Cancer Res 46:6034–6040, 1986.
78. Burke RE, Harris SC, McGuire WL: Lactate dehydrogenase in estrogen responsive human breast cancer cells. Cancer Res 38:2773–2780, 1978
79. Vignon F, Capony F, Chambon M, Freiss L, Garcia M, Rochefort H: Autocrine growth stimulation of the MCF-7 breast cancer cell by the estrogen regulated 52K protein. Endocrinology 118:1537–1545, 1986.
80. Capony F, Morisset M, Barrett AJ, Capony JP, Broquet P, Vignon F, Chambon M, Louisot P, Rochefort H: Phosphorylation, glycosylation, and proteolytic activity of the 52-kD estrogen-induced protein secreted by MCF-7 cells. J. Cell Biol 104:253–262, 1987.
81. Davison NE, Bronzert DA, Chambon P, Gelmann EP, Lippman, ME: Use of two MCF-7 cell variants to evaluate the growth regulatory potential of estrogen-induced products. Cancer Res 46:1904–1908, 1986.

82. Bronzert DA, Greene GL, Lippman ME: Selection and characterization of breast cancer cell line resistant to the antiestrogen LY 117018. Endocrinology 117:1409–1417, 1985.
83. Bronzert DA, Triche TJ, Gleason P, Lippman ME: Isolation and characterization of an estrogen-inhibited variant derived from the MCF-7 breast cancer cell line. Cancer Res 44:3942–3951, 1984.
84. Albini A, Graf JO, Kitten T, Kleinman HK, Martin GR, Veillete A, Lippman ME: Estrogen and v-*ras*^{H} transfection regulate the interactions of MCF-7 breast carcinoma cells to basement membrane. Proc Natl Acad Sci USA 83:8182–8186, 1986.
85. Sapino A, Peitribiasi F, Bussolati G, Marchiso PC: Estrogen and tamoxifen-induced rearrangement of cytoskeletal and adhesion structures in breast cancer MCF-7 cells. Cancer Res 46:2526–2531, 1986.
86. Vignon F, Derocq DF, Chambon M, Rochefort H: Estrogen induced proteins secreted by the MCF-7 human breast cancer cells stimulated their proliferation. CR Acad Sci Paris Endocrinol 296:151–157, 1983.
87. Nowell PC: Mechanisms of tumor progression. Cancer Res 46:2203–2207, 1986.
88. Zwiebel JA, Bano M, Nexo E, Salomon P, Kidwell WR: Partial purification of transforming growth factors from human milk. Cancer Res 46:933–939, 1986.
89. Carpenter G: Epidermal growth factor is a major growth promoting agent in human milk. Science 210:198–199, 1980.
90. Lippman ME, Buzdar A, Tormey DC, McGuire WL: Combining endorcine and chemotherapy any true benefits? Breast Cancer Res Treat 4:251–259, 1985.
91. Jordan VC: Biochemical pharmacology of antiestrogen action. Pharmacol Rev 36:245–276, 1984.
92. Watts CKW, Murphy LC, Sutherland RL: Microsomal binding sites for nonsteroidal antiestrogens in MCF-7 human mammary carcinoma cells. J Biol Chem 259:4223–4229, 1984.
93. Rochefort H: Do antiestrogens and antiprogestins act as hormone antagonists or receptor-targeted drugs in breast cancer? Trends Pharmacol Sci 8:126–128, 1987.
94. O'Brian CA, Liskamp RM, Solomon DH, Weinstein IB: Inhibition of protein kinase C by tamoxifen. Cancer Res 45:2462–2465, 1985.
95. Sutherland RL, Hall RE, Taylor IW: Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on exponentially growing and plateau phase cells. Cancer Res 43:3998–4006, 1983.
96. Osborne CK, Hobbs K, Clark GM: Effects of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 45:584–590, 1985.
97. Osborne CK, Boldt DH, Clark GM, Trent JM: Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G_1 phase. Cancer Res 43:3583–3585, 1983.
98. Robinson SP, Jordan VC. Metabolism of steroid-modifying anticancer agents. Pharmac Therap 36:41–103, 1988.
99. Wakeling AE, Bowler J: Steroidal pure antioestrogens. J Endocrin 112:R7–R10, 1987.
100. Lippman ME: Definition of hormones and growth factors required for optimal proliferation and expression of phenotypic responses in human breast cancer cells In: Cell Culture Methods for Molecular and Cell Biology, Vol. 2, Barnes DW, Sirbasku DA, Sato GH (eds), Alan R. Liss, New York, 1984, pp 183–200.
101. Eisman JA, Martin TJ, MacIntyre I, Framptin RJ, Moseley JM, Whitehead R: 1,25-dihydroxyvitamin D^3 receptor in a cultured breast cancer cell line (MCF-7 cells). Biochem Biophys Res Commun 93:9–18, 1980.
102. Furlanetto RW, DiCarlo JN: Somatomedin C receptors and growth effects in human breast cells maintained in long-term culture. Cancer Res 44:2122–2128, 1984.
103. Myal Y, Shiu RPC, Bhawmick B, Bala M: Receptor binding and growth promoting activity of insulin-like growth factors in human breast cancer cells (T47D) in culture. Cancer Res 44:5486–5490, 1984.
104. Dickson RB, Lippman ME: Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocr Rev 8:29–43, 1987.

105. Setyono-Han B, Henkelman MS, Foekens JA, Klijn JGM: Direct inhibitory effects of somatostatin (analogues) on the growth of human breast cancer cells. Cancer Res 47:1566–1570, 1987.
106. Ikeda T, Sirbasku DA: Purification and properties of a mammary-uterine-pituitary tumor cell growth factor from pregnant sheep uterus. J Biol Chem 259:4049–4964, 1984.
107. Bates SE, McManaway ME, Lippman ME, Dickson RB: Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res 46:1707–1713, 1986.
108. Huseby RA, Maloney TM, McGrath CM: Evidence for a direct growth-stimulating effect of estradiol on human MCF-7 cells *in vivo*. Cancer Res 44:2654–2659, 1984.
109. McGrath CM: Augmentation of the response of normal mammary epithelial cells to estradiol by mammary stroma. Cancer Res 43:1355–1360, 1983.
110. Haslam SZ: Mammary fibroblast influence on normal mouse mammary epithelial cell responses to estrogen *in vitro*. Cancer Res 46:310–316, 1986.
111. Durnberger H, Heuberger B, Schwartz P, Wasner G, Kratochwil, K: Mesenchyme-mediated effect of testosterone on embryonic mammary epithelium. Cancer Res 38:4066–4070, 1978.
112. Jakesz R, Smith CA, Aitken S, Huff KK, Schuette W, Schackney S, Lippman ME: Influence of cell proliferation and cell cycle phase on expression of estrogen receptor in MCF-7 breast cancer cells. Cancer Res 44:619–625, 1984.
113. Dickson RB, Huff KK, Spencer EM, Lippman ME: Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinology 118:138–142, 1986.
114. Dickson RB, McManaway M, Lippman ME: Estrogen induced factors of breast cancer cells partially replace estrogen to promote tumor growth. Science 232:1540–1543, 1986.
115. Smith HS, Scher CD, Todaro GJ: Induction of cell division in medium lacking serum growth factor by SV40. Virology 44:359–370, 1971.
116. Heldin CH, Westermark B: Growth factors: mechanism of action and relations to oncogenes. Cell 37:9–20, 1984.
117. Clemmons DR, Shaw DS: Variables controlling somatomedin production by cultured human fibroblasts. J Cell Physiol 115:137–143, 1983.
118. Clemmons DR, van Wyk JJ: Evidence for a functional role of endogenously produced somatomedin-like peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. J Clin Invest 75:1914–1918, 1986.
119. Cherington PV, Smith BL, Pardee AB: Loss of epidermal growth factor requirement and malignant transformation. Proc Natl Acad Sci USA 76:3937–3942, 1979.
120. Bradshaw GL, Dubes GR: Polyoma virus transformation of rat kidney fibroblasts results in loss of requirement for insulin and retinoic acid. J Gen Virol 64:2311–2315, 1984.
121. Zhan X, Goldfarb M: Growth factor requirements of oncogene-transformed NIH 3T3 and Balb/c 3T3 cells cultured in defined media. Mol Cell Biol 6:3541–3544, 1986.
122. Sporn MB, Todaro GJ: Autocrine secretion and malignant transformation of cells. N Engl J Med 303:878–880, 1980.
123. Delarco JE, Todaro GJ: Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci USA 75:4001–4005, 1978.
124. Freedman VH, Shin S: Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell 3:355–359, 1974.
125. Assoian RK, Grotendorst GR, Miller DM, Sporn MB: Cellular transformation by co-ordinated action of three peptide growth factors from human platelets. Nature (London) 309:804–806, 1984.
126. Massague J, Kelly B, Mottola C: Stimulation by insulin-like growth factors is required for cellular transformation by type transforming growth factor. J Biol Chem 260:4551–4554, 1985.
127. Hammond SL, Ham RG, Stampfer MR: Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary

extract. Proc Natl Acad Sci USA 81:5435–5439, 1984.
128. Tucker RF, Shipley GD, Moses HL, Holley RW: Growth inhibitor from BSC-1 cells closely related to platelet type β transforming growth factor. Science 226:705–707, 1984.
129. Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB: Type β transforming growth factor: a bifunctional regulator of cellular growth. Proc Natl Acad Sci USA 82:119–123, 1985.
130. Halper J, Moses HL: Purification and characterization of a novel transforming growth factor Cancer Res 47:4552–4559, 1987.
131. Salomon DS, Zweibel JA, Bano M, Losonczy I, Felnel P, Kidwell WR: Presence of transforming growth factors in human breast cancer cells. Cancer Res 44:4069–4077, 1984.
132. Knabbe C, Lippman ME, Wakefield L, Flanders K, Kasid A, Derynck R, Dickson RB: Evidence that TGFβ is a hormonally regulated negative growth factor in human breast cancer. Cell 48:417–428, 1987.
133. Nickell KA, Halper J, Moses HL: Transforming growth factors in solid human malignant neoplasms. Cancer Res 43:1966–1971, 1983.
134. Valverius E, Bates SE, Salomon DS, Stampfer M, Clark R, McCormick F, Lippman ME, Dickson RB: Production of transforming growth factor alpha by human mammary epithelial cells and sublines. Proceedings of the Annual Meeting of the Endocrine Society, Indianapolis, Ind, p. 97, 1987 (Abstract #306).
135. Bates SE, Valverius E, Salomon D, Stampfer M, Lippman ME, Dickson RB: Expression and estrogen regulation of transforming growth factor α (TGFα) mRNA in human breast cancer. Proceedings of the Annual Meeting of the American Association for Cancer Research, Atlanta, Ga, 28:240, 1987.
136. Perroteau E, Salomon D, Debortali M, Kidwell W, Hazarika P, Pardue R, Dedman J, Tam J: Immunological detection and quantitation of alpha transforming growth factors in human breast carcinoma cells. Breast Cancer Res Treat 7:201–210, 1986.
137. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV: Human transforming growth factor-α: precursor structure and expression in *E. coli*. Cell 38:287–297, 1984.
138. Ignotz RA, Kelly B, Davis RJ, Massague J: Biologically active precursor for transforming growth factor type α released by retrovirally transformed cells. Proc Natl Acad Sci USA, 83:6307–6311, 1986.
139. Bringman TS, Lindquist PB, Derynck R: Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell 48:429–440, 1987.
140. Gentry LE, Twardzik DR, Lim GJ, Ranchalis JE, Lee DC: Expression and characterization of transforming growth factor precursor protein in transfected mammalian cells. Mol Cell Biol 7:1585–1591, 1987.
141. Derynck R, Roberts AB, Eaton DH, Winkler MC, Goeddel DV: Human transforming growth factor α precursor sequence gene structure, and heterologous expression. In: Cancer Cells 3: Growth factors and transformation, Feramisco J, Ozanne B, Stiles C (eds), Cold Spring Harbor Laboratory, New York pp 79–86, 1985.
142. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD Bringman TS, Berger WH: Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res 47:707–712, 1982.
143. Hackett AJ, Smith HS, Springer EL, Owens RB, Nelson-Rees WA, Riggs JL, Gardner MB: Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines. J Natl Cancer Inst 58:1795–1806, 1977.
144. Bates SE, Davidson NE, Valverius E, Dickson RB, Freter C Kudlow JE, Tam JP, Lippman ME, Salomon D: Expression of transforming growth factor alpha mRNA in human breast cancer: regulation by estrogen. Molec Endocrin, in press, 1988.
145. Sherwin C, Twardzik PR, Bohn WH, Cockley KD, Todaro GJ: High molecular weight transforming growth factor activity in the urine of patients with disseminated cancer. Cancer Res 43:403–407, 1983.

146. Kim M, Warren TC, Kimball ES: Purification and characterization of a low molecular weight transforming growth factor from the urine of melanoma patients. J Biol Chem 260:9237–9243, 1985.
147. Twardzik DR, Kimball ES, Sherwin SA, Ranchalis JE, Todaro GS: Comparison of growth factors functionally related to epidermal growth factor in the urine of normal and human tumor-bearing athymic nude mice. Cancer Res 45:1934–1939, 1985.
148. Kimball ES, Bohn WH, Cockley KD, Warren TC, Sherwin SA: Distinct high-performance liquid chromatography pattern of transforming growth factor activity in urine of cancer patients as compared with that of normal individuals. Cancer Res 44:3613–3619, 1984.
149. Sato M, Yoshida H, Hayashi Y, Miyakami K, Bundo T, Yanagowa T, Yura Y, Azuma M, Ueno A: Expression of epidermal growth factor and transforming growth factor-α in a human salivary gland adenocarcinoma cell line. Cancer Res 45:6160–6167, 1985.
150. Kobrin MS, Samosondar J, Kudlow JE: α-Transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture. J Biol Chem 261:14414–14419, 1986.
151. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R: Expression in rat fibroblasts of a human transforming growth factor α and cDNA results in transformation. Cell 46:301–309, 1986.
152. Stern DF, Hare DL, Cecchini MA, Weinberg RA: Construction of a novel oncogene based on synthetic sequences encoding epidermal growth factor. Science 235:321–324, 1987.
153. Stoscheck CM, King LE: Role of epidermal growth factor in carcinogenesis. Cancer Res 46:1030–1037, 1986.
154. Kurachi H, Okamoto S, Oka T: Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorigenesis. Proc Natl Acad Sci USA 81:5940–5943, 1985.
155. Fitzpatrick SL, LaChance MP, Schultz GS: Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res 44:3442–3447, 1984.
156. Davidson NE, Gelmann EP, Lippman ME, Dickson RB: EGF receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol Endocrinol 1:216–233, 1987.
157. Osborne CK, Hamilton B, Titus G, Livingston RB: Epidermal growth factor stimulation of human breast cancer cells in culture. Cancer Res 40:2361–2366, 1980.
158. Kudlow JE, Cheung CYM, Bjorge JD: Epidermal growth factor stimulates the synthesis of its own receptor in a human breast cancer cell line. J Biol Chem 261:4134–4138, 1986.
159. Sawyer ST, Cohen S: Epidermal growth factor stimulates the phosphorylation of the calcium-dependent 35,000-dalton substrate in intact A-431 cells. J Biol Chem 260:8233–8236, 1985.
160. Eisholtz HP, Mangalam HJ, Potter E, Albert VR, Supowit S, Evans RM, Rosenfeld MG: Two different cis-active elements transfer the transcriptional effects of both EGF and phorbol esters. Science 234–1552–1557, 1986.
161. Schlessinger, J: Allosteric regulation of the epidermal growth factor receptor kinase. J Cell Biology 103:2067–2072, 1986.
162. Tam JP: Physiological effects of transforming growth factor in the newborn mouse. Science 229:673–675, 1985.
163. Schultz G, White M, Mitchell R, Brown G, Lynch J, Twardzik DR, Todaro GJ: Epithelial wound healing enhanced by transforming growth factor α and vaccinia growth factor. Science 235:350–352, 1987.
164. Schreiber AB, Winkler MF, Derynck R: Transforming growth factor-α—a more potent angiogenic mediator than epidermal growth factor. Science 232:1250–1253, 1986.
165. Folkman J, Klagsbrun M: Angiogenic factors. Science 235:442–447, 1987.
166. Jaye M, Howk R, Burgess W, Ricca GA, Chiu HM, Ravera M, O'Brien SJ, Modi WS, Maciag T, Drohan WN: Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. Science 233:541–548, 1986.
167. Lobb R, Sasse J, Sullivan R, Shing Y D'Amore P, Jacobs J, Klagsbrun M: Purification and

characterization of heparin binding endothelial cell growth factors. J Biol Chem 261:1924–1928, 1986.
168. Kurachi K, Davie EW, Strydom DJ, Riordan JF, Vallee BL: Sequence of the cDNA and gene for angiogenin, a human angiogenesis factor. Biochemistry 24:5494–5499, 1985.
169. Maciag, T: Angiogenesis, Progress in Hemostasis and Thrombosis (Grune & Stratton), pp 167–182, 1986.
170. West DC, Hampson IN, Arnold F, Kumar S: Angiogenesis induced by degradation products of hyaluronic acid. Science 228:1324–1326, 1985.
171. Schreiber AB, Kenney J, Kowalski J, Thomas KA, Gimenez-Gallego G, Rios-Candelore M, DiSalvo J, Bamitault D, Courty J, Courtois Y, Moemer M, Loret C, Burgess WH, Mehlman T, Friesel R, Johnson W, Maciag T: A unique family of endothelial cell polypeptide mitogens: the antigenic and receptor cross-activity of bovine endothelial growth factor and eye-derived growth factor-II. J Cell Biol 101:1623–1626, 1985.
172. Gospodarowicz D, Greenburg G, Bialecki H, Zetter BR: Factors involved in the modulation of cell proliferation *in vivo* and *in vitro*: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. *In Vitro* 14:85–113, 1978.
173. Kao RT, Hall J, Engel L, Stern R: The matrix of human breast tumor cells is mitogenic for fibroblasts. Am J Path 115: 109–116, 1984.
174. Tasjian AH, Voelkel EF, Lazzaro M, Singer FR, Roberts AB, Derynck R, Winkler ME, Levine L: α and β human transforming growth factors stimulate prostaglandin production and bone resorption in cultured mouse calvaria. Proc Natl Acad Sci USA 82:4535–4538, 1985.
175. Huff KK, Kaufman D, Gabbay KH, Spencer EM, Lippman ME, Dickson RB: Human breast cancer cells secrete an insulin-like growth factor-1-related polypeptide. Cancer Res 46:4613–4619, 1986.
176. Baxter RC, Maitland JE, Raisur RL, Reddel R, Sutherland RL: High molecular weight somatomedin-C (IGF-I) from T47D human mammary carcinoma cells: immunoreactivity and bioactivity. In: Insulin-like Growth Factors/Somatomedins Spencer, EM (ed), Walter deGruyter Co, Berlin, 1983, pp 615–618.
177. Jansen M, Van Schaik FMA, Ricker AT, Bullock B, Woods RE, Gabbay KH, Nussbaum AL, Sussenback JS, Vander Branch JR: Sequence of cDNA encoding human insulin-like growth factor I precursor. Nature (London), 306:609–611, 1983.
178. Han VK, D'Ercole AJ, Lund PK: Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science 236:193–197, 1987.
179. Huff KK, Knabbe C, Kaufman D, Lippman ME, Dickson RB: Hormonal regulation of insulin-like growth factor I (IGF-I) secretion from MCF-7 human breast cancer cells. J. Cell Biochem Supplement 11A:28, 1987.
180. Huff KK, Knabbe C, Lindsay R, Lippman ME, Dickson RB: Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. Molecular Endocrinology, submitted.
181. Rechler MM, Bruni CB, Yang YWH, Whitfield HJ, Graham DE, Franzio R, Brown AL, Nissley SP: Regulation of insulin-like growth factor gene expression. ICSU Short reports, Vol. 4, Puett D, Ahmad F, Black S, Lopez DM, Melner MH, Scott WA, Whelan, W.J. (eds). Cambridge University Press, 1986, pp 79–82.
182. Rechler MM, Nissley SP: Insulin-like growth factor (IGF)/somatomedin receptor subtypes: structure, function, and relationships to insulin receptor and IGF carrier proteins. Hormone Res 24:152–159, 1986.
183. Ullrich A, Gray A, Tam AW, Yang Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, Jacobs S Francke U, Ramachandran J, Fujita-Yamaguchi Y: Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J 5:2503–2512, 1986.
184. Campisi J, Pardee AB: Post-transcriptional control of the onset of DNA synthesis by an insulin-like growth factor. Mol Cell Biol 4:1807–1814, 1984.

185. Surmacz E, Kaczmarek L, Ronning O, Baserga R: Activation of the ribosomal DNA promoter in cells exposed to insulin-like growth factor I. Mol Cell Biol 7:657–673, 1987.
186. Ellis L, Clauser E, Morgan DO, Ederg M, Roth RA, Rutter WJ: Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 46:721–732, 1986.
187. Clemmons DR, Elgin RG, Han VKM, Casella SJ, D'Ercole AJ, Van Wyk JJ: Cultured fibroblast monolayers secretage a protein that alters the cellular binding of somatomedin-C/insulin-like growth factor I. J Clin Invest 77:1548–1556, 1986.
188. Drop SLS, Kortlene DJ, Guyda HJ: Isolation of a somatomedin-binding protein from preterm amniotic fluid; development of a radioimmunoassay. J Clin Endocrinol Metab 59:899–910, 1984.
189. Devroede MA, Tseng LMH, Katsyannis PG, Nissley SP, Rechler MM: Modulation of insulin-like growth factor I binding to human fibroblast monolayers by insulin-like growth factor carrier proteins released to the incubation media. J Clin Invest 77:602–610, 1986.
190. Nissley SP, Rechler MM: Insulin-like growth factors: biosynthesis receptors and carrier proteins. In: Hormonal Proteins and Peptides, Vol. XII, Li CH (ed). Academic Press, New York, 1984, pp 128–203.
191. Van Wyk JJ: Somatomedins: biological actions and physiologic control mechanisms. In: Hormonal Proteins and Peptides, Vol. XII, Li, CH (ed). Academic Press, New York, 1984, pp 82–125.
192. Minuto F, Del Monte PD, Baweca A, Fortini P, Cariola G, Caterambone G, Giordano G: Evidence for an increased somatomedin C/insulin-like growth factor I content in primary human lung tumors. Cancer Res 46:985–988, 1986.
193. Tricoli JV, Rall LB, Karakousis CP, Herrera L, Petrelli N, Bell GL, Shows TB: Enhanced levels of insulin-like growth factor messenger RNA in human colon carcinomas and liposarcomas. Cancer Res 46:6169–6173, 1986.
194. Reeve AE, Eccles MR, Wilkins RJ, Bell GI, Millon LJ: Expression of insulin-like growth factor II transcripts in Wilms tumor. Nature 317:258–260, 1985.
195. Stiles CD, Capone GT, Scher CD, Antoniades HN, Van Wyk J, Pledger WJ: Dual control of cell growth by somatomedins and platelet derived growth factor. Proc Natl Acad Sci USA 76:1279–1283, 1979.
196. Sporn MB, Roberts AB, Wakefield LM, Assoian RK: Transforming growth factor β: Biological function and chemical structure. Science 233:532–534, 1986.
197. Wang JL, Hsu YM: Negative regulators of cell growth. Trends Bio Sci 11:24–26, 1986.
198. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV: Human transforming growth factor β: Complementary DNA sequence and expression in normal and transformed cells. Nature 316:701–705, 1985.
199. King WJ, Desombre ER, Jensen EV, Greene GI: Comparison of immunocytochemical and steroid binding assays for estrogen receptor in human breast tumors. Cancer Res 45: 294–304, 1985.
200. Greene GL, Sobel NB, King WJ, Jensen EV: Immunological Studies of estrogen receptors. J Steroid Biochem 20:51–56, 1984.
201. Lawrence DA, Pircher R, Krycene-Martineirie A, Lullien P: Normal embryo fibroblasts release transforming growth factors in a latent form. J Cell Physiol 121:184–188, 1984.
202. Keski-Oja F, Lyons RM and Moses HL: Immunodetection and modulation of cellular growth with antibodies against native transforming growth factor β. Cancer Res 47:645–646, 1987.
203. Cheifetz S, Wentherbee JA, Tsang MSS, Anderson JK, Mole JE, Lucas R, Massague J: The tranforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. Cell 48:409–415, 1987.
204. Massague J, Kelly BJ: Internalization of transforming growth factor-β and its receptor in BALB/c 3T3 fibroblasts. J Cell Physiol 128:216–222, 1986.
205. Dickson RB: Endocytosis of polypeptides and their receptors. Trends Pharm Sci 6:164–167, 1985.

206. Leof EB, Proper JA, Goustin AJ, Shipley GD, Dicorleto PE, Moses HL: Induction of c-*sis* mRNA and activity similar to platelet-derived growth factor: A proposed model for indirect mitogenesis involving autocrine activity. Proc Natl Acad Sci USA, 83:2453–2457, 1986.
207. Ignotz RA, Massage J: Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345, 1986.
208. Massague J: Transforming growth factor-β modulates the high affinity receptors for epidermal growth factor and transforming growth factor-. J Cell Biol 100:1508–1514, 1985.
209. Myrdal SE, Twardzik DR, Auersperg N: Cell mediated co-action of transforming growth factors: incubation of Type β with normal rat kidney cells produces a soluble activity that prolongs the ruffling response to type α. J. Cell. Biol 102:1230–1234, 1986.
210. Massague J, Cheifetz S, Endo T, Nadal-Ginard B: Type β transforming growth factor is an inhibitor of myogenic differentiation. Proc Natl Acad Sci USA 83:8206–8210, 1986.
211. Olson EN, Sternberg E, Hu JS, Spizz G, Wilcox C: Regulation of myogenic differentiation by type β transforming growth factor. J Cell Biol 103: 1799–1805, 1986.
212. McMahon JB, Richards WL, del Campo AA, Song M-K, Thorgeirsson SS: Differential effects of transforming growth factor β on proliferation of normal and malignant rat liver epithelial cells in culture. Cancer Res 46:4665–4671, 1986.
213. Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NR, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, Fisher RA, Bertonis JM, Torres G, Wallner BP, Ramachandran KL, Ragin RC, Manganaro TF, MacLaughlin DT, Donahoe PK: Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell 45:685–698, 1987.
214. Heimark RL, Twardzik DR, Schwartz SM: Inhibition of endothelial regeneration by type-beta transforming growth factor from platelets. Science 233:1078–1080, 1986.
215. Takehara K, LeRoy EC, Grotendorst GR: TGFβ inhibition of endothelial cell proliferation alteration of EGF binding and EGF-induced growth regulatory (competence) gene expression. Cell 49:415–422, 1987.
216. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci A: Transforming growth factor type β: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc Natl Acad Sci USA 83:4167–4171, 1986.
217. Ross R, Raines EW, Bowen-Pope DF: The Biology of platelet-derived growth factor. Cell 46:155–169, 1986.
218. Huang JS, Huang SS, Deuel TF: Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell surface receptors. Cell 39: 79–87, 1984.
219. Yeh HJ, Pierce GF, Denel TF: Ultrastructure localization of a platelet-derived growth factor/v-*sis*-related protein(s) in cytoplasm and nucleus of simian sarcoma virus-transformed cells. Proc Natl Acad Sci USA 84:2317–2321, 1987.
220. Rozengurt E: Early signals in the mitogenic response. Science 234: 161–166, 1986.
221. Bronzert DA, Pantazis P, Antoniades HN, Kasid A, Davidson N, Dickson RB, Lippman ME: Synthesis and secretion of PDGF-like growth factor by human breast cancer cell lines. Proc Natl Acad Sci USA, 84:5763–5767, 1987.
222. Peres R, Betsholtz C, Westermark B. and Heldin C-H: Frequent expression of growth factors for mesanchymal cells in human breast carcinoma lines. Cancer Res 47:3425–3429, 1987.
223. Rozengurt E, Sinnett-Smith J, Taylor-Papadimitriou J: Production of PDGF-like growth factor by breast cancer lines. Int J Cancer 36:247–252, 1985.
224. Betsholtz C, Hohnsson A, Heldin CH, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, Knott TJ, Scott J: cDNA sequence and chromosomal localization of human platelet derived growth factor A chain and its expression tumor cell lines. Nature 320:695–700, 1986.
225. Yarden Y, Escobedo JA, Kwang WJ, Yang-Feng TL, Daniel Tremble PM, Chen EY,

Ando ME, Harkins RN, Franks U, Fried VA, Ullrich A, Williams LT: Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factors. Nature 323:226–232, 1986.

226. Ro JY, Holoye PY, Gutterman JU, Block MB: c-*sis* expression in primary human breast carcinoma. Proceedings of the 78th Annual Meeting of the American Association for Cancer Research, Atlanta, Georgia. 1987. Abstract #74.
227. Dembinski TC, Leung CKH, Shiu RPC: Evidence for a novel pituitary factor that poentiates the mitogenic effect of estrogen in human breast cancer cells. Cancer Res 45:3083–3089, 1985.
228. Shiu RPC, Myal Y, Leung CKH, Dmebinski TC, Iwasiow B: Relationship between a pituitary-derived growth factor for breast cancer and insulin-like growth factor 2 (IGF-2). Institute Scientifique Roussel Symposium on 'Hormones, Oncogenes and Growth Factors,' Paris, June, 1986.
229. Dell'Aguilla ML, Pigott DA, Bonaguist DL, Gaffney EV: A factor from plasma-derived human serum that inhibits the growth of the mammary cell line MCF-7: Characterization and purification. J Natl Cancer Inst 72:291–298, 1984.
230. Bano M, Salomon DS, Kidwell WR: Purification of a mammary derived growth factor from human milk and human mammary tumors. J Biol Chem 260:5745–5752, 1985.
231. Halper J, Moses HL: Initial purification and characterization of a epithelial tissue-derived transforming growth factor. J Cell Biol 101:235a, 1986. Abstract #931.
232. Swain S, Dickson RB, Lippman ME: Anchorage independent epithelial colony stimulating activity in human breast cancer cell lines. Proceedings, American Association for Cancer Research Annual Meeting, Los Angeles, CA 1986. Abstract #844.
233. Abraham JA, Whang JL, Tumolo A, Mergia A, Friedman J, Gospodorowicz D, Fiddles JC: Human basic fibroblast growth factor: nucleotide sequence and genomic organization. EMBO J 5: 2523–2528, 1986.
234. Liotta LA, Mandler R, Murano G, Katz DA, Gordon RK, Chiang PK, Schiffman E: Tumor cell autocrine motility factor. Proc Natal Acad Sci USA, 83:3302–3306, 1986.
235. Dickson C, Smith R, Brookes S, Peter G: Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. Cell 37:529–536, 1984.
236. Brown AMC, Wildin RS, Prendergast TJ, Varmus HE: A retroviral vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. Cell 46:1001–1009, 1986.
237. Dickson C, Peters G: Potential oncogene product related to growth factors. Nature 326: 833, 1987.
238. Zarbl H, Sukumar S, Arthur AV, Martin-Zanea D, Barbacid M: Direct mutagenesis of Ha-*ras*-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315:382–385, 1985.
239. Andres AC, Schonenberger CA, Groner B, Hennighausen C, LeMaur M, Gerlinger P: Ha-*ras* oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation and tumor induction in transgenic mice. Proc Natl Acad Sci USA 84: 1299–1303, 1987.
240. Vousden KH, Eccles SA, Purvics H, Marshall CJ: Enhanced spontaneous metastasis of mouse carcinoma cells transfected with an activated c-Ha-*ras*-1 gene. Int J Cancer 37:425–533, 1986.
241. Hynes NE, Jaggi R, Kozma R, Ball R, Muellener D, Wetherall NT, Davis BW, Groner B: New acceptor cell for transfected genomic DNA: oncogene transfer into a mouse mammary epithelial cell line. Mol Cell Biol 5:268–272, 1985.
242. Pulciani S, Santos E, Long LK, Sorrentino V, Barbacid M: *ras* gene amplification and malignant transformation. Mol Cell Biol 5:2836–2841, 1985.
243. Lumpkin CK, Knepper JE, Butel JS, Smith JR, Pereiva-Smith OM: Mitogenic effects of the protooncogene and oncogene forms of c-H-*ras* DNA in human diploid fibroblasts. Mol Cell Biol 6:2990–2993, 1986.

244. McKay IA, Marshall CJ, Cales C, Hall A: Transformation and stimulation of DNA synthesis in NIH-3T3 cells are a titratable function of normal p $21^{N\text{-}ras}$ expression. EMBO J 5:2617–2621, 1986.
245. Greig RG, Koestler TP, Trainer DL, Corwin SP, Miles L, Kline T, Sweet R, Yokoyama S, Poste G: Tumorigenic and metastatic properties of 'normal' and *ras*-transfected NIH-3T3 cells. Proc Natl Acad Sci USA 82:3698–3701, 1985.
246. Egan SE, McClarty GA, Jarolim L, Wright JA, Spiro I, Hager G, Greenberg AH: Expression of H-*ras* correlates with metastatic potential: evidence for direct regulation of the metastatic phenotype in 10 T1/2 and NIH 3T3 cells. Mol Cell Biol 7:830–837, 1987.
247. Stenman G, Delorme EO, Law CC, Sager R: Transfection with plasmid pSV2gpt EJ induces chromosome rearrangements in CHEF cells. Proc Natl Acad Sci USA 84:184–188, 1987.
248. Bishop JM: The molecular genetics of cancer. Science 235:305–311, 1987.
249. Slamon DJ, DeKernion JB, Verma IM, Cline MJ: Expression of cellular oncogenes in human malignancies. Science 224:256–262, 1984.
250. Perucho M, Goldfarb M, Shimizu K, Lama C, Fogh J, Wigler M: Human tumor-derived cell lines contain common and different transforming genes. Cell 27:467–476, 1981.
251. Kraus MH, Yuasa Y, Aaronson SA: A position 12-activated H-*ras* oncogene in all Hs578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proc Natl Acad Sci USA 81:5384–5388, 1984.
252. Graham KA, Richardson CL, Minden MD, Trent JM, Buick RM: Varying degrees of amplification of the N-*ras* oncogene in the human breast cancer cell line MCF-7. Cancer Res 45:2201–2025, 1985.
253. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R: Loss of a c-H-*ras*-1 allele and aggressive human primary breast carcinomas. Cancer Res 46:4776–4781, 1986.
254. Lane MA, Sainten A, Cooper GM: Activation of related transforming genes in mouse and human mammary carcinomas. Proc Natl Acad Sci USA 78:5185–5189, 1981.
255. Becker D, Lane MA, Cooper GM: Identification of an antigen associated with transforming genes of human and mouse mammary carcinomas. Proc Nat Acad Sci USA 79:3315–3319, 1982
256. Fasano O, Birnbaum N, Edlund L, Fogh J, Wigler M: New human transforming genes detected by a tumorigenicity assay. Mol Cell Biol 4:1695–1705, 1984.
257. Young D, Waitches G, Birchmeier C, Fasano O, Wigler M: Isolation and characterization of a new cellular oncogene encoding a protein with multiple potential transmembrane domains. Cell 45:711–719, 1986.
258. Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Stewart T, Taub R. Translocations among antibody genes in human cancer. Science 222:765–771, 1983.
259. Blanchard J-M, Piechaczyk M, Dani C, Chambard C, Franchi A, Pouyssegur J, Jeanteur P: c-*myc* gene is transcribed at high rate in Go-arrested fibroblasts and is post-transcriptionally regulated in response to growth factors. Nature 317:443–445, 1985.
260. Armelin HA, Armelin MCS, Kelly K, Stewart T, Leder P, Cochran BH, Stiles CD: Functional role for c-*myc* in mitogenic response to platelet-derived growth factor. Nature 310: 655–660, 1984.
261. Hann SR, Thompson CB, Eiserman RN: c-*myc* oncogene protein synthesis is independent of the cell cycle in human and avian cells. Nature 314:366–369, 1985.
262. Studzinski GP, Brelvi ZS, Feldman SC, Watt RA: Participation of c-*myc* protein in DNA synthesis of human cells. Science 234:467–470, 1986.
263. King MC, Go RC, Elston RC, Lynch HL, Petrakis NL: Allele increasing susceptibility to human breast cancer may be linked to glutamate-pyruvate transaminase locus. Science 208:406–408, 1980.
264. Kozbor D, Croce CM: Amplification of the c-*myc* oncogene in one of five human breast carcinoma cell lines. Cancer Res 44:438–41, 1984.
265. Modjtahedi N, Lavialle C, Poupon ME, Landin RM, Cassingena R, Monier R, Brison O:

Increased level of amplification of the c-*myc* oncogene in tumors induced in nude mice by a human breast carcinoma cell line. Cancer Res 45:4372–4379, 1985.
266. Stewart TA, Pattengale PK, Leder P: Spontaneous mammary adenocarcinoma in transgenic mice that carry and express MTV/*myc* fusion genes. Cell 38:627–637, 1984.
267. Escot C, Theillet C, Lidereau R, Spyratos E, Champene MH, Gest J, Callahan R: Genetic alterations of the c-*myc* protooncogene (MYC) in human primary breast carcinomas. Proc Nat Acad Sci USA 83:4834–4838, 1986.
268. Wilding G, Lippman ME, Gelmann E: The effects of steroid hormones and peptide growth factors on protooncogene *c-fos* and *s-myc* expression in human breast cancer cells. Cancer Research in press, 1988.
269. Santos GF, Lee W, Benz C: Post-transcriptional regulation of biphasic *c-myc* breast cancer cells. Proceedings of the 78th Annual Meeting of the American Association for Cancer Research, Atlanta, Georgia, Abstract #88, 1987.
270. Bernards R, Dessair SK, Weinberg RA: N-*myc* amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. Cell 47:667–674, 1986.
271. O'Brien SJ, Nash WG, Goodwin JL, Lowy DR, Chang EH: Dispersion of the *ras* family of transforming genes to four different chromosomes in man. Nature 302:839–842, 1983.
272. Hall A, Marshall EJ, Spurr NK, Weiss RA: Identification of transforming gene in two human sarcoma cell lines as a new member of the ras family located on chromosome 1. Nature 303:396–400, 1983.
273. Spandidos DA, Agnantis RJ: Human malignant tumors of the breast, as compared to their respective normal tissue, have elevated expression of the Harvey ras oncogene. Anticancer Res, 4:269–272, 1984.
274. Agnantis NJ, Parissi P, Anagnostakis D, Spandidos DA: Comparative study of Harvey *ras* oncogene expression with conventional clinicopathologic parameters of breast cancer. Oncology 43:36–39, 1986.
275. DeBortoli ME, Abou-Issa H, Haley BE, Cho-Chung YS: Amplified expression of p21 *ras* protein in hormone-dependent mammary carcinomas of humans and rodents. Biochem Biophys Res Commun 127: 699–706, 1985.
276. Hand PH, Thor A, Wunderlich D, Muraro R, Caruso A, Schlom J: Monoclonal antibodies of predefined specificity detect activated *ras* gene expression in human mammary and colon carcinomas. Proc Nat Acad Sci USA 81:5227–5231, 1984.
277. Tanaka T, Slamon D, Battifora H, Cline MJ: Expression of p21 *ras* oncoproteins in human cancers. Cancer Res 46:1465–1470, 1986.
278. Ohuchi N, Thor A, Page DL, Hand PH, Halter S, Schlom J: Expression of the 21,000 molecular weight *ras* protein in a spectrum of benign and malignant human mammary tissues. Cancer Res 46:2511–2519, 1986.
279. Liderau R, Escot C, Theillet C, Champeme MH, Brunet M, Gest J, Callahan R: High frequency of rare alleles of the human c-Ha-*ras*-1 protooncogene in breast cancer patients. J Natl Cancer Inst 77:699–701, 1986.
280. Downward J, Yarden Y, Mayes E, Scrace G, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. Nature 307:521–527, 1984.
281. Coussens L, Yang-Feng TL, Liao YC, Chen E, Schlessinger J, Francke U, Levinson A, Ullrich A: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 320:1132–1139, 1985.
282. Bargmann CI, Hung MC, Weinberg RA: Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. Cell 45:649–657, 1986.
283. King CR, Kraus MH, Aaronson SA: Amplification of a novel v-*erb*B related gene in a human mammary carcinoma. Science 229:974–976, 1985.
284. Yokota J, Toyoshima K, Sugimura T, Yamamoto T, Terada M, Battifora H, Cline MJ: Amplification of c-*erb*B-2 oncogene in human adenocarcinomas *in vivo*. Lancet i:765–766, 1986.

285. Kraus MH, Popescu NC, Amsbaugh SC, King CR: Overexpression of the EGF-receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 6:605–610, 1987.
286. Van de Vijver M, Van de Bersselaar R, Devilec P, Cornelisse C, Peterse J, Nusse R: Amplification of the new (c-*erb*B-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-*erb*A oncogene. Mol Cell Biol 7:2019–2023, 1987.
287. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–182, 1987.
288. Sefton BM, Hunter T, Beeman K, Eckhart W: Evidence that the phosphorylation of tyrosine is essential for transformation by Rous sarcoma virus. Cell 20:807–816, 1980.
289. Jacobs C, Rubsamen H: Expression of pp60c-src protein kinase in adult and fetal human tissue: high activities in some sarcomas and mammary carcinomas. Cancer Res 43:1696–1702, 1983.
290. Rosen N, Bolen JB, Schwartz AM, Cohen P, DeSeau V, Israel MA: Analysis of pp60c-src protein kinase activity in human tumor cell lines and tissues. J Biol Chem 261:13754–13759, 1986.
291. Sainsbury JRC, Farndon JR, Sherbet GV, Harris AL: Epidermal growth factor receptors and oestrogen receptors in human breast cancers. Lancet i:364–366, 1985.
292. Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS: Epidermal growth factor binding by breast tumor biopsies and relationship to oestrogen receptor and progrestin receptor levels. Cancer Res 3448–3453, 1984.
293. Perez R, Pascual M, Macias A Lage A: Epidermal growth factor receptors in human breast cancer. Breast Cancer Res Treat 4:189–193, 1984.
294. Sainsbury JRC, Malcolm AJ, Appleton DR, Farndon JR, Harris AL: Presence of epidermal growth factor receptors as an indicator of poor prognosis in patients with breast cancer. J Clin Path 38:1225–1228, 1985.
295. Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS: Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen receptor and progesterone receptor levels. Cancer Res 44:3448–3453, 1984.
296. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, Harris AL: Epidermal growth factor receptor status of human breast cancer is related to early recurrence and death. Lancet 1:1398–1402, 1987.
297. Filmus J, Pollak MN, Cailleau R, Buick RN: MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified GF receptor gene and is growth inhibited by EGF. Biochem Biophys Res Comm 128:88–905, 1985.
298. Filmus J, Trent JM, Polak MN, Buick RN: Epidermal growth factor receptor gene-amplified MDA-468 breast cancer cell line and its non-amplified variants. Mol Cell Biol 7:251–257, 1987.
299. Santon JB, Cronin MT, Macleod CL, Mendelsohn J, Masui H, Gill GN: Effects of epidermal growth factor receptor concentration or tumorigenicity of A431 cells in nude mice. Cancer Res 46: 4701–4705, 1986.
300. Neal DE, Bennett MK, Hall, RR, Marsh C, Abel PD, Sainsbury JRC, Harris AL: Epidermal growth factor receptors in human bladder cancer: comparison of invasive and superficial tumors. Lancet i:366–368, 1985.
301. Real FX, Rettig WJ, Chesa PG, Mclamed MR, Old LJ, Mendelsohn J: Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and tissues: relationship to cell lineage and stage of differentiation. Cancer Res 46:4726–4731, 1986.
302. Stampfer MR, Bartley JC: Induction of transformation and continuous cell lines from normal human mammary epithelial cells after cells exposure to benzo[a]pyrene. Proc Natl Acad Sci USA 82:2394–2398, 1985.

303. Clark R, Stampfer MR, Milley R, O'Rourke C, Walen KH, Kriegler M, Kopplin J and McCormick F: Transformation of human mammary epithelial cells by oncogenic viruses. Cancer Res, in press, 1988.
304. Garcia I, Sordat B, Raucicio-Farnin E, Dunand M, Kraehenbuhl JP Diggelmann H: Establishment of two rabbit mammary epithelial cell lines with distinct oncogenic potential and differentiated phenotype after microinjection of transforming genes. Mol Cell Biol 6:1974–1982, 1986.
305. Kasid A, Lippman ME, Papageorge AG, Lowy DR, Gelmann EP: Transfection of v-*ras*^{H} DNA into MCF-7 cells bypasses their dependence on estrogen for tumorigenicity. Science 228:725–728, 1985.
306. Worland PJ, Bronzert DA, Dickson RB, Lippman ME, Thorgeirsson SS, Wirth PJ: Cellular polypeptide patterns of MCF-7 and transfected MCF-7 (*ras*) human breast cancer cells. J Cell Biochem Supplement 11A, p 84, 1987. Abstract #A293.
307. Bell RM: Protein kinase C activation by diaglycerol second messengers. Cell 45:631–632, 1986.
308. Parker PJ, Coussens L, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield MD, Ullrich A: The complete primary structure of protein kinase C—the major phorbol ester receptor. Science 233:853–858, 1986.
309. Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke U, Ullrich A: Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science 233:859–866, 1986.
310. Wakelum MJO, Davies SA, Houslay MD, McKay I, Marshall CJ, Hall A: Normal $p^{21N\text{-}ras}$ couples bombesin and other growth factors to inositol phosphate production. Nature 323: 173–176, 1986.
311. Dickson RB, Kasid A, Huff KK, Bates S, Knabbe C, Bronzert D, Gelmann EP, Lippman ME: Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17β-estradiol or v-*ras*^{H} oncogene. Proc Natl Acad Sci USA 84:837–841, 1987.
312. Salomon DS, Perroteau I, Kidwell WR, Tam JS, Derynck R: Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha-transforming growth factors in *ras*-transformed mouse mammary epithelial cells. J Cell Physiol 130:397–409, 1987.
313. Masui H, Kawamoto T, Sato JP, Wolt B, Sato G, Mendelsohn J: Growth inhibition of human tumor cells in athymic mice by antiepidermal growth factor receptor monoclonal antibodies. Cancer Res 44:1002–1012, 1984.
314. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fisher A, Minna J: Bombesin-like peptides can function as autocrine growth factors in small cell lung cancer. Nature 316:823–827, 1985.
315. Vitella ES, Uhv JW: Immunotoxins: redirecting nature's poisons. Cell 41:653–665, 1985.
316. Pastan I, Willingham MC, Fitzgerald DP: Immunotoxins. Cell 47:641–648, 1986.

7. Actions of pituitary prolactin and insulin-like growth factor II in human breast cancer

Robert P.C. Shiu, Leigh C. Murphy, Yvonne Myal, Thomas C. Dembinski, Deborah Tsuyuki, and Barbara M. Iwasiow

1. Pituitary influence on the mammary gland

The pituitary gland plays a central role in the regulation of mammary gland development. It secretes a variety of hormones that directly or indirectly influence the mammary gland. The best known mammogenic hormone of the pituitary gland is prolactin which directly promotes development and differentiated function (e.g., milk synthesis) of the normal mammary gland. Also, the pituitary gland can indirectly influence mammary gland development via the production of gonadotropins which stimulate the ovary to produce steroids such as estrogen and progesterone; these ovarian steroids are indispensible in mammary gland development. Furthermore, pituitary hormones regulate the production of thyroid hormones and adrenal glucocorticoids which are important in the performance of the mammary gland.

Apart from the production of classical hormones, there is increasing evidence to indicate that the pituitary gland is a source of a variety of peptide growth factors. For example, basic and acidic fibroblast growth factors produced in the pituitary are potent mitogens for a variety of cell types *in vitro* [1]. Insulin-like growth factor II immunoreactive peptides were found in the extract of human pituitary glands [2]. Furthermore, several other growth promoting activities have been reported to be produced by the pituitary gland [3–5]. However, the physiology and frequently the chemistry of many of the reported pituitary-derived growth factors are poorly understood. Whether any one of these growth factors has a physiological role in the mammary gland is unknown.

After malignant transformation, breast tumor cells frequently retain their responsiveness to many of the hormones that influence their normal counterparts. Thus, breast tumors that respond to pituitary hormonal influences (directly or indirectly) are well documented both in experimental animals and in man. In the carcinogen-induced mammary tumors of the rat, their growth requires pituitary prolactin in addition to ovarian estradiol [6]. Also, there is a considerable body of evidence from experimental and clinical studies which indicates that estrogen's action on normal and tumorous mammary gland requires the presence of the pituitary gland [7–12]. In particular, the authors of

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

two clinical studies [11, 12] have suggested that a pituitary-derived growth factor, unrelated to prolactin and growth hormone, may mediate, or synergize with, the effect of estrogen in stimulating the proliferation of human breast cancer *in vivo*. Thus, the well-known efficacy of hypophysectomy in the treatment of hormone-responsive human breast cancer may, in part, be due to the removal of such a pituitary growth factor. In this chapter, we shall discuss aspects of our work which aim at gaining insight into the functions of pituitary prolactin and insulin-like growth factor in human breast cancer.

2. Actions of prolactin in human breast cancer

The importance of prolactin in rodent breast tumors has provided, in part, the impetus to search for a function for prolactin in human breast cancer. Unfortunately, epidemiological and clinical studies have failed to identify an unequivocal function of prolactin [13–15]. However, as much as 60% of human breast tumor biopsies have been shown to possess membrane receptors for prolactin [16], and a large number of human breast cancer cell lines also contain prolactin receptors [17], suggesting that prolactin may influence the physiology of at least some human breast cancers. An early study indicated that enzyme activity of the pentose monophosphate pathway was stimulated by prolactin in human breast tumor biopsies cultured *in vitro* [18]. Further, prolactin has been reported to stimulate anchorage-dependent and -independent proliferation of human breast cancer cells [19–23], although in many of these studies, the specificity of the prolactin action has not been rigorously tested. Despite these findings, the precise role of prolactin and its molecular mechanism of action in human breast cancer have not yet been explored.

In order to undertake studies that might shed light on the above issues, we embarked upon the identification of a quantifiable biological end point of prolactin action in human breast cancer cell lines. Since receptors are obligatory in mediating the biological effects of hormones, our initial efforts led to the identification of membrane receptors for prolactin in many human breast cancer cell lines [17]. The prolactin receptors in these cell lines, like those in human breast tumor biopsies and other prolactin target tissues, bind not only human prolactin but also other members of the lactogenic hormone family. Thus, human growth hormone, and to a lesser extent human placental lactogen, also bind to the prolactin receptors in the human breast cancer cell lines. One such cell line, T–47D, was found to contain the highest number of prolactin receptors and it was therefore chosen for further studies. Physiological doses of human prolactin or human growth hormone (ng/ml range), in the presence of supraphysiological concentrations (ug/ml range) of glucocorticoid was found to produce a variety of changes in the T–47D cells. Prominent amongst these changes are increased lipid synthesis, altered morphology, decreased cell-substratum interaction and induction of a unique secretory protein [24, 25]. Because this secretory protein was induced by pro-

lactin (or by human growth hormone binding to the prolactin receptors), it was termed *P*rolactin *I*nducible *P*rotein (PIP) [25]. PIP could serve as a useful marker for further study on the molecular action of prolactin in human breast cancer.

3. Regulation of a prolactin-inducible gene in human breast cancer

PIP exists in both the glycosylated (14–16 kDa) and non-glycosylated (11 kDa) forms (Figure 1) which have been purified to homogeneity and antibodies raised to them in rabbits (25). The anti-PIP antibodies were first used to identify and quantitate the precursor form of PIP by immunoprecipitation of proteins translated *in vitro* using poly (A)+ RNA isolated from T–47D cells (Figure 2). This analysis revealed that treatment of T–47D cells by prolactin and hydrocortisone resulted in >10-fold increase in the amount of translatable PIP mRNA. This finding provided the first indication that prolactin and glucocorticoid may regulate the expression of the PIP gene. To gain further insight into how prolactin and glucocorticoid regulate the expression of the PIP gene in human breast cancer, it was necessary to clone the gene that encodes PIP. Further, molecular cloning of the PIP gene may allow us to efficiently deduce the structure, and possibly the function, of PIP in human breast cancer. A cDNA library was therefore generated in the expression vector lambda gt11 using poly (A)+ RNA isolated from prolactin and glucocorticoid-treated T–47D cells [26]. After screening this library with the rabbit anti-PIP antiserum, several positive clones were identified. The longest cloned cDNA was 577 bases long, and after subcloning into the plasmid vector pBR322, was used for most of our studies. Northern hybridization analysis revealed that the PIP mRNA transcript is approx. 0.9 kb in length [26].

The nature of multihormonal regulation of PIP gene expression in the T–47D cells was first studied. Northern and cytoplasmic dot-blot hybridization analyses (which measure the accumulation of PIP mRNA) were used to assess the steroid specificity of induction of PIP gene expression. Human prolactin and human growth hormone were equipotent [26]. However, the most active steroid that synergized with prolactin/growth hormone in the induction of PIP mRNA was the androgen, dihydrotestosterone. Indeed, glucocorticoid was 4 orders of magnitude less potent than androgen (Figure 3). All other steroid hormones tested, including estrogens and progestins, had minimal effect [26]. Indeed, androgen is the only steroid effective at physiological concentrations between 1 to 100 pM. These results therefore suggest that, under physiological circumstances, androgen and prolactin or growth hormone exert the major influence in the regulation of PIP gene expression in breast cancer.

Since the increase in PIP mRNA could be due to an increase in the rate of transcription and/or in the stability of the mRNA transcripts, further studies were carried out to ascertain the mechanism(s) by which prolactin and androgen influence the expression of the PIP gene. The nuclear run-on tran-

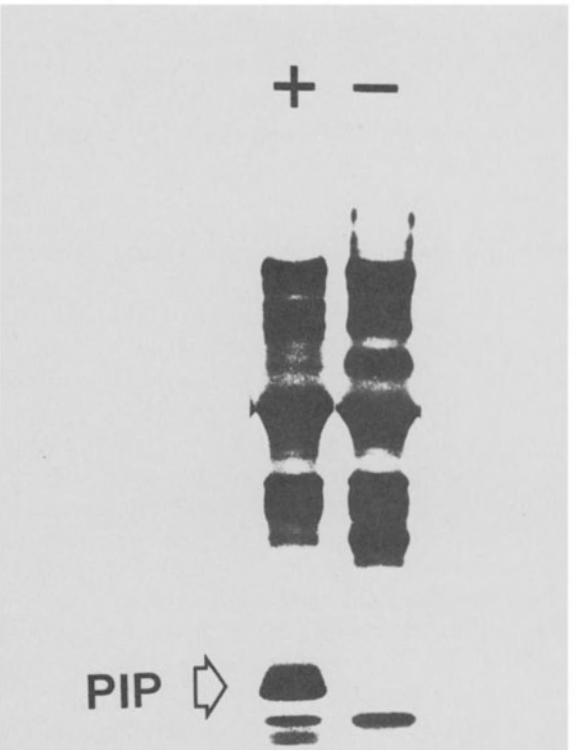


Figure 1. Prolactin inducible proteins (PIP) in T-47D human breast cancer cells. ^{35}S-methionine labeled proteins in the medium of cells treated with human prolactin and glucocorticoid (+ lane) and that of cells not exposed to the hormones (− lane), separated by SDS-polyacrylamide gel, are shown in this fluorogram. The glycosylated forms (14-16 kDa) and non-glycosylated form (11 kDa) of PIP are indicated [modified from 25].

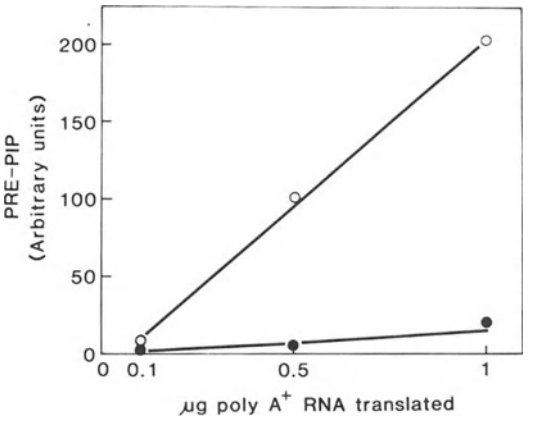


Figure 2. Quantitation of precursor of PIP from *in vitro* translation of poly (A)+ RNA isolated from control (●) and prolactin/glucocorticoid-treated (○) T-47D cells. After immunoprecipitation with anti-PIP antiserum followed by SDS-polyacrylamide gel electrophoresis and fluorography, the pre-PIP peptide was quantitated by densitometry [modified from 25].

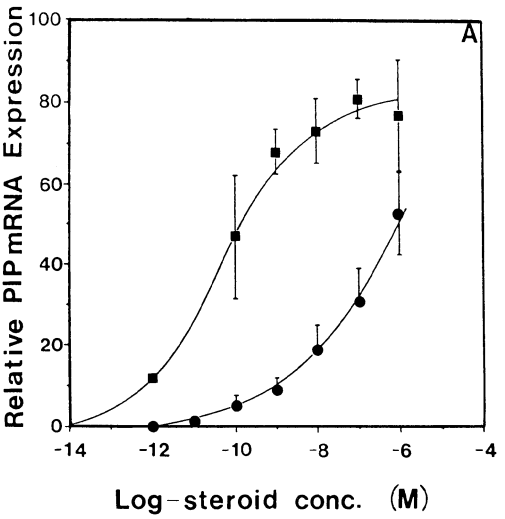


Figure 3. Dose-response of androgen (■) and glucocorticoid (●) in the induction of PIP mRNA in T-47D cells in the presence of prolactin. RNA isolated from T-47D cells 24 hrs. after hormone treatment was subjected to Northern hybridization analysis using the PIP cDNA insert [26]. The hybridization signals were quantitated by densitometry [modified from 26].

scription assay was performed on nuclei isolated from T–47D cells treated with human growth hormone or androgen or both to estimate the rate of transcription of the PIP gene [26]. Table 1 shows that human growth hormone treatment had no effect on the transcription rate of the PIP gene although this hormone led to a 5-fold accumulation of PIP mRNA. Dihydrotestosterone by itself increased transcription by approx. 4-fold which could account for the 4-fold increase in PIP mRNA under its influence. The combination of androgen and human growth hormone did not further increase the rate of transcription of the gene, but resulted in approx. 15-fold increase in PIP mRNA. These results therefore indicate that androgen regulates the expression of PIP gene at the transcriptional level while prolactin/human growth hormone exert their effect at the posttranscriptional level, possibly by increasing the half-life of PIP mRNA.

Table 1. Effects of human growth hormone (hGH) and dihydrotestosterone (DHT) on PIP mRNA levels and PIP gene transcription in T-47D cells. Northern analysis and nuclear run-off transcription assay [26] were used to measure PIP mRNA levels and PIP gene transcription, respectively. Values are expressed as fold increase above control cells.

Hormone	PIP mRNA levels	PIP transcription rate
None (control)	1	1
hGH	5	1
DHT	4	4
hGH + DHT	15	4

Thus, the regulation of PIP gene expression in human breast cancer and that of the expression of casein (milk protein) genes in the rodent mammary gland [27, 28] share interesting similarities and differences. The expression of the two genes are regulated synergistically by prolactin and a steroid hormone—androgen in the case of PIP and glucocorticoid in the case of caseins. For both genes, the steroid hormones exert their effects at the transcriptional level while prolactin acts post-transcriptionally. Therefore, both the PIP and casein genes are excellent models to study the molecular mechanism of action of prolactin in target cells. For example, it will be of interest to compare any cis-acting elements and trans-acting factors that are important for the regulation of expression of these genes.

While many eukaryotic genes are known to be transcriptionally regulated by estrogen, progesterone and glucocorticoid, very few genes have been shown to be regulated in this manner by androgen. Thus, the PIP gene should be very useful for such studies as the identification of DNA sequences with which the androgen receptor can interact.

4. Prolactin-inducible gene expression as a prognostic and diagnostic marker of human breast diseases?

In addition to the study of basic mechanisms of prolactin and androgen action, the PIP system may allow additional insight into the role of prolactin/growth hormone and steroid hormones in human breast tumorigenesis. Despite its undesirable side-effects of producing masculinizing features, androgen has been used effectively in the treatment of a subgroup of breast cancer patients, including some who did not benefit from tamoxifen treatment [29]. Therefore, PIP expression may be a useful index for androgen and prolactin/growth hormone responsiveness and may be a positive prognostic factor. According to this hypothesis, PIP expression is not a growth marker but is a differentiation marker. This is consistent with our finding that prolactin/growth hormone failed to stimulate the growth of T–47D cells either in cell culture [24, 30] or in nude mice [31]. These findings appear not to support a significant mitogenic role of prolactin/growth hormone in *human* breast cancer. On the other hand, we have gathered evidence to suggest that PIP expression may still be related to the actions of other growth-promoting factors such as estradiol in human breast cancer. First, PIP is expressed only in human breast cancer cell lines that are estrogen receptor (ER)-positive and estradiol-responsive. No ER-negative cell line so far examined expressed PIP [26]. Second, PIP mRNA was detected in approx. 60% of human breast cancer biopsies, indicating that PIP is expressed *in vivo*. There was a highly significant correlation ($p < 0.01$) between PIP mRNA and ER levels in the 51 tumor specimens analysed [32]. Since ER content is a useful predictor of estrogen-responsiveness and a significant prognostic marker of human breast cancer, PIP expression may be an additional useful marker of hormone-

responsiveness. Tumors that express high levels of ER and PIP may belong to a subgroup that are most responsive to hormonal therapy and therefore have the most favorable prognosis.

We failed to detect PIP mRNA in many other forms of human cancers such as tumor of the lung, kidney, pituitary, colon and others [32]. However, PIP mRNA was abundant in benign gross cystic breast diseases [32], indicating that PIP may not be a marker for breast cancer. Nevertheless, PIP expression may be a tale-tell sign of abnormal development of the human breast. The appearance of elevated PIP in the circulation, which can be measured by radioimmunoassay [32], may afford diagnosis of early symptoms of benign or malignant breast diseases.

5. In search of an identity for the prolactin-inducible gene product

Since the function of PIP is unknown, knowledge of its sequence may reveal homology with other known gene products. Nucleotide sequence analysis of the PIP cDNA [26] revealed that it encodes a polypeptide of 146 amino acids with a calculated molecular mass of 16.5 kilodaltons. The predicted amino-terminal sequence is rich in hydrophobic residues indicative of a signal peptide which, after cleavage, generates the mature protein of 123 amino acids. One glycosylation signal (Asn-X-Thr) is present, consistent with the previous finding [25] that PIP exists mainly as a secreted glycoprotein.

After the completion of the sequence of PIP, we became aware of the work of Haagensen and his colleagues [33]. These investigators have for some years been studying a 15 kDa protein which was isolated from the fluid of human gross cystic breast disease. They termed this protein the gross cystic disease protein (GCDP-15). They have also found GCDP-15 to be produced by the T-47D breast cancer cell line and have deduced the amino acid sequence for GCDP-15. Our predicted amino acid sequence of PIP is identical to that of GCDP-15. This would account for our finding that PIP mRNA is found in gross cystic breast disease tissues. Although Haagensen and co-workers have not studied the expression of GCDP-15 in breast tumor tissues, they did observe that the serum levels of GCDP-15 in breast cancer patients were comparable to that of the protein in patients with benign gross cystic breast diseases.

The establishment of the identity of PIP with GCDP-15, however, did not provide any information on the function of this protein. Therefore, we compared the nucleotide sequence of PIP with existing sequence information stored in GenBank. This search revealed significant homology between PIP and a gene which was expressed in the mouse submaxillary gland [34]. Comparison of the predicted amino acid sequences encoded by the two genes revealed an overall 40% homology [26]. When only two-thirds (the middle and carboxyl terminus) of the sequences of the two gene products was compared, 51% of the amino acid residues are identical, and this homology

becomes 67% when conservative amino acid changes are also considered. This mouse submaxillary product may be the mouse equivalent of PIP, and raises the possibilities that PIP may also be expressed in the human submaxillary gland and that the submaxillary glands in both man and animal are targets for prolactin and androgen. Indeed, the mouse submaxillary gland is an androgen target tissue and its production of epidermal growth factor is regulated by androgen [35]. That prolactin may regulate the function of the submaxillary gland is a concept that requires experimental verification. In any event, the study of the molecular action of prolactin in human breast cancer may lead to new avenues of research in the identification of potentially novel functions of the pituitary hormone prolactin.

6. Pituitary-mediated proliferation of human breast cancer

As eluded to in a previous section, we have not been able to demonstrate an *in vitro* mitogenic effect of prolactin or growth hormone on any of the established human breast cancer cell lines [24, 30]. First, we reasoned that this could be due to the inadequacy of the culture conditions, and decided to evaluate the growth in *vivo* of T-47D cells transplanted into the immunodeficient athymic nude mice. Nude mice not only lack thymic function but are also deficient in many aspects of endocrine function: insufficient production of gonadotropins leading to steroid hormone deficiency and thus to sterility. For this reason, it was not surprising to find that the estrogen-dependent T-47D human breast cancer cells did not proliferate at all in these animals [31, 36] (Figure 4). Upon supplementation with estradiol, moderate but sustained proliferation of the T-47D tumor was observed. The administration of purified prolactin and growth hormone by daily injections or by continuous infusion with osmotic minipumps did not alter the growth of the T-47D human tumor in the estrogenized nude mice. However, when the estrogenized animals also received xenografts of normal rat pituitary or GH_3 rat pituitary tumor cells, the rate of growth of the T-47D human breast tumor was increased approximately 4-fold. Again, the T-47D tumor failed to grow in the nude mice in the absence of estradiol supplement in spite of the presence of rat pituitary grafts. Subsequently, this phenomenon was reproduced by Welsch and colleagues [37] employing another human breast cancer cell line MCF-7. These results indicated that the normal or tumorous pituitary gland is essential for the estrogen-dependent growth of human breast cancer. This pituitary influence was not due to prolactin or growth hormone. We therefore hypothesized that the pituitary gland secretes a factor that potentiates the mitogenic effect of estrogen on human breast cancer. A similar hypothesis was proposed in 1959 and 1960 based on the results of two clinical studies [11, 12] which demonstrated that estrogen, but not prolactin or growth hormone, injected into breast cancer patients stimulated the growth of the tumors, whereas it failed to do so when injected into the same patients after hypophy-

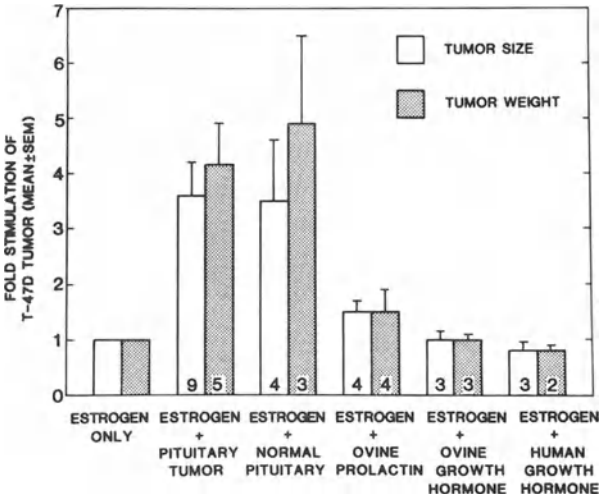


Figure 4. Effects of xenografts of normal and tumorous rat pituitary cells and the administration of purified pituitary hormones on the growth T-47D human breast tumor in estrogenized athymic nude mice. The experimental details were described in [31]. The T-47D tumor size and weight were determined 49 days after transplantation, and the number in each bar indicates the number of separate experiments performed [reproduced from 31].

sectomy. The authors of these studies suggested that a pituitary factor either mediated or was required for estrogen action in breast cancer patients. Thus, the requirement of a pituitary-derived growth factor for estrogen action in human breast cancer was suggested by both laboratory and clinical studies. Because this pituitary factor appears to potentiate the mitogenic effect of estrogen (although their roles could theoretically be reversed), we therefore refer to it as the "estrogen potentiating factor" or EPF.

7. Relationship between pituitary mitogen and insulin-like growth factor II

An *in vitro* assay monitoring the proliferation of T-47D human breast tumor cells in the presence or absence of estradiol was used to measure EPF activity [31]. Extracts of rat and human pituitary glands also contained EPF activity. EPF activity was present in the anterior but not the posterior lobe of the human pituitary gland (Figure 5). EPF activity can be detected in the conditioned media of some rat pituitary tumor cell lines such as GH_3 and GH_1 [31] (Figure 6) and of primary rat pituitary cell cultures (unpublished). While we were comparing conditioned media of a variety of cell lines for EPF activity, we fortuitously found that the Buffalo rat liver cell line of Dulak and Temin [38], BRL-3A, secreted potent EPF activity into the medium. The BRL-3A cells are well-known for their production of rat multiplication stimulating activity which, because of its homology with human insulin-like growth factor

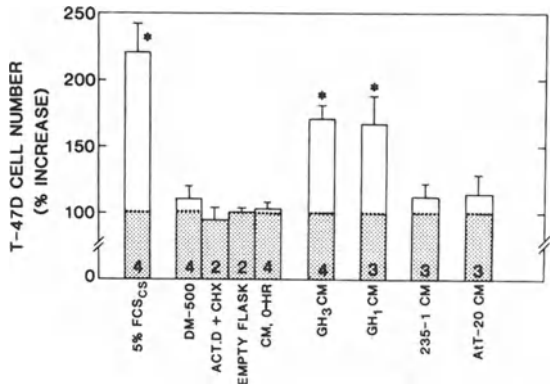


Figure 5. Effect of conditioned media from pituitary tumor cell lines on the proliferation *in vitro* of T-47D cells in the absence (shaded) and presence (open) of 10^{-10}M estradiol. With exception to the positive control (5% FCS_{cs}), all other conditions were conducted in the serum-free medium containing 500 ug/ml bovine serum albumin (DM-500). EPF activity was present in conditioned media of GH_3 and GH_1 rat pituitary tumor cell lines but not of the rat 235-1 and mouse AtT-20 pituitary tumor cell lines. Also, EPF activity was absent in medium of GH_3 previously treated with actinomycin D and cycloheximide, in medium harvested from flask with no cells (empty flask) and in medium that was added to GH_3 cells but was immediately removed (CM, 0-hr) [reproduced from 31].

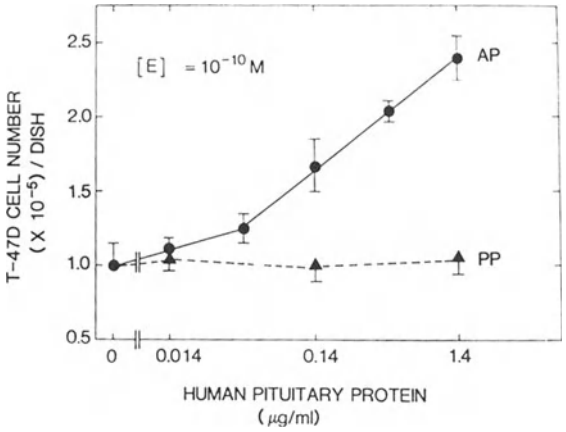


Figure 6. Effect of extracts of human anterior pituitary (AP) and posterior pituitary (PP) on the growth *in vitro* of T-47D cells. The two lobes of the pituitary gland were quickly dissected after thawing of the frozen gland and homogenized in phosphate buffered saline. Increasing amounts of proteins in the 100,000 × g supernatant were added to T-47D cells. The cell proliferation assay was conducted in the absence of serum. Estradiol (10^{-10}M) was present in each dish. Extract of AP, but not of PP, contained EPF activity.

II (IGF-II), is now commonly referred to as rat IGF-II [39]. The BRL-3A derived IGF-II is a heterogeneous mixture of related peptides differing in their molecular weights but displaying overlapping biological and immunological properties [38, 40, 41]. Because of the similarity between the breast cancer stimulating activity produced by rat pituitary cells and that secreted by the Buffalo rat liver cells, we wondered whether or not the pituitary EPF is an IGF-II like growth factor. To test this hypothesis, we had to show that the rat pituitary gland secretes IGF-II peptides and that purified IGF-II is mitogenic in human breast cancer cells.

8. Characterization of IGF-II peptides secreted by the rat pituitary gland

Binoux *et al.* [42] had previously used a competitive protein binding assay to detect insulin-like growth factor activity from rat pituitary glands. In this study, however, the investigators were not able to determine whether IGF-I/somatomedin C or IGF-II was actually produced. Hasselbacher *et al.* [2] had also reported that extracts of human pituitary glands contained immunoreactive IGF-II peptides although the nature of the IGF-II peptide(s) was not studied. Nevertheless, these reports encouraged us to investigate the molecular forms of IGF-II secreted by the rat pituitary glands. In this study [43], primary pituitary monolayer cultures were incubated with ^{35}S-cysteine, and the labeled media were subjected to immunoprecipitation using a rabbit anti-rat IGF-II antiserum donated by Dr. S. Peter Nissley (N.I.H.). The immunoreactive peptides were analyzed by polyacrylamide gel electrophoresis and visualized by fluorography. Figure 7 shows that the predominent IGF-II peptides secreted by the pituitary cultures were the 10 and 8 kDa species. When the IGF-II peptides from the BRL-3A cell line were similarly analysed, a 7 kDa peptide in addition to the 8 and 10 kDa peptides were detected. These results indicate that the pituitary cells were unable to secrete the 7kDa species which is a prominent species produced by the BRL-3A cells. Also, several larger peptides were also immunoprecipitated from both the BRL-3A and pituitary cells; a 20 kDa species may be the precursor product (pro-IGF-II), while the 33 kDa species has been tentatively identified as the carrier protein for IGF [44], co-precipitated with the IGF-II. At present, the identity of several other immunoreactive peptides with apparent mass of 64 to 100 kDa is not known. These results are consistent with early reports [38, 40, 41] which showed that rat IGF-II is a heterogeneous mixture of immunologically related peptides derived either from the translation of different mRNA transcripts and/or from post-translational processing. Differences in either of these pathways in the rat pituitary gland and the BRL-3A cells may account for our observation that the former tissue failed to produce the small (7 kDa) IGF-II peptide.

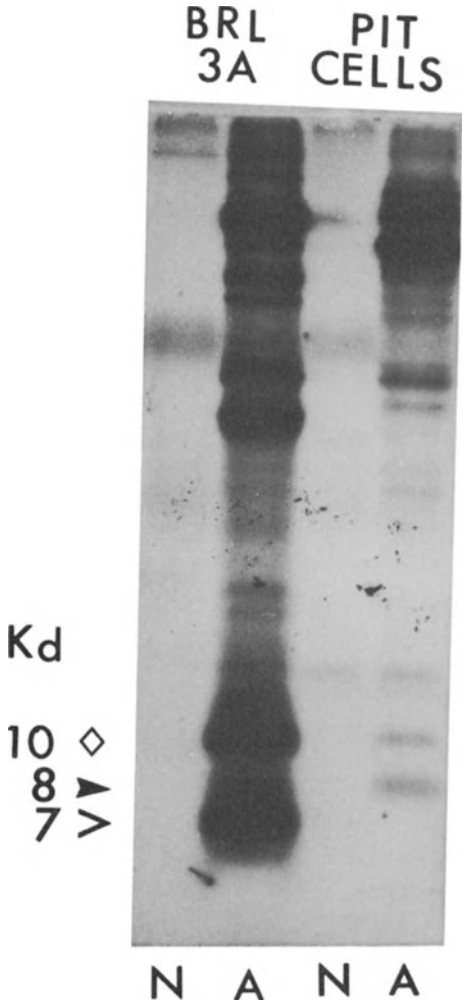


Figure 7. Immunoreactive rat IGF-II peptides secreted by the Buffalo rat liver cell line (BRL-3A) and rat pituitary cell cultures (pit cells). ^{35}S-cysteine labeled proteins secreted by each cell culture were incubated with either non-immune rabbit serum (NS) or rabbit anti-rat IGF-II serum (AS). The immunoprecipitated proteins were analyzed by SDS- polyacrylamide gel electrophoresis followed by fluorography. Note the absence of the 7,000-dalton IGF-II species in the pituitary cells [modified from 43].

9. Response of human breast cancer cells to IGF-II

Our finding that the pituitary gland secretes IGF-II provides only circumstantial evidence to support the thesis that the pituitary-derived mitogen for breast cancer is IGF-II. It was therefore necessary to show that purified rat IGF-II peptides were indeed mitogenic in human breast cancer cells. Again,

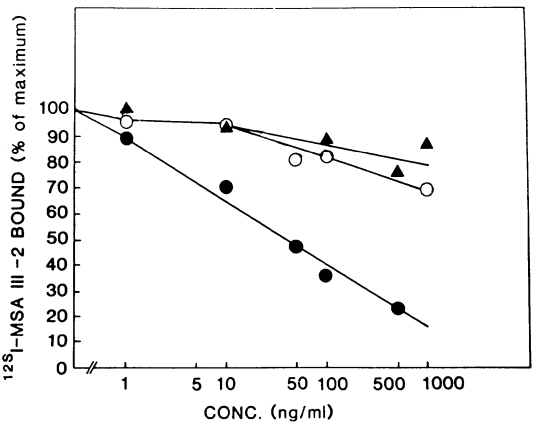


Figure 8. Binding of ^{125}I-MSA-III-2 (rat IGF-II) and its displacement by unlabeled MSA-III-2 (●), human IGF-I/Somatomedin C (○) and bovine insulin (▲) in T-47D cells [reproduced from 45].

Dr. S. Peter Nissley has generously provided the purified rat IGF-II preparations which enabled us to perform several studies *in vitro* [45]. Figure 8 shows that T-47D human breast cancer cells possessed specific receptor sites for rat IGF-II; the binding of ^{125}I-IGF-II (MSA-III-2 of Nissley, which presumably corresponds to the 7 kd peptide) was inhibited effectively by the corresponding unlabeled peptide, while human IGF-I and insulin did not inhibit IGF-II binding significantly. More importantly, rat IGF-II peptides were potent mitogens for the T-47D cells *in vitro* (Figure 9). The homogeneous IGF-II preparation (MSA-III-2) was approx. 100-fold more effective than insulin in stimulating the proliferation of T-47D cells grown on type I collagen gel; cells grown on plastic also responded to IGF-II albeit less dramatically [45]. Another IGF-II preparation (MSA-II, the 8 kDa peptide was the major species) was also active in stimulating the growth of T-47D cells, although less effectively than the MSA-III-2 preparation. Nevertheless, these IGF-II peptides were active in the range of 10–500 ng/ml. In man these levels are considered physiological as 600–900 ng/ml of IGF-II was found in human plasma [46]. These circulating levels of IGF-II should be more than adequate to promote the growth of human breast cancer *in situ*. Experiments are underway to evaluate the efficacy of IGF-II in promoting the growth of T-47D human breast cancer cells transplanted into estrogenized athymic mice.

10. Concluding remarks

The hormonal regulation of breast cancer is undoubtedly very complex, involving endocrine, paracrine and autocrine mechanisms. These mechanisms will encompass traditional hormones and growth factors. Our efforts in the recent past have been devoted to elucidating the role that pituitary hormones

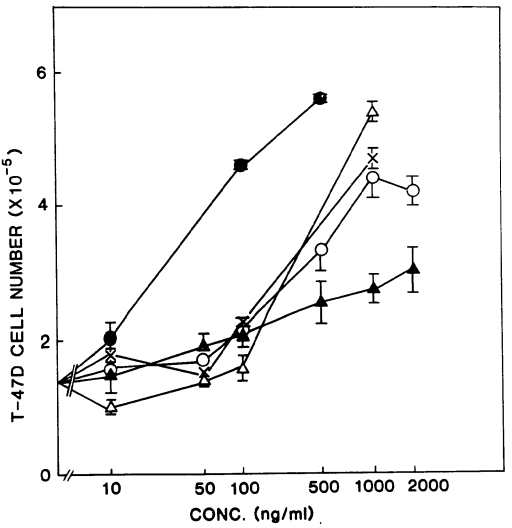


Figure 9. Effect of rat IGF-II preparations (MSA-III-2, •; MSA-II, X; and CR-MSA, △), human IGF-I (○) and bovine insulin (▲) on the proliferation of T-47D cells cultured on collagen gels in the absence of serum. MSA-III-2 and MSA-II were generous gifts of Dr. S.P. Nissley, N.I.H. and CR-MSA was purchased from Collaborative Research, Mass. Human IGF-I was prepared from the laboratory of Dr. M. Bala, University of Saskatchewan [reproduced from 45].

and growth factors may play in human breast tumorigenesis. Our efforts have led to the identification and characterization of a human breast cancer gene which is regulated by prolactin and androgen. The study of this gene and the product it encodes may allow us to: (1) address the nature of multihormonal regulation of human breast cancer involving both polypeptide and steroid hormones; (2) explore the mechanism of action at the molecular level of prolactin and androgen in target cells; (3) evaluate the expression of the prolactin/androgen inducible gene as a potential prognostic and/or diagnostic marker for hormone responsive human breast cancer. Further, we have added IGF-II to the list of peptide hormones that are secreted by the pituitary gland, and have provided preliminary evidence to suggest that IGF-II may be a physiologically important growth factor for human breast cancer. This forms the basis for future directions to address the *in vivo* action of IGF-II in human breast cancer. Based on the work of ours and of others, we favor the notion that the important role of the pituitary gland in human breast cancer is not merely to influence the secretion of ovarian and adrenal steroids via the production of the gonadotropins and adrenocorticotropin, but also to provide direct stimuli on the breast cancer cells via the production of prolactin and insulin-like growth factor II. The balance between the actions of these two pituitary-derived hormones, as well as that of the endocrine steroids and growth factors [47–50; also see chapter by Dickson *et al.* in this volume], will influence the progression of human breast cancer.

Acknowledgments

This work was supported by the Medical Research Council of Canada, the National Cancer Institute of Canada, the Manitoba Health Research Council and the St. Boniface Hospital Research Foundation. RPCS is a MRC Scientist, LCM is a MRC Fellow, TCD and YM are recipients of a postdoctoral fellowship and studentship, respectively, from the St. Boniface Hospital Research Foundation. The authors thank Dr. S. Peter Nissley, N.I.H., for the rabbit anti-rat IGF-II serum and the rat IGF-II preparations.

References

1. Gospodarowicz D, Neufeld G, Schweigerer L: Fibroblast growth factor. Mol Cell Endocrinol 46:187–204.
2. Haselbacher GK, Schwab ME, Pasi A, Humbel E: Insulin-like growth factor II in human brain: regional distribution of IGF-II and of higher molecular mass forms. Proc Natl Acad Sci USA 82:2153–2157, 1985.
3. Brockes JP, Lemke GE, Balzer DR, Jr: Purification and preliminary characterization of a glial growth factor from the bovine pituitary. J Biol Chem 255:8374–8377, 1980.
4. Lau DCW, Roncari DAK, Yip DK, Kindler S, Nilsen SGE: Purification of a pituitary polypeptide that stimulates the replication of adipocyte precursors in culture. FEBS Lett 153:395–398, 1983.
5. Kudlow JE, Kobrin MS: Secretion of epidermal growth factor-like mitogens by cultured cells from bovine anterior pituitary glands. Endocrinology 115:911–917, 1984.
6. Welsch CW: Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res 45:3415–3443, 1985.
7. Reece RP, Leonard SL: Effect of estrogens, gonadotropins and growth hormone on mammary glands of hypophysectomized rats. Endocrinology 29:297–305, 1941.
8. Lyon WR: Hormonal synergizm in mammary growth. Proc Roy Soc (London) Series B 149:303–325, 1958.
9. Clifton KH, Furth J: Ducto-alveolar growth in mammary glands of adrenogonadectomized male rats bearing mammotropic pituitary tumors. Endocrinology 66:893–897, 1960.
10. Meites J: Control of mammary growth and lactation. In: Neuroendocrinology, Vol. 1 Martin L and Ganong WF, (eds), Academic Press, New York, pp 669–701.
11. Pearson OH, Bronson SR: Results of hypophysectomy in the treatment of metastatic mammary carcinoma. Cancer (Phila) 12:85–92, 1959.
12. Lipsett MB, Bergenstal DM: Lack of effect of human growth hormone and ovine prolactin on cancer in man. Cancer Res 20:1172–1178, 1960.
13. Nagasawa H: Prolactin and human breast cancer: a review. Eur J Cancer 15:267–279, 1979.
14. Vorherr H: Breast Cancer: Epidemiology, Endocrinology, Biochemistry and Pathology, Urban & Schwarzenberg, Baltimore. pp 107–119, 1980.
15. Henderson BE, Pike MC: Prolactin—an important hormone in breast neoplasm? In: Banbury Report 8: Hormones and Breast Cancer, Pike MC, Siiteri PK, and Welsch CW (eds), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. pp 115–130, 1981.
16. Pearson OH, Manni A, Chambers M, Brodkey J, Marshall JS: Role of pituitary hormones in the growth of human breast cancer. Cancer Res 38:4323–4326, 1978.
17. Shiu RPC, Murphy LC, Tsuyuki D, Myal Y, Lee-Wing M, Iwasiow B: Biological actions of prolactin in human breast cancer. Rec Prog Horm Res 43:277–303, 1987.
18. Salih H, Flax M, Brander W, Hobbs JR: Prolactin dependence in human breast cancers. Lancet 2:1103–1105, 1972.

19. Shafie SM, Brooks SC: Effect of prolactin on growth and the estrogen receptor level of human breast cancer cells (MCF-7). Cancer Res 37:792–799, 1977.
20. Leung BS, Potter AH, Qureshi S: Interaction of prolactin, estrogen and progesterone in a human mammary carcinoma cell line, CAMA-1. Cell growth and thymidine uptake. J Steroid Biochem 15:421–427, 1981.
21. Malarkey WB, Kennedy M, Allred LE, Milo G: Physiological concentrations of prolactin can promote the growth of human breast tumor cells in culture. J Clin Endocrinal Metab 56:673–677, 1983.
22. Simon WE, Pahnke VG, Holzel F: *In vitro* modulation of prolactin binding to human mammary carcinoma cells by steroid hormones and prolactin. J. Clin. Endocr. Metab. 60:1243–1249, 1985.
23. Manni A, Wright C, Davis G, Glenn J, Joehl R, Feil P: Promotion of prolactin of the growth of human breast neoplasms cultured *in vitro* in the soft agar clonogenic assay. Cancer Res 46: 1669–1672, 1986.
24. Shiu RPC, Paterson JA: Alteration of cell shape, adhesion and lipid accumulation by prolactin in human breast cancer cells. Cancer Res 44:1178–1186, 1984.
25. Shiu RPC, Iwasiow BM: Prolactin-inducible proteins in human breast cancer cells. J Biol Chem 260:11307–11313, 1985.
26. Murphy LC, Tsuyuki D, Myal Y, Shiu RPC: Isolation and sequencing of a cDNA clone for a prolactin-inducible protein (PIP): Regulation of PIP gene expression in the human breast cancer cell line T-47D. J Biol Chem 262:15236–15241, 1987.
27. Rosen JM, Matusik RJ, Richards DA, Gupta P, Rodgers JR: Multihormonal regulation of casein gene expression at the transcriptional and posttranscriptional levels in the mammary gland. Recent Progr Horm Res 36:157–187, 1980.
28. Banerjee MR, Ganguly R, Mehta NM, Ganguly N: Hormonal regulation of casein gene expression in normal and neoplastic cells in murine mammary glands. In: Hormonal Regulation of Mammary Tumors, Vol. 2 Leung BS (ed), Eden Press, Montreal, pp 229–283, 1982.
29. Arafah BM, Pearson OH: Endocrine treatment of advanced breast cancer. In: Estrogen/ antiestrogen Action and Breast Cancer Therapy Jordan VC (ed), The University of Wisconsin Press, pp 417–430, 1986.
30. Shiu RPC: Prolactin, pituitary hormones and breast cancer. In: Banbury Report 8: Hormones and Breast Cancer Pike MC, Siiteri PK, Welsch CW (eds), Cold Spring Harbor Laboratory, Cold Spring Harbor, pp 185–196, New York, 1980.
31. Dembinski TC, Leung CKH, Shiu RPC: Evidence for a novel pituitary factor that potentiates the mitogenic effect of estrogen in human breast cancer cells. Cancer Res 45:3083–3089, 1985.
32. Murphy LC, Lee-Wing M, Goldenberg GJ, Shiu RPC: Expression of the gene encoding a prolactin-inducible protein (PIP) by human breast cancers *in vivo*: correlation with steroid receptor status. Cancer Res 47:4160–4164, 1987.
33. Haagensen DE, Jr, Mazoujian G: Biochemistry and immunohistochemistry of fluid proteins of the breast in gross cystic disease. In: Disease of the Breast Haagensen CD, (ed), WB Saunders Co., Philadelphia, pp 474–500, 1986.
34. Windass JD, Mullins JJ, Beecroft LJ, George H, Meacock PA, Williams BRG, Brammer WJ: Molecular cloning of cDNAs from androgen-independent mRNA species of DBA/2 mouse submaxillary glands. Nucleic Acid Res 12:1361–1376, 1984.
35. Byyny RL, Orth DN, Cohen S, Doyne ES: Epidermal growth factor: effects of androgens and andrenergic agents. Endocrinology 95:776–782, 1974.
36. Leung CKH, Shiu RPC: Required presence of both estrogen and pituitary factors for the growth of human breast cancer cells in athymic nude mice. Cancer Res 41:546–551, 1981.
37. Welsch CW, Shim EL, McManus MJ, White AC, McGrath CM: Estrogen induced growth of human breast cancer cells (MCF-7) in athymic nude mice is enhanced by secretions from a transplantable pituitary tumor. Cancer Lett 14:309–316, 1981.
38. Dulak, NC, Temin HM: Multiplication-stimulating activity for chicken embryo fibroblasts

from rat liver cell conditioned medium: a family of small polypeptides. J Cell Physiol 81:161–170, 1973.
39. Marquardt H, Todaro GJ, Henderson LE, Oroszlan S: Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures: homology with human insulin-like growth factor II. J Biol Chem: 256:6859–6863, 1981.
40. Moses AC, Nissley SP, Short PA, Rechler MM, Podskalny JM: Purification and characterization of multiplication-stimulating activity: insulin-like growth factors purified from rat-liver-cell-conditioned medium. Eur J Biochem 103:387–400, 1980.
41. Moses AC, Nissley SP, Short PA, Rechler MM: Immunological cross-reactivity of multiplication-stimulating activity polypeptides. Eur J Biochem 103:401–408, 1980.
42. Binoux M, Hossenlopp P, Lassarre C, Hardouin N: Production of insulin-like growth factors and their carrier by rat pituitary gland and brain explants in culture. FEBS Lett 124:178–184, 1981.
43. Shiu RPC, Iwasiow B: Insulin-like growth factor II peptides secreted by the rat pituitary gland. (Submitted for publication, 1988.)
44. Romanus JA, Terrell JE, Yang YWH, Nissley SP, Rechler MM: Insulin-like growth factor carrier proteins in neonatal and adult rat serum are immunologically different: demonstration using a new radioimmunoassay for the carrier protein from BRL-3A rat liver cells. Endocrinology 118:1743–1758, 1986.
45. Myal Y, Shiu RPC, Bhaumick B, Bala M: Receptor binding and growth-promoting activity of insulin-like growth factors in human breast cancer cells (T-47D) in culture. Cancer Res 44:5486–5490, 1984.
46. Zapf J, Froesch ER, Humbel RE: The insulin-like growth factor (IGF) of human serum: chemical and biological characterization and aspects of their physiological role. Curr Top Cell Regul 19:257–309, 1981.
47. Imai Y, Leung CKH, Friesen HG, Shiu RPC: Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture. Cancer Res 42:4394–4398, 1982.
48. Solomon DS, Zwiebel JA, Bano M, Losonczy I, Fehnel P, Kidwell WR: Presence of transforming growth factors in human breast cancer cells. Cancer Res 44:4069–4077, 1984.
49. Dickson RB, Bates SE, McManaway M, Lippman ME: Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res 46:1707–1713, 1986.
50. Lippman ME, Dickson RB, Kasid A, Gelmann E, Davidson N, McManaway M, Huff K, Bronzert D, Bates S, Swain S, Knabbe C: Autocrine and paracrine growth regulation of human breast cancer. J Steroid Biochem 24:147–154, 1986.

8. Structure and function of the pS2 gene and estrogen receptor in human breast cancer cells

G. Stack, V. Kumar, S. Green, M. Ponglikitmongkol, M. Berry, M.C. Rio, A.M. Nunez, M. Roberts, C. Koehl, P. Bellocq, B. Gairard, R. Renaud, and P. Chambon

1. Introduction

A significant percentage of breast cancers are estrogen-dependent. These tumors undergo regression when deprived of estrogen or when treated with anti-estrogens [1]. The basis for this hormonal dependence in these cancers is the presence of estrogen receptors (ER). However, the function of the estrogen-receptor complex in promoting the growth of cancer cells is incompletely understood. The MCF-7 cell line, which is derived from a pleural effusion of a human breast cancer [2], contains ER [3] and is dependent on estrogen for optimal growth [4, 5]. In addition, the levels of a variety of mRNAs, proteins and secreted growth factors are under estrogen control in MCF-7 cells [6–10]. As such, these cells represent a good *in vitro* model system for hormone-dependent human breast cancer. Using these cells we have taken a two-pronged approach to study the molecular basis of estrogen action: 1) the molecular cloning of estrogen-responsive MCF-7 cell gene(s) and 2) the isolation of the human ER cDNA. Of interest is both the function of the gene product of an estrogen-responsive gene and its relationship to cell growth, as well as the mechanism by which estrogen and its receptor control the expression of that gene. We will discuss in this chapter our analysis of one such gene, named pS2, and the polypeptide for which it codes. Included will be a discussion of the localization of the estrogen-responsive DNA element (ERE) to the 5′-flanking region of the pS2 gene. In addition, a structure/function analysis of the ER, made possible by the expression and site-directed mutagenesis of the human ER cDNA, has provided information concerning how the ER activates this and other EREs.

2. pS2 is a small secretory polypeptide induced by estrogen

Originally, pS2 was the designation given to a cDNA clone corresponding to a polyadenylated RNA species whose level was stimulated by estradiol treatment of hormone-deprived MCF-7 cells [11]. The induction of pS2 RNA is an estrogen-specific response since other steroid hormones, such as the progestins, glucocorticoids, and androgens, have no effect.

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

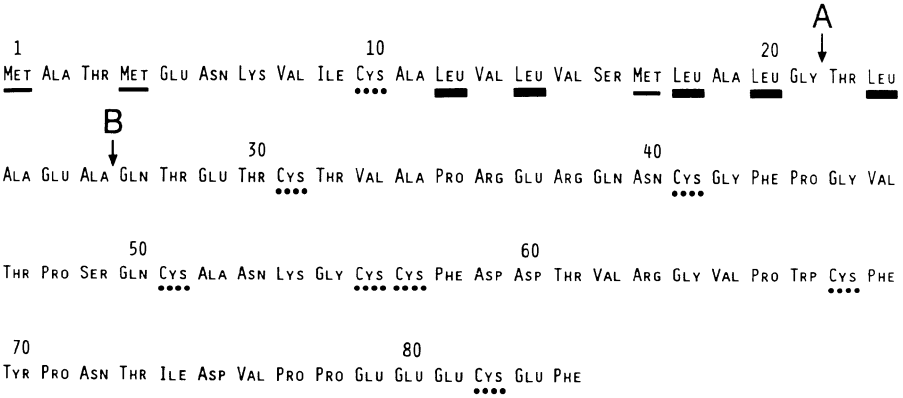


Figure 1. pS2 protein amino acid sequence deduced from that of pS2 mRNA. The cysteine, leucine and methionine residues are underlined. The putative cleavage sites of the signal peptide are indicated by arrows A and B [12, 13].

The pS2 gene product is a small secreted polypeptide. The primary structure of pS2, as deduced from the sequence of a full-length cDNA [12], is shown in Figure 1. It is composed of 84 amino acids, has a signal peptide at the amino terminus, and its corresponding molecular weight is 9140 daltons. Rabbit antiserum prepared against a synthetic polypeptide corresponding to the last 31 amino acids of the carboxyl end of the predicted sequence was used to demonstrate the estrogen-dependent presence of pS2 in the media of MCF-7 cell cultures [13]. The synthesis and secretion of pS2 is induced by estradiol and phenol red, but not by the anti-estrogen tamoxifen (Figure 2). The mature protein produced after processing of the signal peptide has an apparent size of approximately 7,000 daltons. The longer nascent polypeptide can only be detected as the product of the *in vitro* translation of poly(A) RNA from MCF-7 cells enriched in pS2 mRNA [13]. The exact site of cleavage of the signal peptide has not been determined. The location of two potential peptidase recognition sites of the form ala-X-B ↓ [14], where B is usually ala, gly, or ser, indicate that cleavage could be occurring either after glycine 21 or alanine 26. However, because the mature form of pS2 cannot be labelled with ^{14}C-leucine, the cleavage must be occurring after residue 23 (see Figure 1). Thus, the signal peptide has a likely length of 26 amino-acids. It follows that the mature pS2 protein is probably 58 amino-acids long with a true molecular weight of 6450 daltons.

3. Putative function of the pS2 protein

No close sequence homologies have been found to pS2 among existing nucleic acid or protein sequence databases. However, pS2 has general similarities to growth factors such as human insulin-like growth factors I and II (IGFI and

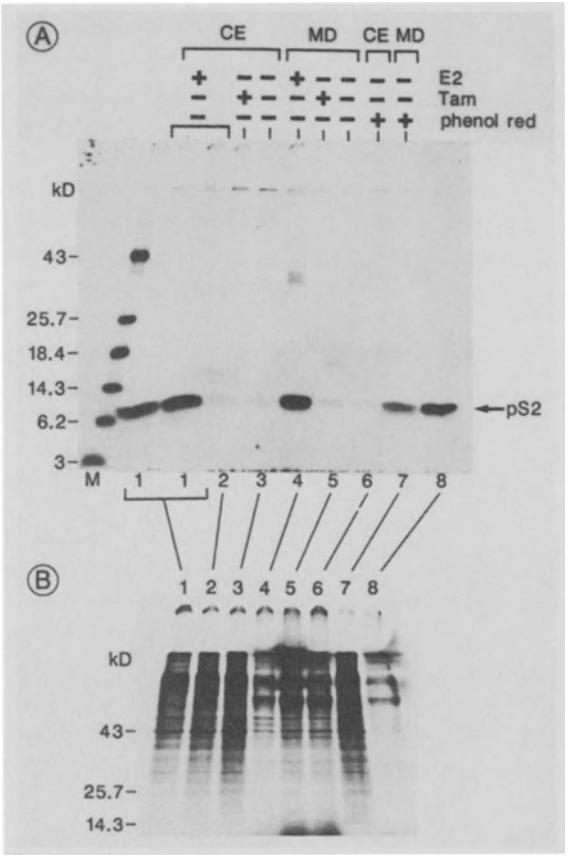


Figure 2. Effect of estradiol, tamoxifen and phenol red on pS2 protein synthesis and secretion in MCF-7 cells. Cells grown in the absence (lanes 1 to 6) or presence (lanes 7 and 8) of phenol red were supplemented with estradiol (E2, lanes 1 and 4) or tamoxifen (Tam, lanes 2 and 5). After labelling in the presence of ^{35}S-cysteine for 9 hrs., aliquots of the cell extracts (panel A, CE, lanes 1, 2, 3 and 7; lanes 1 correspond to duplicates) containing approximately 150,000 TCA-precipitable counts were analyzed by 10–25% gradient SDS-PAGE after immunopurification using pS2 antiserum. Aliquots of the media corresponding to approximately 150,000 TCA-precipitable counts of cell extracts were treated under the same conditions (panel A, MD, lanes 4, 5, 6 and 8). In panel B, aliquots of cell extracts (lanes 1, 2, 3 and 7) and media (lanes 4, 5, 6 and 8) corresponding to approximately 15,000 TCA-precipitable counts of cell extracts were electrophoresed without immunopurification. M, molecular weight markers,CE, cell extract and MD, medium [13].

II). These are all small, secreted polypeptides. IGF-I and II, for comparison, are 67 and 70 amino-acids long, respectively [15, 16]. In addition, the mature form of pS2, like the IGFs, is rich in cysteine residues and capable of forming three disulfide bonds [see reference 12 for further discussion]. Accordingly, we are tempted to speculate that pS2 may be an estrogen-inducible growth factor or estromedin [17]. Estrogen is known to stimulate MCF-7 cells to secrete a variety of putative growth factors, such as epidermal growth factor-

like (or transforming growth factor alpha-like) activities [8] and a 52 kilodalton glycoprotein with mitogenic properties [18]. It has been proposed that these secreted growth factor activities may play an autocrine or paracrine role in the regulation of human breast cancer growth [18, 19]. pS2 could be a related, but previously uncharacterized, growth factor. Functional studies must be performed to test this possibility.

4. Clinical correlations: pS2 gene expression is a possible marker for hormone-dependent breast cancers

pS2 RNA has been detected only in MCF-7 cells and some human breast cancer biopsies [20, 21]. No pS2 RNA has been found in other human cells in culture (HeLa cells, fibroblasts) or in other human cells or tissues (lymphocytes, placenta, liver, endometrium, normal breast tissues, benign breast tumors). On the basis of this initial tissue screening it appears that pS2 gene expression is breast cancer-specific. However, not all breast cancers express the pS2 gene. For this reason we have been interested to examine whether pS2 gene expression may identify a subset of cancers with therapeutic or prognostic significance.

Approximately 50 to 65% of all ER positive [ER(+)] breast cancers are responsive to anti-estrogen therapy [1, 22]. A better predictor of tumor responsiveness to anti-hormone therapy is the simultaneous presence of ER and progesterone receptor (PR); that is, about 80% of ER(+) and PR(+) breast cancers are hormonally responsive [1]. The presence of PR can be thought to reflect the responsiveness of the cells to estrogen because of the ability of estrogen to stimulate PR levels in MCF-7 and a variety of other cells [23, 24]. Thus, the ER(+) and PR(+) cells not only have estrogen receptors, but these receptors may be functional in mediating a response. By similar reasoning pS2 could also be a useful marker for hormone-dependent breast cancer.

One hundred twenty-five breast cancer biopsies were screened for the presence of ER, PR and pS2 RNA. The receptors were measured with radioligand assays and in some cases the presence of ER RNA was measured in RNA blots using the human ER cDNA as a probe. Overall, expression of the pS2 gene correlated positively with the presence of both ER and PR. Nevertheless, all possible combinations of the three markers were found, thereby subdividing the tumors into eight subclasses (Figure 3). Sixty-three percent of all biopsies were ER(+). The majority of these (48% overall) were PR(+). Slightly more than 80% of biopsies which were both ER(+) and PR(+) were also positive for pS2 RNA. This MCF-7-like subclass of breast cancer was the largest in our study (40% overall). Interestingly, 17% of ER(+) and PR(+) biopsies were pS2(−). This situation is reminiscent of another breast cancer cell line, T47D [25], which is ER(+), has a high constitutive level of PR which is estrogen insensitive, and has no detectable pS2 gene expression with or without estrogen [20, 26]. This T47D-like subclass could constitute the 20%

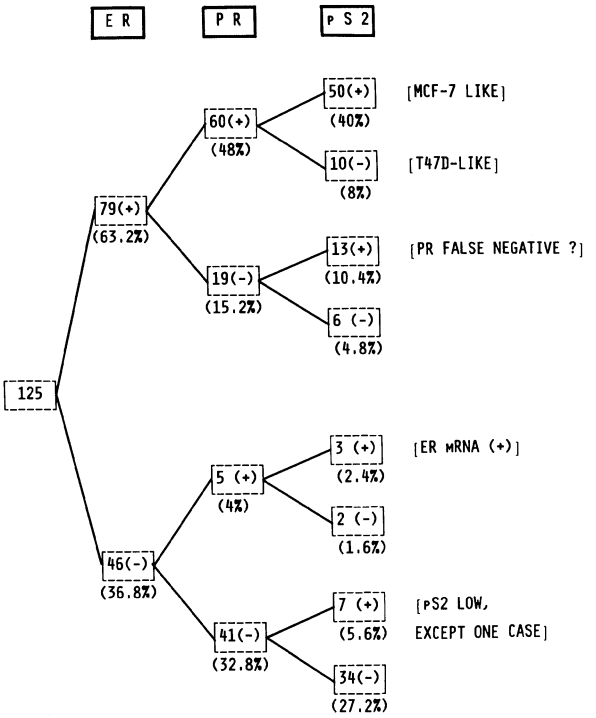


Figure 3. Breast cancer subclassification resulting from pS2 screening. Biopsies from 125 breast cancers were screened for ER and PR by classical radioligand assays on cytoplasmic fractions and for pS2 RNA by RNA blot analysis using a pS2 cDNA hybridization probe. The numbers in boxes indicate how many biopsies were positive (+) or negative (−) for the marker indicated at the top of each column. The numbers in parentheses represent the percentage of the total (125) biopsies having the indicated marker combination. Assays of ER RNA using the human ER cDNA as a probe were used to supplement the ER binding assays for the tumor subgroups which were ER(−) but pS2(+).

of breast cancers which are resistant to hormone therapy despite being both ER(+) and PR(+). If so, pS2 screening may provide the means to identify this important group. Another 5% of all biopsies were ER(+), but both PR(−) and pS2(−). Among ER(+) tumors, these should be most unlikely to respond to hormone therapy.

In ER(+) and PR(−) biopsies, the majority were unexpectedly pS2(+) (10.4% overall). Whether these constitute a true subclass or are PR false negatives remains to be determined. For this reason it will be important to screen for PR RNA, which should be a more sensitive test than the receptor binding assay. On the other hand, these tumors could be incompletely estrogen-responsive or perhaps estrogen-insensitive, but pS2-constitutive.

Among ER(−) biopsies the majority were both PR(−) and pS2(−) or low in pS2 RNA. Thus, the majority of hormone-independent cancers cannot be due to a constitutive expression of pS2. Three ER(−), PR(+) and pS2(+)

biopsies proved to be ER false negatives when ER RNA was measured to supplement the receptor binding assay. Finally, two biopsies were apparently constitutive for PR, while ER(−) and pS2(−).

Clearly, pS2 screening provides the basis for establishing new biochemical subclasses of breast cancers. Further studies are underway to determine the actual hormonal responsiveness and prognosis of breast cancers in relation to pS2 gene expression. The results will indicate whether this new subclassification scheme can be used to fine tune the prediction of the responsiveness of breast cancers to hormonal therapy.

5. Structure of the pS2 gene and transcript

The pS2 gene was isolated from both MCF-7 cells and human placenta [27]. It is organized into three exons (I-III) of 125, 153 and 212 base pairs, respectively, interrupted by two introns of approximately 3.1 kb (Intron A) and 0.77 kb (Intron B) (Figure 4). The complete unpolyadenylated gene transcript is 490 nucleotides long of which 40 nucleotides at the 5′-end and 198 nucleotides at the 3′-end are untranslated. After polyadenylation the pS2 mRNA has a size of about 600 nucleotides [11, 28]. The organization within and around the pS2 gene is the same in MCF-7 cells and normal human tissue [27]. The exon and adjacent intron sequences derived from human placenta and MCF-7 cells are identical. Based on a Southern analysis the gene organization and dosage are the same in human liver, placenta, and MCF-7 cells. Moreover, the 5′-flanking regions are identical in the genomic clones isolated from the human placenta and MCF-7 cells at least to position −328 with the exception of two base transitions at −185 and −304. Thus, no obvious rearrangement or amplification of the pS2 gene has occurred in MCF-7 cells, despite the fact that these cells are known to be polyploid and to have undergone extensive chromosomal translocations [29]. However, large translocations affecting the pS2 gene have not been ruled out.

The proximal 5′-flanking region contains DNA elements typical of many RNA polymerase B(II)-transcribed genes, namely a TATA box at position −23 to −29 and a CAAT element at −64 to −72 (Figure 4). A GC-rich motif (5′-GGGCGG-3′), with which the transcription factor Spl interacts [30], is usually located at −7 to −12 between the TATA box and cap site. The significance, if any, of this arrangement is not known. Computer-assisted searches failed to reveal any extensive homologies between the 5′-flanking sequence of the pS2 gene and that of other estrogen-responsive genes, most of which are of avian or amphibian origin [27]. In particular, there is no sequence identical to the well-characterized *Xenopus* vitellogenin estrogen-responsive elements (EREs) [31, 32]. Although several regions of homology (approximately 80% over 14 to 20 base pairs) were found to the upstream regions adjacent to other estrogen-responsive genes, these were also found in the 5′-flanking regions of genes whose expression is not known to be regulated

Figure 4. The nucleotide sequence of the pS2 gene. Sequences were determined from clones of both MCF-7 cell and placental origin. The sequence obtained from the MCF-7 cell clone is shown. The cap site (+1) in indicated as well as the exon/intron boundaries (open triangles). The two base differences found (up to −328) between the MCF-7 cell clone and the placental clone are indicated below the sequence. The 5′ flanking region has been numbered negatively (a dot is present every 10 nucleotides). Three sequences are underlined: the CAAT-like box (dashed line), the TATA box and the ATTAAA polyadenylation signal (full line). Closed circles indicate the possible polyadenylation sites. The deduced pS2 protein sequence is given along with the exons [27].

by estrogens. Thus, the physiological significance of these regions is not clear.

6. The 5′-flanking region of the pS2 gene contains an estrogen-responsive element

From run-on transcription assays in nuclei from MCF-7 cells, it is clear that expression of the pS2 gene is controlled by estrogen at the transcriptional level [26]. Moreover, the induction of transcription occurs rapidly (≤15 min.) after estrogen treatment of MCF-7 cells and occurs even in the absence of *de novo* protein synthesis. Thus, pS2 gene transcription represents a primary response to estrogen.

This transcriptional response is mediated by an ERE located in the 5′-flanking region of the pS2 gene [33]. A chimeric recombinant (pS2-*neo*) containing the Tn5 neomycin-resistance gene under the control of the pS2 promoter and upstream sequences from ~−3000 to +10 relative to the cap site was stably integrated into the genome of MCF-7 cells. Expression of the *neo* gene renders eukaryotic cells resistant to the drug G-418. The transformed clones exhibited resistance to G-418 only in the presence of estradiol. According to a northern analysis of cytoplasmic RNA from transformants, estradiol clearly stimulated transcription of the *neo* gene while having no effect on an internal control (the constitutively expressed 3A5 RNA [11]) (Figure 5A). In fact, the stimulation of expression of the integrated pS2-neo gene closely mimics that of the endogenous pS2 gene (Figure 5B). Therefore, an ERE is located in the ~3 kb fragment upstream of the pS2 gene cap site.

Further characterization of the pS2 ERE has been performed using transient transfection assays in cells into which the human ER has been introduced by co-transfection of an ER expression vector. The coding region of the human ER cDNA was subcloned into the eukaryotic expression vector pKCR2 [34] downstream of the SV40 early promoter region to create the human ER expression vector, HEO (originally pKCR2–ER [35]). This expression vector made possible the introduction of the human ER into cell lines of choice. For example, when HeLa cells are transfected with HEO, they produce receptors with a size and hormone-binding characteristics indistinguishable from that produced normally in MCF-7 cells [35]. When HeLa cells are co-transfected with HEO and a reporter plasmid in which expression of the *E. coli* chloramphenicol acetyltransferase (CAT) gene is controlled by a promoter region containing an ERE, CAT activity becomes estrogen-dependent. This was initially demonstrated with the reporter plasmid *vit*-tk-CAT in which the CAT gene was inserted downstream of a chimeric promoter region consisting of the *Xenopus* vitellogenin A2 upstream region (−331 to −87, *vit*) fused to the *Herpes simplex* virus thymidine kinase (tk) promoter (−105 to +51) [36]. This transient co-transfection assay provides the advantage of reducing estrogen-responsiveness to two, well-defined,

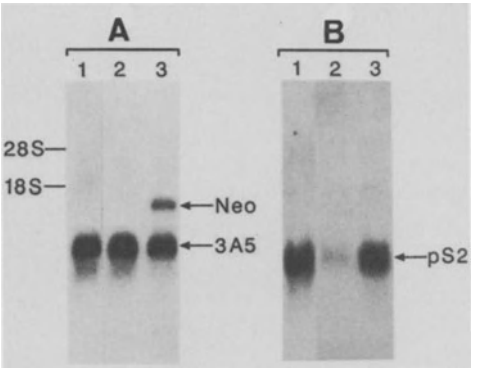


Figure 5. Northern analysis of cytoplasmic RNA isolated from MCF-7 transformants containing copies of a pS2-*neo* recombinant stably integrated into the genome. After growing cells in the absence of steroids for at least 5 days, the medium was replaced with withdrawn medium only (lane 2) or withdrawn medium plus 5×10^{-8}M estradiol (lane 1, 3). Cytoplasmic RNA was isolated by Nonidet P40-lysis 48 hours later. Fifteen μg of cytoplasmic RNA isolated from heterogenous populations of MCF-7 transformants (lanes 2 and 3), and wild-type MCF-7 cells (lane 1) were fractionated on a 1.5% agarose gel, transferred to nitrocellulose filters, and hybridized with three different probes specific for *neo* and 3A5 (see text) encoded transcripts (panel A) and endogenous pS2-gene encoded transcripts (panel B) [33].

required components: 1) an ERE upstream of a promoter which can be activated in the cell type being used and 2) an expression vector coding for the estrogen receptor of recombinant DNA origin. This assay can be used to study the structure and function of both the receptor (see section 7) and an ERE.

Recombinant plasmids containing the CAT gene under the control of pS2 gene 5′-flanking segments from +10 to either approximately −3,000 (pS2CAT−3,000), −1,000 (pS2CAT−1,000), −400 (pS2CAT−400) or −300 (pS2CAT−300) were constructed and co-transfected with HEO into HeLa cells cultured in the presence or absence of estradiol. CAT gene expression was stimulated by estradiol for pS2CAT−3,000, pS2CAT−1,000 and pS2CAT−400, but not for pS2CAT−300, demonstrating that an ERE is present in the −300 to −400 region of the pS2 gene (our unpublished results). Additional experiments using a recombinant (pS2-tk-CAT) into which the pS2 gene −84 to −400 5′-flanking segment was inserted upstream to tk-CAT, as described above, have shown that the pS2 ERE is located within the −84 to −400 5′-flanking region (our unpublished results). A precise identification of the pS2 ERE at the base level is in progress.

7. The structure and function of the human estrogen receptor

The ER is one member of a family of related gene regulatory molecules which includes receptor proteins for glucocorticoids, progestins, vitamin D3,

thyroid hormone, almost certainly the other steroid hormones, and probably other ligands [37, 38]. Each receptor binds specifically and with high affinity to its cognate ligand. The binding of estrogen to its receptor results in ill-defined physical changes within the ER, causes the ER to associate more tightly with the nucleus, and activates the transcription-regulating function of the receptor [39]. A key question to be answered regarding the mechanism of estrogen action in breast cancer and normal tissues, therefore, is how the receptor works. To this end, we have studied the structure and function of the human ER.

From a comparison of the human and chicken ER cDNAs, the receptor protein can be divided into six distinct regions (designated A through F) of varying homology (Figure 6) [40]. Three regions, A, C and E, are highly conserved between chicken and man (87%, 100% and 94% amino-acid homology, respectively). To a lesser extent regions C and E, but not A, are conserved between the various steroid and thyroid hormone receptors. The three regions B (56%), D (38%) and F (41%) show relatively low homology. The conservation of structure implies important functions for the regions A, C and E. To test the roles of these and the other regions of the ER a variety of expression vectors coding for receptor deletion mutants were constructed by site-directed mutagenesis on the human ER cDNA (Figure 6) [41].

The mutant receptors were expressed in HeLa cells and analyzed for three functions: 1) hormone binding; 2) tight nuclear binding; and 3) the ability to stimulate transcription via the pS2 and *Xenopus* vitellogenin A2 EREs. Tight nuclear binding was determined as those nuclear estrogen-receptor complexes which were resistant to low salt extraction (i.e. 0.15 M NaCl), but extractable with high salt (0.4 M KC1). The transcription-activating function was measured in transient co-transfection assays using either of two reporter plasmids, *vit*-tk-CAT or pS2CAT-1,000 (hereafter designated pS2CAT) (see section 6). The *vit*-tk-CAT reporter plasmid has the advantage of containing a well-characterized ERE which is located from position −297 to −331 upstream of the *Xenopus* vitellogenin A2 gene cap site and contains the palindromic sequence 5′-*GGTCACAGTGACC*− 3′ [31]. On the other hand, pS2CAT provides a completely homologous system; that is, the pS2 ERE is upstream of its natural promoter and both pS2 and the ER are of human and MCF-7 cell origin. Transcriptional activation was determined by CAT assays, supplemented in some cases by nuclease S1 mapping RNA analyses to verify correct transcription start-sites. Immuno-precipitation studies using the human ER monoclonal antibodies H222 and H226 from Abbott Laboratories were performed to verify that approximately equal amounts of mutant receptors of the expected size were expressed [41]. This was the case for all of mutants HE1 though HE25 (Figure 6) with the exception of HE7 for which a smaller than expected ER antigen was detected (possibly due to proteolysis) and HE20 which did not contain the epitopes for these antibodies. The results of this structure/function analysis, which are summarized in Figure 6, indicate that the structural domains suggested from sequence homologies correspond to discrete functional domains.

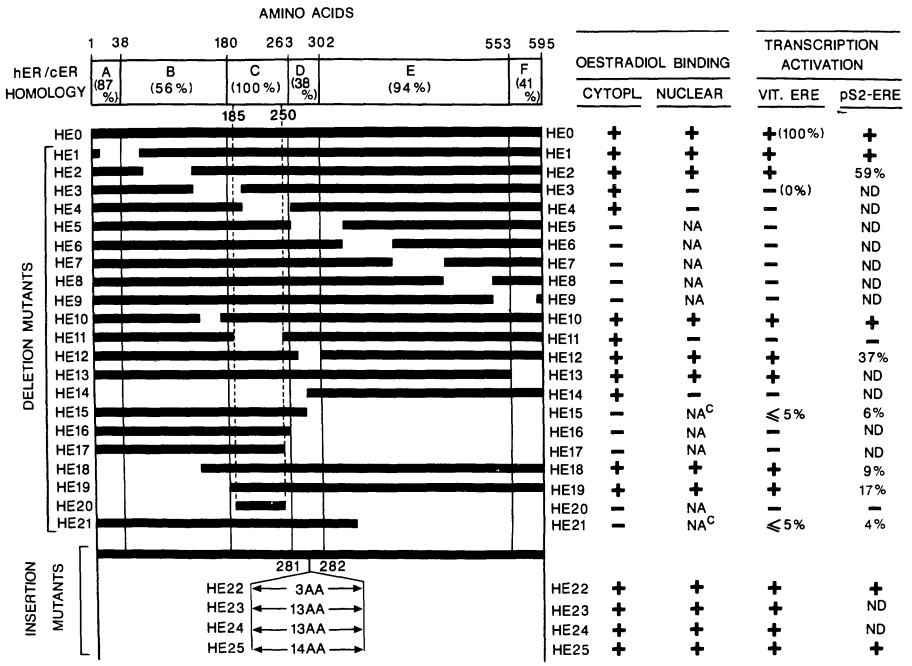


Figure 6. Structure/function analysis of human ER. Each of a variety of human ER mutants were analyzed for estradiol binding, in both the cytoplasm and nucleus, and for their abilities to activate transcription of the two reporter genes *vit*-tk-CAT (*vit* ERE) and pS2-CAT (pS2 ERE). The division of the human ER into 6 regions (A-F); [40] is shown at the top of the figure together with the percentage homologies between the human and chicken ERs in these regions. Other numbers refer to amino acid positions. HEO represents the wild-type human ER as indicated by the solid line from amino acids 1 to 595. A gap indicates a deletion and the arrows for mutants HE22 to HE25 indicate insertions between amino acids 281 and 282. The sequence of the insertions is as follows: HE22:SRA; HE23:SRALALAIDISRA; HE24:SRGMGMGIDISRA; HE25:SRPIDSLLSLLSRA (single letter amino acid code). The results of the analysis of the mutants are shown along the right hand side of the figure. A plus sign (+) indicates wild type activity and a negative sign (−), no activity. Values which fell between these two extremes are indicated as percentages of wild-type (100%). The suffix 'c' for mutants HE15 and HE21 in the column for nuclear estradiol-binding indicate that these two mutants compete with HEO in the transcription competition assay (see text). N.D., not determined. N.A., not applicable.

7.1 Region E (amino acids 303 to 552) is the estrogen-binding domain of the receptor

Any of a series of contiguous deletions which impinged on region E (HE5 through HE9) eliminated estrogen binding (Figure 6). All other deletions including those directly on the boundaries of this region (HE12 and HE13) left hormone-binding ability intact. Moreover, the hormone-binding domain can act independently of the remainder of the protein since the expression of only regions E and F (HE14) produced a protein with estradiol-binding properties indistinguishable from the intact receptor. Region E is, on average, more hydrophobic than the remainder of the receptor [35]. Thus, this region may

form a large hydrophobic pocket for the estrogen molecule whose structure is destroyed by any large internal deletion.

7.2 Region C (amino acids 180–262) is the nuclear tight binding domain and provides target gene specificity

Deletions in region C (HE3, HE4, HE11 and HE14) eliminated the tight nuclear binding of the estrogen-receptor complex (note: the hormone binding domain E needed to be intact in these experiments for the receptor to be detectable). On the other hand, deletions in regions A, B and F had no effect on nuclear binding. Mutant receptors with a deletion of most of region D (HE12) gave about 20% of the wild-type (HEO) level of tight nuclear binding (data not shown). The effect of the remaining amino-end of domain D (amino acids 263–270) has not been investigated. Thus, region C, perhaps with some contribution from region D, is that part of the receptor which presumably binds to a nuclear component.

The homologous region of the glucocorticoid receptor (GR) is responsible for the DNA binding of that receptor to the glucocorticoid-responsive element (GRE) [42, 43]. By analogy we assume that tight nuclear binding represents or correlates with DNA binding, presumably to the ERE. This is supported by the results obtained with a chimeric estrogen/glucocorticoid receptor molecule [44]. The highly conserved, 66-amino-acid, cysteine-rich segment of region C (amino acids 185–250) of the ER was replaced by its counterpart from the human GR (amino acids 421–486). The remainder of the ER was left intact. In a co-transfection experiment the transiently expressed chimeric receptor was activated by estrogen to stimulate transcription of a CAT reporter gene downstream from the GREs of the mouse mammary tumor virus long terminal repeat (MMTV LTR from −631 to +125). The chimeric receptor no longer could activate transcription using the ERE of the *vit*-tk-CAT reporter plasmid. Thus, this portion of region C provides the specificity for recognition of the hormone-responsive element of a target gene.

7.3 Region C has homology to DNA-binding fingers

That region C is a DNA-binding domain also is suggested by the potential of this cysteine-rich region to form at least two zinc-stabilized 'DNA-binding fingers' analogous to those proposed for the *Xenopus* 5S rRNA transcription factor TFIIIA [45, 46], the protein products of the *Drosophila* developmental genes Krüppel [47] and Serendipity [48], and the yeast regulatory protein ADR1 [49]. The proposed basic structure of the DNA-binding fingers consists of pairs of cysteine and histidine residues tetrahydrally-coordinated with a Zn^{2+} ion with intervening amino acids forming a loop or finger which would make specific contacts with the DNA. The N-terminal half of the putative DNA-binding domains of the ER and other steroid receptors contains two pairs of cysteine residues spaced by 13 amino acids (Figure 7), similar to the

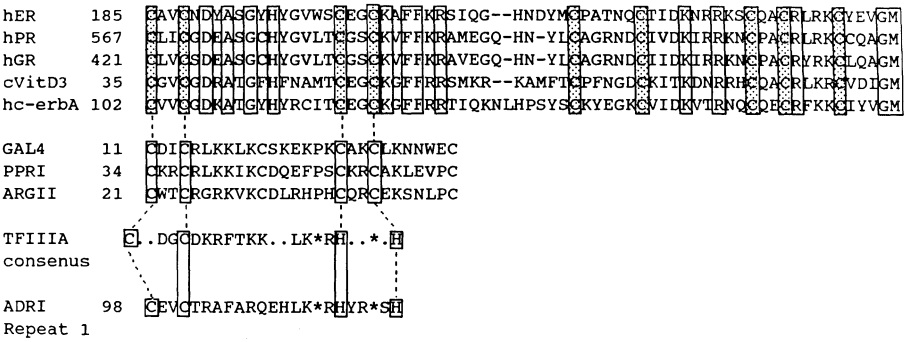


Figure 7. Alignment of the putative DNA-binding domains of the human estrogen (hER), progesterone (hPR) and glucocorticoid (hGR) receptors together with homologous regions of the chicken vitamin D3 (cVitD3) receptor and the human c-*erb* = A (hc-*erb* = A) gene product. Boxed areas indicate complete amino acid identity between all the sequences and dashes indicate amino acid gaps for optimal alignment. Conserved cysteines are shaded. The Cys-$(X)_2$-Cys$(X)_{13}$-Cys$(X)_2$-Cys motif present in the putative DNA-binding domain of the GAL4, PPR1 and ARGRII yeast regulatory proteins is aligned with the corresponding motif of the steroid hormone receptors and *erb*-A gene product. The consensus motif of the *Xenopus* transcription factor TFIIIA and the corresponding repeat 1 of the yeast regulatory protein ADR1 are also shown. The pairs of cysteine and histidine residues dictating the postulated 'finger' structures are boxed. Stars indicate positions where amino acid insertions may occur, and dots in the consensus sequence indicate variable residues. Numbers indicate the position of amino acid residues in each sequence.

TFIIIA and ADR1 arrangements and identical to the cysteine spacing in the presumptive DNA-binding domains of three yeast regulatory proteins GAL4 [50], PPR1 [51, 52] and ARGRII [53]. It has been proposed that, as with two cysteine and histidine pairs, a 'finger' could be formed with these four cysteine residues binding a Zn^{2+} ion [40, 49]. To test this possibility in the human ER, point mutations were introduced to replace one cysteine pair (residus 202 and 205) with histidines [44]. The resulting mutant (HE27) bound estradiol, but could not activate gene transcription. Such a negative result was inconclusive, however. These replacements could have caused a deleterious alteration of the finger structure or else the expected finger may not be present in the ER. The C-terminal half of the putative DNA-binding domain also has the potential to form loop structures of different lengths involving cysteine and histidine residues. Because the cysteine structural arrangement is highly conserved between the different steroid receptors, it is the less-conserved intervening 'finger' residues which presumably provide the specificity for the interaction of a receptor with its hormone-responsive element.

7.4 Regions C and E are essential for efficient activation of transcription

The deletions in region C which had eliminated tight nuclear binding of the ER (HE3, HE4, HE11 and HE14) also completely destroyed the ability of

these mutants to activate gene transcription. This is not surprising given the role of this region in determining the receptor's target gene specificity. The mutants with deletions in regions B (HE10) and D (HE12) just adjacent to region C retained activity, thereby demarcating the putative DNA-binding domain as one region of the ER important for transcription activation.

The receptors containing large deletions within the hormone-binding domain (region E; HE5 to HE9) also failed to activate transcription. Thus, region E is a discrete domain required not only for hormone binding, but also for activating transcription. There are three possible mechanisms to account for this requirement. First, region E could itself be a 'transcription-activating domain', for example, by interacting with some component of the transcriptional machinery. Second, estrogen binding to this region could induce a conformational change in the receptor which 'creates' active ERE-binding and/or 'transcription-activating domains' elsewhere. Third, estrogen binding to region E may induce a structural change which 'unmasks' pre-existing, active ERE-binding and/or 'transcription-activating domains' elsewhere in the receptor. In the latter case the unoccupied hormone binding domain would only have a 'masking' function and the removal of this region from the receptor, theoretically, may create a constitutively-active transcription regulator. Alternatively, there may be a combination of 'unmasking' and 'creation' of 'active' domains following estrogen binding.

We tested the 'masking' model by examining whether the N-terminal half of the ER containing the putative DNA-binding domain, but lacking all or most of the hormone-binding domain, could constitutively stimulate transcription. The mutants HE15, HE16 and HE17 which lack regions E and F, HE21 which lacks much of region E and all of region F were constructed for this purpose (see Figure 6). Using the *vit*-tk-CAT reporter gene, HE15 and HE21 had a low constitutive activity in the absence of estradiol, corresponding to not more than 5% of that of the wild-type (HEO) receptor in the presence of hormone. This constitutive activity was more clearly seen with the pS2CAT reporter plasmid than with *vit*-tk-CAT due to the higher basal CAT activity of the latter provided by the tk promoter. Although quite low, the constitutive activity of HE15 and HE21 is clear when compared to the activity of HE11 (which lacks the nuclear tight binding domain) or the hormone-free HEO. The mutants HE16 and HE17, which were slightly further truncated to the border and slightly within region C, respectively, had no transcriptional activity. Likewise, a severely-deleted receptor mutant (HE20), consisting of only the 66-amino-acid segment of region C which confers target gene specificity, did not stimulate transcription. These results suggest that region C together with a small amount of region D may contain at least an element of what is required for gene activation. However, a much greater activation of transcription is obtained with the addition of a hormone-bound region E.

The region E, nevertheless, is apparently not required for the receptor to interact with the ERE. Competition experiments were performed by co-transfecting the C-terminal truncation mutants (HE15, HE16, HE17, HE20

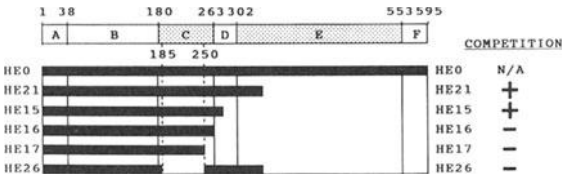


Figure 8. Competition of HEO-induced *vit*-tk-CAT activity. Expression vectors coding for the indicated C-terminal truncation mutants were co-transfected into HeLa cells with HEO (which codes for the wild-type ER) and a *vit*-tk-CAT reporter plasmid. A plus sign (+) indicates that the mutant receptor competed with and inhibited the ability of the wild-type estrogen-receptor complexes to activate CAT gene expression; a minus sign (−) indicates no competition. The receptor mutants are represented as in Figure 6 (see Figure 6 legend). N.A., not applicable.

or HE21) with HEO and *vit*-tk-CAT (Figure 8). HE15 and HE21, but not the others, can efficiently inhibit the ability of HEO to stimulate the expression of the reporter gene. This competition with HEO was eliminated by deleting the cysteine-rich region C of the competing receptor expression vector (HE26). We interpret these results to mean that HE15 and HE21 efficiently interact with (i.e. bind to) the ERE, but since they are poor transcriptional activators they reduce the stimulation seen with HEO. Thus, binding or interaction with the ERE is necessary, but not sufficient, for transcriptional activation. While our results support the possibility that the unoccupied hormone-binding domain may 'mask' a pre-formed DNA-binding domain, they also indicate that the hormone-binding domain plays a more direct role in the activation of transcription. It may either contain a "transcription-activating domain" or induce such a domain elsewhere in the ER following estrogen binding.

7.5 Region A/B (amino acids 1–179) is not absolutely required for transcriptional activation, but does optimize the activation of the pS2 ERE

N-terminal deletions covering part (HE1, HE2, HE10, HE18) or all (HE19) of regions A and B had no effect on the ability of the receptor to stimulate transcription using *vit*-tk-CAT (Figure 6). However, the activities of several of these mutants were significantly depressed using the pS2 ERE. An internal deletion in region B (HE2) had only about 60% of the stimulatory activity of HEO, while the N-terminal truncations, HE18 and HE19, stimulated the activity of the pS2 ERE present in pS2CAT (see above) by only 9% and 17%, respectively. A measurement of the ability of the mutant hormone-receptor complexes to bind tightly to the nucleus revealed that HE18 and HE19 had actual nuclear levels approximately 40% and 70% of HEO, respectively. Should the ER bind with lower affinity to the pS2 ERE than to the vitellogenin ERE, then the expression of pS2CAT could be more sensitive to such an impairment in nuclear (DNA) binding ability. This could explain the lower activity of these mutants with the pS2 ERE without invoking a direct role of region A/B in transcriptional activation. However, we observed the same

relative decrease in transcriptional activity of pS2CAT using different amounts of the transfected mutant receptor expression vectors, which argues against this explanation. Further studies are required to establish definitively whether this differential effectiveness reflects additional receptor requirements for activation of the pS2 promoter and/or for a different ERE (pS2 versus vitellogenin ERE), or other possibilities. Moreover, all of the studies have been performed up to now using transient co-transfection assays in which the EREs are not integrated in the genome and most likely not in a 'normal' chromatin structure. A role of region A/B may be more clearly revealed only when using a responsive gene in a more physiological genomic environment. Nevertheless, since region A/B is dispensable when using the *vit*-tk-CAT reporter gene, we can conclude that this region is not absolutely required for the activation of transcription.

7.6 The integrity of the proposed hinge region D (amino acids 263–301) is not a strict requirement.

Region D is the stretch of 39 amino acids linking the two critical regions of the receptor, the nuclear- and hormone-binding domains. Should there be allosteric changes in the receptor activating the DNA-binding and/or the 'transcription-activating domains' as a result of hormone binding to region E, then these changes would have to be transmitted either 'through' or 'around' region D. Because there is little conservation of amino-acid sequence between the chicken and human ER in this region (38%), and no conservation of sequence or length among the other related receptors [40], it is unlikely that such a structural change would be propagated through region D. As an alternative, structural transitions could correspond to juxtapositional changes of regions C and E with region D acting as a hinge.

To test the role of region D we have investigated the effect of varying its length on receptor function (Figure 6). A large deletion (30 amino acids; HE12) had no effect on the ability of the receptor to activate *vit*-tk-CAT; however, the activation of pS2CAT transcription was only about 37% that seen with HEO. This mutant receptor bound estradiol with the same affinity as HEO, but the nuclear tight binding of HE12 was only about 20% of that observed with HEO. Mutants with insertions of between 3 and 14 amino acids (HE22 through HE25) in the middle of region D bound estradiol as effectively as HEO and the corresponding estrogen-receptor complexes were tightly associated with the nucleus. These insertions had no effect on the ability of the receptor to activate the transcription of either *vit*-tk-CAT or pS2CAT. Thus, region D can be lengthened or shortened considerably without a loss of receptor function. Although HE12 is somewhat impaired in its ability to bind tightly to the nucleus and to activate pS2 gene transcription, that it works at all indicates that the exact distance between the DNA- and hormone-binding domains is not critical. This argues against estrogen-induced structural changes which would be transmitted through region D.

7.7 The role of estrogen in receptor activation: possible mechanisms

To summarize, the estrogen receptor is composed of at least two physically and functionally independent domains, one which recognizes the ERE and determines target gene specificity and one which binds hormone. In addition, ERE DNA binding and transcription activation are separable functions of the ER, both of which apparently require hormone binding *in vivo* [37]. The mechanism by which hormone binding causes 'receptor activation', which we define as the triggering of these two functions, remains unknown. From the competition experiments of section 7.4, it is evident that the DNA-binding domain can interact with the ERE when the hormone-binding domain is removed. We interpret this to mean that the presence of the unoccupied hormone-binding domain 'masks' the DNA-binding domain and that the role of the ligand is to induce a conformational change in the ER that results in an 'unmasking.' We use the term 'mask' to mean that in the absence of hormone the DNA-binding domain may be either fully pre-formed and physically 'masked', or its structure may be in some way distorted which prevents it from binding to DNA. The 'masking' could be due to a direct interaction of the hormone-binding domain with the DNA-binding domain or due to an *in vivo* complex of the receptor with some protein(s) or structure(s) which requires an unoccupied hormone-binding domain. In the latter case binding of the hormone to the receptor would 'unmask' the DNA-binding domain by inducing the dissociation of such a complex. It has been suggested that the 90 kilodalton heat shock protein [37] or the nuclear matrix [54] may serve this function.

Although ERE DNA binding must certainly be a prerequisite for transcription activation, it is not enough. There is an additional 'transcription-activating function' which requires an estrogen-occupied, hormone-binding domain for full activity. We presume that transcription activation involves an interaction of the ER with the transcriptional machinery. Accordingly, hormone binding may induce a conformational change in the receptor which allows some part of the ER (the 'transcription-activating domain') to have this interaction. It is presently not clear which region of the receptor is a 'transcription activating domain.' It could be region C, region E, or both . In addition, region A/B may play a role at least with the pS2 ERE. Clearly, although much has been learned about the structure of the ER, a complete model of steroid hormone and receptor action is not yet possible.

8. Mutant receptors and EREs have carcinogenic potential

A mutation of the ER potentially could be a crucial element in gene deregulation and carcinogenesis. For example, we have seen that two C-terminal truncation mutants lacking a hormone-binding domain (HE15 and HE21) can block the activity of wild-type receptors in competition experiments, yet at the same time have a low level of constitutive activity (see section 7.4). The

analogous glucocorticoid receptor mutants, in fact, have a high level of constitutive activity [42, 43]. These mutants illustrate two possible scenarios for transcriptional regulation gone away [37, 40, 55, 56]. First, an aberrant receptor with a truncation in the hormone-binding domain could bind to DNA and constitutively activate some key gene(s) for proliferation in the absence of hormone. Second, a truncated mutant receptor might bind to the hormone-responsive DNA element in a sterile fashion and thereby block the action of the normal receptor. The resulting gene deregulation, perhaps in conjunction with the action of another oncogene product or environmental insult, could induce a neoplastic transformation.

The prototypic mutant receptor/oncogene is the v-*erb*-A oncogene of the avian erythroblastosis virus. The gene product of its most closely related, normal cellular counterpart is a thyroid hormone receptor, which is a member of the same family of transcriptional regulatory proteins as the steroid hormone receptors [37, 57, 58]. The avian v-*erb*-A oncogene cannot by itself induce cell transformation, but enhances the erythroblast transforming potential of the v-*erb*-B primary oncogene [59, 60]. The v-*erb*-A gene product completely inhibits erythroblast differentiation into erythrocytes and allows transformed erythroblasts to grow in standard tissue culture media [59–61]. It has been proposed that v-*erb*-A achieves this result by interfering with ion transport systems and regulatory factors of differentiation [57]. Apparently due to the many mutations of the putative hormone-binding domain, v-*erb*-A cannot bind thyroid hormone [57], yet it seems that because of other mutations v-*erb*-A has acquired the ability to bind 'constitutively' to responsive elements of target genes [61]. As a result, v-*erb*-A could possibly mimic or competitively inhibit the effect of c-*erb*-A (thyroid hormone receptor). It is also possible that two amino-acid substitutions in the putative DNA-binding domain of v-*erb*-A compared to c-*erb*-A may have changed its target gene specificity [57].

The discovery of the hepatitis B viral genome inserted into an *erb*-A-related gene of a human hepatoma further suggests a role of the steroid receptor family of regulatory molecules in carcinogenesis [62]. Given these findings we have been interested to determine whether altered ERs can be implicated in any human breast cancers. We have sequenced the exons of the human ER gene in clones isolated from a placental genomic library. No significant differences were noticed between these placental exonic sequences and the sequence of the ER cDNA cloned from MCF-7 cells, indicating that the MCF-7 cell ER is not mutated (our unpublished results). Moreover, at this time we have been unable to detect any ER mRNA of anomalous size in RNA blots from human breast cancer biopsies (our unpublished results). Thus, it remains to be seen whether mutant ERs can act as oncogenes. In addition to receptor mutations, alterations or transpositions of the EREs of target genes have the potential to disrupt normal cellular regulation. Whether this plays a role in oncogenesis also remains speculation.

9. Summary

The role of estrogen in the growth of human breast cancers has been investigated at two levels. First, we have studied the pS2 gene, whose transcription is stimulated by estrogen in the human breast cancer cell line, MCF-7. The pS2 gene product is a small, secreted polypeptide currently of unknown function, but with structural features similar to some growth factors. The expression of the pS2 gene has so far been detected only in MCF-7 cells and some breast cancer biopsies. Preliminary studies indicate that pS2 is a potential marker for hormone-dependent breast cancer. Ongoing studies will continue to focus on the implicated role of pS2 in the estrogen-mediated growth of breast cancers and its possible use as a marker for estrogen-dependent tumors.

Second, we have analyzed the structure and function of the human ER. The receptor stimulates pS2 gene transcription by interacting with an ERE in the 5′-flanking region of that gene. A mutational analysis of the receptor protein has localized a DNA-binding domain, which determines target gene specificity, and a hormone-binding domain. These domains appear to be the only two regions of the receptor which are absolutely required for the transcription-activating function of the ER in transfection assays with reporter plasmids. The N-terminal region of the protein (regions A and B), which is necessary for increasing the efficiency of gene expression using the pS2 ERE, but not a vitellogenin ERE, may also play a role in transcription activation. Further progress in the characterization of the ER functional domains will require studies on target genes in a more physiological chromatin environment, as well as detailed physical analyses of receptor structure.

Acknowledgments

MCF-7 cells were kindly provided by the Michigan Cancer Foundation. We are grateful to our numerous colleagues who have participated in various aspects of this work since 1982. We gratefully acknowledge the help of the secretarial staff and of B. Boulay and C. Werlé in the preparation of the figures. This work was supported by grants from the CNRS (A.I.V.), the INSERM (grants CNAMTS), the Ministère de la Recherche et de la Technologie, the Fondation pour la Recherche Médicale Française and the Association pour la Recherche sur le Cancer.

References

1. Jensen EV, Greene GL, Closs LE, DeSombre ER, Nadji M: Receptors considered: a 20-year perspective. Recent Prog Horm Res 38:1–34, 1982.
2. Soule HD, Vasquez J, Long A, Albert S, Brennan MJ: A human cell line from a pleural

effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409– 1416, 1973.
3. Brooks SC, Locke ER, Soule HD: Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 248:6251–6253, 1973.
4. Lippman M, Bolan G, Huff K: The effects of estrogens and antiestrogens on hormone responsive human breast cancer in long term tissue culture. Cancer Res 36:4595–4601, 1976.
5. Darbre P, Yates J, Curtis S, King RJB: Effect of estradiol on human breast cancer cells in culture. Cancer Res 43:349–354, 1983.
6. Adams DJ, Edwards PD, McGuire WL: Hormone action in human breast cancer: estrogen regulation of specific proteins. In: Regulation of Gene Expression by Hormones, McKerns KW (ed), Plenum Press, New York, pp 1–25, 1983.
7. Rochefort H: Modulation of an estrogen-induced protein in the MCF-7 human mammary cancer cell. *In*: Regulation of Gene Expression of Hormones, McKerns, KW, (ed) Plenum Press, New York, 1983, pp 27–37.
8. Dickson RB, Huff KK, Spencer EM, Lippman ME: Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinology 118:138–142, 1986.
9. Kasid A, Davidson NE, Gelmann EP, Lippman ME: Transcriptional control of thymidine kinase gene expression by estrogen and antiestrogens in MCF-7 human breast cancer cells. J Biol Chem 261:5562– 5567, 1986.
10. Sheen YY, Katzenellenbogen BS: Antiestrogen stimulation of the production of a 37,000 molecular weight secreted protein and estrogen stimulation of the production of a 32,000 molecular weight secreted protein in MCF-7 human breast cancer cells. Endocrinology 120:1140–1141, 1987.
11. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P: Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. Nucleic Acids Res 10:7895–7903, 1982.
12. Jakowlev SB, Breathnach R, Jeltsch JM, Masiakowski P, Chambon P: Sequence of the pS2 mRNA induced by estrogen in the human breast cancer cell line MCF-7. Nucleic Acids Res 12:2861–2878, 1984.
13. Nunez AM, Jakowlev S, Briand JP, Gaire M, Krust A, Rio MC, Chambon P: Characterization of the oestrogen-induced pS2 protein secreted by the human breast cancer cell line MCF-7. Endocrinology 121:1759–1765, 1987.
14. Perlman D, Halvorson HO: A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J Mol Biol 167:391–409, 1983.
15. Rinderknecht E, Humbel RE: The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 253:2768–2776, 1978.
16. Rinderknecht E, Humbel RE: Primary structure of human insulin-like growth factor II. FEBS Lett 89:283–286, 1978.
17. Sirbasku DA, Leland FE: Estrogen-inducible growth factors: proposal of new mechanisms of estrogen-promoted tumor cell growth. In: Biochemical Actions of Hormones, Vol. IX, Litwack G (ed), Academic Press, New York, 1982, pp 115–140.
18. Vignon F, Capony F, Chambon M, Freiss G, Garcia M, Rochefort H: Autocrine growth stimulation of the MCF-7 breast cancer cells by the estrogen-regulated 52K protein. Endocrinology 118:1537–1545, 1986.
19. Lippman ME, Dickson RB, Kasid A, Gelmann E, Davidson N, McManaway M, Huff K, Bronzert D, Bates S, Swain S, Knabbe C: Autocrine and paracrine growth regulation of Human breast cancer. J Steroid Biochem 24:147–154, 1986.
20. Chambon P, Dierich A, Gaub MP, Jakowlev S, Jongstra J, Krust A, LePennec JP, Oudet P, Reudelhuber T: Promoter elements of genes coding for proteins and modulation of transcription by estrogens and progesterone. Recent Prog Horm Res 40:1–42, 1984.
21. Gairard B, Krust A, Rio MC, Koehl C, Bellocq JP, Chambon P, Renaud R: Estrogen-inducible gene in human breast cancer. In: Endocrinology of the Breast: Basic and Clinical Aspects, Annals of the New York Academy of Sciences, Vol. 464, Angeli A, Bradlow HL, Dogliotti L (eds), The New York Academy of Sciences, 1986, pp 443–447.

22. Shimada A, Kimura S, Abe K, Nagasaki K, Adachi I, Yamaguchi K, Suzuki M, Nakajima T, Miller LS: Immunocytochemical staining of estrogen receptor in paraffin antibody: comparison with that in frozen sections. Proc Natl Acad Sci USA 82:4802–4807, 1985.
23. Horwitz KB, McGuire WL: Estrogen control of progesterone receptor in human breast cancer. J Biol Chem 248:6351–6353, 1978.
24. Kassis JA, Walent JH, Gorski J: Estrogen receptors in cultured rat uterine cells: induction of progesterone receptors in the absence of estrogen receptor processing. Endocrinology 118:603–608, 1986.
25. Keydar I, Chou L, Karby S, Weiss FR, Delasea J, Radu M, Chaitcik S, Brenner HJ: Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer 15:659–670, 1979.
26. Brown AMC, Jeltsch JM, Roberts M, Chambon P: Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. Proc Natl Acad Sci USA 81:6344–6348, 1984.
27. Jeltsch JM, Roberts M, Schatz C, Garnier JM, Brown AMC, Chambon P: Structure of the human estrogen-responsive gene pS2. Nucleic Acids Res 15:1401–1414, 1987.
28. Prud'homme JF, Fridlansky F, LeCunff M, Atger M, Mercier-Bodart C, Pichon M-F, Milgrom E: Cloning of a gene expressed in human breast cancer and regulated by estrogen in MCF-7 cells. DNA 4:11–21, 1985.
29. Cowan KH, Goldsmith ME, Levine RM, Aitken SC, Douglass E, Clendeninn N, Nienhuis AW, Lippmann ME: Dihydrofolate reductase gene amplification and possible rearrangement in estrogen-responsive methotrexate-resistant human breast cancer cells. J Biol Chem 257:15079–15086, 1982.
30. Kadonaga JT, Jones KA, Tjian R: Promoter-specific activation of RNA Polymerase II transcription of Sp1. Trends Biochem Sci 11:20–23, 1986.
31. Klein-Hitpass L, Schorpp M, Wagner U, Ryffel GU: An estrogen-responsive element derived from the 5′ flanking region of the *Xenopus* vitellogenin A2 gene functions in transfected human cells. Cell 46:1053–1061, 1986.
32. Seiler-Tuyns A, Walker P, Martinez E, Mérillat AM, Givel F, Wahli W: Identification of estrogen responsive DNA sequences by transient expression experiments in a human breast cancer cell line. Nucleic Acids Res 14:8755–8770, 1986.
33. Roberts M, Wallace J, Jeltsch JM, Berry M, Chambon P: The 5′ flanking region of the human pS2 gene mediates its transcriptional activation by estrogen in MCF-7 cells. Biochem Biophys Res Comm, 1987.
34. Breathnach R, Harris BA: Plasmids for the cloning and expression of full-length double-stranded cDNAs under control of the SV40 early or late gene promoter. Nucleic Acids Res 11:7119–7136, 1983.
35. Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P: Human estrogen receptor cDNA: sequence, expression and homology to v-*erb*-A. Nature 320:134–139, 1986.
36. Druege PM, Klein-Hitpass L, Green S, Stack G, Chambon P, Ryffel GU: Introduction of estrogen-responsiveness into mammalian cell lines. Nucleic Acids Res 14:9329–9337, 1986.
37. Green S, Chambon P: A superfamily of potentially oncogenic hormone receptors. Nature 324:615–617, 1986.
38. McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR, O'Malley BW: Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235:1214–1217, 1987.
39. Gorski J, Welshons WV, Sakai D, Hansen J, Walent J, Kassis J, Shull J, Stack G, Campen C: Evolution of a model of estrogen action. Recent Prog Horm Res 42:297–329, 1986.
40. Krust A, Green S, Argos P, Kumar V, Walter P, Bornert JM, Chambon P: The chicken estrogen receptor sequence: homology with v-*erb*-A and the human estrogen and glucocorticoid receptors. EMBO J 5:891–897, 1986.
41. Kumar V, Green S, Staub A, Chambon P: Localization of the estradiol-binding and putative DNA-binding domains of the human estrogen receptor. EMBO J 5:2231–2236, 1986.
42. Godowski PJ, Rusconi S, Miesfeld R, Yamamoto KR: Glucocorticoid receptor mutants that

are constitutive activators of transcriptional enhancement. Nature 325:365–368, 1987.
43. Hollenberg SM, Giguere V, Segui P, Evans RM: Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell 49:39–46, 1987.
44. Green S, Chambon P: Estradiol induction of a glucocorticoid-responsive gene by a chimeric receptor. Nature 325:75–78, 1987.
45. Miller J, McLachlan AD, Klug A: Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J 4:1609–1614, 1985.
46. Brown RS, Sander C, Argos P: The primary structure of transcription factor TFIIIA has 12 consecutive repeats. FEBS Lett 186:271–274, 1985.
47. Rosenberg UB, Schröder C, Preiss A, Kienlin A, Côté S, Riede I, Jäckle H: Structural homology of the product of the *Drosophila* Krüppel gene with *Xenopus* transcription factor IIIA. Nature 319:336–339, 1986.
48. Vincent A, Colot HV, Rosbash M: Sequence and structure of the Serendipity locus of *Drosophila melanogaster*. J Mol Biol 186:149–166, 1985.
49. Hartshorne TA, Blumberg H, Young ET: Sequence homology of the yeast regulatory protein ADR1 with *Xenopus* transcription factor TFIIIA. Nature 320:283–287, 1986.
50. Laughon A, Gesteland RF: Primary structure of the *Saccharomyces cerevisiae* GAL4 gene. Mol Cell Biol 4:260–267, 1984.
51. Kammerer B, Guyonvarch A, Hubert JC: Yeast regulatory gene PPR1. I. Nucleotide sequence, restriction map of codon usage. J Mol Biol 180:239–250, 1984.
52. Liljdund P, Losson R, Kammerer B, Lacroute F: Yeast regulatory gene PPR1. II. Chromosomal localization, meiotic map, suppressibility, dominance/recessivity and dosage effect. J Mol Biol 180:251–265, 1984.
53. Messenguy F, Dubois E, Deschamps F: Nucleotide sequence of the ARGRII regulatory gene and amino acid sequence homologies between ARGRII, PPR1 and GAL4 regulatory proteins. Eur J Biochem 157:77–81, 1986.
54. Gorski J, Welshons W, Sakai D: Remodeling the estrogen receptor model. Mol Cell Endocrinol 36:11–15, 1984.
55. Sluyser M, Mester J: Oncogenes homologous to steroid receptors? Nature 315:546, 1985.
56. Bishop JM: Oncogenes as hormone receptors. Nature 321:112, 1986.
57. Sap J, Munoz A, Dam K, Ghysdael J, Leutz A, Beug H, Vennström B: The c-*erb*-A protein is a high affinity receptor for thyroid hormone. Nature 324:635–640, 1986.
58. Weinberger C, Thompson CC, Ong ES, Lebo R, Evans RM: The c-*erb*-A gene encodes a thyroid hormone receptor. Nature 324:641–646, 1986.
59. Frykberg L, Palmieri S, Beug H, Graf T, Hayman MJ, Vennström B: Transforming capacities of avian erythroblastosis virus mutants deleted in the *erb*-A or *erb*-B oncogenes. Cell 32:227–238, 1983.
60. Kahn P, Frykberg L, Brady C, Stanley IJ, Beug H, Vennström B, Graf T: V-*erb*-A cooperates with sarcoma oncogenes in leukemia cell transformation. Cell 45:349–356, 1986.
61. Damm K, Beug H, Graf T, Vennström B: A single point mutation in *erb*-A restores the erythroid transforming potential of a mutant avian erythroblastosis virus (AEV) defective in both *erb*-A and *erb*-B oncogenes. EMBO J 6:375–382, 1987.
62. Dejean A, Bougueleret L, Grzeschik KH, Tiollais P: Hepatitis B virus DNA integration in a sequence homologous to v-*erb*-A and steroid receptor genes in a hepatocellular carcinoma. Nature 322:70–72, 1986.

9. The 52K cathepsin-D of breast cancer: structure, regulation, function and clinical value

Henri Rochefort, Patrick Augereau, Françoise Capony, Marcel Garcia, Vincent Cavailles, Gilles Freiss, Muriel Morisset, and Françoise Vignon

1. Introduction

A major question in cancer research is: why do cancer cells continuously proliferate and invade adjacent tissue. Breast cancer cells are particularly suitable for studying this question since when they are differentiated, they contain estrogen receptors, and estrogen, specifically, triggers their growth. One approach to understanding the estrogen-induced growth stimulation in this model is to identify the estrogen-induced factors involved in the control of cell proliferation [1–6]. Here, we illustrate this approach in a study of growth regulation of MCF_7 cells by estrogen, in which we found a secreted 52K protein with mitogenic activity. It was identified as a protease that is also possibly involved in the process of tumor cell metastasis.

2. Estrogen-induced autocrine mitogens

Steroid hormones regulate specific gene expression within minutes following their interaction with specific nuclear receptors [7]. By contrast, the mitogenic effect of sex steroids is observed only after a lag of approximately 1 day; during this time, the transcription of several genes has been stimulated and different proteins have been induced. Among them, the proteins secreted by breast cancer cells are good candidates for mediating the effect of estrogens by interacting in turn on the plasma membrane of the same cells via an estrogen-regulated autocrine mechanism [8, 9]. An autocrine mechanism has been proposed for cancer cells which acquire the ability to make and respond to their own growth factors [10]. We have applied this concept to breast cancer cells which remain under the control of sex-steroid hormones during the first steps of tumor progression.

One way to define growth factors and other mitogens involved in this autocrine mechanism is to characterize the estrogen-induced proteins and peptides that are secreted by hormone-dependent human breast cancer cells since estrogens are the only steroids with mitogenic activity in these cells [2–4].

The estrogen-regulated autocrine mechanism was supported by showing

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

that glycoprotein fractions prepared from serum-free media conditioned by estrogen-stimulated MCF_7 cells increase the growth of resting MCF_7 cells, while similar fractions from the conditioned media of estrogen-stripped MCF_7 cells are inactive [9]. The mitogenic activity of media conditioned by estrogen-treated cells has been confirmed by different groups [11, 12]. Following the labeling of newly synthesized proteins by $|^{35}S|$ methionine or $|^{35}S|$ cysteine and the analysis of the labeled proteins by SDS-polyacryamide gel electrophoresis, several estrogen-regulated proteins have been detected [see 6, 13] and there is a general consensus that estrogen-induced proteins, or factors present in these conditioned media are at least partly responsible for the stimulation of the cancer cells.

One of the major challenges in the field of hormone-dependent cancer is to define the protein(s) or peptide(s) responsible for controlling proliferation and invasion by these cancers. To this end, two strategies were used. The first was to characterize classical growth and transforming factor activities and to show that some of these growth factors, such as EGF, IGF1 and PDGF like peptides, are induced by estrogens in breast cancer cell lines and are potential autocrine mitogens [14, 15]. The second was to purify and identify the major secreted proteins which were actually found to be synthesized and regulated by estrogens in these cells. This approach led us to identify a lysosomal protease with mitogenic and invasive potential.

3. Regulation and purification of the 52K protein

The 52K protein was first described in 1979 [16, 17] by labeling MCF_7 cell proteins with $|^{35}S|$ methionine and analyzing the secreted proteins by SDS-polyacryamide gel electrophoresis. While estradiol increased by 2 to 3 fold the total amount of secreted proteins, normalization for constant TCA precipitable material showed that some of the proteins (mainly a 160K and a 52K species) were more specifically increased by estradiol at concentrations as low as 1 to 10 picomole.

The 52K protein is secreted in small amounts into the culture medium by estrogen-treated MCF_7 cells (5 ng/10^6 cells/hour) and by other ER-positive breast cancer cells under estrogen control ($T_{47}D$, ZR_{75-1}). It is constitutively produced without the influence of estrogen in ER-negative cell lines (MDA-MB231, BT20) [18]. In ER-positive cells, the protein is specifically regulated by hormones (estrogens and high doses of androgens) that can bind to and activate the estrogen receptor, but not by glucocorticoids, progestins, or androgens at low concentrations [17]. The effects of the antiestrogens, tamoxifen and hydroxytamoxifen, suggested that this protein was in some way related to the mitogenic activity of estrogens. In wild type MCF_7 cells, the antiestrogens totally inhibited the synthesis of the protein, whereas they partially stimulated the synthesis of the progesterone receptor. By contrast, in the antiestrogen-resistant variants of MCF_7 cells (R27 and RT×6) cloned for

their ability to grow in 1 μM tamoxifen, the antiestrogens became able, like estrogens, to increase the production of the 52K protein, but remained unable, as in wild type MCF_7 cells, to stimulate the production of the estrogen-regulated 160K secreted protein and of pS2 mRNA [19]. In these cell lines, the 52K protein was therefore a better candidate for being a mitogen than the pS2 or 160K proteins. A possible mechanism for the anti-estrogen resistance was that cells had acquired a growth advantage, due to the tamoxifen induced increased production of autocrine growth factors such as the 52K protein.

We applied a three-step strategy to purify the 52K glycoprotein. Using concanavalin-A sepharose chromatography, we partially purified it from 22 liters of conditioned medium from MCF_7 cell cultures. From this partially purified fraction, we obtained several mouse monoclonal antibodies to the 52K protein [20] (Table 1). Finally, using an immunoaffinity column, we purified the 52K protein to apparent homogeneity (1,000-fold purification) both in its secreted and cellular forms [21]. The purification of 52K protein made it possible to study its activity on cell growth, determine its structure and identity, and finally clone its corresponding cDNA sequences.

By immunopurification without detergent, a homogeneous secreted 52K protein was obtained, which was shown to stimulate the growth of estrogen-

Table 1. Characterization of the MAbs to the 52K protein

	Specificity	Affinity (KD in nM)	Immuno-precipitation	Immunoblot
Site 1				
M1G8		0.43	+	+
M1H11		2.86	+	+
M6H10		1.43	+	+
D8F5	52K	1.25	+	+
D11E2	+	1.00	+	+
	48K			
Site 2	+			
D7E3	34K	0.83	+	+
Site 3				
M4A3		0.58	+	+
Site 4				
M2E8		0.18	+	ND
	52K only			
Site 5				
D9H8		0.96	+	ND

All the monoclonal antibodies (MAbs) are IgG1 and purified from a single fusion. The antibodies of sites 1 to 3 (20) recognize the 34K part of the molecule and can be used to assay total 52K-cathepsin-D in cell extracts. The antibodies of sites 4 and 5 recognize only the pro-fragment and can be used to assay the precursor alone (secreted and cellular of the 52K-cathepsin-D) (41).

+ indicates the specificity of the antigen-antibody recognition. ND: not determined.

deprived recipient MCF_7 cells [22]. This stimulation was dose-dependent and occurred at concentrations (1 to 10 nM) similar to those found in the culture medium. However, time-course experiments indicated that both estradiol and the 52K protein required the same (18 h) lag before stimulating |^{3}H|thymidine incorporation. Like estradiol, the 52K protein was also able to stimulate the number and length of microvilli on the cell surface. This mitogenic activity of the purified 52K protein could be intrinsic or could be due to a contaminant not visible by silver staining overloaded gels. However, |^{35}S| cysteine-labeling experiments have excluded that the activity could be produced by newly synthesized peptides incorporating cysteine residues such as TGFα, pS2 protein, or IGF1 [22]. The *in vitro* mitogenic activity of the purified 52K protein was in agreement with an estrogen-regulated autocrine mechanism.

4. Structure and identification as a lysosomal protease

Study of the co- and post-translational modifications of the 52K protein helped us to define its structure and enzymatic activity. After exposure of cultured MCF_7 cells to ^{32}P, the 52K protein is intensely labeled. However, most of this label can be removed by endoglycosidase-H treatment, which deletes two N-glycosylated chains of the protein (Figure 1). Mannose-6-P signals were then identified on these chains [23–24]. Pulse-chase experiments and Western blot analysis showed that the 52K protein is the precursor of a lysosomal enzyme which accumulates in lysosomes as a stable 34K protein. About 40% of the cellular 52K precursor is secreted while about 60% is successively processed into a 48K and a 34K + 14K protein [21, 25]. Part of the secreted 52K protein can be taken up and processed by MCF_7 cells, but its binding is specifically inhibited by mannose-6-P and not by other sugars [24].

The presence of mannose-6-P signals indicated that the protein is normally routed to lysosomes where it exerts its usual function [27]. In testing several enzymatic activities corresponding to lysosomal hydrolases of similar molecular weight, we found that both the purified secreted 52K protein and the corresponding cellular proteins (52K, 48K, 34K+ 14K) displayed a strong proteolytic activity at acidic pH, which was mostly inhibited by pepstatin (Figure 2) [23, 24]. There is a strong homology with cathepsin-D [27]. Antibodies to the 52K protein interact with liver cathepsin-D, while anti-cathepsin-D immunoprecipitates the 52K protein [24]. The pH and inhibitor sensitivities of the two proteases are very similar, as are their molecular weights. In addition, the first 15 amino acids determined by micro-sequencing the N-terminal of the molecules are identical (P. Ferrara, unpublished).

The differences with the previously characterized cathepsin-D of normal tissue at present appear, to the mostly quantitative and linked to its hormonal regulation. The concentration of this enzyme is very high in some breast cancers and melanoma (see clinical studies), and its precursor is secreted in greater amounts by breast cancer cells than by normal mammary epithelial

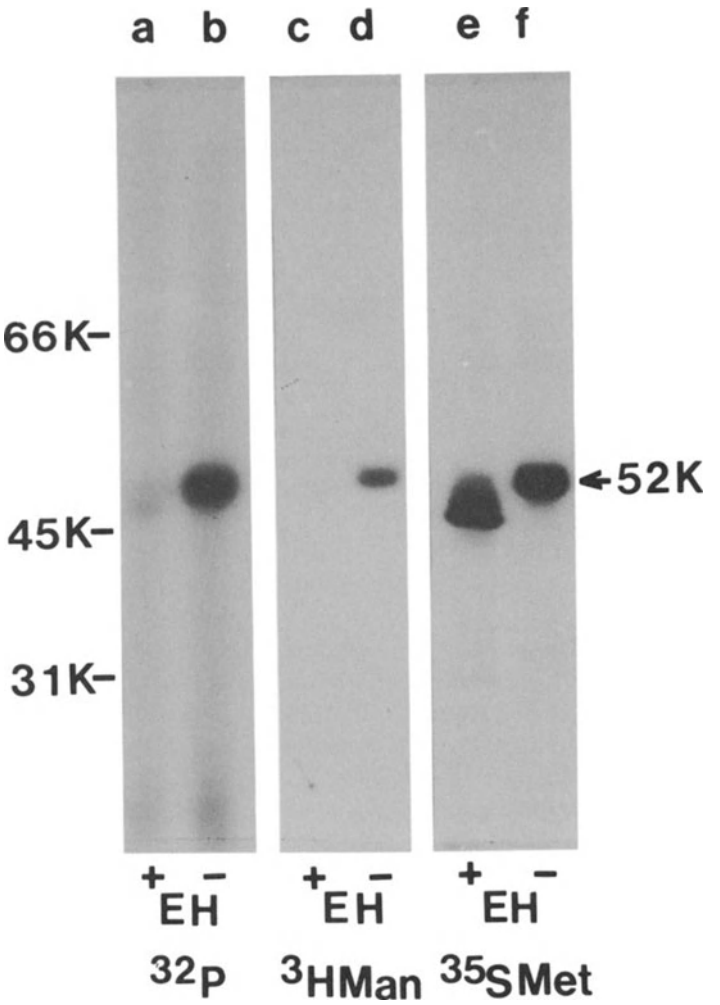


Figure 1. Estrogen-treated MCF_7 cells were labeled with either $|^{35}S|$methionine, $^{32}P\ H_3PO_4$, or $|^{3}H|$mannose.

Media were immunoprecipitated with the M1G8 antiboty to the 52K protein and analyzed by SDS-polyacrylamide gel electrophoresis. The immunoprecipitated secreted 52K protein was digested (+) or not (−) with endoglycosidase H (E.H.) and the TCA-precipitated proteins were electrophoresed.

[Modified by permission from 24.]

cells or fibroblasts in culture (F. Capony *et al.*, in preparation). Until now, a specific regulation of cathepsin-D by estrogens has never been observed.

Using monoclonal antibodies to the 52K protein and synthetic oligonucleotides prepared from the sequence of the N-terminal end of the 52K protein, we screened a cDNA library expressed in λgt11 (gift of Dr. P. Chambon) and

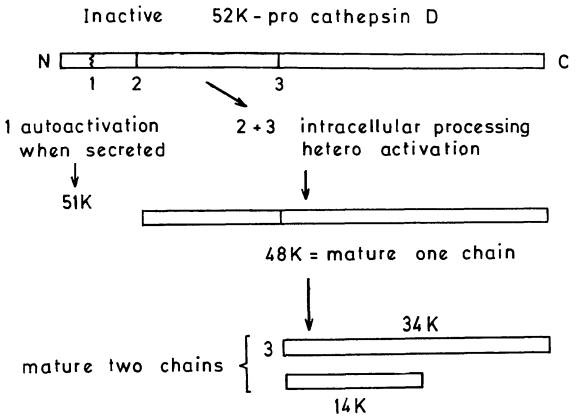


Figure 2. Structure and processing of the 52K-cathepsin-D.

The MCF_7-52K protein is an inactive pro-cathepsin-D [23, 24], which can be autoactivated when secreted or processed by cysteine proteinase successively into an intermediate (48K) form and final mature enzyme (34K + 14 K). The molecular weights and immunoreactivities of the processed forms of cathepsin-D in normal fibroblasts [26] and in MCF_7 breast cancer cells are very similar if not identical.

cloned several cDNA probes corresponding to the whole mRNA of the pre-, pro-cathepsin-D of MCF_7 cells [28]. Partial sequencing indicated a strong homology with cathepsin-D of normal kidney [29] with a few differences whose biological significance is now being studied (P. Augereau *et al.*, in preparation). Using the monoclonal antibodies, we showed that the intracellular cathepsin-D was also regulated by estrogens [25] and, using a cDNA probe, that the level of a 2.2 kb 52K pre-pro-cathepsin-D mRNA was rapidly and specifically increased by 6- to 10-fold following estradiol treatment [28, 30] (Figure 3). It is therefore likely that as in the case of other estrogen-regulated genes [7], the 52K-cathepsin-D is transcriptionally regulated by estrogens.

5. Potential role of the 52K-cathepsin-D protease in mammary carcinogenesis

Some of the characteristics of the 52K-cathepsin-D, i.e. its high concentration in proliferative and tumoral cells, its induction by estrogen, and the large proportion of its secreted form, suggest that it may have major function(s) in mammary carcinogenesis by stimulating tumor growth and/or invasion via its proteolytic activity (Figure 4). The mechanism of the mitogenic action of the 52K-cathepsin-D-like enzyme is unknown. As for other proteases, cathepsin-D may acts indirectly by releasing growth factors from precursors or from the extracellular matrix via their enzymatic activity and/or by activating growth factor receptors. For instance, proteolytic cleavages are needed to detach the TGFα precursor from the membrane [31] and may be involved in the activation of TGFβ [32]. The proteases responsible for these cleavages are

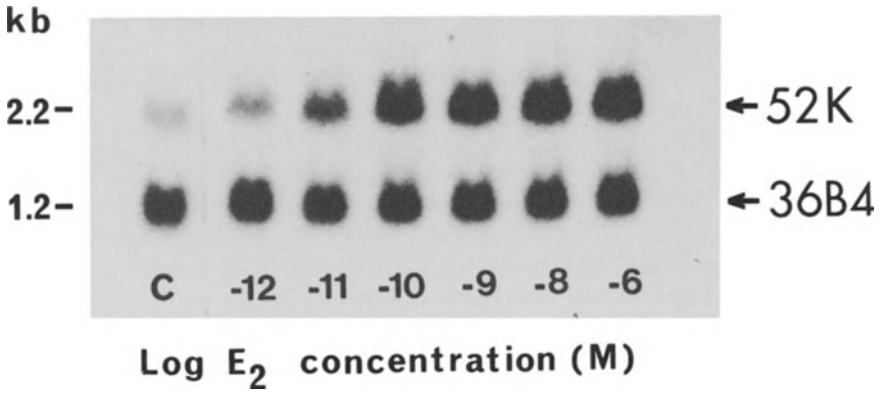


Figure 3. Effect of estradiol on the 52K protease mRNA in MCF_7 cells.

Six hours following addition of increasing concentrations of estradiol (E_2) or solvent (C) to MCF_7 cells, RNA was purified from MCF_7 cells and analyzed by 1% agarose gel electrophoresis. The 52K-cathepsin-D mRNA was detected by hybridization with one cloned cDNA (λP9) isolated from a λgt11 cDNA library of estrogen-treated MCF_7 cells (28, 30). The molecular weight of the markers is shown in kilobases, 36B4 is a cDNA corresponding to the expression of a hormone independent gene (7). (V. Cavailles et al., submitted for publication).

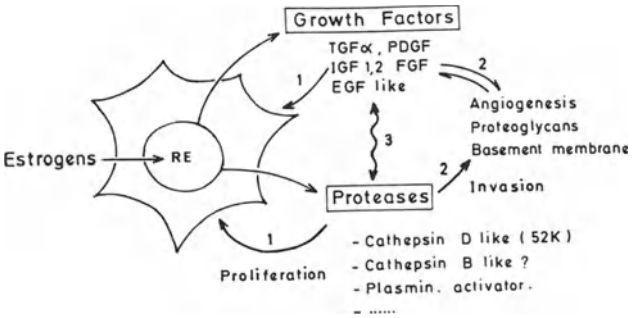


Figure 4. Putative functions of estrogen-regulated secreted proteins, and peptides secreted by breast cancer cells.

Estrogens, *via* their nuclear receptors (RE), induce several proteins and factors that are secreted by breast cancer cells. One category (growth factors) may act as autocrine factors which stimulate the growth of the same cells [10]. Other proteins, such as proteases, can potentially act as mitogens either directly or *via* the activation of growth factors. Proteases may also facilitate tumor invasion and angiogenesis *via* their proteolytic activity.

unknown. A more direct action via specific receptors as in the case of thrombin [33] is however not excluded.

The major normal functions of cathepsins occur in lysosomes at very low pH, where they degrade endogenous proteins [34]. Since a pro-cathepsin-D enzyme is secreted in large amounts at the periphery of cancer cells, the enzyme may acquire abnormal functions, such as facilitating cancer cell migra-

tion and invasion by digesting basement membrane, extracellular matrix, and connective tissues. Cathepsin-D is secreted as an inactive proenzyme, but at acidic pH, it can be auto-activated by the removal of a small part of the N-terminal pro-fragment [35]. The same is true of the breast cancer 52K protein [24]. Culture media conditioned under serum-free conditions by estradiol-treated MCF_7 cells contain potential proteolytic activities that can digest methemoglobin [27] and proteoglycans [24, 36]. It is conceivable that under certain circumstances, breast cancer cells develop a sufficiently acidic micro-environment to allow the autoactivation of the secreted 52K-cathepsin-D [36]. Recent clinical studies based on the use of monoclonal antibodies strongly support this hypothesis.

6. Immunoperoxidase staining of the 52K-cathepsin-D in frozen sections

Using monoclonal antibodies directed to the mature 34K enzyme [20] (Table 1), we have examined frozen sections of several human tissues [37] with the peroxidase-anti-peroxidase technique of Sternberger. Most of the staining was granular in the cytoplasm and corresponded to lysosomes. No plasma membrane staining was detected. Among the different normal tissues studied, the protein appeared to be mostly concentrated in sweat glands and liver, but not in normal uterus or normal resting mammary glands collected by reduction mammoplasties. By contrast, immunostaining was observed in 43% of 127 biopsies of benign mastopathies [37]. Gynecomastias, fibroadenomas, fibrous lesions, and lobular structures (adenosis, sclerosing adenosis, atypical lobular hyperplasia) were usually negative. The two groups of mastopathies that were highly stained consisted of cysts over 3 mm in diameter and ductal hyperplasias.

When the different histological types were pooled into proliferative (high-risk) and nonproliferative (low-risk) lesions, according to criteria defined by Dupont and Page [38], we found a significant correlation between proliferation and 52K protein staining. Among the 23% of the lesions that were proliferative, nearly 80% were positive for the 52K protein. The negative cases were all lobular hyperplasia. In the non-proliferative group, only 32% of the tumors were stained. This positivity was due mainly to the presence of cysts. The use of 52K protein staining in predicting high-risk (proliferative) mastopathies may therefore be useful and complementary to histopathology, since 91.5% of the 52K-protein-negative lesions were non-proliferative and 60.5% of the 52K-protein-positive (non cystic) lesions were proliferative.

We also studied 52K-immunostaining of breast cancer tissue in an attempt to correlate its positivity with steroid-receptor content and other prognostic markers. We studied 232 primary breast carcinomas collected from April to September 1985, in three French cancer centers (Institut Gustave Roussy, Villejuif; Centre Antoine Lacassagne, Nice; Centre Paul Lamarque, Montpellier). The 52K protein was evaluated in frozen sections by immunohisto-

chemistry, and the concentration of estrogen receptors and progesterone receptors were assayed in the cytosol by the classical dextran-coated charcoal method [18]. Altogether, the 52K protein was detected in 64% of the 232 tumor biopsies. In 80% of the 52K-positive tumors, the staining was very heterogeneous and affected only 1–30% of the epithelial tumor cells. The staining was generally less homogeneous in breast cancer cells than in proliferative benign mastopathies and in cells bordering the lumen of large cysts. In the four categories of tumors defined according to their positivity for estrogen and progesterone receptors, a similar proportion of tumors was found to be positive for the 52K protein [18]. The estrogen receptor (RE) positive and progesterone receptor (RP) positive group was not significantly different from the RE negative and the RP negative group, or the total population (64%), with respect to 52K-protein immunostaining. Moreover, when the percentage of the 52K-protein-stained cells was plotted against the cytosolic RE and RP concentrations, no statistical correlation, either positive or negative, was observed between the estrogen or progesterone receptor concentration and the amount of 52K-positive cells.

The absence of correlation between these two types of markers was also shown directly using fine-needle aspirates of breast carcinomas to perform double immunohistochemical staining of the nuclear NE and cytoplasmic 52K protein in the same sample [39]. The first results from 35 patients also indicate that the two markers are not correlated. There were tumor cells with 52K protein and without RE staining, and others with RE and without 52K protein. These data suggest that the amount of 52K protein is not correlated with that of the estrogen receptor in breast cancer cells.

Among other cancers examined, some melanomas were found to be positive, while benign nevi were generally negative [37] suggesting that this marker will be useful in melanoma but further studies are required.

To obtain a better quantification of the concentration of the 52K protein, we used a solid phase double-determinant immunoassay of the 52K-cathepsin-D.

7. Immunoassay of the total and cathepsin-D-like enzyme and its precursor (52K) in soluble extracts

Using two associations of antibodies that recognize two domains of the proenzyme [20, 40, 41], both the precursor (52K) and its cellular products (48K + 34K) were assayed (Table 1). The technique was applied both in the culture medium and in the cytosol, to assay the secreted and total cellular forms, respectively. The concentration of the 52K protein was determined by reference to a standard purified 52K protein assayed by silver staining.

The production and secretion of the 52K protein were compared in several RE positive and RE negative cell lines cultured in 10% fetal calf serum containing estrogens [18]. All RE-positive cell lines tested at confluency pro-

duced the 52K protein in the presence of estradiol, but its secretion varied markedly according to the cell line and the subspecies. For instance, the secretion of 52K protein by two MCF_7 subspecies was quite different.

Interestingly, the 52K-cathepsin-D was also produced and secreted by the two RE-negative breast cancer cell lines (BT20, MDA-MB231). The secretion of 52K protein by RE-negative cell lines and their intracellular concentrations were even higher than in some RE-positive cell lines but were not regulated by estrogens. The high production of 52K protein by hormone-independent breast cancer is in full agreement with its lack of correlation with the estrogen receptor.

Since breast cancer cytosol is routinely used to determine estrogen and progesterone receptor levels, we recently assayed in this cytosol the level of the total cathepsin-D-like protein (using the antibodies to the mature 34K enzyme) [40] and that of the 52K precursor alone (using the antibodies to the pro-fragment) [41]. Each essay was a solid phase double-determinant immunoenzymatic assay. Two studies have been completed. The first is a prospective study on 183 cytosols of primary breast cancers [42]. The results are summarized in Table 2. The concentration of total cathepsin-D-like enzyme varied markedly according to the patient, as did the proportion of the 52K precursor. The absence of correlation with the estrogen receptor was confirmed. The slight correlation with axillary lymph node invasion indicates that the assay of total enzyme in cytosol may be valuable as a prognostic marker for predicting the degree of invasiveness of breast cancers.

These results suggested some relationship in primary tumors between high concentrations of 52K-cathepsin-D and the degree of invasiveness. A second clinical study was retrospective and included on 150 post-menopausal patients of the Danish Breast Cancer Cooperative Group, we assayed total 52K en-

Table 2. IEMA of 52K-cathepsin-D in cytosol from 183 primitive breast cancers

1. A prospective study starting February 1985. Pre- and post-menopausal patients (Centre Paul Lamarque, Montpellier and other Languedoc Hospitals).
2. Concentration of total 52K protein (secreted + cellular) from 52 to 2,800 U/mg cytosol protein (mean = 40).
3. No correlation with RE ($r = 0.15$) and a slight correlation with RP ($r = 0.41$).
4. High concentrations of 52K (>700 U/mg proteins) are correlated with lymph node invasion ($p < 0.01$) and RE.
5. Variable proportion of the 52K precursor 0% to 42% of the total 52K cellular proteins (52 + 48 + 34).
6. Not correlated with Scarff and Bloom grading, tumor size, or age of patients.

Cytosol was prepared from non-irradiated breast cancers. The concentration of the 52K protein (toatl and precursor) was measured by a double-determinant immunoenzymatic assay using our first series of antibodies (Table 1). The concentration of RE was determined by a similar enzymatic assay using Abbott antibodies (kit ER-EIA) and that of RP sites by the classical charcoal assay. Axillary lymph node invasion was defined by the pathologist following radical mastectomy.

Detailed information in Reference [42].

zyme in cytosol collected at surgery 6 to 10 years earlier and kept at −70°C in the Fibiger Center—Finsen Institute by Dr. Susan Thorpe [43]. A significant correlation was found between patients with high (400 μ/mg protein) 52K protein concentrations and short recurrence-free survival. The 52K protein parameter was found in this study to be independent of the other prognostic parameters.

The first clinical studies strongly suggest a role of this protease in the process of tumor invasion and metastasis, which is the major cause of death in breast cancer. However, these results will have to be confirmed and extended in further studies and compared with those of other new prognostic markers [44].

8. Conclusions and perspectives

It has long been proposed that proteases are involved in the process of invasion and metastasis by cancer cells [see 45]. Collagenases [46] and plasminogen activator [47] have been the most extensively studied, and have been considered to be involved in the process of metastasis. However, at present, clinical studies do not show a correlation between plasminogen activator activity and bad prognosis of breast cancer [48]. By contrast, tissue plasminogen activator appears to be more correlated with estrogen and progesterone receptor sites than with prognosis [49, 50]. On the other hand, there is converging evidence favoring the idea that cathepsins are closely related to the metastatic and invasive processes in cancer. Cell transformation and several mitogens have been shown to stimulate the secretion of the precursors of MEP-cathepsin-L [51], cathepsin-B [52], and the 52K-pro-cathepsin-D.

Moreover, the first clinical results indicate that high concentrations of the 52K-cathepsin-D antigen in breast cancer cytosol is associated with invasive breast cancers. These results strongly support the idea that secreted lysosomal proteases as well as other secreted proteases [45] are involved in the process of tumor growth and invasion. We propose that the increased synthesis and secretion of 52K-cathepsin-D under estrogen control in well differentiated tumor, and constitutively in the hormone independent tumor may facilitate mammary tumor invasion and metastases [23, 53]. In support of this hypothesis, estradiol appears to stimulate the migration of MCF_7 cells through a reconstituted basement membrane [54]. Other cancers may also be controlled by this protease, but at present our immunoperoxidase staining has revealed high concentrations of it mostly in breast cancer cells and melanoma [37], suggesting that this cathepsin may act more specifically in these two types of cancer, which frequently result in metastases.

The involvement of proteases in the control of cancer growth has important therapeutic implications [45]. Since the 52K protease, like other autocrine growth factors, is produced by both hormone-dependent and -independent breast cancer cells, the neutralization of its activity (by antibodies, analogs,

enzyme inhibitors, etc.) could be of considerable interest in treating breast cancer and melanoma and in preventing metastasis, in the same way as small-cell lung cancer is treated by neutralizing bombesin-like growth factors [55]. These antimitogenic treatments would be of more general use than antihormone therapy, whose efficacy is restricted to hormone-dependent cancers. Further studies are needed to prove the biological function of these proteases in carcinogenesis and to discover how to inhibit their putative destructive effects.

Acknowledgments

We would like to thank members of our laboratory who have contributed to several parts of this work and to E. Barrié and M. Egéa of their skillful preparation of the manuscript. We are grateful to Clin Midy Sanofi Laboratory (B. Pau, F, Paolucci) for monoclonal antibodies, P. Chambon (Strasbourg) and F. Rougeon (Pasteur, Paris) for 52K-cDNA cloning, P. Ferrara, P. Louisot, A. Barrett, for protein structure studies and to several scientists (Drs. M. Lippman, M. Rich, I. Keydar, and the Mason Research Institute) for their gifts of mammary cell lines. Clinical studies have been performed with the help of several clinical Centers in Copenhagen (Finsen Institute, Dr. S. Thorpe), Montpellier (Prs. H. Pujol, J.L. Lamarque, F. Laffargue), Villejuif (Dr. G. Contesso) and Nice (Dr. Duplay).
This work was supported by the 'Institut National de la Santé et de la Recherche Médicale', the Faculty of Medicine of Montpellier, the 'Association pour la Recherche sur le Cancer', the 'Fédération Nationale des Centres de Lutte contre le Cancer' and a grant INSERM-SANOFI n°81039.3.

Editors Note:
Following the submission of this manuscript a report appeared in the literature concerning the cloning and sequencing of a cathepsin D from a human breast cancer cell live [56]. This gene appear to encode the 52K protein or a closely related protein.

References

1. Sirbasku DA, Benson RH: Estrogen-inducible growth factors that may act as mediators (estromedins) of estrogen-promoted tumor cell growth. In: Hormones and Cell Culture, Vol. 6, Sato JH and Ross R (eds), Cold Spring Harbor Lab., pp 477–497, 1979.
2. Lippman ME, Bolan G, Huff K: The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res 36:4595–4601, 1976.
3. Chalbos D, Vignon F, Keydar I, Rochefort H: Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). J Clin Endocrind Metab 55:276–283, 1982.
4. Darbre P, Yates J, Curtis S, King RJB: Effect of estradiol on human breast cancer cells in culture. Cancer Res 43:349–354, 1983.
5. Adams DJ, Edwards DP, McGuire WL: Estrogen regulation of specific proteins as a mode of hormone action in human breast cancer. In: Biomembranes, Vol. 11; p 389, 1983.

6. Rochefort H, Chalbos D, Capony F, Garcia M, Veith F, Vignon F, Westley B: Effect of estrogen in breast cancer cells in culture: released proteins and control of cell proliferation. In: Hormones and Cancer, Vol. 142, Gurpide E, Calandra R, Levy C, Soto RJ (eds), Alan R. Liss Inc., New York, pp 37–51, 1984.
7. Chambon P, Dierich A, Gaub MP, Jakowlew S, Jongstra J, Krust A, Lepennec JP, Oudet P, Reudelhuber T: Promoter elements of genes coding for proteins and modulation of transcription by estrogens and progesterone. In: Recent Progress in Hormone Research, Vol. 40, Greep O, (ed), Academic Press, New York, pp 1–42, 1984.
8. Rochefort H, Coezy E, Joly E, Westley B, Vignon F: Hormonal control of breast cancer in cell culture. In: Hormones and Cancer, Vol. 14 Iacobelli S. *et al.*, (eds), Raven Press, New York, pp 21–29, 1980.
9. Vignon F, Derocq D, Chambon M, Rochefort H. Les protéines oestrogéno-induites sécrétées par les cellules mammaires cancéreuses humaines MCF7 stimulent leur proliferation. C R Acad Sci Paris 296:151–156, 1983.
10. De Larco JE, Todaro GJ: Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci 75:4001–4005, 1978.
11. Dickson RB, Huff KK, Spencer Em, Lippman ME: Induction of epiderman growth factor-related polypeptides by 17β-estradiol in MCF7 human breast cancer cells. Endocrinology 118, 138–142, 1986.
12. Manni A, Wright C, Feil P, Baranao L, Demers L, Garcia M, Rochefort H: Autocrine stimulation by estradiol-regulated growth factors of rat hormone-responsive mammary cancer: interaction with the polyamine pathway. Cancer Res 46:1594–1599, 1986.
13. Vignon F, Rochefort H: The regulation by estradiol of proteins released by breast cancer cells. In: Hormone Responsive Tumors, Hollander VP (ed), Academic Press, New York, pp 135–153, 1985.
14. Lippman ME, Dickson RB, Bates S, Knabbe C, Huff K, Swain S, McManaway M, Bronzert D, Kasid A, Gelmann EP: Autocrine and paracrine growth regulation of human breast cancer. Breast Cancer Res Treat 1:59–70, 1986.
15. Salomon DS, Zwiebel JA, Bano M, Lasonczy L, Fehnel P, Kidwell WR: Presence of transforming growth factors in human breast cancer cells. Cancer Res 44:4069–4077, 1984.
16. Westley B, Rochefort H: Estradiol induced proteins in the MCF7 human breast cancer cell line. Biochem Biophys Res Commun 90:410–416, 1979.
17. Westley B, Rochefort H: A secreted glycoprotein induced by estrogen in human breast cancer cell lines. Cell 20:352–362, 1980.
18. Garcia M, Lacombe MJ, Duplay H, Cavailles V, Derocq D, Delarue JC, Krebs B, Contesso G, Sancho-Garnier H, Richer G, Domergue J, Namer M, Rochefort H: Immunohistochemical distribution of the 52-kDa protein in mammary tumors: A marker associated with cell proliferation rather than with hormone responsiveness. J Steroid Biochem 27:439–445, 1987.
19. Westley B, May FEB, Brown AMC, Krust A, Chambon P, Lippman ME, Rochefort H: Effects of antiestrogens on the estrogen regulated pS2 RNA, 52-kDa and 160-kDa protein in MCF7 cells and two tamoxifen resistant sublines. J Biol Chem 259:10030–10035, 1984.
20. Garcia M, Capony F, Derocq D, Simon D, Pau B, Rochefort H: Monoclonal antibodies to the estrogen-regulated Mr 52,000 glycoprotein: Characterization and immunodetection in MCF7 cells. Cancer Res 45:709–716, 1985.
21. Capony F, Garcia M, Capdevielle J, Rougeot C, Ferrara P, Rochefort H: Purification and characterization of the secreted and cellular 52-kDa proteins regulated by estrogens in human breast cancer cells. Eur J Biochem. 161:505–512, 1986.
22. Vignon F, Capony F, Chambon M, Freiss G, Garcia M, Rochefort H: Autocrine growth stimulation of the MCF7 breast cancer cells by the estrogen-regulated 52K protein. Endocrinology 118:1537–1545, 1986.
23. Morisset M, Capony F, Rochefort H: The 52-kDa estrogen-induced protein secreted by MCF7 cells is a lysosomal acidic protease. Biochem Biophys Res Commun 138:102–109, 1986.
24. Capony F, Morisset M, Barrett AJ, Capony JP, Broquet P, Vignon F, Chambon M, Louisot

P, Rochefort H: Phosphorylation, glycosylation and proteolytic activity of the 52K estrogen-induced protein secreted by MCF7 cells. J Cell Biol 104:253–262, 1987.
25. Morisset M, Capony F, Rochefort H: Processing and estrogen regulation of the 52-kDa protein inside MCF7 breast cancer cells. Endocrinology 119:2773–2783, 1986.
26. Von Figura K, Hasilik A: Lysosomal enzymes and their receptors. Ann Rev Biochem 55:167–193, 1985.
27. Barrett AJ: Purification of isoenzymes from human and chicken liver. Biochem J 117:601–607, 1970.
28. Augereau P, Garcia M, Mattei MG, Cavailles V, Depadova F, Derocq D, Capony F, Ferrara P, Rochefort H: Cloning and sequencing of the 52K cathepsin D cDNA of MCF7 breast cancer cells and mapping on chromosome 11. Mol Endocrinol 2:186–192, 1988.
29. Faust PL, Kornfeld S, Chirgwin JM: Cloning and sequence analysis of cDNA for human cathepsin D. Proc Natl Acad Sci USA 82:4910–4914, 1985.
30. Cavailles V, Augereau P, Garcia M, Rochefort H: Estrogens and growth factors induce the mRNA of the 52K-pro-cathepsin-D secreted by breast cancer cells. Nucleic Acids Res. In press, 1988.
31. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV: Human transforming growth factor-: precursor structure and expression. E Coli Cell 38:287–297, 1984.
32. Lawrence DA, Pircher R, Jullien P: Conversion of a high molecular weight latent β-TGF from chicken embryo fibroblasts into a low molecular weight active β-TGF under acidic conditions. Biochem Biophys Res Commun 133:1026–1034, 1985.
33. Low DA, Wiley HS, Cunningham DD: Role of cell surface thrombin-binding sites in mitogenesis. In: Cancer Cells 3, Growth Factors and Transformation, Feramisco J, Ozanne B, Stiles B (eds), Cold Spring Harbor Lab. Cold Spring Harbor, NY, pp 401–408, 1985.
34. De Duve C: In: A guided tour of the living cell, Scientific American Books Inc., New York, Vol. 1. 1984.
35. Hasilik A, Von Figura K, Conzelmann E, Nehrkorn H, Sandhoff K: Lysosomal enzyme precursors in human fibroblasts: activation of cathepsin D precursor *in vitro* and activity of β-hexosaminidase. A precursor towards ganglioside GM2. Eur J Biochem 125:317–321, 1982.
36. Briozzo P, Morisset M, Capony F, Rochefort H: In vitro degradation of extracellular matrix with 52K cathepsin D secreted by breast cancer cells. Cancer Res, in press, 1988.
37. Garcia M, Salazar-Retana G, Pages A, Richer G, Domergue J, Pages AM, Cavalié G, Martin JM, Lamarque JL, Pau B, Pujol H, Rochefort H: Distribution of the Mr 52,000 estrogen-regulated protein in benign breast diseases and other tissues by immunohistochemistry. Cancer Res 46:3734–3738, 1986.
38. Dupont WD, Page DL: Risk factors for breast cancer in women with proliferative breast disease. N Engl J Med 312:146–151, 1985.
39. Cavailles V, Garcia M, Salazar G, Domergue J, Simony J, Pujol H, Rochefort H: Immunodetection of estrogen receptor and 52K protein in fine needle aspirates of breast cancer. J Natl Cancer Inst 79:245–252, 1987.
40. Rogier H, Freiss G, Besse MG, Cavalié-Barthez G, Garcia M, Pau B, Rochefort H, Paolucci F: Two-site immunoenzymometric assay of the 52-kDa-catheps in D cytosols of breast cancer tissues. Clin Chem, In press, 1988.
41. Freiss G, Rochefort H, Maudelonde T, Cavalié G, Khalaf S, Vignon F: Characterization and properties of two monoclonal antibodies to the pro-fragment of the 52K estrogen-regulated protease. In: Proceedings of the Endocrine Society, The Endocrine Society, Bethesda, MD. 1987. Abstract 953
42. Maudelonde T, Khalaf S, Garcia M, Freiss G, Duporté J, Benatia M, Rogier H, Paolucci F, Simony J, Pujol H, Pau B, Rochefort H: Immunoenzymatic assay of 52K cathepsin D in 182 breast cancer cytosols. Low correlation with other prognostic parameters. Cancer Res 48: 462–466, 1988.
43. Thorpe SM, Rochefort H, Garcia M, Freiss G, Christensen IJ, Khalaf S, Paolucci F, Pau B, Rasmussen BB, Rose C. Submitted for publication, 1988.

44. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182, 1987.
45. Goldfarb RH: Proteolytic enzymes in tumor invasion and degradation of host extracellular matrices. In: Mechanisms of Cancer Metastasis: Honn KV, Powers WE, Sloane BF (eds), Martinus Nijhoff Publishers, Boston, pp 341–375, 1986.
46. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284:67–68, 1980.
47. Ossowski L, Reich E: Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 35:611–619, 1983.
48. O'Grady P, Lijnen HR, Duffy MJ: Multiple forms of plasminogen activator in human breast tumors. Cancer Res 45:6216–6218, 1985.
49. Thorsen T: Associations of plasminogen activator activity and steroid receptors in human breast cancer. Eur J Cancer Clin Oncol 18:129–132, 1982.
50. Magdelenat H, Bieth J, Coppey J, Durand JC: Plasminogen activator and progesterone receptor are correlated in human mammary tumors. Breast Cancer Res Treat 3:383–384, 1983.
51. Gal S, Gottesman MM: The major excreted protein of transformed fibroblasts in an activable acid-protease. J Biol Chem 261:1760–1765, 1986.
52. Poole AR: Tumor lysosomal enzymes and invasive growth. In: Lysosomes in Biology and Pathology, Dingle JT and Fell HB (eds), American Elsevier Publishing Co., New York, pp 304–337, 1979.
53. Rochefort H, Capony F, Cavalié G, Chambon M, Freiss G, Garcia M, Morisset M, Vignon F: A 52K estrogen-induced protease secreted by breast cancer cells with autocrine mitogenic activity. In: Hormonal Manipulation of Cancer, Klijn JGM (ed), Raven Press, New York, pp 407–414, 1987.
54. Albini A, Graf J, Kitten GT, Kleinman HK, Martin GR, Veillette A, Lippman ME: 17β-estradiol regulates and v-Ha-ras transfection constitutively enhances MCF7 breast cancer cell interactions with basement membrane. Proc Natl Acad Sci USA 83:8182–8186, 1986.
55. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A, Minna JE: Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature 316:823–826, 1985.
56. Westley BR, May FEB: Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cells. Nucleic Acids Research 15:3773–3783, 1987.

10. Tumor invasion and metastases: biochemical mechanisms

Lance A. Liotta, and Mary L. Stracke

1. Introduction

Tumor invasion and metastases is the major cause of treatment failure for cancer patients. Approximately 30% of patients with newly diagnosed solid tumors (excluding skin cancers other than melanoma) already have clinically detectable metastases. This percentage has increased somewhat in recent years due to the widespread adoption of new imaging technology resulting in the detection of metastases at an earlier stage in their growth. Of those 60% of cancer patients which are clinically free of metastases, approximately half can be cured by local tumor therapy alone [1]. The remaining patients have clinically occult micrometastases which ultimately become manifest. The patient with metastatic disease succumbs to the direct anatomic compromise caused by the metastases or to complications associated with therapy.

2. Multistep cascade of metastases

A metastatic colony is the end result of a complicated series of tumor host interactions (Figure 1). Primary tumor initiation and progression is followed by the transition from *in situ* to locally invasive cancer and angiogenesis [3–6]. Newly formed tumor vessels are often defective and easily invaded by tumor cells within the primary mass. At the invasion front tumor cells also invade pre-established host blood vessels. Tumor cells are discharged into the venous drainage in single cell form and in clumps. For rapidly growing tumors 1 cm in size, millions of tumor cells can be shed into the circulation every day. Fortunately for the patient, only a very small percentage (<.01%) of circulating tumor cells initiate metastatic colonies. Tumors generally lack a well-formed lymphatic network. Therefore, communication of tumor cells with lymphatic channels occurs only at the tumor periphery and not within the tumor mass. Tumor cells entering the lymphatic drainage are carried to regional lymph nodes where they arrest in the large lymphatics of the subcapsular sinus. Within 10 to 60 minutes after initial arrest in the lymph node, a

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

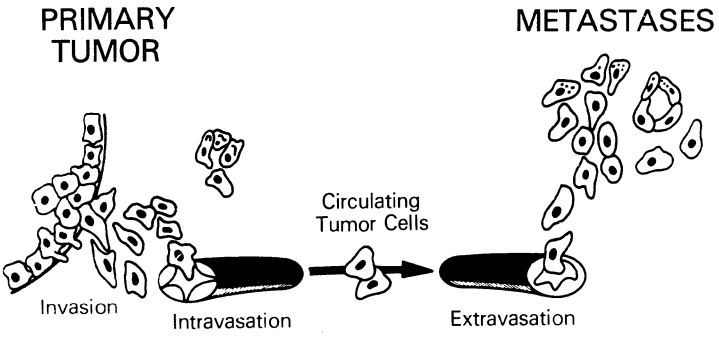


Figure 1. Multistep metastatic cascade. Following transition from *in situ* to invastive carcinoma, tumor cells gain access of host blood vessels and lymphatics. Tumor cells directly (or indirectly via lymphatic-hematogenous communications) enter the blood stream and are carried to the distant organ site. Here they arrest in the vascular bed, extravasate, and initiate a metastatic colony. Continued growth of the metastases requires angiogenesis and escape from host defenses.

significant fraction of the tumor cells detach and enter the afferent lymphatics. These tumor cells eventually end up in the regional or systemic venous drainage due to the existence of numerous lymphatic-hematogenous communications. Thus, the regional lymph node does not function as a true mechanical barrier to tumor dissemination. Lymphatic and hematogenous dissemination occurs in parallel.

Circulating tumor cells utilize a variety of means to arrest in the vessels of the target organ where they will initiate metastatic colonies. The fate and time course of the arrested tumor cells differs depending on the mechanism and location of lodgement. Approximately 80% of the circulating tumor cells are in single cell form and directly attach to the intact endothelial surface or to pre-existing regions of exposed subendothelial basement membrane. Clumps of circulating tumor cells or tumor cells aggregated with host leukocytes, fibrin, or platelets can directly embolize, in the pre-capillary venules by mechanical impaction or can adhere to the endothelial lumenal surface of arterioles. Tumor cells adherent to the surface of venule or capillary endothelium rapidly (1–4 hrs) induce the active retraction of the endothelial cells. The tumor cell then attaches avidly to the exposed basement membrane. Once the tumor cells have attached, the adjacent endothelial cells extend over the tumor cell and separate it from the blood stream. Tumor cells located between the endothelium and the basement membrane are held up in this location for 8 to 24 hrs. Local dissolution of the basement membrane is then observed in association with tumor cell pseudopodia traversing the basement membrane. This step is soon followed by complete extravasation of the tumor cell and quite often re-establishment of blood flow in the breached vessel. Tumor cells arrested in the arterial tree can remain in this location for two or three weeks. Endothelial retraction does not occur following arterial arrest. Intra-arterial tumor cells can actually proliferate and expand as colonies. As

the tumor colonies enlarge, they become covered by a host endothelial surface which lacks a basement membrane. Once the tumor colony fills the arteriole, mechanical damage to the endothelium occurs, and this exposes the basement membrane. Tumor cells at the periphery of the interarterial colony then invade through the basement membrane and the elastic lamina of the arteriole wall to gain an extravascular position.

At all stages of the metastatic cascade, tumor cells must overcome host defenses [3–5]. Although tumor-specific antigens have been identified in animal models, it still remains unclear whether similar antigens play a role in human tumors, and whether the recognition of these antigens can be boosted by adjuvant immunotherapy. Limited effectiveness of adjuvent immunotherapy of metastases may be due to tumor antigen heterogeneity, tumor antigen shedding, or absence of tumor cell immunogenicity. "Nonspecific" host defenses such as macrophages and natural killer cells may be more effective against heterogeneous tumor cell populations. In animal models these effector cells play an important role in the elimination of circulating tumor cells, and destruction of micrometastases.

Extravasated tumor cells proliferate as colonies, but require a new vascular supply to grow larger than 0.5 mm. Thus angiogenesis is necessary at the beginning and the end of the metastatic cascade. Metastases can themselves metastasize, further amplifying the level of disease progression. Numerous clinical reports provide circumstantial evidence for the existence of dormant metastases [1]. Up to one third of the mortality from breast cancer, for instance, occurs more than 5 years after removal of the primary tumor. Three potential mechanisms of tumor dormancy have been distinguished in animal models: 1) immunologic restraint such that the tumor population death rate equals its growth rate, 2) constitutive dependency of tumor cells on host growth factors, and 3) avascularity causing the metastasis to be limited in size due to deficiency in nutrient diffusion.

3. Organ tropism for metastases

The distribution of metastases varies widely depending on the histologic type and anatomic location of the primary tumor (Table 1). The most frequent organ location of distant metastasis in many types of cancer appears to be the first capillary bed encountered by the circulating cells. Major pathways of metastases determined directly by anatomic considerations are listed as follows:

1. Sarcomas arising in the extremities metastasize primarily to the lungs. Sarcoma cells entering the tumor venous drainage are carried into the inferior vena cava, enter the right heart, and are carried via the pulmonary artery to the lungs.
2. Lung cancer disseminates widely to multiple organs including brain. Lung

Table 1. Autopsy incidence of metastases

Organ of metastases	Primary tumor (%)			
	Lung	Colon	Breast	Melanoma
Liver	30–50	50–60	40–60	58–70
Lung	20–40	25–40	60–80	66–80
Bone	30–45	5–10	50–90	30–48
Brain	15–43	0–1	15–30	40–55
Adrenal	17–38	14	38–54	40–47
Pituitary	0–2	0–1	20	18
Ovary	0–2	14	15–30	10–15
Kidney	16–23	8	13	31–35
Spleen	9	5	17	31

cancer is the only tumor that has direct access to the general arterial circulation via the pulmonary vein through the left heart ventricle [3].

3. Colorectal carcinomas tend to metastasize to the liver. Colorectal carcinoma cells enter the mesenteric lymphatics and portal venous system and are carried to the liver [1, 3].
4. Tumors of the testicle metastasize via the lymphatics to lymph nodes of the periaortic area and then enter the subclavian veins by lymphatic-hematogenous communications. Tumor cells entering the subclavian veins go to the right heart and then to the lungs [1–3].
5. Prostate cancer metastasizes primarily (90%) to vertebral bone. The anatomic route is via Batson's plexus of paravertebral veins [2]. Tumor cells entering the prostatic plexus of veins are carried to the veins about the sacrum, ilium and the lumbar spine.
6. Patterns of head and neck cancer metastases correspond primarily to the regions of local lymphatic drainage [1].
7. Ovarian cancer remains confined for long periods of time in the abdominal cavity. Local spread occurs to the peritoneal surfaces, the posterior gutters, and the diaphragm. These tumors invade the liver in only a small percentage of cases at a very late stage. Liver invasion is usually by direct invasion from omental disease or by mesenteric venous emboli derived from omental implants. [1].
8. Breast cancer metastases are frequently found in vertebral bone. Based on dye injection studies, it has been demonstrated that the mammary venous drainage can communicate with Batson's plexus of paravertebral veins [2]. When dye was injected into a small mammary vein, the dye was found in the clavicles, intercostal veins, head of the humerus, cervical vertebrae and transverse cranial sinuses.

However, there are many metastatic sites which cannot be predicted based on anatomic considerations alone, and can be considered examples of organ

tropism. For example, clear cell carcinoma of the kidney often metastasizes to bone and thyroid, and ocular melanoma frequently metastasizes to the liver. Theoretical mechanisms for organ tropism include the following [3, 5]: 1) tumor cells disseminate equally in all organs, but preferentially grow only in specific organs. Preferential growth may be induced by local growth factors or hormones present in the target organ for metastases; 2) circulating tumor cells may adhere preferentially to the endothelial lumenal surface only in the target organ for metastases. This hypothesis predicts organ-specific endothelial determinants; 3) circulating tumor cells may respond to soluble factors diffusing locally out of the target organ. Such factors could act in a chemotactic fashion to attract the tumor cells to extravasate. They could also cause the circulating tumor cells to aggregate and therefore embolize in the target organ. Research with animal models indicates that all of these mechanisms play a role to various degrees depending on the tumor model system [3, 5].

4. Tumor cell interaction with the extracellular matrix

The mammalian organism is composed of a series of tissue compartments separated from each other by two types of extracellular matrix: basement membranes and interstitial stroma [4]. The matrix determines tissue architecture, has important biologic functions, and exists as a mechanical barrier to invasion. During the transition from *in situ* to invasive carcinoma, tumor cells penetrate the epithelial basement membrane and enter the underlying interstitial stroma. Once the tumor cells enter the stroma they gain access to lymphatics and blood vessels for further dissemination. Fibrosarcomas and angiosarcomas, developing from stromal cells, invade surrounding muscle basement membrane and destroy myocytes. Tumor cells must cross basement membranes to invade nerve and most types of organ parenchyma. During intravasation or extravasation the tumor cells of any histologic origin must penetrate the subendothelial basement membrane. In the distant organ where metastases colonies are initiated, extravasated tumor cells must migrate through the perivascular interstitial stroma before tumor colony growth occurs in the organ parenchyma. Therefore, tumor cell interaction with the extracellular matrix occurs at multiple stages in the metastatic cascade.

General and widespread changes occur in the organization, distribution and quantity of the epithelial basement membrane during the transition from benign to invasive carcinoma. The human breast is a particular example. Benign proliferative disorders of the breast such as fibrocystic disease, sclerosing adenosis, intraductal hyperplasia, fibroadenoma and intraductal papilloma are all characterized by disorganization of the normal epithelial stromal architecture. Extreme forms can mimic the appearance of invasive carcinoma. However, no matter how extensive the architectural disorganization, these benign disorders are always characterized by a continuous basement membrane separating the epithelium from the stroma. In contrast, invasive ductal

carcinoma, invasive lobular carcinoma and tubular carcinoma, consistently possess a defective extracellular basement membrane with zones of basement membrane loss around the invading tumor cells. The basement membrane is also markedly defective adjacent to tumor cells in lymph node and organ metastases. In some focal regions of well differentiated carcinoma, partial basement membrane formation by differentiated structures can be identified. These findings are of direct application to diagnostic problems in surgical pathology such as the differentiation of tangential sections of *in situ* lesions from true invasion, or differentiating severe adenosis from invasive carcinoma. Loss of basement membranes in human carcinomas significantly correlates with increased incidence of metastases and poor 5-year survival.

5. Three-step theory of invasion

A three-step hypothesis has been proposed to describing the sequence of biochemical events during tumor cell invasion of extracellular matrix. The first step is tumor cell attachment to the matrix. Attachment may be mediated by tumor cell plasma membrane receptors that interact with specific glycoproteins such as laminin and fibronectin. Following attachment, the tumor cell secretes hydrolytic enzymes (or induces host cells to secrete enzymes) which can locally degrade the matrix (including degradation of the attachment glycoproteins). Matrix lysis most likely takes place in a highly localized region close to the tumor cell surface where the amount of active enzyme outbalances the natural protease inhibitors present in the serum and in the matrix itself. In contrast to the invasive tumor cell, when the normal cell or benign tumor cell attaches to the matrix it may respond by shifting into a resting or differentiated state. The third step is tumor cell locomotion into the region of the matrix modified by proteolysis. The direction of the locomotion may be influenced by chemotactic factors and autocrine motility factors. Autocrine motility factors (AMF) are a newly described class of proteins (see section below) which bind to a cell surface receptor and profoundly stimulate motility [4]. They are distinct from known growth factors, and their mechanism of action involves the membrane G protein pathway inhibited by pertussis toxin. The chemotactic factors derived from serum, organ parenchyma, or the matrix itself [3, 6] may influence the organ specificity of metastases. Continued invasion of the matrix may take place by cyclic repetition of these three steps.

6. Autocrine motility factors

Cell motility is necessary for tumor cells to traverse many stages in the complex cascade of invasion. Such stages could include the detachment and subsequent infiltration of cells from the primary tumor into adjacent tissue, the migration of the cells through the vascular wall into the circulation (intravasa-

tion), and the extravasation of the cells to a secondary site. The movement of cells through biologic barriers such as the endothelial basement membranes of the vasculature may well occur by means of chemotactic mechanisms. Indeed, studies on *in vitro* chemotaxis of some tumor cells report that a variety of compounds such as complement-derived materials, collagen peptides, formyl peptides, and certain connective tissue components can act as chemoattractants [7, 8]. While these agents may well contribute to the directional aspects of a motile response, they are not sufficient to initiate the intrinsic locomotion of tumor cells. The availability of soluble attractants to the tumor cell is greatly dependent upon the host even in those cases in which the production of attractants is the result of tumor cell-host tissue interaction. At best, it seems that the cell would have access to such motility stimuli at sporadic and irregular intervals, conditions unfavorable to a sustained migration of highly invasive cells. With these considerations in mind and stimulated by the studies of Todaro, Sporn and co-workers [9], in which they demonstrated autocrine growth factors for transformed cells, we investigated the possibility that such cells could elaborate autocrine motility factors. The action of these substances might, in part, explain both the markedly invasive character and the metastatic property of malignant cells. Thus, under the influence of such an autocrine material, a tumor cell might move out into the surrounding host tissue and also exert a 'recruiting' effect on adjacent tumor cells in the presence of a gradient of attractant (Figure 2). Conceivably, such factors might also attract fibroblastic cells of the host, resulting in the phenomenon of desmoplasia, characteristic of invasive tumors.

We have found that the human melanoma cell line A2058 and human breast carcinoma cells produce in culture a material that markedly stimulates their own motility [10]. These cells respond in a dose-dependent manner to various concentrations of conditioned medium obtained by incubating confluent cells in serum-free medium, an indication that the motility factor is derived from the cell. Motility was measured by the modified Boyden chamber procedure. Using this assay and the 'checkerboard' analysis [11], we have also found that the conditioned medium factor has both chemotactic (directional) and chemo-kinetic (randomly motile) properties.

7. Isolation and characterization of a human melanoma autocrine motility factor

Conditioned protein-free medium which elicited both large (10–15% of the cells migrated) dose-dependent chemotactic and randomly motile responses (checkerboard analysis) was used to isolate AMF. The conditioned medium, after concentration (Amicon), was subjected to molecular sieve chromatography. AMF emerged as a broad major peak between 40 and 65 kDa (data not shown). The AMF was further isolated by fast-performance liquid chromatography.

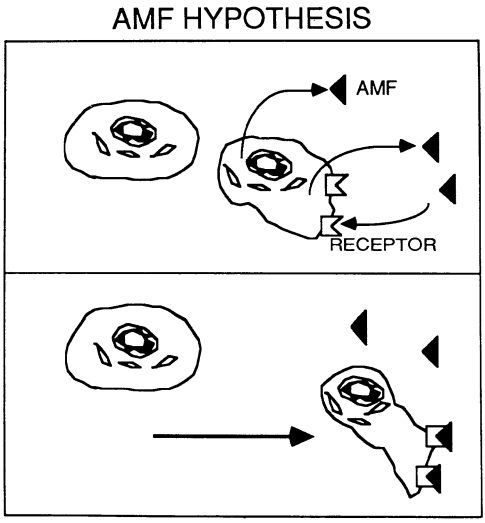


Figure 2. AMF hypothesis: cells produce a protein factor (AMF) which binds to specific cell surface receptors resulting in the triggering of an intrinsic motile response. The factor or fators can also recruit neighboring cells via a paracrine mechanism.

The AMF activity was iodinated and found to comprise a single major component (electrophoresis) of ≈55 kDa without reduction of disulfide bonds. Upon reduction with 5 mM dithiothreitol, the migration of this component on the gel became slower, indicating the existence of interchain disulfide bonds. A maximal chemotactic response to the purified AMF was elicited at a concentration of 10 mM. Amino acid analysis of AMF revealed a high content of glycine, serine, glutamic acid, and aspartic acid residues. Both tyrosine and cysteine are present, the latter concordant with the existence of interchain disulfide bonds as indicated by the altered electrophoretic mobility of AMF before and after treatment with dithiothreitol.

8. Transduction of the chemical signal in the motile response of tumor cells

Because some cells require ongoing protein synthesis to develop a motile response, we determined whether inhibition of protein synthesis affected the response of the melanoma cells to its autocrine factor. We found that concentrations of cycloheximide that eliminated *de novo* protein synthesis had no effect on stimulated cell motility. Therefore, the cell protein components required for developing a motile response appear to be stable for the duration of migration (4 hrs).

Studies with leukocytes [12, 13] have implicated a guanine nucleotide protein (G-protein) in the receptor-mediated initiation of a motile response in these cells. The evidence is convincing that the locomotion of certain tumor

cells also directly involves a G-protein [14]. Pertussis toxin, known to inhibit action of the Gi protein of the adenylate cyclase pathway [15], profoundly and rapidly inhibited the AMF-stimulated migration *in vitro* of A2058 melanoma cells [14] and two breast cancer cell lines [R. Guirguis *et al.*, submitted]. In the melanoma cell line 0.5 μg/ml of pertussis toxin completely blocked motility without affecting growth in culture [M.L. Stracke, unpublished results]. However, the adenylate cyclase pathway does not appear to be directly involved in the motility response since agents which selectively modulate or have a role in this pathway, e.g., cholera toxin, forksolin, the cyclic AMP analogue 8-bromoadenosine 3′:5′-cyclic monophosphate, and the cyclase inhibitor 2′,5′-dideoxyadenosine, all had minor effects on cell migration. It is likely, then, that effector systems other than adenylate cyclase are mediated by a G-protein in producing tumor cell motility. G proteins have been shown to act in a variety of second messenger pathways including phospholipase A2 [16], phospholipase C [17], and activation of calcium channels [18]. Specifically, in the neutrophil pertussis toxin inhibits both lipase enzymes as well as motility [19, 20]. Evidence that suggests a role for phospholipase A2 in tumor cell locomotion has been obtained with the melanoma cell. Quinacrine, an agent that inhibits phospholipase A2, markedly reduced AMF-stimulated migration. Additionally, deaza-adenosine, an inhibitor of biological methylation [21], was found to reduce both membrane phospholipid methylation and AMF-stimulated motility, whereas AMF itself caused a sustained increase in the methylation of phosphatidyl choline (Ptd Cho) in melanoma cells. Since Ptd Cho is the major substrate for phospholipase A2, these findings are consistent with a role for this enzyme in tumor cell motility. Studies with a murine tumor cell thus, BU-L, suggest that metabolism of arachidonic acid, a product of the lipase reaction, may play a role in tumor cell motility [22]. Lipoxygenase inhibitors such as quercetin, nordihydroguaretic acid, and nafazatrom significantly reduced stimulated motility, but indomethacin, a cyclo-oxygenase blocking agent, had no effect. Calm-idazolium also substantially inhibited motility. Collectively, these results are in accord with both the lipoxygenase pathway for arachidonate metabolism and a calmodulin-mediated mobilization of calcium participating in migration of certain tumor cells. However, a role for the cyclo-oxygenase pathway cannot be ruled out. It has been reported that phorbol myristate acetate and laminin-stimulated motility in murine fibrosarcoma cells are inhibited by prostaglandins of the E series [23]. Preliminary studies [M. Stracke, unpublished material] with human melanoma cells indicate that calcium channel blocking agents inhibit AMF-stimulated motility. On the other hand, calcium ionophores were found to stimulate motility. These results are consistent with the participation of phospholipase C (PDE Figure) and the generation of IP_3 in initiating motility. Preliminary experiments clearly demonstrate that lithium, an inhibitor of the IP_3 pathway, significantly reduces AMF-induced motility [Kohn *et al.*, in preparation].

From these considerations, it is likely that the generation of a motile re-

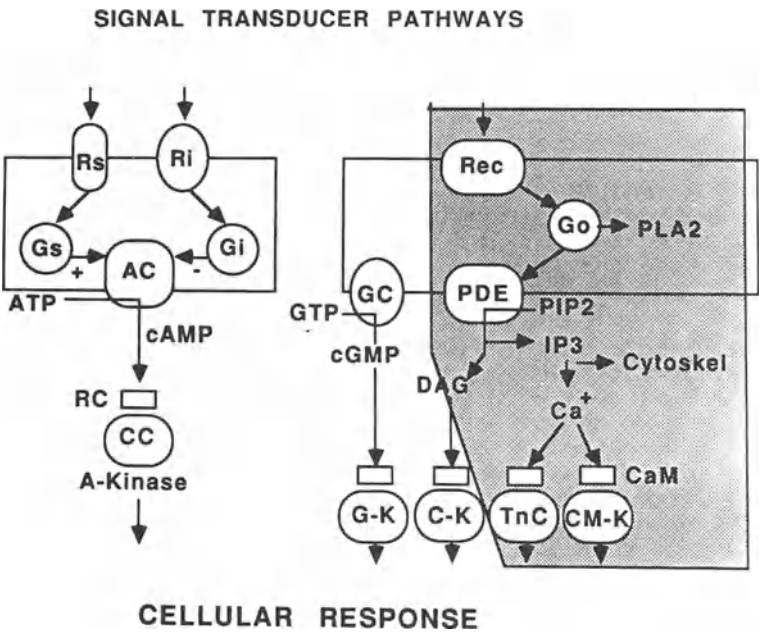


Figure 3. Signal transducer pathways. Three major classes of second messenger pathways are depicted; the cyclic nucleotide pathway includes cyclic AMP (cAMP) and cyclic GMP (cGMP) generated adenylcyclase (AC) and guanyl cyclase (GC), respectively. G proteins can regulate the production for both nucleotides through a stimulatory (Gs) and inhibitory (Gi) arms. A separate series of effector pathways involves stimulation of phospholipase A_2 (PLA2) and phospholipase C (PLC) are also regulated by G proteins (G_0). PLC results in the activation of the calcium mobilizing factor IP_3 and diacycl glycerol. All the experiments to date indicate that the cAMP and the cGMP pathway is not centrally involved in the generation of a motile response in tumor cells. The pertussis toxin sensitive G_0 pathway plays a necessary role in the motile response. This pathway may mediate changes in membrane fluidity through PLA_2 and changes in cytoskeleton organization through PDE and the IP_3 pathway which is known to alter actin binding calcium regulated events such as gelsolin action.

sponse in tumor cells initially involves a direct role for a G-protein which interacts with an activated receptor and then transduces the signal to effector systems such as the phosphodiesterase IP_3 pathway and phospholipase A2 (Figure 3). The subsequent production of arachidonate and its metabolism via lipoxygenase may contribute to the mobilization of calcium by IP_3 and DAG, which could also be required for changes in the cytoskeleton that are essential for locomotion. With respect to a role for cathepsin B, it is conceivable that AMF may stimulate its activity within the membrane to cause a specific cleavage of a pro-enzyme whose active form, e.g., protein kinase C [24], is required for the motile response.

Early events in migration may involve pseudopodia protrusion. During the course of invasion, the same tumor cell must interact with a variety of extracellular matrix proteins as it traverses each tissue barrier. For example, the

tumor cell encounters laminin and type IV collagen when it penetrates the basement membrane, and type I collagen and fibronectin when it crosses the interstitial stroma. It has recently been shown that cells express specific cell surface receptors which recognize extracellular matrix proteins. The first example of such a receptor is the laminin receptor which binds to laminin with nanomolar affinity. Laminin receptors have been shown to be augmented in actively invading tumor cells, and may play an important role in tumor cell interaction with the basement membrane. RGD recognition receptors are another class of cell surface proteins which bind extracellular matrix proteins which in turn contain the protein sequence arg-gly-asp [8]. Such proteins include fibronectin, collagen type I, and vitronectin. The process of cell migration undoubtedly requires a series of adhesion and detachment steps resulting in traction and propulsion. Studies using the AMF stimulated motility as a model system have revealed an important role of pseudopodia protrusion in this process. AMF stimulates motility on a variety of different substrata. Therefore, its action is independent of the mechanism of attachment. Furthermore, AMF induces the rapid protrusion of pseudopodia in a time and dose dependent manner [25]. Isolation of the induced pseudopodia reveals that they are highly enriched in their content of laminin and fibronectin matrix receptors. Since cell pseudopodia formation is known to be a prominent feature of actively motile cells, we can now set forth a working hypothesis to explain the early events in cell motility. Cytokines such as AMF which stimulate intrinsic motility may induce exploratory pseudopodia prior to cell translocation. Such pseudopodia may express augmented levels of matrix receptors (and possibly proteinases). The protruding pseudopodia may serve multiple functions including acting as 'sense organs' to interact with the extracellular matrix proteins and thereby locate directional cues, provide propulsive traction for locomotion, and even induce local matrix proteolysis to assist the penetration of the matrix.

9. Laminin receptors

Cell surface receptors for the basement membrane glycoprotein laminin mediate adhesion of tumor cells to the basement membrane prior to invasion [4, 26]. Laminin as visualized by rotary shadowing electron microscopy has a distinctive cruciform shape with three short arms (35 nm) and one long arm (75 nm). All arms have globular end regions. The specialized structure of the laminin molecule may contribute to its multiple biologic functions. Laminin plays a role in cell attachment, cell spreading, mitogenesis, neurite outgrowth, morphogenesis, and cell movement. Many types of neoplastic cells contain high affinity (nM kd) cell surface binding sites (laminin receptors) for laminin. The molecular weight of the isolated receptor is 65 kDa [26]. The laminin receptor binds to the 'B' chain (short arm) region of the laminin molecule. Laminin receptors may be altered in number or degree of occu-

pancy in human carcinomas. This may be the indirect result of defective basement membrane organization in the carcinomas. Breast carcinoma and colon carcinoma tissue contain higher numbers of exposed (unoccupied) receptors compared to benign lesions. The laminin receptors of normal epithelium may be polarized at the basal surface and occupied with laminin in the basement membrane. In contrast, the laminin receptors on invading carcinoma cells are amplified and may be distributed over the entire surface of the cell. The laminin receptor can be shown experimentally to play a role in hematogenous metastases. Treating tumor cells with the receptor binding fragment of laminin at very low concentrations markedly inhibits or abolishes lung metastases from hematogenously introduced tumor cells. The mechanism of action involves blocking the adhesion of circulating tumor cells to the subendothelial basement membrane (Figure 2).

10. RGD recognition receptors

A family of cell surface glycoproteins, termed "integrins" has been identified which bind with low affinity (uM kd) to a variety of adhesion proteins including fibronectin, von Willebrand factor, fibrin, vitronectin, type I collagen and thrombospondin. The integrins are a complex of alpha (140 kDa) and beta (95 kDa) subunit proteins [27]. The functions of several of the integrins are inhibited by peptides related to the Arg-Gly-Asp (RGD) sequence of fibronectin. RGD sequences present on a wide variety of proteins may serve as the recognition site for binding of the integrins. It is likely that specific ligand sequences adjacent to the RGD site may confer preferential recognition of one type of adhesion protein by certain members of the integrin family. Integrin proteins are thought to align adhesion proteins such as fibronectin on the cell surface with cytoskeletal components such as talin and actin, thus altering cell shape. Integrin type proteins may play an adhesive role in platelet-tumor cell interactions, binding of lymphoid cells to endothelium, and the interaction of circulating tumor cells with endothelial surfaces, fibrin, von Willibrand factor or thrombospondin. In keeping with this concept, it has been reported that co-injection of tumor cells with large quantities of RGD peptides will inhibit metastases formation in animal models. The RGD peptides may interfere with the adhesion of tumor cells to the endothelial surface which may be mediated directly or indirectly through integrin proteins.

11. Tumor cell proteinases

In vitro studies of tumor cell invasion of the extracellular matrix have shown that cell proliferation is not absolutely required. Invasion of the matrix is not merely due to passive growth pressure, but requires active biochemical mechanisms. Inhibitors of protein synthesis or inhibitors of proteinases block invasion of the matrix [4]. Many research groups have proposed that invasive

Table 2.

Metastatic cascade event	Potential mechanisms
1. Tumor initiation	Carcinogenic insult, oncogene activation or derepression, chromosome rearrangement
2. Promotion and progression	Karyotypic, genetic, and epigenetic instability, gene amplification; promotion associated genes and hormones
3. Uncontrolled proliferation	Autocrine growth factors or their receptors, receptors for host hormones such as estrogen
4. Angiogenesis	Multiple angiogenesis factors including known growth factors
5. Invasion of local tissues, blood and lymphatic vessels	Serum chemoattractants, autocrine motility factors, attachment receptors, degradative enzymes
6. Circulating tumor cell arrest and extravasation	Tumor cell homotypic or heterotypic aggregation
a. adherence to endothelium	Tumor cell interaction with fibrin, platelets, and clotting factors, adhesion to RGD type receptors
b. retraction of endothelium	Platelet factors, tumor cell factors
c. adhesion to basement membrane	laminin receptor, thrombospondin receptor
d. dissolution of basement membrane	degradative proteases, type IV collagenase, heparanase, cathepsins
e. locomotion	antocrine motility factors, chemotaxis factors
7. Colony formation at secondary site	Receptors for local tissue growth factors, angiogenesis factors
8. Evasion of host defenses and resistance to therapy	Resistance to killing by host macrophages, natural killer cells and activated T cells, failure to express, or blocking of, tumor specific antigens, amplification of drug resistance genes

tumor cells secrete matrix degrading proteinases. Collagen is an important substrate because it constitutes the structural scaffolding upon which the other components of the matrix are assembled. Tumor derived collagenases which degrade interstitial collagen types I, II and III have been characterized by a number of investigators. They are metal ion (calcium and zinc) dependent enzymes which function at neutral pH. Classic collagenase produces a single cleavage in the collagen molecule (interstitial collagen types I, II and III) producing 3/4 and 1/4 size fragments (75% of the distance from the N terminus). Tumor cells can degrade both collagenous and non-collagenous components of the basement membrane [4, 5, 29]. Basement membrane specific collagen types IV and V are *not* susceptible to classic collagenase which degrades collagen types I, II and III. A separate family of collagenolytic enzymes (type IV collagenase) [4, 29] cleaves the type IV collagen chain 1/4 of the distance from the amino terminus. Type IV collagenases are augmented in highly metastatic tumor cells and in endothelial cells during angiogenesis. Antibodies prepared against type IV collagenase react with invading breast carcinoma cells and breast carcinoma lymph node metastases by immunohistology. Amplification of type IV collagenase production is biochemi-

cally linked to the genetic induction of metastases in experimental models [29].

12. Molecular genetics of metastases

It is apparent that interactions in the complicated metastatic process involve multiple gene products. A cascade or coordinated group of gene products expressed above a certain threshold level may be required for a tumor cell to successfully traverse the successive steps in the metastatic process. The crucial gene products may regulate host immune recognition of the tumor cells, cell growth, attachment, proteolysis, locomotion, and differentiation. The specific family of gene products necessary for metastases may be different for each histologic type of tumor.

The evidence linking oncogenes to the induction or maintenance of human malignancies has become increasingly compelling. In the past, oncogenes have been linked to unrestrained tumor growth. Recently, two different types of experimental approaches [5, 27, 28] have indicated that certain classes or combinations of oncogenes may play a role in the metastatic behavior of tumors. In the first type of experimental approach, human tumor DNA samples are surveyed for the level of oncogene expression and this is correlated with disease stage. In the second type of approach, tumor DNA or isolated oncogenes are transfected into recipient cells. The transfected cells are then studied for their metastatic propensity. Notable examples of these two approaches are work with the HER-2/*neu* oncogene in human breast cancer [28] and transfection of the *ras* oncogene in rodent systems [29].

The HER-2/*neu* (*neu*) oncogene (also termed c-*erb*B-2 and HER-2) encodes a protein which is a member of the tyrosine kinase family, and is related to, but distinct from, the EGF receptor gene. At the time of this writing, the ligand for the *neu* oncogene encoded receptor protein had not yet been identified. A significant increase in the incidence of *neu* gene amplification is noted in breast cancer patients with >3 axillary lymph nodes positive for metastases. Amplification of *neu* was also highly correlated with disease relapse (actuarial survival) as well as tumor size. Thus, even though the function of *neu* is unknown, its level of expression may provide important prognostic information for breast cancer.

Transfection of members of the *ras* oncogene family into suitable rodent recipient cells, including diploid rat embryo fibroblasts, can induce these cells to rapidly progress to express the complete metastatic phenotype [5, 29]. Other oncogenes, including *myc*, *svc*, and *fos*, failed to induce metastases in rodent cells. Furthermore, when ras was transfected in combination with the abenovirus type 2 EIA oncogene, the recipient cells became very tumorigenic but totally non-metastatic [29]. Thus, some genes can suppress the action of ras to induce metastases. The current working hypothesis is that induction of the metastatic phenotype requires at least two (and possibly more) comple-

mentary genes or gene products. In the correct rodent cells, one of these genes may be the activated form of the *ras* oncogene. When these genes interact in the correct fashion, a cascade of specific gene products are elaborated which confer the metastatic phenotype.

References

1. Sugarbaker EV: Patterns of metastasis in human malignancies. Cancer Biol Rev 2:235, 1981.
2. Weiss L, Gilbert HA: Bone Metastases. Boston, GK Hall, Medical Publishers, 1981.
3. Schirrmacher V: Cancer metastasis: Experimental approaches, theoretical concepts, and impacts for treatment strategies. Advances Cancer Res 43:1–73, 1985.
4. Liotta LA: Tumor invasion and metastases—role of the extracellular matrix: Rhoads Memorial Award Lecture. Cancer Res 46:1–7, 1986.
5. Nicolson GL: Tumor cell instability, diversification, and progression to the metastatic phenotype: From oncogene to oncofetal expression. Cancer Res 47:1473, 1987.
6. Furcht LT: Editorial: Critical factors controlling angiogenesis: Cell products, cell matrix, and growth factors. Lab Invest 55:505, 1986.
7. Lam WC, Delikatny JE, Orr FW, Wass J, Varani J, Ward PA: The chemotactic response of tumor cells: A model for cancer metastasis. Am J Pathol 104:69–76, 1981.
8. McCarthy JB, Basara ML, Palm SL, Sas DF, Furcht LT: Stimulation of haptotaxis and migration of tumor cells by serum spreading factor. Cancer Metastasis Rev 4:125–152, 1985.
9. Anzano MA, Roberts AB, Smith JM, Sporn MB, De Larco JE: Sarcoma growth factors from conditioned media of virally transformed cells composed of both type α and type β growth factors. Proc Natl Acad Sci USA 80:6264–6268, 1983.
10. Liotta LA, Mandler R, Murano G, Katz DA, Gordon RK, Chiang PK, Schiffmann E: Tumor cell autocrine motility factor. Proc Natl Acad Sci USA 83:3302–3306, 1986.
11. Zigmond SH, Hirsch JG: Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of cell-derived chemotactic factor. J Exp Med 137:387–410, 1973.
12. Bokoch GM, Gilman AG: Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. Cell 39:301–308, 1984.
13. Smith CD, Cox CC, Snyderman R: Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. Science 232:97–100, 1986.
14. Stracke ML, Guirguis R, Liotta LA, Schiffmann E: Pertussis toxin inhibits stimulated motility independently of the adenylate cyclase pathway in human melanoma cells. Biochem Biophys Res Commun 146:339–345, 1987.
15. Katada T, Ui M: Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc Natl Acad Sci USA 79:3129–3133, 1982.
16. Okajima F, Ui M: ADP-ribosylation of the specific membrane protein by islet-activating protein, pertussis toxin, associated with inhibition of a chemotactic peptide-induced arachidonate release in neutrophils. A possible role of the toxin substrate in Ca2+-mobilizing biosignaling. J Biol Chem 259:13863–13871, 1984.
17. Kikuchi A, Kozawa O, Kaibuchi K, Katada T, Ui M, Takai Y: Direct evidence for involvement of a guanine nucleotide-binding protein in chemotactic peptide-stimulated formation of inositol bisphosphate and trisphosphate in differentiated human leukemia (HL-60) cells. Reconstitution with Gi or Go of the plasma membranes ADP-ribosylated by pertussis toxin. J Biol Chem 261:11558–11562, 1986.
18. Hescheler J, Rosenthal W, Trautwein W, Schultz G: The GTP-binding protein, Go, regulates neuronal calcium channels. Nature 325:445–447, 1987.
19. Molski TF, Naccache PH, Marsh ML, Kermode J, Becker EL, Sha'afi RI: Pertussis toxin inhibits the rise in the intracellular concentration of free calcium that is induced by

chemotactic factors in rabbit neutrophils: possible role of the 'G proteins' in calcium mobilization. Biochem Biophys Res Commun 124:644–650, 1984.
20. Lad PM, Olson CV, Grewal IS, Scott SJ: A pertussis toxin-sensitive GTP-binding protein in the human neutrophil regulates multiple receptors, calcium mobilization, and lectin-induced capping. Proc Natl Acad Sci USA 82:8643–8647, 1985.
21. Guranowski A, Montgomery JA, Cantoni GL, Chiang PK: Adenosine analogues as substrates and inhibitors of S-adenosylhomocysteine hydrolase. Biochemistry 20:110–115, 1981.
22. Boike GM, Sloane BF, Deppe G, Stracke M, Schiffmann E, Liotta LA, Honn KV: The role of calcium and arachidonic acid metabolism in the chemotaxis of a new murine tumor line. Am Assoc Cancer Res 28:82, 1987.
23. He XM, Fligiel SE, Varani J: Modulation of tumor cell motility by prostaglandins and inhibitors of prostaglandin synthesis. Exp Cell Biol 54:128–137, 1986.
24. Pontremoli S, Melloni E, Michetti M, Sacco O, Salamino F, Separatore B, Horecker BL: Biochemical responses in activated human neutrophils mediated by protein kinase C and a Ca2+-requiring proteinase. J Biol Chem 261:8309–8313, 1986.
25. Guirguis R, Margulier I, Taraboletti G, Schiffmann E, Liotta L: Cytokine-induced pseudopodial protrusion is coupled to tumor cell migration. Nature 329:261–263, 1987.
26. Wewer UM, Liotta LA, Jaye M, Ricca GA, Drohan WN, Claysmith AP, Rao CN Wirth P, Coligan JE, Albrechtsen R, Mudryj M, Sobel ME: Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. Proc Natl Acad Sci USA 83:7137–7141, 1986.
27. Hynes RO: Integrins: A family of cell surface receptors. Cell 48:549, 1987.
28. Slamon DJ, Clark GM, Wong SG, *et al*: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177, 1987.
29. Garbisa S, Pozzatti R, Muschel RJ, Saffiotti U, Ballin M, Goldfarb RH, Khoury G, Liotta LA: Secretion of type IV collagenolytic protease and metastatic phenotype: Induction by transfection with c-Ha-*ras* but not c-Ha-*ras* plus Ad2-Ela. Cancer Res 47:1523–1528, 1987.

11. Factors regulation basement membrane invasion by tumor cells

E.W. Thompson, R. Reich, G.R. Martin, and A. Albini

1. Introduction

Breast cancer cells, initially dependent on estrogen for growth, frequently progress to a more aggressive, estrogen-independent state [1, 2]. Such a change in their phenotype is associated with the potential of the tumor cells to form metastatic lesions and with a poorer prognosis for the patient [3]. Current concepts suggest that the formation of metastases by tumor cells requires the completion of a complex series of events by which certain tumor cells leave the primary lesion, escape immune surveillance in the circulation, and penetrate and proliferate in normal tissue at a distant site [4, 5]. These are not random events but require distinct biochemical activities possessed by a limited population of cells which arises during tumor progression. The characterization of metastatic cells and their activities has been greatly facilitated by the isolation of cell lines with high metastatic capacities. The initial metastatic cell lines were murine melanoma-derived [6–8]. A number of different breast cancer lines also now exist representing various stages of malignant development [9, 10]. Many malignant cells possess the ability to degrade and traverse basement membranes and such invasive behavior is thought to be a necessary step in their metastasis [11]. Some pertinent steps in tumor cell invasion through basement membrane have been identified (Table 1). Studies on breast cancer cells have shown that their invasive behavior can be modulated [12].

2. The basement membrane: barrier to metastases

The interaction of tumor cells with basement membranes is a critical step in metastasis, since the cells encounter and pass numerous basement membranes as they disseminate through the body [11]. Basement membranes are sheets of extracellular matrix which surround epithelial tissues, nerves and muscles, providing physical support to these tissues [13]. Their major components, collagen IV, laminin, and heparan sulfate proteoglycan are common to all basement membranes studied [14] and the ubiquitous occurrence of their common components may explain why similar biochemical events are asso-

M.E. Lippman and R.B. Dickson (eds), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

Table 1. Rate limiting steps in tumor cell invasion of basement membranes

Step	Mechanism
1. Penetration of the Endothelial Cell Barrier	Adherence: Ligands Unknown
2. Attachment to Basement Membrane	Laminin Receptor on Cell Binds to YIGSR Site of Laminin
3. Degradation of Basement Membrane	Plasminogen Activator Activates Plasmin, Plasmin Activates Collagenase IV, Collagenase IV Degrades Basement Membrane
3. Motility	Chemoattractants and Motility Factors Activate Cell Movement
5. Proliferation	Tissue Derived and Autocrine Factors Sustain Growth

ciated with the behavior of a variety of metastatic cells. Invasion through basement membrane initiates with tumor cell attachment to its surface [15] thereby inducing the production of a cascade of degradative enzymes [16, 17]. The migration of the tumor cells may also involve matrix molecules and/or their fragments acting as chemotactic or haptotactic factors [18]. These, plus chemoattractants produced by normal cells [19] as well as tumor cells [20], increase the mobility of metastatic cells and their invasion into normal tissue. It is the purpose of this article to review studies on the interaction of tumor cells with basement membranes and the rate limiting steps in the dissemination of metastases. Much of this information appears to be applicable to malignant breast cancer cells.

3. Components of basement membranes: collagen IV, laminin and heparan sulfate proteoglycan

Collagen IV is the principal structural element of basement membranes. It forms a continuous, cross-linked network around which the other components are organized [13]. The collagen IV molecule has a triple-helical structure but the helix has frequent interruptions which increase its flexibility and also serve as sites where other components bind [14]. Collagen IV and the network it forms are not susceptible to the collagenases that degrade fibrous collagens. Instead, a specific collagenase IV is associated with the digestion of this collagen [21]. Due to the nonhelical sequences in the protein, however, it is possible that other enzymes could play a role in its removal. As discussed below, the degradation of collagen IV by metastatic cells depends on production of collagenase IV and invasion can be prevented by inhibitors of this enzyme.

Laminin, a very large glycoprotein (Mr = 800,000) is one of the most

abundant components of all basement membranes [22]. A current model of laminin based on electron microscopy and chemical studies arranges its three chains in a cruciform shape, (Figure 1) [23]. Disulfide bonds, as well as the interaction of α-helices present in those portions of the chains forming the long arm, are believed to hold the chains together. Laminin is found only in basement membranes, probably due to its binding to collagen IV and to heparan sulfate proteoglycan whose occurrence are also limited to these structures. Laminin appears to mediate many of the interactions of cells with basement membranes by promoting their attachment, proliferation and differentiation [24]. One of the major cell attachment sites in laminin has been recently identified [25]. The minimal sequence with attachment activity —tyrosine—isoleucine—glycine—serine—arginine (YIGSR), is present in the B1 chain of laminin in proximity to the intersection of the chains in the cross. Synthetic peptides with the YIGSR sequence, when coated onto plastic petri dishes, will support the attachment of MCF-7 breast carcinoma cells, CHO cells, PAM 212-epidermal cells, B16 melanoma cells and HT1080 fibrosarcoma cells [25, Graf, in preparation]. If laminin is used as a substrate, the YIGSR peptide is able to inhibit cells from attaching to it, presumably by competing for cell surface receptors.

One of the principal laminin receptors (Mr = 67,000) is a membrane-associated protein with a high affinity for laminin [26, 27]. This receptor is most commonly isolated by chromatography of a detergent extract of cell membranes on a laminin-Sepharose column. Peptides from the B1 chain containing the YIGSR sequence are able to elute the laminin receptor from a laminin affinity column [25] suggesting that this sequence is a major determinant

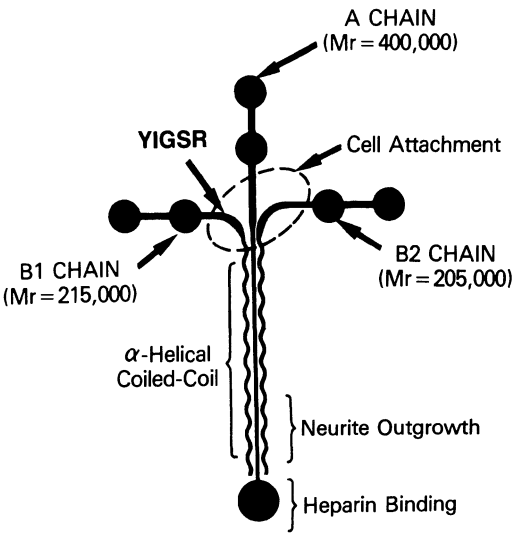


Figure 1. Schematic diagram of the laminin molecule showing the structural and functional domains.

of receptor binding. There are undoubtedly other receptor binding sites in laminin however, such as a region at the end of the long arm which causes neural cells to form axonal-like processes [28]. Presumably the neural cells have a specific receptor which recognizes this region of laminin.

A laminin receptor from human breast cancer cells has been isolated [26], cloned [29] and partially characterized. This receptor is widely distributed among cells and is involved in both the chemotactic and attachment responses of cells to laminin [25].

Several lines of evidence suggest that laminin and the laminin receptor are involved in the formation of metastases. For example, tumor cells of high metastatic potential bind better to laminin than to fibronectin [15]. Highly metastatic cells show increased laminin binding and have greater number of laminin receptors than their benign counterparts [29, 30]. Antibodies to laminin [15, 31], its cell attachment peptide (YIGSR) [32] and protease-derived laminin fragments [33], are capable of binding to the laminin receptor, and are antimetastatic in an experimental, intravenous model of metastasis. Metastatic tumor cells exposed to laminin in culture produce more lesions *in vivo* [31]. Current concepts suggest that the high affinity that the metastatic cells have for laminin favors their binding to basement membranes and that the interaction between laminin and the laminin receptor triggers the production of enzymes that degrade the basement membrane [34].

The heparan sulfate proteoglycan in basement membrane is a large protein with 3 or 4 heparan sulfate chains clustered at one end [14, 35]. Presumably the large protein tail is needed to fix the proteoglycan firmly to the basement membrane, while the heparan sulfate chains are arrayed along the surface of the basement membrane producing an anionic shield. These anionic groups are believed to resist the passage of negatively charged macromolecules and probably also impede the passage of cells [35]. Specific heparan sulfate degrading enzymes have been found to be produced by tumor cells [36, 37] and inhibitors of these enzymes inhibit tumor cell invasion [38].

4. *In vitro* systems for assaying tumor cell invasiveness

A variety of *in vitro* systems have been devised to assess the invasiveness of tumor cells. Typically, a devitalized tissue such as the lens [39], bladder [8, 40] or amnion [41] is placed on flat filter separating two chambers. Cells placed above the tissue, if invasive, are able to cross through it over the course of 2 or 3 days, while normal cells and benign tumor cells can not.

More recently components of basement membrane, such as collagen IV or laminin or mixtures of these components have been used to form barriers on top of a porous filter separating the two chambers [42, 43] of a Boyden chamber. The advantages of these barriers are that they are chemically defined, require only a few hours for invasive cells to cross and the cells crossing the barrier can be reisolated for further study.

5. Role of plasminogen activator and collagenase IV in invasion

Tumor cells are known to secrete large quantities of proteolytic enzymes. In breast cancer the production of these enzymes appears to be hormonally regulated. Estrogens have been reported to increase plasminogen activator activity in DMBA-induced murine mammary tumors both in vivo and in cultured cells [44] and in human carcinoma cell lines including MCF-7, ZR75-1 and UCT-Br 1 cells [45–49]. Tamoxifen does not induce plasminogen activator but is effective in inhibiting the estrogen-induced enzyme in MCF-7 cells [48]. A high level of plasminogen activator is associated with malignant breast lesions [50]. Although plasminogen activator is also produced by non-malignant cell lines, it is higher in the invasive lines.

Using the amnion as a barrier, Mignatti *et al.* [51], showed that metastatic melanoma cells secrete collagenase I and that inhibitors of this enzyme prevented the passage of tumor cells across this barrier. This is not unexpected because the amnion is composed of stromal tissue as well as a basement membrane. While collagenase I does not degrade basement membrane collagen, it is possible that collagenase IV could show a similar sensitivity to the inhibitors used in this study. Additionally, it was found that collagenase I was secreted in an inactive form and that its activation involved plasminogen activator and plasmin. The authors [51] proposed that a 'proteinase cascade' was involved in the invasion of the amnion.

To assess the importance of specific enzymes in the invasion process, we have added proteases inhibitors to the *in vitro* assay measuring the ability of tumor cells to cross basement membrane barriers and found evidence for a coordinated cascade of proteases (Figure 2). Inhibitors of serine proteases and of collagenase IV, but not elastase inhibitors, blocked the penetration of

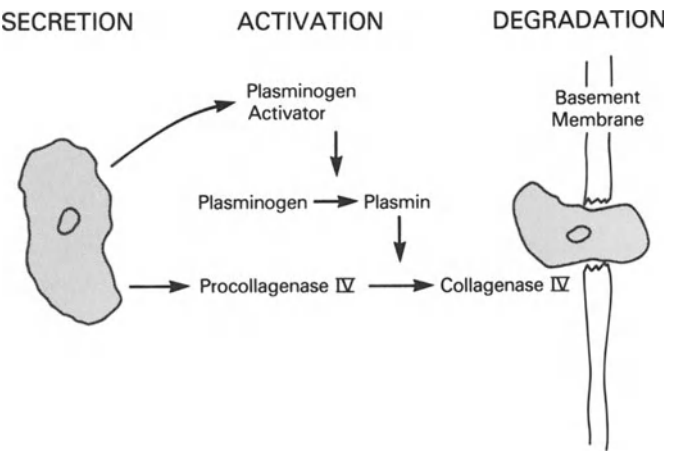


Figure 2. Schematic representation of the proteinase cascade required to produce active type IV collagenase for basement membrane degradation.

the cells (Reich, in preparation). Collagenase IV was found to be secreted as an inactive precursor which was activated via a plasminogen activator-plasmin cascade as suggested previously [51]. Since none of these inhibitors altered the movement of the tumor cells in the absence of the basement membrane barrier (Reich, in preparation), these results indicate that collagen IV is the critical component of the barrier restricting the migration of the cells. A comparison of various invasive and non-invasive cells showed that all lines produced plasminogen activator but only the invasive cells produced collagenase IV. Disruption of the collagen IV network probably represents the key step in the tumor cell degradation of basement membrane. Specific enzymes appear to be involved in the breakdown of heparan sulfate proteoglycan as well as other components of the basement membrane and to be required for invasion of basement membrane [38].

6. Chemoattractants

Tumor cells respond to a variety of factors by directed movement, i.e. chemotaxis. Chemotactic factors for tumor cells include inflammatory mediators [52], certain growth factors [53], matrix molecules such as fibronectin and laminin [18], factors secreted by the tumor cells themselves [20], and proteins from normal cells [19] or from the tissues to which they metastasize (Table 2) [54]. The chemotactic response of the cells to these substances is thought to be a receptor-mediated event. The reaction between receptor and attractant creates a redistribution of receptors on the surface of the cell which allows the gradient to be detected and chemical reactions triggered by the occupied receptor initiate the movement of the cells. The effect of various chemoattractants on the invasive activity of tumor cells is readily demonstrated *in vitro*. The presence of chemoattractants increases the penetration of tumor cells across basement membranes some 5–10 fold [19] providing that the cells have the capacity to degrade this barrier [42]. It is interesting that mitogens can induce a strong chemotactic response in certain cells. For example, PDGF stimulates both the proliferation and the chemotaxis of mesenchymal cells, including many transformed fibroblasts [55]. Reaction of these mitoattractants with their receptors has been shown to modulate or activate the phosphotidylinositol pathway and suggests that this pathway is involved in activating chemotactic as well as mitotic responses.

Tissue-specific attractants have been identified in extracts of brain, liver

Table 2. Chemoattractants in tumor cell invasion

Mitoattractants	PDGF, TGFα, bombesin components of conditioned medium
Matrix molecules	Laminin, fibronectin
Tissue specific chemoattractants	Proteins not identified

and lung which preferentially attract the tumor cells that metastasize to those particular sites [54]. This suggests that the unique pattern of dissemination of tumor cells may be due in part to responses of the cells to local chemoattractants [56]. Due to the semipermeable property of basement membrane, attractants could be trapped in this structure and serve as a unique recognition signal which elicits invasion following binding of the cells to its surface. Specificity has also been noted in the avidity with which metastatic cells bind to endothelial cells suggesting that this also is a regulating factor. Some data suggests that the tumor cells require a substantial amount of time (1 to 2 days) to penetrate through the endothelial cell layer [57].

7. Behavior of breast cancer cells in the invasion assay

Some of the assays described above have been carried out with breast cancer derived cell lines. Estrogen dependent breast cancer cell lines, such as the MCF-7 cells, show a low rate of growth and little or no ability to invade basement membrane barriers when deprived of estrogen for prolonged periods (Table 3) [12]. Estrogen stimulates both the growth of these cells and their ability to cross a basement membrane barrier. The implication is that the occupied estrogen receptor modulates those responses necessary for invasion.

Breast cancer cells which have progressed to an estrogen independent state, such as MDA-MB231, HS578T, HBL100, BT483 and BT549 cells or MCF-7 cells transfected with the ras oncogene, show a much higher degree of invasiveness and even higher levels of laminin receptors and chemotactic responsiveness [12]. These parameters correlate well with the malignant potential of the cells, and suggest that the quantitation of these responses could be useful in the clinical staging of these lesions.

The effect of estrogen in these systems is not yet well defined at the molecular level. Estrogen causes a moderate increase in the invasiveness of estrogen dependent MCF-7 cell [12]. This effect is labile in that the onset is rapid, (less

Table 3. Variations in the invasive activity of breast cancer cells with Estrogen, Estrogen antagonists and *ras* transfection.

Cell	Growth	Invasion
MCF-7 Cells – Estrogen Deprived*	+	+
" + Estrogen (10^{-9}M)*	+++	+++
" + Tamoxifen (10^{-6}M)*	+	++
" + LY 117018 (10^{-7}M)*	+	+
" *Ras* transfected	++	++++
MDA-MB-231 Cells	++++	+++++

* Cells were maintained in charcoal stripped serum containing medium (steroid free) and treated for 5 days as indicated.

than 6 hrs.) and the effect is readily lost when estrogen is removed from the cells (Thompson, unpublished). In addition, invasiveness is greatest in cells grown to high density as is the chemotactic response of cells. Such observations suggest that the invasive activity of estrogen dependent breast cancer cells is variable and expressed most strongly when growth is minimal (E. Thompson, unpublished). Once progression has occurred the cells become very invasive and this activity is expressed constitutively.

Anti-estrogens with partial agonist activity, such as tamoxifen and hydroxytamoxifen, can also elicit an invasive state in estrogen-dependent breast cancer cells. LY 117018, LY 156758 and ICI 164384, anti-estrogens with little agonist activity [58, 59] reduce both the growth of cells and their invasiveness. The data suggest that antiestrogens with minimal agonist activity may be more effective in suppressing the growth and spread of breast cancer than compounds with agonistic activity. The results further indicate that the effects of estrogens and anti-estrogens on growth are separable from their effects on invasiveness.

8. Summary

Basement membranes serve as significant barriers to the passage of tumor cells but ones which metastatic cells can pass. This involves the production of a cascade of proteases leading to the activation of a specific collagenase that degrades the unique collagen network in basement membrane. Breast cancer cells, when estrogen dependent, show a requirement for estrogen for invasive activity. However, when these cells progress to an estrogen independent state and increased malignancy, they express an invasive phenotype constitutively. Studies with various anti-estrogens suggest that these responses are mediated via the estrogen receptor. Anti-estrogens lacking agonist activity suppress invasiveness as well as growth of the breast cancer cells.

Acknowledgments

E.W. Thompson, and portions of this work, were supported by the Breast Cancer Study Group, Medical Breast Cancer Section, Medicine Branch, NCI, NIH.

References

1. Maass H, Jonat W, Stolzenbach G, Trams G: The problem of nonresponding estrogen receptor-positive patients with advanced breast cancer. Cancer 46:2835–2837, 1980.
2. Rubens RD, Hayward JL: Estrogen receptors and response to endocrine therapy and cytotoxic chemotherapy in advanced breast cancer. Cancer 46:2922–2924, 1980.

3. DeVita VT Jr, Hellman S, Rosenberg SA: Cancer principles and Practice of Oncology. JB. Lippencott, Philadelphia PA. 1985.
4. Fidler IJ: Tumor heterogeneity and the biology of cancer invasion and metastasis. Cancer Res 38:2651–2660, 1978.
5. Liotta LA: Tumor invasion and metastases: Role of the basement membrane. Am J Path 117:339–348, 1984.
6. Fidler IJ: Selection of successive tumor lines for metastasis. Nature (New Biol) 242:148–149, 1973.
7. Fidler IJ, Nicolson GL: Organ selectivity for implantation, survival and growth of B16 melanoma variant tumor lines. J Natl Cancer Inst 57:1199–1202, 1976.
8. Poste G, Doll J, Hart IR, Fidler IJ: *In vitro* selection of murine B16 melanoma variants with enhanced tissue-invasive properties. Cancer Res 40:1636–1644, 1980.
9. Engel LW, Young NA: Human breast carcinoma cells in continuous culture: a review. Cancer Res 38:4327–4339, 1978.
10. Dickson RB, Lippman ME: Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocr Rev 8:29–43, 1987.
11. Terranova VP, Hujanen ES, Martin GR: Basement membrane and the invasive activity of metastatic tumor cells. J Natl Cancer Inst 77:311–316, 1986.
12. Albini A, Graf J, Kitten GT, Kleinman HK, Martin GR, Veillette A, Lippman ME: 17 beta-estradiol regulates and v-Ha-*ras* transfection constitutively enhances MCF7 breast cancer cell interactions with basement membrane. Proc Natl Acad Sci USA 83:8182–8186, 1986.
13. Leblond CP, Laurie GW: Morphological features of connective tissue In: Connective Tissue: Biological and Clinical Aspects. Rheumatology 10 Kuhn K, Krieg T (eds), Kager, Basel, 1986 p 1–28.
14. Timpl R, Dziadek M: Structure, development and molecular pathology of basement membrane. Int Rev Exp Pathol 29:1–112, 1986.
15. Terranova VP, Liotta LA, Russo RG, Martin GR: Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res 42:2265–2269, 1982.
16. Liotta LA, Thorgeirsson UP, Garbisa S: Role of collagenases in tumor cell invasion. Cancer Met Rev 1:277–288, 1982.
17. Nakajima M, Welch DR, Irimura T, Nicolson GL: Basement membrane degradative enzymes as possible markers of tumor metastasis. In: Cancer Metastasis: Experimental and Clinical Strategies. Welch DR, Bhuyan BK, Liotta LA (eds), Alan R. Liss, Inc. NY, 1986 p 113–122.
18. McCarthy JB, Basara ML, Palm DF Sas, Furcht LT: The role of cell adhesion proteins—laminin and fibronectin—in the movement of malignant and metastatic cells. Cancer Metastasis Rev 4:125–152.
19. Mensing H, Albini A, Krieg T, Pontz B, Muller PK: Enhanced chemotaxis of tumor-derived and virus-transformed cells to fibronectin and fibroblast-conditioned medium. Int J Cancer 33:43–48, 1984.
20. Liotta LA, Mandler R, Murano G, Katz DA, Gordon RK, Chiang PK, Schiffmann E: Tumor cell autocrine motility factor. Proc Natl Acad Sci USA 83:3302–3306, 1986.
21. Liotta LA, Abe S, Gehron-Robey P, Martin GR: Preferential digestion of basement membrane collagen by an enyzme derived from a metastatic murine tumor. Proc Natl Acad Sci USA 76:2268–2272, 1979.
22. Timpl R, Rohde H, Gehron-Robey P, Rennard SI, Foidart JM, Martin GR: Laminin-a glycoprotein from basement membranes. J Biol Chem 254:9933–9937, 1979.
23. Martin GR, Timpl R: Laminin and other basement membrane proteins. Ann Rev Cell Biol. In press.
24. Kleinman HK, Cannon FB, Laurie GW, Hassell JR, Aumailley M, Terranova VP, Martin GR, Dubois-Dalcq M: Biological activities of laminin. J Cell Biochem 27:317–325, 1985.
25. Graf J, Iwamoto Y, Sasaki M, Martin GR, Kleinman HK, Robey FA, Yamada Y: Identification of an amino and sequence in laminin mediating cell attachment, chemotaxis and receptor binding. Cell 48:989–996, 1987.
26. Liotta LA, Wewer UM, Rao CN, Bryant G: Laminin receptors in The Cell in Contact,

Edelman GM, Thiery JP (ed), John Wiley, NY 1985, p 334–344.
27. von der Mark K, Kuhl U: Laminin and its receptor. Biochim Biophys Acta 823:147–160, 1985.
28. Edgar D, Timpl R, Thoenen H: The heparan-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J 3:1463–1468, 1984.
29. Wewer UM, Liotta LA, Jaye M, Ricca GA, Drohan WN, Claysmith AP, Rao CN, Wirth P, Coligan JE, Albrechtsen R, Mudryj M, Sobel ME: Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. Proc Natl Acad Sci USA 83:7137–7141, 1986.
30. Terranova VP, Rao CN, Kalebic T, Margulies IM, Liotta LA: Laminin receptor on human breast carcinoma cells. Proc Natl Acad Sci USA 80:444–448, 1983.
31. Terranova VP, Williams JE, Liotta LA, Martin GR: Modulation of the metastatic activity of melanoma cells by laminin and fibronectin. Science 226:982–985, 1984.
32. Iwamoto Y, Robey FA, Graf J, Sasaki M, Kleinman HK, Yamada Y, Martin GR: YIGSR, a synthetic laminin pentapeptide inhibits experimental metastasis formation. Science 238: 1132–1134, 1987.
33. Barsky SH, Rao CN, Williams JE, Liotta LA: Laminin molecular domains which alter metastasis in a murine model. J Clin Invest 74:843–848, 1984.
34. Turpeenniemi-Hujanen T, Thorgeirsson UP, Rao CN, Liotta LA: Laminin increases the release of type IV collagenase from malignant cells. J Biol Chem 261:1883–1889, 1986.
35. Hassell JR, Kimura JH, Hascall VC: Proteoglycan core protein families. Ann Rev Biochem 55:539–567, 1986.
36. Nakajima M, Irimura T, DiFerrante N, Nicolson GL: Metastatic melanoma cell heparanase. Characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. J Biol Chem 25:2283–2290, 1984.
37. Vlodovsky I, Fuks Z, Bar-Ner M, Ariav Y, Schirmacher V: Lymphoma cell-mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: relationship to tumor cell metastasis. Cancer Res 43:2704–2711, 1983.
38. Irimura T, Nakajima M, Nicolson GL: Chemically modified heparins as inhibitors of heparan sulfate specific endo-β-glucuronidase (heparanase) of metastatic melanoma cells. Biochemistry 25:5322–5328, 1986.
39. Starkey JR, Hosick HL, Stanford DR: Interaction of metastatic tumor cells with bovine lens capsule basement membrane. Cancer Res 44:1585–1594, 1984.
40. Hart IR: The selection and characterization of an invasive varient of B16 melanoma. Am J Pathol 97:587–592, 1979.
41. Liotta LA, Lee CW, Morakis DJ: New method for preparing large surfaces of intact human basement membrane for tumor invasion studies. Cancer Lett 11:141–152, 1980.
42. Terranova VP, Hujanen ES, Loeb DM, Martin GR, Thornberg L, Glushko V: Use of a reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells. Proc Natl Acad Sci USA 83:465–469, 1986.
43. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson S, Kozlowski J, McEwan R: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res 47: 3239–3245, 1987.
44. Yamashita J, Horiuchi S, Shigaki N, Akagi M: Estrogen-dependent plasminogen activator in 7.12-dimethylbenz[a]anthracene-induced rat mammary tumors *in vivo* and *in vitro*. Gann 75:681–689, 1984.
45. Butler WB, Kirkland WL, Jorgensen TL: Induction of plasminogen activator by estrogen in a human breast cancer. Biochem Biophys Res Commun 90:1328–1334, 1979.
46. Butler WB, Kirkland WL, Gargala TL, Goran N, Kelsey WH, Berlinski PJ: Steroid stimulation of plasminogen activator production in a human breast cancer cell line (MCF-7). Cancer Res 43:1637–1641, 1983.
47. Wilson EL, Dutlow C, Dowdle EB: In: Growth of Cells in Hormonally Defined Media. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 849–854, 1982.
48. Katzenellenbogen BS, Norman MJ, Eckert RL, Peltz SW, Mangel WF: Bioactivities, estro-

gen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxy-tamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res 44:112–119, 1984.
49. Huff KK, Lippman ME: Hormonal control of plasminogen activator secretion in ZR-75-1 human breast cancer cells in culture. Endocrinology 114:1702–1710, 1984.
50. Clavel C, Chavanel G, Birembaut P: Detection of the plasmin system in human mammary pathology using immunofluorescence. Cancer Res 46:5743–5747, 1986.
51. Mignatti P, Robbins E, Rifkin DB: Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 47:487–498, 1986.
52. Romualdez AG Jr, Ward PA: A unique complement derived chemotactic factor for tumor cells. Proc Natl Acad Sci USA: 72:4128–4132, 1975.
53. Grotendorst GR: Alteration of the chemotactic response of NIH/3T3 cells to PDGF by growth factors, transformation and tumor promoters. Cell 36:279–285, 1984.
54. Hujanen ES, Terranova VP: Migration of tumor cells to organ-derived chemoattractants. Cancer Res 45:3517–3521, 1985.
55. Grotendorst GR, Martin GR: Cell movements in wound healing and fibrosis. In: Connective Tissue:Biological and Clinical Aspects. Rheumatology, Vol. 10, Kuhn K, Krieg T (eds), Karger Basel, p. 385–403, 1986.
56. Nicholson GL, Dulski K, Basson C, Welch DR: Preferential organ attachment and invasion *in vitro* by B16 melanoma cells selected for differing metastatic colonization and invasive properties. Invasion Metastasis 5:144–158, 1985.
57. Crissman JD, Hatfield M, Schaldenbrand M, Sloane BF, Honn KV: Arrest and extravasion of B16 amelanotic melanoma in murine lungs. Lab Invest 53:570–478.
58. Black LJ, Goode RL: Evidence for biological action of the antiestrogens LY117018 and tamoxifen by different mechanisms. Endocrinology 109:987–989, 1981.
59. Scholl SM, Huff KK, Lipmann ME: Antiestrogenic effects of LY117018 in MCF-7 cells. Endocrinology 113:611–617, 1983.

12. Regulation of development of the normal mammary gland by hormones and growth factors

Barbara K. Vonderhaar

1. Introduction

During the life cycle of a female mammal, the mammary glands (or breasts) undergo a dramatic series of cyclical changes. The glands must first undergo morphogenesis to prepare for pregnancy-induced lactogenesis, followed by lactation and involution. This cycle continues and repeats with each subsequent pregnancy. Brief periods of cyclic growth and regression of the epithelial component of the gland occur during the estrous or menstrual cycle in the adult female's nonpregnant intervals.

The mouse mammary gland is an excellent system for studying hormonal and growth factor control as the mammary glands go through these natural development cycles of growth and morphogenesis, development and functional differentiation, and involution *in vivo*. In addition, these events can be mimicked *in vitro* under controlled growth factor and hormonal conditions by using the whole-gland organ culture [1–4].

2. Morphogenesis *in vivo*

2.1 Fetal mammary development

In the embryonic state, the mouse mammary epithelial bud develops as a distinct entity within the primitive mammary fat pad. The regulatory mechanisms involved in the process of fetal mammary development generally involve epithelial-mesenchymal (stromal) interactions, with little defined hormonal involvement. At about day 11 of gestation in the mouse, the mammary crest begins to appear on either side of the trunk in the form of a zone of raised epiderm [5]. In the mammary rudiment, mitosis occurs at a rate lower than that found in the surrounding epidermis. Thus, early fetal mammary development is achieved by morphogenic movement of epidermal cells and displacement of neighboring ectodermal cells [6]. The mammary bud then goes into a brief resting phase lasting 1 to 3 days, after which epithelial proliferation increases. This burst of growth results in formation of the primitive

M.E. Lippman and R.B. Dickson (eds), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

ductal gland by day 19 of gestation. No further development of the mammary bud occurs until the postnatal period [7].

The hormonal controls involved in the process of nipple and primitive ductal formation are limited when compared with the adult animal. Sexual dimorphism is apparent in early fetal life. Between days 13 and 15 of gestation in the mouse, growth of the epithelium is inhibited, and the nipple attachment is severed in the male fetus [8–10]. This step coincides with the emergence of androgens from the developing fetal testes [11, 12]. The destruction of the mammary rudiment in the male mouse is a precisely timed biological event involving epithelial-mesenchymal interactions modified by hormones and growth factors [13]. These interactions can be mimicked in explant culture *in vitro* [14–17].

Development of the mammary bud in the female mouse fetus is independent of ovarian function [18], although estrogen receptors are present in the mesenchyme but not the epithelium of 16-day female embryonic mammary tissue [19]. Exogenous estrogens can accelerate differentiation of the nipple and induce extensive proliferation of the surrounding mesenchyme at this time [20]. Pituitary hormones are not involved in normal morphogenesis of fetal mammary glands in either sex [21].

2.2 Postnatal mammary development

Although most organs of the body have completed morphogenesis at birth and subsequently develop by enlargement or replication of preexisting structures, the mammary gland undergoes most of its morphogenesis in the subadult and adult animal under precise hormonal and growth factor control of both the epithelial and mesenchymal elements.

Little change is observed in the gross morphology of the female mammary gland during the first 3 weeks of life. The gland at this time consists of the primary duct and a few primitive branched ducts emanating from the nipple and embedded in the fat pad (Figure 1a). At the beginning of postnatal week 4, the ovaries begin to function in the female mouse and affect the development of the mammary gland. Small dense end buds (Figure 1b) appear at the ends of the terminal ducts. These structures, which are believed to be the growing points of the glands [22–24], consist of layers of actively dividing epithelial and myoepithelial cells [25]. The growth and position of the end buds at the ends of the terminal ducts and at points of ductal branching regulate the ultimate spacing of the mammary tree [23]. The growth of the epithelial component occurs in an ordered fashion and is restricted by the dimensions of the fat pad. There is an inhibitory zone of unoccupied fat between each duct into which other ducts do not normally penetrate [24] so that, upon reaching sexual maturity (postnatal week 8–10), the female mammary gland's epithelial component has filled the entire fat pad with a highly branched network of ducts with many growing ends.

Several hormones and growth factors appear to be involved in the morpho-

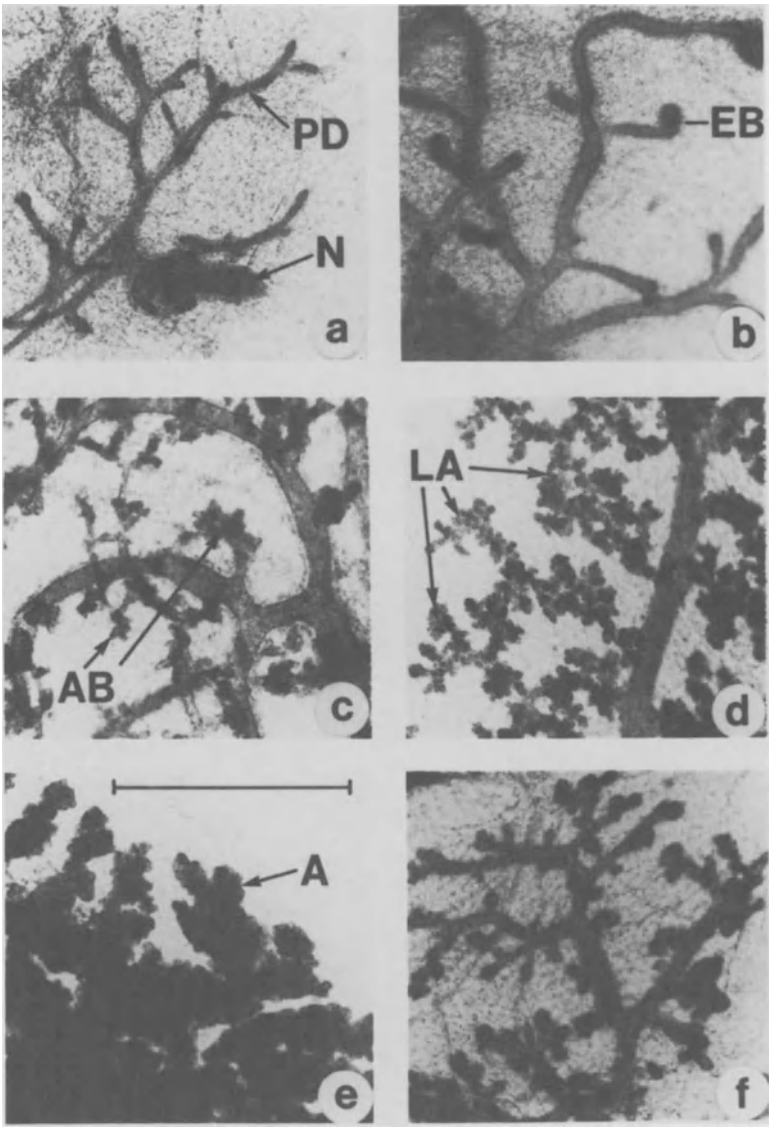


Figure 1. Whole-mount preparations from number 4 abdominal mammary glands from 15-day-old (a), 4-week-old (b), 3-month-old sexually mature (c), 8-day pregnant (d), 20-day pregnant (e), and 8-month postlactational (f) female C3H/HeN mice. Glands were stained with hematoxylin. N, nipple; PD, primitive duct; EB, end bud; AB, alveolar bud; LA, lobulo-alveolar structures; A, mature alveoli within lobules. Bar = 1 mm.

logical development of the mammary gland of the subadult animal. Ovariectomy causes regression of the end buds and cessation of growth within a few days [25]. This effect is easily reversed with exogenous estrogen. However, in triply operated animals (i.e., ovariectomy, adrenalectomy, and hypophysec-

tomy), estrogen treatment is without effect. Maximal ductal stimulation is achieved with a combination of estrogen and growth hormone. This effect, however, is not fully equivalent to that seen in the endocrine intact animal [25], thus pointing out the complex interplay of several hormones, and possibly undefined growth factors, in ductal branching morphogenesis. Richards *et al.* [26] have demonstrated a dependence on epidermal growth factor (EGF) for *in vitro* growth of isolated end buds from subadult mouse mammary glands.

2.3 The adult mammary gland

2.3.1 The estrous cycle After full sexual maturity is reached (age 3 to 4 months), the epithelial component of the mammary gland undergoes cyclic variations during the estrous cycle. During the routine 4- to 5-day cycle of the mouse, the concentrations of estrogen and progesterone, as well as follicle-stimulating hormone, luteinizing hormone, and prolactin, vary in a cyclical manner [27]. The mammary glands, in response to these various hormonal stimuli, go through cycles of growth and regression. During proestrus, the estrogenic phase of the cycle [28], epithelial DNA synthesis is maximal. Mitosis occurs during late estrus and metestrus [29, 30] and results in small increments of growth with each subsequent cycle. The cumulative effect is extensive ductal side branching and, in some strains [22, 25, 31], the appearance of isolated alveolar structures (Figure 1c). The difference in the ability of the alveolar buds to develop seems to be related to the presence of endogenous mammary tumor virus [32]. The estrogen responsiveness of the epithelium appears to result from epithelial-stromal cell interactions [33].

2.3.2 Pregnancy and lactation When the sexually mature female becomes pregnant, the mammary glands begin a pronounced cycle of lobulo-alveolar development that ultimately results in full functional differentiation and the production of milk. Proliferation of the epithelial component is a controlled event that occurs at the expense of the surrounding fat cells [34, 35]. Within 3 days after coitus, in response to the pregnancy-induced sustained elevated levels of estrogen and progesterone [27, 36], increased ductal branching and extensive alveolar formation begin. Alveolar buds begin to form along the lateral walls of the mammary ducts and their numerous side branches. By day 6–8 of gestation, the alveoli have begun to cluster to form true lobulo-alveolar structures surrounding small lumina (Figure 1d). Toward the end of the second week of pregnancy, lobulo-alveolar development slows as these structures begin to dominate the glands. The lobules are well formed and composed of many alveoli.

Cellular synthesis of the secretory products is initiated after the middle of pregnancy as the levels of prolactin begin to rise [37, 38] and in response to adrenal steroids [39]. This synthesis results in a marked increase in the size of the individual epithelial cells as the secretion products accumulate [40]. Thus, alveoli expand and unfold, resulting in a considerable increase in the thick-

ness of the glands of the 20-day pregnant mouse (Figure 1e). This predominance of the alveoli continues throughout pregnancy and active lactation.

Maintenance of the alveoli in pregnant mice requires intact ovaries and pituitary gland [25, 41, 42]. In ovariectomized hypophysectomized animals, estrogen, progesterone, and growth hormone can maintain alveoli. Thyroid hormones, in concert with prolactin, stimulate the formation of alveoli in the mouse [43–46]. Maintenance of alveoli in the mouse is not affected by adrenalectomy [25, 41], although adrenal steroids are important in lobule formation [47]. In triply operated mice, development of lobulo-alveolar structures requires prolonged treatment with estrogen, progesterone, deoxycorticosterone acetate, and prolactin and/or growth hormone [25, 48].

At parturition, the pregnancy-induced elevated levels of estrogen and progesterone [27, 49] abruptly drop, and active lactation begins [50]. Although the secretory cell population has increased dramatically during gestation (an estimated 78% of mammary cell growth takes place during this period [51]), there is a transient surge of cell proliferation between 3 and 5 days postpartum [52–54]. At this time, the secretory process reaches a very high level, engorging the entire gland with milk. By 10 to 12 days postpartum, lactation is maximal. This appearance of the glands remains virtually unaltered throughout the remainder of the 21- to 22-day lactational period in the mouse.

2.3.3 Involution When the nursing young are weaned from the dam, the involution phase of the developmental cycle begins. Within a few days, breakdown of the basement membrane around the alveoli occurs [55], and degeneration of the alveolar structures follows. Within 5 days, milk accumulation has completely ceased, and extensive cellular debris is present in the lumen. By day 10 of involution, the lobules and alveoli have significantly decreased in number, and fat cells again appear in large number. The glands eventually regress until only a highly branched ductal system with a few alveoli remains (Figure 1f). The glands are similar in appearance to those of the sexually mature virgin female animal. The involuted glands continue to undergo periods of limited growth and regression as the postlactational animal goes through estrous cycles until another pregnancy intervenes, and the developmental cycle of lobulo-alveolar development, lactation, and involution occurs again.

3. Mammary development in vitro

3.1 Hormonal requirements

The developmental cycle of growth and mammogenesis, lobulo-alveolar development, and alveolar loss during glandular involution can be mimicked in vitro by using the serum-free whole-organ culture method [1, 2]. Full lobulo-alveolar development is achieved in culture by using chemically defined hormonally supplemented medium and the entire second thoracic gland

from 3- to 4-week old female Balb/c mice primed with 17β estradiol and progesterone (E+P) for 9 days (Figure 2) [56]. The length of time required for priming to allow for a full response *in vitro* varies among strains of mice [57] and is dependent on the hormones and growth factors in the medium [1, 4, 58]. Initial results, using glands from mice primed for 9 days, demonstrated an absolute requirement for a combination of insulin, prolactin, aldosterone, and hydrocortisone (IPr1AH) in the medium for a minimum of 5 to 6 days to achieve a single round of development [1, 58]. Both estrogen and progesterone are required during the priming process (Table 1).

Addition of estrogen and progesterone to the medium is not required, although these ovarian hormones are required for maintenance of alveolar structures in the sexually mature female mouse. Addition of E+P to the medium along with the other four hormones is not sufficient to allow unprimed glands to develop lobulo-alveolar structures in culture (Table 1).

Once development has been achieved *in vitro*, subsequent cultivation of the glands in a medium containing insulin, prolactin, and hydrocortisone only results in the induction of milk-protein synthesis [59]. Viability of the ducts requires that only insulin be present in the culture medium [60], so that withdrawing all hormones except insulin for an additional 9 days results in full alveolar regression similar to involution *in vivo* [61].

3.2 Epidermal growth factor

After complete regression *in vitro*, a second round of development can only be initated if epidermal growth factor (EGF) is added to the culture medium

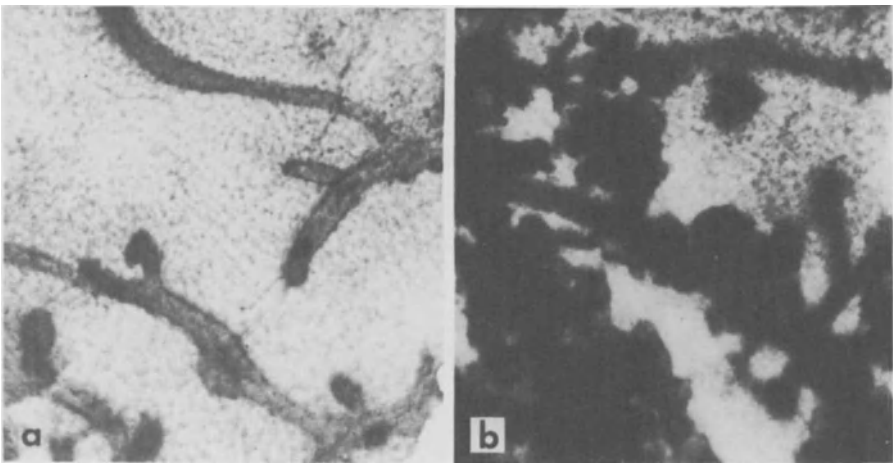


Figure 2. Whole-mount preparations of second thoracic glands from 5-week-old female Balb/c mice primed with estradiol and progesterone for 9 days. Primed glands were either uncultured (a) or cultured for 9 additional days in the presence of IPrlAH (b). Glands were stained with hematoxylin [56].

Table 1. Hormones required during priming

Four-week-ago Balb/c mice were either unprimed or primed with cholesterol-based slow release pellets containing estradiol-17β (E), progesterone (P), or testosterone (T) alone or in various combinations [4]. The second thoracic glands were then cultured in serum-free medium containing IPrlAH and, where indicated, estradiol-17β (10^{-9}M) and progesterone (10^{-7}M). After 9 days of culture, glands were removed, fixed, stained with hematoxylin, and scored for lobulo-alveolar development. Glands with more than 20% lobulo-alveolar development were scored as positive [4]. Results are given as number of developed glands per total number of glands cultured.

Priming conditions		Hormones in culture	
Hormone	Days	IPrlAH	IPrlAH + E+P
none	—	0/15	0/9
E+P	3	0/9	—
E+P	6	0/9	—
E+P	9	15/15	—
E	9	0/9	—
P	9	0/9	—
T	9	0/6	—
T+E	9	0/6	—
T+P	9	0/6	—
T+E+P	9	6/6	—

along with IPrlAH [3, 62]. The second round of development can only be achieved if the mice are primed with E+P for a minimum of 9 days before the onset of culture [3]. The requirement for EGF cannot be met by either platelet-derived growth factor or fibroblast growth factor [3]. The lack of a similar requirement for EGF in the initial round of lobulo-alveolar development is believed to reflect the presence of EGF or EGF-like growth factors carried into the culture by the primed tissue.

When glands are removed from animals that have been primed for only 6 days, full lobulo-alveolar development is achieved in culture only if EGF or a related growth factor is added to the four hormone mammogenic combination (Table 2). Glands from unprimed animals or animals primed for only 3 days are unable to develop even in the presence of EGF [4].

3.2.1 Induction of mammary-derived growth factor The estrogen-progesterone priming does not simply allow for carry-over of the steroids into the culture because unprimed glands cannot respond in culture in the presence of the IPrlAH and E+P (Table 1). The role of priming appears to be twofold. After only 3 days of priming, significant binding of EGF can be seen in the epithelial-rich region of mammary glands of primed animals. No binding is detected in unprimed glands or in the epithelial-free region of the glands from primed animals [4]. It is not clear whether these induced receptors are located on epithelial or stromal cells, both of which are targets for the growth factor [63–65]. Daniel and Silberstein [24] recently reported that, in ovarian intact

Table 2. Induction of lobulo-alveolar development *in vitro* by EGF and EGF-like growth factor in glands primed for 6 days with estradiol-17β and progesterone
See Table 1 for description of glandular development in culture.

Culture conditions	Number of developed glands/ Total number of glands cultured
IPrIAH	0/30
IPrIAH + EGF (high)[a]	22/24
IPrIAH + EGF (low)[a]	2/6
IPrIAH + MDGF (primed)[b]	8/11
IPrIAH + MDGF (unprimed)[c]	0/5
IPrIAH + αTGF (high)[d]	13/13
IPrIAH + αTGF (low)[d]	13/13

[a]High = 60 ng/ml; low = 20 ng/ml.
[b]0.3 ng EGF equivalents per milliliter.
[c]No EGF equivalents per milliliter.
[d]High = 110 ng/ml (recombinant) or 75 ng/ml (chemically synthesized); low = 11 ng/ml (recombinant) or 7.5 ng/ml (chemically synthesized).

subadult mice, EGF receptors are located in stromal cells surrounding the growing end buds.

The estrogen-progesterone priming results in increased submaxillary gland EGF content [4]. However, this increased EGF synthesis alone is not sufficient to prepare the glands to respond in culture to IPrlAH with lobulo-alveolar development. As shown in Table 1, 9 days of priming with testosterone, which elevates the submaxillary EGF content of a female mouse to that of a male mouse [66], does not allow the glands to respond to the four hormones in culture. Addition of estrogen or progesterone along with the testosterone is also ineffective. EGF itself, released over a 10-day period via an Alzet pump, also is ineffective (not shown).

Acid-alcohol extracts of glands of mice primed by estrogen and progesterone for 9 days do not contain immunologically detectable EGF [4]. However, these same extracts contain an activity that competes for EGF binding to its receptors. Table 2 shows that, when added with the four hormone mammogenic combination to cultures of glands from animals primed with estrogen and progesterone for 6 days, extracts of primed glands promote full lobulo-alveolar development. This mammary-derived growth factor(s) (MDGF) is extracted from the epithelial-rich region of primed glands and is not present in similar extracts from glands of unprimed animals. Detectable levels of MDGF are present in extracts of glands as early as 3 days after the initiation of estrogen-progesterone priming and continue to rise for at least 9 days [4].

MDGF is more effective than EGF, since levels as low as 0.3 ng EGF binding equivalents per milliliter result in morphological development equal to that attained with 60 ng EGF/ml. Thus, it may appear that estrogen-progesterone priming induces an EGF-like growth factor in the mammary glands of mice as well as inducing EGF receptors in those same glands.

3.2.2 Transforming growth factor Alpha transforming growth factor (TGFα) is also a member of the family of peptides that are like EGF [67, 68]. This factor competes for EGF receptors on target tissues [69] but, in addition, promotes anchorage-independent cell growth and promotes the loss of contact-inhibited cell movement and growth [6, 7, 70, 71]. Because TGFα has been isolated from non-neoplastic normal tissues [72–76], as well as from neoplastic tissues [67, 75, 77–79], this member of the EGF family was also tested for its ability to promote full lobulo-alveolar development *in vitro*. As shown in Table 2, TGFα (either recombinant from Genentech [San Francisco, CA] [80] or chemically synthesized by Peninsula Laboratories [Belmont, CA]) was fully active at high (equivalent to 60 ng EGF/ml in an EGF receptor-competing assay) as well as low (one-tenth the high value) doses in promoting full lobulo-alveolar development of 6-day primed glands in the presence of IPrlAH. EGF at one-third of the high dose was only partially effective in culture, suggesting a greater sensitivity of the glands to the action of αTGF.

4. Direct local effects of growth factors

These same growth factors promote localized lobulo-alveolar development of the mammary epithelium when inserted directly into the growing gland *in vivo*. The use of slow-release pellets inserted directly into the glands helps to assess the effects of various mammotrophic agents *in vivo*, in that local effects can be more easily distinguished from systemic effects. Ductal morphogenesis in subadult animals can be stimulated locally by using Elvax plastic pellets to directly insert growth-promoting agents, such as deoxycorticosterone acetate, dibutyryl cAMP, and cholera toxin, into the glands [81, 82].

Slow-release cholesterol-based pellets [4] inserted directly into the gland were used to assess the effects of EGF, MDGF, and TGFα *in vivo*. Four days after insertion of the growth factor-containing pellets, the mammary glands were examined for branching morphogenesis and lobulo-alveolar development using the whole-mount technique. Figure 3b shows the localized growth obtained in Balb/c mice treated with EGF pellets. This localized growth was not seen in the contralateral glands containing pellets made with cholesterol only (Figure 3a). In contrast to the growth factor-stimulated glands, which show definite alveolar structures and many branch ducts in the area where the pellet had resided, the area around the cholesterol-only pellet contains only crude ducts with prominent end buds similar to the structures present in untreated glands. The effect is only local, since areas distant from the site of the growth factor-containing pellet contain only crude ducts (see Figure 3b, arrow) similar to those of untreated or cholesterol-only-treated glands.

The local growth effects obtained with EGF or MDGF require the simultaneous presence of a subcutaneous pellet containing estrogen and progesterone (Table 3). The effect of the estrogen and progesterone is systemic in

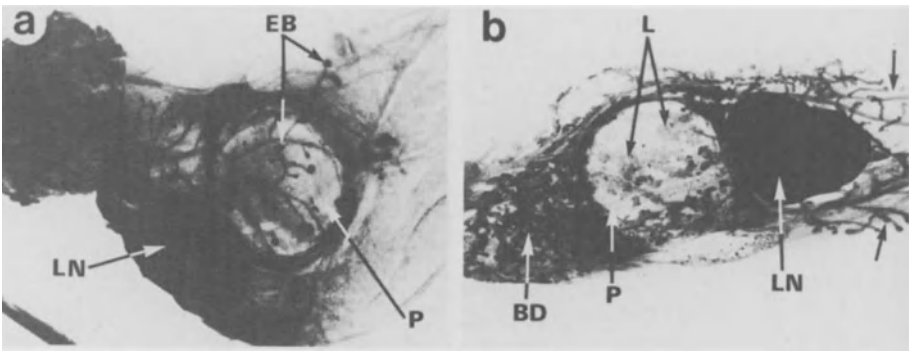


Figure 3. Local *in vivo* effects of EGF on mammary gland lobulo-alveolar development in female Balb/c mice 32-35 days old. Control pellets of cholesterol only (a) or containing 10 μg EGF (b) were placed directly in the number 4 abdominal mammary fat pad near the growing end of the gland, directly posterior to the central lymph node (day 0). Animals simultaneously received an estrogen-progesterone pellet subcutaneously [4]. On day 4, when >80 of the growth factor had been released from the pellets, the glands from each animal were whole-mounted and stained with hematoxylin. Large circular area (P) was site of pellet implantation. Arrow shows undeveloped area distant from site of growth factor-containing pellet. LN, lymph node; EB, end bud; L, lobules, BD, branched ducts.

this case but is not sufficient in itself to cause proliferation of the gland during the 4 days of these experiments [4]. MDGF from 9-day estrogen-progesterone-primed glands, but not from unprimed glands, also promoted local development (Table 3).

In a similar system that used 5-week-old C3H/HeN mice, TGFα gave somewhat different results. This growth factor also stimulated localized growth, but without the steroid supplement (Table 3). The degree of development was somewhat diminished in the animals without the subcutaneous estrogen-progesterone pellets but was consistently observed. A lower dose of EGF equal to the dose of αTGF used in these experiments (i.e., 2 μg EGF/10-mg pellet) was ineffective, even with the steroid supplement. Lower doses of αTGF were not tested.

The circulating levels of estrogen and progesterone in these ovary-intact subadult animals are not sufficient to support the local growth effects of EGF or the crude MDGF, but they can support, to a limited extent, the growth-promoting action of TGFα. However, the αTGF is maximally effective in the presence of the hormonal supplement. This apparent increase in sensitivity of the glands to the TGFα appears to be regulated by the female steroid hormones, possibly by induction of the growth factor receptor in the epithelial-rich region of the gland [4]. Whether these effects on growth factor induction and receptor regulation are due to estrodiol alone, progesterone alone, or both hormones in combination needs to be elucidated. The nature of the lobulo-alveolar development-promoting agent(s) in the crude MDGF preparations is not known. Although it competes with EGF for receptor binding, MDGF is immunologically distinct from EGF, and its relationship to TGFα is

Table 3. Local lobulo-alveolar development of the mammary glands of subadult mice induced by EGF-like growth factors

All glands treated with growth factor-containing or cholesterol-only pellets were examined by the whole-mount technique 4 days after insertion of the pellets as described in the legend to Figure 3. Each animal received a cholesterol-only pellet in the gland contralateral to the gland receiving the growth factor-containing pellet. MDGF was prepared from glands of animals treated with a subcutaneous pellet of estrogen and progesterone for 10 days (primed) or from untreated animals (unprimed) by the acid-alcohol extraction method and used without further purification [4].

	Number of developed glands/ Total number of glands examined	
Pellet in gland	No subcutaneous pellet	E+P subcutaneous pellet
A. Balb/c mice cholesterol only	0/33	0/34
+ EGF (10 μg)	0/18	14/19
+ MDGF (primed)	9/10	8/10
+ MDGF (umprimed)	0/5	0/5
B. C3H/HeN mice cholesterol only	0/7	0/13
+ EGF (10 μg)	0/2	4/4
+ EGF (2 μg)	N.T.[a]	0/4
+ αTGF (2 μg)	5/5	5/5

[a] N.T. = not tested.

untested as yet. The site of synthesis as well as the actual target cells within the mammary gland are also unknown at this point. Whether MDGF's mode of action is autocrine or paracrine [83, 84], as well as whether it is present in growing mammary tissue during other stages of development or in mammary preneoplasias and/or tumors, remains to be shown. The whole-organ mammary culture and slow-release pellets *in vivo* provide excellent models for answering these questions and, thus, shedding light on the events involved in the natural developmental cycles of the mammary gland.

5. Conclusions

In vivo, the mammary glands go through natural developmental cycles of growth, morphogenesis, differentiation, and involution under the influences of a variety of hormones and growth factors. These cycles can be mimicked in culture using thoracic glands from subadult female mice primed with estrogen and progesterone. This priming induces mammary gland EGF receptors and the production of a mammary-derived, EGF-like growth factor, MDGF. EGF, TGFα, and MDGF are able to promote lobulo-alveolar development of glands from primed mice *in vitro* in the presence of the four mammogenic hormones: insulin, prolactin, aldosterone, and hydrocortisone. The same growth factors are able to promote local lobulo-alveolar development *in vivo* when inserted directly into the mouse mammary gland in slow-release

cholesterol-based pellets. Both *in vitro* and *in vivo*, the mouse mammary gland is more sensitive to TGFα than to EGF in terms of lobulo-alveolar development.

Acknowledgments

The author thanks Erika Ginsburg, Elizabeth Andrew, Lisa Hanna, and Richard Sinda for technical assistance.

References

1. Ichinose RR, Nandi S: Influence of hormones on lobulo-alveolar differentiation of mouse mammary glands *in vitro*. J Endocrinol 35:331–340, 1966.
2. Banerjee MR, Wood BG, Kinder DL: Whole mammary gland organ culture: Selection of appropriate gland. *In Vitro* 9:129–133, 1973.
3. Tonelli QJ, Sorof S: Epidermal growth factor requirement for development of cultured mammary gland, Nature 285:250–252, 1980.
4. Vonderhaar BK: Hormones and growth factors in mammary gland development. In: Control of Cell Growth and Proliferation Veneziale CM, (ed), Van Nostrand-Reinhold, Princeton, NJ, pp 11–33, 1984.
5. Propper AY: Wandering epithelial cells in the rabbit embryo milk line. Dev Biol 67:225–231, 1978.
6. Balinsky BI: On the pre-natal growth of the mammary gland rudiment in the mouse. J Anat 84:227–235, 1950.
7. Balinksy BI: On the developmental processes in mammary glands and other epidermal structures, Trans R Soc Edinburgh 62:1–32, 1950.
8. Raynaud A, Raynaud J, Dobrovolskaia N, Rudali G, Adamoff N, Defoort J: Sur l'etat de developpement des ebauches mammaires chez des souris nouveau-nees appartenant a cinq lignees selectionnees elevees a l'Institut du Radium de Paris. Bull Cancer 57:447–476, 1970.
9. Raynaud A, Raynaud J: Les principales etapes de la separation, d'avec l'epiderme, des ebauches mammaires des foetus male de souris; recherches sur les processus de la rupture de la tige du bourgeon mammaire. CR Soc Biol 147:1872–1876, 1953.
10. Colard C, Gomot L: Etude comparee de l'ultrastructure des bourgeons mammaries primaries d'embryons de souris males et femelles au stade de la differenciation sexuelle. CR Acad Sci Ser D 280:1821–1824, 1975.
11. Raynaud A, Frilley M: Destruction des glands genitales, de l'embryon de souris, par une irradiation au moyen des rayons X, a l'age de treize jours. Ann Endocrinol 8:400–419, 1947.
12. Raynaud A: Morphogenesis of the mammary gland. In: Milk: The Mammary Gland and Its Secretion, Volume 1 Kon SK and Cowie AT, (eds), Academic Press, New York, pp 3–46, 1961.
13. Sakakura T: Mammary embryogenesis. In: *The Mammary Gland: Development, Regulation and Function* Daniel CW and Neville MC, (eds), Plenum Publishing Co., New York, pp 37–66, 1987.
14. Kratochwil K: *In vitro* analysis of the hormonal basis for the sexual dimorphism in the embryonic development of the mouse mammary gland, J Embryol Exp Morphol 25:141–153, 1971.
15. Kratochwil K: Development and loss of androgen responsiveness in the embryonic rudiment of the mouse mammary gland, Dev Biol 61:358–365, 1977.

16. Kratochwil K, Schwartz P: Tissue interaction in androgen response of embryonic mammary rudiment of mouse: Identification of target tissue for testosterone. Proc Natl Acad Sci USA 73:4041–4044, 1976.
17. Drews U, Drews U: Regression of mouse mammary gland anlagen in recombinants of TfM and wild-type tissue: Testosterone acts via the mesenchyme. Cell 10:401–404, 1977.
18. Raynaud A: Recherches experimentales sur le developpement de l'appareil genital et le fonctionnement des glandes endocrines des foetus de souris et de mulot. Arch Anta Microsc Morphol Exp 39:518–576, 1950.
19. Narbaitz R, Stumpf WE, Sar M: Estrogen receptors in mammary gland primordia of fetal mouse. Anat Embryol 158:161–166, 1980.
20. Raynaud A: Observations sur les modifications provoquees par les hormones oestrogenes, du mode de developpement des mamelons des foetus de souris. C R Acad Sci 240:674–676, 1955.
21. Raynaud A. Foetal development of the mammary gland and hormonal effects on its morphogenesis. In: Lactation Falconer IR, (ed), Butterworths, London, pp 3–29, 1971.
22. Turner CW, Gomez ET: The normal development of the mammary gland of the male and female albino mouse. Univ Mo. Columbia Coll Agric Agric Exp Stn Res Bull 182:1–43, 1933.
23. Bresciani F. Topography of DNA synthesis in the mammary gland of the C3H mouse and its control by ovarian hormones: An autoradiographic study. Cell Tissue Kinet 1:51–63, 1968.
24. Daniel CW, Silberstein GB: Postnatal development of the rodent mammary gland. In: The Mammary Gland: Development, Regulation and Function Daniel CW and Neville MC (eds), Plenum Publishing Co., New York, pp 3–36, 1987.
25. Nandi S: Endocrine control of mammary-gland development and function in the C3H/HeCrgl mouse: J Natl Cancer Inst 21:1039–1063, 1958.
26. Richards J, Guzman R, Konrad M, Yang J, Nandi S: Growth of mouse mammary gland end buds cultured in a collagen gel matrix, Exp Cell Res 141:433–443, 1982.
27. Bronson FH, Dagg CP, Snell GD: Reproduction. In: Biology of the Laboratory Mouse, 2nd ed., Green EL, (ed), Dover Publications, New York, pp 187–204, 1975.
28. Sutton H, Suhrbier K: The estrous cycle and DNA synthesis in the mammary gland. *Argonne Natl Lab US Atomic Energy Commission Annual Report*, pp 157–158, 1967.
29. Grahame RE, Bertalanffy FD: Cell division in normal and neoplastic mammary gland tissue in the rat. Anat Rec 174:1–7, 1972.
30. Dulbecco R, Henahan M, Armstrong B: Cell types and morphogenesis in the mammary gland. Proc Natl Acad Sci USA 79:7346–7350, 1982.
31. Hummel KP, Richardson FL, Fekete E. Anatomy. In: Biology of the Laboratory Mouse, 2nd ed., Green EL, (ed), Dover Publications, New York, pp 247–307, 1975.
32. Squartini F, Basolo F, Bistocchi M. Lobuloalveolar differentiation and tumorigenesis: Two separate activities for mouse mammary tumor virus. Cancer Res 43:5879–5882, 1983.
33. Haslam SZ. Mammary fibroblast influence on normal mouse mammary epithelial cell responses to estrogen *in vitro*. Cancer Res 46:310–316, 1986.
34. Elias JJ, Pitelka DR, Armstrong RC. Changes in fat cell morphology during lactation in the mouse. Anat Rec 177:533–548, 1973.
35. Kidwell WR, Knazek RA, Vonderhaar BK, Losonczy I: Effects of unsaturated fatty acids on the development and proliferation of normal and neoplastic breast epithelium. In: Molecular Interrelations of Nutrition and Cancer Arnott MS, vanEys J, Wang YM, (eds), Raven Press, New York, pp 219–236, 1982.
36. Murr SM, Bradford GE, Geschwind II. Plasma luteinizing hormone, follicle-stimulating hormone and prolactin during pregnancy in the mouse. Endocrinology 94:112–116, 1974.
37. Lyons WR, Li CH, Johnson RE: The hormonal control of mammary growth and lactation. Recent Prog Horm Res 14:219–250, 1958.
38. Djiane J, Kann G, Delouis C, Martal J. Placental lactogenic hormone. Study of its role on mammogenesis and lactogenesis in the ewe. In: International Symposium on Growth Hormone Related Peptides Pecile A and Müller EE (eds), University of Milan, Milan, pp 128–129.

39. Tucker HA: Physiological control of mammary growth, lactogenesis, and lactation. J Dairy Sci 64:1403–1421, 1981.
40. Foster RC: Changes in mouse mammary epithelial cell size during mammary gland development. Cell Differ 6:1–8, 1977.
41. Turner CW, Gomez ET. The experimental development of the mammary gland. I. The male and female albino mouse. Univ Mo Columbia 1934.
42. Nandi S, Bern HA: The hormones responsible for lactogenesis in BALB/cHeCrgl mouse. Gen Comp Endocrinol 1:195–210, 1961.
43. Mixner JP, Turner CW: Influence of thyroxin upon mammary lobule-alveolar growth. Endocrinology 31:345–348, 1942.
44. Dubnik CS, Morris HP, Dalton AJ: Inhibition of mammary-gland development and mammary-tumor formation in female C3H mice following ingestion of thiouracil. J Natl Cancer Inst 10:815–841, 1950.
45. Vonderhaar BK, Greco AE., Lobulo-alveolar development of mouse mammary glands is regulated by thyroid hormones. Endocrinology 104:409–418, 1979.
46. Vonderhaar BK: Effect of thyroid hormones on mammary tumor induction and growth. In: *Hormonal Regulation of Experimental Mammary Tumors*, Volume II Leung BS (ed), Eden Press, Montreal, pp 138–154, 1982.
47. Banerjee MR: Responses of mammary cells to hormones. Int Rev Cytol 46:1–97, 1976.
48. Nandi S, Bern HA: Relation between mammary-gland responses to lactogenic hormone combinations and tumor susceptibility in various strains of mice. J Natl Cancer Inst 24:907–931, 1960.
49. Murr SM, Stabenfeldt GH, Bradford GE, Geschwind II: Plasma progesterone during pregnancy in the mouse. Endocrinology 94:1209–1211, 1974.
50. Kuhn NJ: Lactogenesis. The search for trigger mechanisms in different species. In: Comparative Aspects of Lactation Peaker M (ed), Academic Press, New York, pp. 165–192, 1977.
51. Brookreson AD, Turner CW: Normal growth of mammary gland in pregnant and lactating mice: Proc Soc Exp Biol Med 102:744–745, 1959.
52. Traurig HH: Cell proliferation in the mammary gland during late pregnancy and lactation. Anat Rec 157:489–503, 1967.
53. Traurig HH: A radioautographic study of cell proliferation in the mammary gland of the pregnant mouse. Anat Rec 159:239–244, 1967.
54. Knight CH, Peaker M: Mammary cell proliferation in mice during pregnancy and lactation in relation to milk yield. Q J Exp Physiol 67:165–177, 1982.
55. Wicha MS, Liotta LA, Vonderhaar BK, Kidwell WR: Effect of inhibition of basement membrane collagen deposition on rat mammary development. Dev Biol 80:253–266, 1980.
56. Vonderhaar BK, Bhattacharjee M: The mammary gland: A model for hormonal control of differentiation and pre-neoplasia. In: *Biological Responses in Cancer* Mihich E, (ed), Plenum Publishing Corp New York, pp 125–159, 1985.
57. Singh DV, DeOme KB, Bern HA: Strain differences in response of the mouse mammary gland to hormones *in vitro*. J Natl Cancer Inst 45:657–675, 1970.
58. Ichinose RR, Nandi S: Lobuloalveolar differentiation in mouse mammary tissue *in vitro*. Science 145:496–497, 1964.
59. Terry PM, Ball EM, Ganguly R, Banerjee MR: An indirect radioimmunoassay for mouse casein using ^{125}I-labeled antigen, J Immunol Methods 9:123–134, 1975.
60. Mehta RG, Banerjee MR: Action of growth promoting hormones on macromolecular biosynthesis during lobuloalveolar development of the entire mammary gland in organ culture. Acta Endocrinol 80:501–516, 1975.
61. Wood BG, Washburn LL, Mukherjee AS, Banerjee MR. Hormonal regulation of lobuloalveolar growth, functional differentiation and regression of whole mouse mammary gland in organ culture, J. Endocrinol 65:1–6, 1975.
62. Tonelli QJ, Sorof S: Expression of a phenotype of normal differentiation in cultured mammary glands is promoted by epidermal growth factor and blocked by cyclic adenine

nucleotide and prostaglandins. Differentiation 20:253–259, 1981.
63. Taketani Y, Oka T: Biological action of epidermal growth factor and its functional receptors in normal mammary epithelial cells. Proc Natl Acad Sci USA 80:2647–2650, 1983.
64. Taketani Y, Oka T: Epidermal growth factor stimulates cell proliferation and inhibits functional differentiation of mouse mammary epithelial cells in culture. Endocrinology 113:871–877, 1983.
65. McNeill JM, Reed MJ, Beranek PA, Newton CJ, Ghilchik MW, James VHT. The effect of epidermal growth factor, transforming growth factor and breast tumor homogenates on the activity of oestradiol 17β hydroxy-steroid dehydrogenase in cultured adipose tissue. Cancer Lett 31:213–219, 1986.
66. Ginsburg E, Vonderhaar BK: Epidermal growth factor stimulates the growth of A431 tumors in athymic mice. Cancer Lett 28:143–150, 1985.
67. Todaro GJ, DeLarco JE, Fryling C, Johnson PA, Sporn MB. Transforming growth factors (TGFs): Properties and possible mechanisms of action. J Supramol Struct Cell Biochem 15:287–301, 1981.
68. Lawrence DA: Transforming growth factors—an overview. Biol Cell 53:93–98, 1985.
69. Massague J: Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J Biol Chem 258:13614–13620, 1983.
70. Moses HL, Branum EL, Proper JA, Robinson RA: Transforming growth factor production by chemically transformed cells, Cancer Res 41:2842–2848. 1981.
71. Anzano MA, Roberts AB, Smith JM, Sporn MB, DeLarco JE: Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and type β transforming growth factors: Proc Natl Acad Sci USA 80:6264–6268, 1983.
72. Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB: New class of transforming growth factors potentiated by epidermal growth factor: Isolation from non-neoplastic tissues. Proc Natl Acad Sci USA 78:5339–5343, 1981.
73. Proper JA, Bjornson CL, Moses HL: Mouse embryos contain polypeptide growth factor(s) capable of inducing a reversible neoplastic phenotype in nontransformed cells in culture: J Cell Physiol 110:169–174, 1982.
74. Matrisian LM, Pathak M, Magun BE: Identification of an epidermal growth factor-related transforming growth factor from rat fetuses. Biochem Biophys Res Commun 107:761–769, 1982.
75. Eckert K, Lubbe L, Schon R, Grosse R: Demonstration of transforming growth factor activity in mammary epithelial tissues, Biochem Int 11:441–451, 1985.
76. Zwiebel JA, Bano M, Nexo E, Salomon DW, Kidwell WR: Partial purification of transforming growth factors from human milk. Cancer Res 46:933–939, 1986.
77. Salomon DS, Zwiebel JA, Bano M, Losonczy I, Fehnel P, Kidwell WR: Presence of transforming growth factors in human breast cancer cells. Cancer Res 44:4069–4077, 1984.
78. Dickson RB, Huff KK, Spencer EM, Lippman ME. Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinology 118:138–142, 1985.
79. Perroteau I, Salomon D, DeBartoli M, Kidwell W, Hazarika P, Pardue R, Dedman J, Tam J: Immunological detection and quantitation of alpha-transforming growth factor in human breast carcinoma cells. Breast Cancer Res Treat 7:201–210, 1986.
80. Schreiber AB, Winkler ME, Derynck R: Transforming growth factor-α: A more potent angiogenesis mediator than epidermal growth factor, *Science* 232:1250–1253, 1986.
81. Silberstein GB, Daniel CW: Elvax 40P implants: Sustained, local release of bioactive molecules influencing mammary ductal development. Dev Biol 93:272–278, 1982.
82. Silberstein GB, Strickland P, Trumpbour V, Coleman S, Daniel CW. *In vivo*, cAMP stimulates growth and morphogenesis of mouse mammary ducts. Proc Natl Acad Sci USA 81: 4950–4954, 1984.
83. Bradshaw RA, Sporn MB: Polypeptide growth factors and the regulation of cell growth and differentiation. Fed Proc 42:2590–2591, 1983.

84. Lippman ME, Dickson RB, Bates S, Knabbe C, Huff K, Swain S, McManaway M, Bronzert D, Kasid A, Gelmann EP: Autocrine and paracrine growth regulation in human breast cancer. Breast Cancer Res Treat 7:59–70, 1986.

13. MMTV as a model for gene expression in mammary tissue

Gordon L. Hager

1. Introduction

Studies on mechanisms of gene regulation in mammalian cells have been greatly aided by the use of viral model systems. The small-to-intermediate sized DNA viruses (SV40 and Adenovirus) have been particularly useful because their genomes are relatively simple and they rely to a great extent on components of the host cell transcription apparatus for expression of their genetic information. Members of the retrovirus family, although studied primarily because of their oncogenic activity, offer an additional utility as models of gene regulation. When integrated in the host cell chromosome, these viruses adopt a low copy template structure (provirus) more similar to host cell genes than that of the high copy DNA viruses.

One member of this family, mouse mammary tumor virus (MMTV), has become a fruitful model for the study of hormone regulation of gene expression at the transcriptional level. MMTV, the etiologic agent of mammary adenocarcinomas in murine strains harboring infectious virus, was discovered in early investigations to be responsive to regulation by glucocorticoid hormones, and was the first system in which this regulation was rigoroously shown to occur primarily at the transcriptional level [1, 2]. Shortly after recombinant clones of the virus were isolated [3], cis-acting regulatory sequences required for hormone regulation were shown to be encoded within viral DNA [4, 5, 6]. These experiments eliminated any major role for host DNA sequences (adjacent to the proviral integration site) in the hormone response, and set the stage for a series of rapid advances on the molecular mechanisms involved in steroid hormone regulation.

Both the general biology of MMTV [7], and regulation of viral expression [8, 9] have been the subject of several reviews. The purpose of this discussion is to examine mechanisms of MMTV transcriptional regulation in the context of the nucleoprotein structure acquired by proviral DNA in the living cell. Recent experimentation indicates that the viral LTR, which harbors the cis-

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

acting regulatory sequences, assumes a specific and reproducible chromatin organization when introduced into cells by a variety of routes. This nucleoprotein structure also undergoes specific alterations in response to promoter activation. Our knowledge of the diffusible protein components involved in transcriptional activation at this promoter is sufficiently advanced to begin a characterization of the template in the living cell with which these components interact. In particular, the question of whether the template is involved in any significant way in the actual mechanism of transcriptional regulation can now be addressed.

2. Primary sequence structure of the MMTV promoter

The organization of the MMTV promoter in the context of proviral DNA is presented in Figure 1. The promoter resides within the long terminal repeat (LTR), which is particularly large, 1328 bp [10], compared to other retroviruses. The molecular basis for this large size is unknown, but the U3 region encodes an open reading frame for a 37kd protein, in addition to the promoter and regulatory sequences. The role of this 'fourth gene' in the retrovirus life cycle has yet to be discovered, although it must be functional, since the reading frame has been maintained in all strains of MMTV that have been sequence [11–15].

Initiation of RNA synthesis from proviral DNA occurs at bp 1195 of the LTR [10, 14]. Polyadenylation of MMTV transcripts occurs in the downstream (or 3′) LTR at position. The inferred signal for this event is the AGTAAA at position 1186, a deviation from the standard AATAAA [13, 14]. The resulting terminal redundancy (or R sequence) in the MMTV genome is only 15 nucleotides in length (by far the smallest for any of the retroviruses), defining thus far the minimal size sequence necessary for reverse transcriptase to transfer (or 'jump') templates.

3. Promoter regulatory elements

Well-characterized cis-acting regulatory elements of the MMTV promoter are summarized in Figure 1. The promoter contains a TATA motif at position -34 relative to the site for transcription initiation. This signal is thought to direct the binding of a protein, common to the great majority of mammalian promoters, that is involved directly in formation of the initiation site complex. Such a protein has actually been visualized in vivo on the MMTV promoter (discussed further below).

Upstream of the TATA position, between bp -58 and -79, a site exists for a DNA binding activity originally described either as a nuclear protein from chick oviduct, the TGGCA protein [16], or as a DNA replication factor

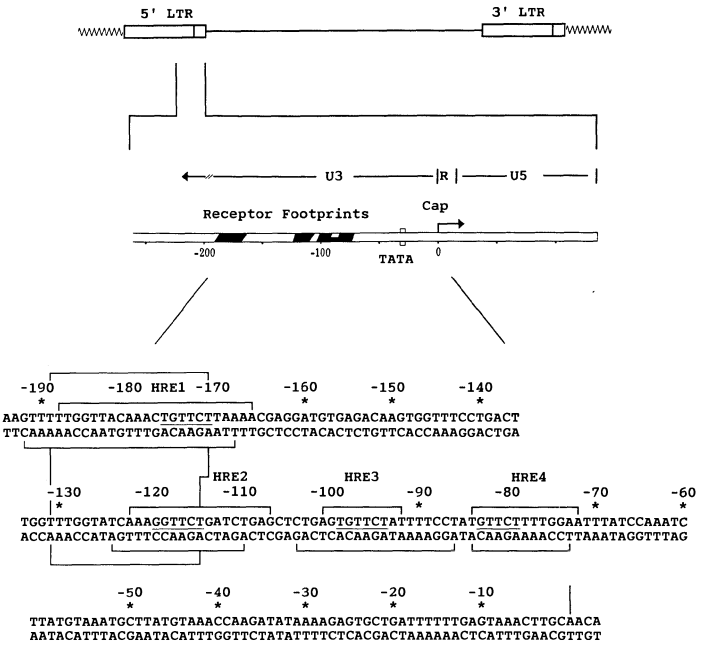


Figure 1. Structure of MMTV proviral DNA. The region of the LTR containing the promoter elements is described schematically. MMTV sequences [10] protected by glucocorticoid receptor [20, 21] are overlined (sense strand) and underlined (antisense strand). The TGTTCT consensus motifs are indicated by the lines between the strands. Individual receptor binding sites distinguishable by ExoIII mapping *in vitro* [23] are designated HRE's 1, 2, 3, and 4. The box encloses sequences that represent a direct repeat.

isolated from Adenovirus infected HeLa cells, NF1 [17]. The precise characterization of these proteins is incomplete, but both are currently thought to be identical to a factor (CTF [18]), also from HeLa cells, isolated as a transcription-stimulating activity. A mouse cell homologue of NF1 has been clearly identified as a transcription factor for MMTV [19], and shown to bind to the promoter in a hormone-dependent fashion (see below).

Located further upstream are two regions identified by DNaseI footprinting *in vitro* as binding regions for activated glucocorticoid receptor-hormone complex [20, 21]. There is slight disagreement in reported DNaseI footprints, but the two regions described in Figure 1 are common in the protection experiments, fit well with linker scanning analysis of the hormone response [22], and also correlate with exonuclease III (ExoIII) foot-printing *in vitro* with purified receptor [23]. These domains have been termed glucocorticoid response elements (or GRE's), based on their receptor binding properties and their ability to function at some distance from the promoter [24, 25]. They will be referred to heretofore in this discussion as the distal (-192 to -166) and proximal (-125 to -72) HRE's (hormone response element), because of recent findings that several different steroid receptors can activate the MMTV promoter by binding to overlapping regions within these elements [23].

4. Additional promoter sequence elements

In addition to the well characterized promoter elements described above, several poorly understood features of the MMTV promoter have emerged from recent studies. Using the highly sensitive techniques now available to observe the interaction of DNA-binding proteins with high-affinity sites *in vitro*, factors have been detected that bind to specific sequences in the MMTV LTR. One of these, F-i [19], binds to the TATA region, and will be discussed further below. A second protein, MP2, protects sequences between −195 and −215 (Figure 2), and is present in nuclei from both uninduced and hormone-treated cells in apparently equal concentrations (Cordingley and Hager, submitted). The significance of this factor awaits further investigation.

A binding activity has also been observed (Figure 3) that protects sequences −115 to −140, and more weakly sequences in the −190 to −170 region. The positions of these protections are of some interest in that they correlate with a set of sequences directly repeated at the left edge of the distal and proximal HRE's (see boxed region, Figures 1 and 3), even though the repeated sequence in HRE2 is not protected by receptor. As shown in Figure 3, each of these sites contains a core sequence with homology to the observed consensus for NF1, and therefore represent potential secondary binding sites for this factor. The NF-1 protein binds to the −70 MMTV region with extraordinary affinity *in vitro*, and one must be cautious in the interpretation of binding events with cross homology to the NF1 site. Since the factor(s) that

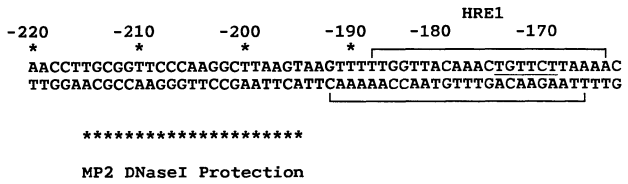


Figure 2. DNaseI footprint of MP2. Sequences protected from DNaseI digestion by factor MP2 are indicated by the asterisks.

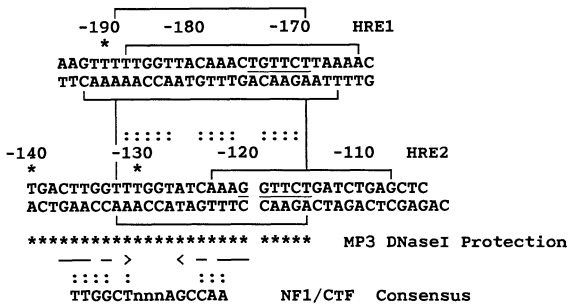


Figure 3. DNaseI footprint of MP3. Sequences protected from DNaseI digestion by factor MP3 are indicated by the asterisks. The protected sequence includes the direct repeat described in Figure 1.

bind to these sites have not been purified, or otherwise characterized, their potential role in transcription at the MMTV promoter remains speculative at this time.

5. Tissue specific expression

It is also clear that the MMTV promoter manifests some element of tissue specificity. The promoter responds weakly in many cell types when fused to reporter genes [4, 26], despite relatively efficient levels of expression observed in cell lines derived from MMTV virus-induced tumors. Perhaps the most striking evidence of cell-specific expression is provided by the transgenic experiments of Mitchell and Leder [27], who found that animals harboring myc sequences expressed from the MMTV promoter manifested a myc tissue expression profile remarkably similar to that for MMTV expression in animals infected with the virus. Elements of the MMTV promoter associated with this tissue specificity have yet to be identified.

6. Chromatin structure of the MMTV LTR

The DNA of eucaryotic cells is condensed via its association with histones into a repetitive array of nucleosomes. This polynucleosomal array can be further compacted into increasingly ordered structures, permitting the large amounts of DNA found in these cells to be packaged in the smallest possible volume. We now know that these highly ordered structures are incompatible with active gene expression, and are dissociated in regions where active transcription is underway. These regions are characterized by an increased sensitivity to DNaseI, and are often referred to as active chromatin.

In addition to the increased sensitivity of transcribed chromatin to nucleolytic attack, highly localized regions of 'hypersensitivity' are frequently observed. The underlying mechanism responsible for the phenomenon of hypersensitivity has been hypothesized to result either from a region of nucleosome-free DNA [28], or to the development of regions subject to unusual torsional stress [20]. Whatever the mechanism responsible, it is clear that these hypersensitive sites frequently correspond to binding sites for regulatory molecules.

Activation of the MMTV promoter is accompanied by the acquisition of such an hypersensitive site the the LTR [30, 31, 32]. We have carried out a series of experiments to explore the nature of this hormone-dependent hypersensitivity, and to investigate its potential role in promoter activation. To facilitate a detailed examination of LTR chromatin structure, we developed a series of cell lines with the MMTV promoter mobilized on bovine papilloma virus (BPV) episomes. This permits an examination of the promoter both in the amplified state, and isolated from potential position effects of adjacent

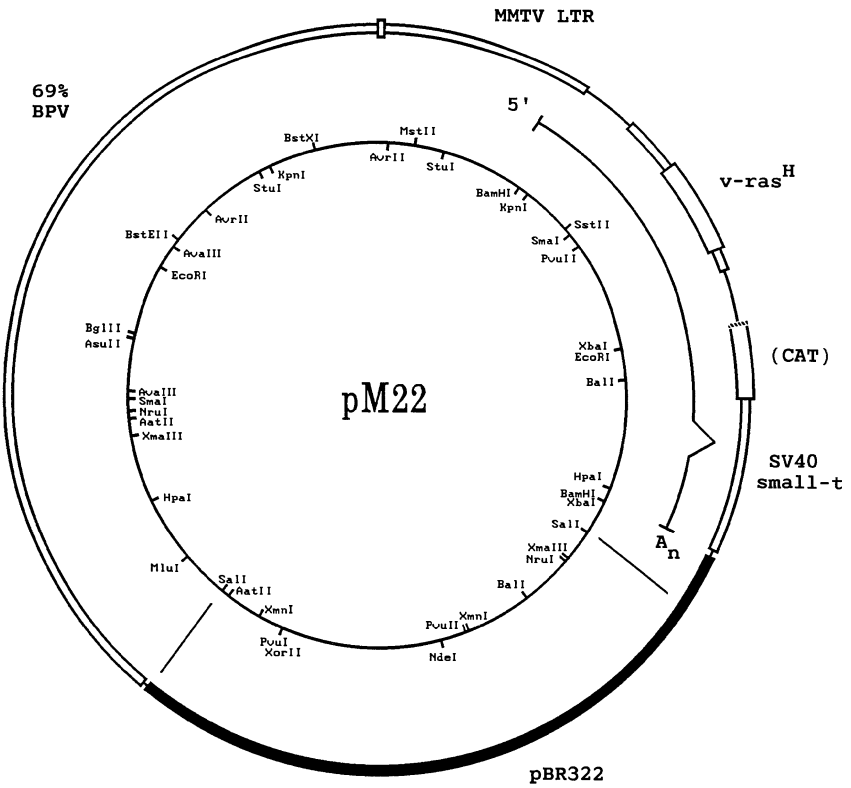


Figure 4. Structure of episome pM22. A schematic representation of a typical MMTV-BPV chimeric episome. This molecule contains the v-*ras*^{H} gene downstream of the MMTV promoter, followed by a a 3′ terminal region of the CAT gene, SV40 small-t splice donor and acceptor sites, and the SV40 early polyadenylation signal. Prior to transfection into mammalian cells, pBR322 sequences are deleted by restriction at the two SalI sites and religation.

cellular DNA sequences. In addition, the copy number of BPV-based episomes (50–200) is not so high as to titrate critical components of the transcription apparatus.

The development of the minichromosome system was successful, in that stable cell lines with non-rearranged episomes could be isolated, and hormone activation of transcription could be demonstrated [33]. We have utilized this system to examine nucleoprotein organization of the MMTV LTR, and hormone-dependent changes to that structure. The structure of a typical BPV-based episome is presented in Figure 4. In this particular molecule, the v-*ras*^{H} oncogene has been placed under control of the MMTV promoter. The transcript also contains a 3′ portion of the chloramphenicol-acetyl-transferase (CAT) gene, SV40 small-t antigen splice donor and acceptor signals, and the SV40 early message polyadenylation signal. In cells containing such episomal chimeras, MMTV-v-H-*ras*-SV40 fusion transcripts are correctly initiated and strongly responsive to hormone regulation [33, 19].

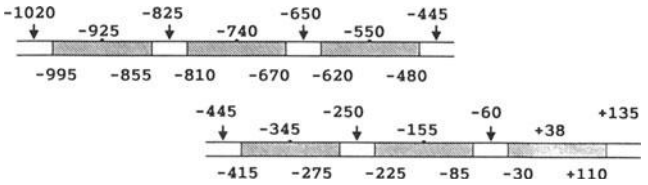


Figure 5. Nucleosome boundaries for MMTV LTR sequences. Site-specific localization of nucleosomes over the MMTV LTR, as inferred from MPE and micrococcal nuclease digestion studies [32], is summarized. Cross-hatched areas represent sequences associated with nucleosome cores. Positions above the lines indicate respectively the centers of linker regions, and the centers of core regions. Positions below the lines represent core boundaries.

The organization of nucleosomes over the MMTV LTR in these episomes was examined to determine if promoter sequences were phased or random with regard to nucleosome position. Utilizing both micrococcal nuclease and methidium-propyl-EDTA (Fe^{++}) as probes of linker-specific DNA, it was found [32] that nucleosome boundaries on the episomes were site-specific over the complete LTR region, as well as adjacent sequences insofar as examined. Furthermore, these boundaries were invariant with regard to the orientation of the LTR in the BPV vector, suggesting that phasing was an intrinsic property of LTR DNA. A summary of inferred nucleosome boundaries is presented in Figure 5.

It is to be emphasized that the nucleosome positions indicated in Figure 5 represent a pattern that is highly reproducible, irrespective of: a) the type of gene driven from the LTR promoter, b) the type of cell in which the episome is replicating, c) the copy number of the episome, or d) the orientation of the LTR cassette in the vector. In addition, we have recently determined (Richard-Foy and Hager, unpublished) that nucleosomes are similarly positioned on the LTR in a cell line with a single, integrated copy of the MMTV sequence. We conclude that LTR sequences acquire a phased array of nucleosomes, irrespective of the method by which the sequences are introduced into the cell and unaffected by sequences adjacent to the LTR. The nucleoprotein structure of LTR chromatin, as deduced from this series of experiments, is depicted schematically in Figure 6.

When the MMTV promoter is induced, a broad hypersensitive site appears in LTR chromatin between positions −50 and −200 [30, 31, 32]. This site is clearly associated with the binding of glucocorticoid receptor to the LTR, since both HRE elements are located within the boundaries of hypersensitivity. What emerges from the analysis of chromatin structure, however, is the striking parallel between the region of hypersensitivity and the position of nucleosome B in the phased array. Given only the correlation between the position of the HREs and the hypersensitive site, one might argue a local change in torsional stress contributes to the mechanism of hypersensitivity. However, knowing the structure of LTR chromatin, it is more likely that the hypersensitive site results from the displacement of nucleosome B from chromatin, as depicted in Figure 6.

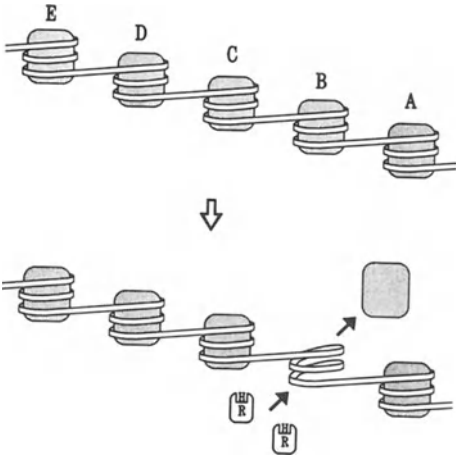


Figure 6. MMTV LTR chromatin structure. The proposed structure of LTR chromatin is indicated schematically, letters indicating phased nucleosomes whose boundaries are summarized in Figure 5. The figure also indicates the proposed model for generation of the hormone-dependent hypersensitive site.

The model of LTR nucleoprotein structure and hormone-dependent hypersensitivity proposed in Figure 6 is supported by evidence based on restriction enzyme access to sequences in MMTV chromatin. The ability of a restriction endonuclease to cleave at its recognition site in chromatin is also dependent on nucleosome position. We have determined accessibility of LTR sequences in minichromosome chromatin for a number of restriction enzymes. These data are summarized in Figure 7. Enzymes whose ability to cleave chromatin is unaffected by hormone treatment of cells are indicated above the line, while enzymes whose access is strongly hormone dependent are listed below the line. It is seen that the enzyme access profile is strikingly consistent with the nucleosome positioning data. If an enzyme site is present in linker DNA, access is relatively efficient, and unaffected by hormone stimulation. If the recognition site is present on nucleosome B, however, access is quite dependent on promoter activation. Data for enzymes whose sites are located on nucleosomes further upstream are not included, since these enzymes do not cleave chromatin under any condition, and a positive control is not available. These results are exactly as would be predicted by the nucleosome displacement model.

7. Formation of an active transcription initiation complex

The availability of the minichromosome system described above has also permitted us to investigate hormone dependent binding of proteins to LTR DNA *in vivo*. If LTR chromatin in isolated nuclei is first digested with a restriction endonuclease whose access is unaffected by hormone treatment (as

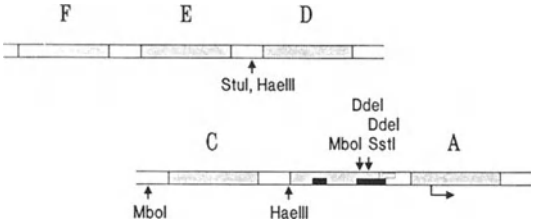


Figure 7. Restriction enzyme access to MMTV LTR chromatin. The susceptibility of LTR DNA to restriction enzyme cleavage when organized in chromatin is summarized. Cross-hatched areas represent sequences associated with nucleosome cores, as in Figure 5. Enzymes listed above the lines demonstrate hormone-dependent cleavage of LTR DNA, while enzymes listed below the line digest LTR chromatin from induced and noninduced cells with equal efficiency.

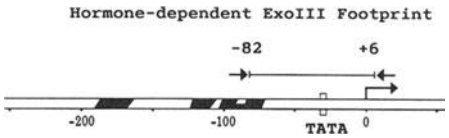


Figure 8. Hormone-dependent ExoIII footprint. Boundaries resistant to exonuclease III digestion that form over LTR DNA sequences in response to hormone stimulation [19] are depicted schematically.

described above), then exonuclease III (ExoIII) is allowed to enter chromatin at the restriction site, one can map boundaries that appear in regions adjacent to the restriction site (ExoIII footprinting; Figure 8, [34]). These boundaries provide unique information concerning the molecular events which occur at the promoter in response to transcriptional activation. Not only can proteins that interact with a given DNA be identified, but the subnuclear localization of these factors can be monitored at various stages of gene activation.

Binding of proteins to the MMTV promoter was investigated by digesting minichromosome chromatin upstream of the promoter at a HaeIII site, or downstream at a BamHI site (Figure 8). Using appropriate indirect end-labelled probes, boundaries could be located over promoter sequences. A very pronounced ExoIII footprint was detected in these experiments [19] with boundaries at bp −82/83 and +6 relative to the cap site (Figure 8). This footprint was present on the promoter only in cells that had been induced by hormone, indicating that a transcription initiation complex was assembled on the promoter in response to hormone stimulation. Promoter chromatin from non-stimulated cells was completely free of ExoIII resistant boundaries. These results indicated that the process of promoter activation by glucocorticoid receptor resulted in the formation of an initiation complex, rather than the attraction of RNA polymerase to a preexisting preinitiation complex.

The large size of the ExoIII footprint also suggested the participation of several components. Subsequent comparison of the *in vivo* footprint with partially purified factors from crude extracts has provided the following information (see Figure 9). The −82 to +6 footprint is composed of at least two

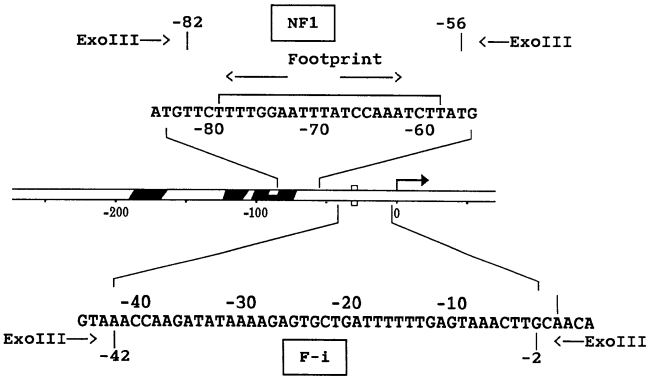


Figure 9. Components of the ExoIII footprint. The ExoIII boundaries formed in vitro by the two known components [19; Cordingley and Hager, submitted] of the *in vivo* ExoIII footprint are shown. In addition, the DNaseI resistance footprint for NF1 is indicated.

separate factors. The 5′ boundary corresponds to the mouse cell homologue of NF1 (see introduction); the 3′ boundary results from a factor we have designated F-i [19], which probably corresponds to a TATA binding activity common to many promoters.

Two further observations from this series of experiments are of particular interest. First and most surprising was the absence of a detectable boundary by glucocorticoid receptor at either of the putative receptor binding sites. One of two possibilities are the likely explanation for this observation. It is conceivable that the binding of receptor to its site is transitory, or that it follows a two-stage process, with initial DNA-binding followed by a shift into a multimeric protein complex composed of factors with which the receptor interacts (possibly the transcription initiation complex itself). Alternatively, the receptor may simply be unusually sensitive to ExoIII displacement. This possibility seems unlikely, given that ExoIII footprints have been obtained *in vitro* with purified glucocorticoid receptor [23], but it cannot be excluded.

A second observation from these experiments is also of considerable interest. For both of the factors involved in the −82 to +6 footprint, no change in either the affinity of the protein for promoter DNA, or nuclear concentration of the protein, could be detected in response to hormone stimulation (Cordingley and Hager, submitted; [19]). Thus, both factors are present in the uninduced nucleus in a form with high affinity for the promoter, yet are excluded from MMTV chromatin. This is particularly striking for NF1, which binds very tightly to MMTV DNA *in vitro*.

8. Mechanism of steroid hormone action

Hormone activation of the MMTV promoter occurs via the formation of a transcription initiation complex that is assembled on the promoter in direct

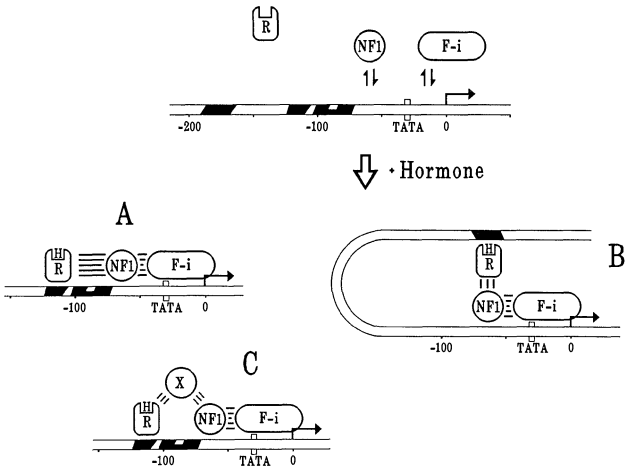


Figure 10. Recruitment of factors to a promoter by protein-protein interactions. Alternative models are presented for the formation of a transcription initiation complex at a target promoter by interaction between receptor and other factors. A. Direct interaction between receptor and an adjacent promoter site, as in the MMTV case. B. Interaction between receptor and a promoter at a distance. C. Recruitment of a transcription complex via a bridging protein.

response to binding of activated glucocoritcoid receptor. This complex is composed of a TATA-binding activity, and a mouse cell protein that is either identical to, or very similar to, the NF1 (CTF) protein from HeLa cells. The mechanism of steroid hormone action, insofar as the MMTV model can be generalized, therefore focuses on the molecular events involved in receptor-stimulated formation of the NF1-Fi complex. Assuming that protein-protein interactions [35] are crucial, a search for direct interactions between receptor and the NF1-TFIID complex, or possibly a third 'bridging' protein would seem appropriate (Figure 10). Although mutational analysis of steroid receptor proteins has not yet identified a region of the molecule involved in such interactions, it is possible that a protein interaction region could be closely associated with other critical features, not easily separable, and that more detailed analysis will eventually identify such a region.

The highly organized nucleoprotein structure of the MMTV promoter also invites further attention to the potential role of chromatin organization in promoter activation. It is remarkable that NF1, which has such a high affinity for the −70 MMTV region, is completely excluded from MMTV chromatin *in vivo*. One might imagine that the −70 site is to some extent sequestered by the presence of nucleosome B, limiting the access of NF1 to its site as it exists in chromatin (Figure 11). Such a mechanism would provide the cell with a certain economy, in that general transcription factors such as NF1 (CTF), which undoubtedly interact with a large number of cellular promoters, would be completely excluded from chromatin in the absence of 'triggering' factors specific for each promoter, or class of promoters.

A more general consideration relative to chromatin participation in gene

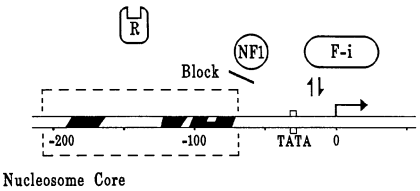


Figure 11. Potential role of chromatin in modulating factor access. The possible role of nucleosomes in blocking access of factors whose binding sites are unfavorably positioned in the polynucleosomal array is described.

activation concerns the enormous stimulation observed for some enhancers *in vivo*. The SV40 enhancer, for example, has been estimated to stimulate the SV40 early promoter by a factor of at least 2,000-fold. It is possible that the polynucleosomal arrays with which the transcription components interact *in vivo* are much more refractive to 'non-induced', or 'leaky' transcription in the absence of the appropriate stimulating factor, and the use of such templates *in vitro* would provide more pronounced stimulation ratios. As our understanding of chromatin structure advances, and the factors involved in specific promoter activation are identified, these more sophisticated (and more difficult) issues can be addressed.

9. The interaction of regulatory molecules with chromatin

The studies that have been reviewed here, while directed at understanding the mechanism of steroid hormone action, have produced findings that bear on a more general question. While the repeating nucleosomal structure of chromatin has been understood for some time, only recently has it become generally accepted that nucleosomes can be specifically positioned, or phased, over some cellular DNA sequences [36, 37]. With this acceptance comes a renewed interest in the extent to which chromatin structure (i.e., nucleosome positioning) can effect the binding of site-specific regulatory proteins.

It has been suggested [38] that a competition could exist between nucleosome core particles and site-specific binding factors. Under this model, nucleosomes could exclude a site—specific factor except during DNA replication, when the nucleosomal array is temporarily dissociated. According to this model, nucleosome displacement and the generation of a resultant hypersensitive site would require both the appearance of a developmentally regulated site-specific factor in the nucleus, and at least one round of DNA replication.

Our data suggests this model is not appropriate for the interaction of receptor with LTR chromatin. If the structure that protects the −60 to −220 region of the LTR is in fact a nucleosome, then one is forced to conclude that glucocorticoid receptor can actually displace an existing nucleosome core from its

position. Activation of MMTV transcription, and concomitant induction of the hypersensitive site, is quite rapid and can occur in the absence of DNA replication.

Following this line of argument, a further suggestion is that some DNA binding proteins (glucocorticoid receptor, for example) can successfully interact with their recognition sites even when organized in nucleosomes, while others (NF1 as a possibility) cannot. Alternatively, the exact 'path of the DNA' on the face of the nucleosome could critically determine if recognition sites are available for interaction.

Studies on the interaction of site-specific DNA binding proteins with chromatin are still in their infancy. The preparation of DNA templates *in vitro* that are organized in polynucleosomal arrays with the same phasing pattern found *in vivo* has not been accomplished, or even attempted. Whether such templates can be extracted from cells in sufficient quantity to be useful remains a daunting goal. It is unlikely that a complete understanding of the interaction of regulatory molecules with eucaryotic genomes can be achieved until these reagents can be successfully manipulated in the test tube.

References

1. Scolnick EM, Young HA, Parks WP: Biochemical and physiological mechanisms in glucocorticoid hormone induction of mouse mammary tumor virus. Virology 69:148–156, 1976.
2. Ringold G, Lasfargues EY, Bishop JM, Varmus HE: Production of mouse mammary tumor virus by cultured cells in the absence and presence of hormones: assay by molecular hybridization. Virology 65:135–147, 1975.
3. Donehower LA, Andre J, Berard DS, Wolford RG, Hager GL: Construction and characterization of molecular clones containing integrated mouse mamary tumor virus sequences. Cold Spring Harbor Symp. Quant. Biol 44 (Pt 2): 1153–1159, 1980.
4. Huang AL, Ostrowski MC, Berard D, Hager GL: glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumor virus. Cell 27 (Pt 1): 245–255, 1981.
5. Hager GL, Huang AL, Bassin RH, Ostrowski MC: Analysis of glucocorticoid regulation by linkage of the mouse mammary tumor virus promoter to a viral oncogene. In: Eukaryotic Viral Vectors, Gluzman Y (ed), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp 165–169, 1982.
6. Lee F, Mulligan R, Berg P, Ringold G: Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. Nature 294:228–232, 1981.
7. Young HA, Hager GL: Mouse mammary tumor virus and glucocorticoids. In: Steroid Receptors and the Management of Cancer, Thompson EB and Lippman M (eds), C.R.C. Press, Inc., Cleveland, Ohio, pp 45–58, 1979.
8. Ringold GM: Glucocorticoid regulation of mouse mammary tumor virus gene expression. Biochim Biophys Acta 560:487–508, 1979.
9. Yamamoto KR: Steroid receptor regulated transcription of specific genes and gene networks. Ann Rev Genet 19:209–252, 1985.
10. Donehower LA, Huang AL, Hager GL: Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy. J Virology 37:226–238, 1981.
11. Hager GL, Donehower LA: Observations on the DNA sequence of the extended terminal

redundancy and adjacent host sequences for integrated mouse mammary tumor virus. In: Animal Virus Genetics, ICN-UCLA Symp on Mol and Cell Biol, Vol. 18, pp 241–253, 1980.
12. Donehower LA, Fleurdelys B, Hager GL: Further evidence for the protein coding potential of the mouse mammary tumor virus long terminal repeat: nucleotide sequence of an endogenous proviral long terminal repeat. J Virology 45:941–949, 1983.
13. Fasel N, Pearson K, Buetti E, Diggelmann H: The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long coding sequence and signals for hormonally regulated transcription. EMBO J 1:3–7, 1982.
14. Klemenz R, Reinhardt M, Diggelmann H: Sequence determination of the 3′ end of mouse mammary tumor virus RNA. RNA Virus Topics 7(1–3):123–126, 1981.
15. Wheeler DA, Butel JS, Medina D, Cardiff RD, Hager GL: Transcription of mouse mammary tumor virus: identification of a candidate mRNA for the long terminal repeat gene product. J Virology 46:42–49, 1983.
16. Nowock J, Borgmeyer U, P: uschel AW, Rupp RA, Sippel AE: The TGGCA protein binds to the MMTV-LTR, the adenovirus origin of replication, and the BK virus enhancer. Nucleic Acids Res 13:2045–2061, 1985.
17. Rosenfeld PJ, Kelly TJ: Purification of nuclear factor I by DNA recognition site affinity chromatography. J Biol Chem 261:1398–1408, 1986.
18. Cato AC, Miksicek R, Schutz G, Arnemann J, Beato M: The hormone regulatory element of mouse mammary tumour virus mediates progesterone induction. EMBO 5:2237–2240, 1986.
19. Cordingley MG, Riegel AT, Hager GL: Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary tumor virus *in vivo*. Cell 48:1–10, 1986.
20. Scheidereit C, Geisse S, Westphal HM, Beato M: The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumour virus. Nature 304:749–752, 1983.
21. Payvar F, DeFranco D, Firestone GL, Edgar B, Wrange O, Okret S, Gustafsson JA, Yamamoto KR: Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35 (Pt 1):381–392, 1983.
22. Buetti E, Kuhnel B: Distinct sequence elements involved in the glucocorticoid regulation of the mouse mammary tumor virus promoter identified by linker scanning mutagenesis. J Mol Biol 190:379–389, 1986.
23. von der Ahe D, Janich S, Scheidereit C, Renkawitz R, Schutz G, Beato M: Glucocorticoid and progesterone receptors bind to the same sites in two hormonally regulated promoters. Nature 313:706–709, 1985.
24. Chandler VL, Maler BA, Yamamoto KR: DNA sequences bound specifically by glucocorticoid receptor *in vitro* render a heterologous promoter hormone responsive *in vivo*. Cell 33:489–499.
25. Ponta H, Kennedy N, Skroch P, Hynes NE, Groner B: Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc Natl Acad Sci USA 82:1020–1024, 1985.
26. Ostrowski MC, Huang AL, Kessel M, Wolford RG, Hager GL: Modulation of enhancer activity by the hormone responsive regulatory element from mouse mammary tumor virus. EMBO J 3:1891–1899, 1984.
27. Stewart TA, Pattengale PK, Leder P: Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 38:627–637, 1984.
28. Bogenhagen DF, Wormington WM, Brown DD: Stable transcription complexes of Xenopus 5S RNA genes: a means to maintain the differentiated state. Cell 28:413–421, 1982.
29. Weintraub H: A dominant role for DNA secondary structure in forming hypersensitive structures in chromatin. Cell 32:1191–1023, 1983.
30. Zaret KS, Yamamoto KR: Reversible and persistent changes in chromath structure accompany activation of a glucocorticoid-dependent enhancer element. Cell 38:29–38, 1984.
31. Hager GL, Richard-Foy H, Kessel M, Wheeler D, Lichtler AC, Ostrowski MC: The mouse

mammary tumor virus Model in studies of glucocorticoid regulation. Recent Prog Horm Res 40:121–142, 1984.
32. Richard-Foy H, Hager GL: Sequence specific positioning of nucleosomes over the steroid-inducible MMTV promoter. EMBO J 6:2321–2328, 1987.
33. Ostrowski MC, Richard-Foy H, Wolford RG, Berard DS, Hager GL: Glucocorticoid regulation of transcription at an amplified, episomal promoter. Mol Cell Biol 3:2045–2057, 1983.
34. Guo LH, Wu R: Exonuclease III: use for DNA sequence analysis and in specific deletions of nucleotides. Methods Enzymol 100:60–96, 1983.
35. Ptashne M: Gene regulation by proteins acting nearby and at a distance. Nature 322:697–701, 1986.
36. Eissenberg JC, Cartwright IL, Thomas GH, Elgin SC: Selected topics in chromatin structure. Ann Rev Genet 19:485–536, 1985.
37. Pederson DS, Thoma F, Simpson RT: Core particle, fiber, and transcriptionaly active chromatin structure. Ann Rev Cell Biol 2:117–147, 1986.
38. Schlissel MS, Brown DD: The transcriptional regulation of Xenopus 5s RNA genes in chromatin: the roles of active stable transcription complexes and histone H1. Cell 37:903–913, 1984.

14. The activation of cellular oncogenes by proviral insertion in murine mammary cancer

Roel Nusse

1. Introduction

In this chapter, I shall review our current knowledge of the mechanism of mammary oncogenesis by the Mouse Mammary Tumor Virus (MMTV), with particular emphasis on the activation of cellular oncogenes by insertion of MMTV proviral DNA. The properties of these newly discovered cellular oncogenes will be described in some detail, and finally, I shall speculate a little on the mechanism of action of these genes. As an introduction, some of the relevant earlier discoveries on MMTV will be summarized to provide a framework of the biology of viral mammary tumorigenesis. More encyclopedic overviews of the biology and molecular biology of MMTV are found in references 1, 2 and 3.

2. The mouse mammary tumor virus

MMTV was one of the earliest tumor viruses discovered, as a milk factor inducing mammary tumors in mice [reviewed in 2]. Despite its importance as a causative agent in an animal model of a prevalent form of human cancer, MMTV has for a long time been a stepchild in the large family of RNA tumor viruses. This was not due to a lack of interest, but was caused by difficulties in handling MMTV in *in vitro* cell systems and the lack of rapid and reliable assays for biologically active virus. Even the best virus preparations, which are highly oncogenic in susceptible mice, are very poorly infectious in cultured cells. Consequently, MMTV has never been biologically cloned, nor have variants of MMTV been separated from each other in a rigorous way. The genetics of MMTV is therefore virtually nonexistant and limited to the description of properties of virus variants isolated from different strains of mice, assayed in a time consuming tumorigenicity experiment [4].

With the advent of molecular cloning in bacterial plasmid vectors, a cir-

M.E. Lippman and R.B. Dickson (eds), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

cumvention of this problem seemed within reach, but to make things worse, MMTV proviral DNA appeared to be refractile to molecular cloning. Most attempts to isolate the MMTV genome as recombinant DNA have yielded molecular clones lacking a part of the viral genome thought to contain a socalled poisonous sequence [5–8]. Some recombinant bacteriophages with a complete viral genome have been obtained and were useful to establish the nucleotide sequence [9, 10], but plasmid subclones of the part with the poisonous sequence usually had sustained mutations. It has recently been shown that these mutations often are caused by insertion of bacterial transposable elements [11]. Only a few apparently complete forms of proviral DNA have been reported to be stable upon propagation in bacterial hosts. When transfected in eukaryotic cells, these recombinant DNA molecules give rise to viral proteins and particles [12–14], but it has not been shown that such viruses are infectious or tumorigenic. For a properly designed reverse genetic experiment—manipulation of cloned DNA and assaying the biology of the resulting virus—it is imperative to generate oncogenic virus by transfection.

Thus, in an era of rapid development of tumor virology, resulting in detailed insight in the replication cycle of retroviruses [1], progress in understanding MMTV propagation has been very slow. The genetic content of many tumor viruses has been described in detail by the availability of mutants and the nucleotide sequence of the genome, but we still have no formal genetic proof of any gene in the MMTV genome. Only very recently has its complete nucleotide sequence been established [10]. Let me give an example of the practical consequences of these difficulties: the Long Terminal Repeat (LTR) of MMTV, which is unusually long for a retrovirus, contains an open reading frame, encoding a protein with no homology to other viral or cellular genes, called *orf* [6, 15–19] (see Figure 1). The function of this novel gene could have been delineated by generating site-specific mutations in the coding domain and testing the replication and oncogenicity of such mutant viruses. Largely because of the inability to generate infectious, oncogenic wild-type virus from cloned MMTV DNA, is is still unknown what the function of the *orf* gene is.

There are nevertheless a number of unique properties of MMTV which have significantly advanced our knowledge of tumor virology and molecular biology in several areas.

1. It was recognized early in the history of tumor virology that MMTV could be inherited as a Mendelian gene, from parents to offspring without replication through the cycle of milk-borne transmission [20]. This was shown before the introduction of molecular hybridization techniques [21], by careful genetic experiments on mammary tumor incidence in crosses involving the GR strain of mice. These results provided a strong argument to postulate the existence of a provirus, an integrated DNA copy of the viral genome.

2. MMTV has been an ideal model system to study steroid hormone induced transcription. Virus production in some tumor cell lines and from infected cells can be stimulated by glucocorticoids [22–24], pointing to the

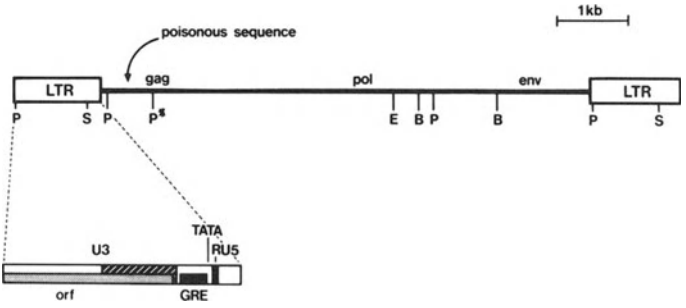


Figure 1. A map of the MMTV genome from the virus of the C3H strain. The position of some restriction sites is given (R:EcoRI; P:PstI: S:SacI; B:BamHI) and the position of genes encoded by the viral genome. P* is the PstI site not present in some endogenous MMTV variants. *gag* codes for the viral core proteins, *pol* for the reverse transcriptase, *env* for the viral envelope proteins, and *orf* indicates the gene of the viral long terminal repeat (LTR) with an unknown function, indicated by a stippled area. U3 and U5 are the parts of the LTR derived from the 3′ and 5′ end of the viral RNA, respectively. R is the repeat at the termini of the viral RNA and the start point of transcription of the provirus. The cross-hatched area in the LTR indicated the domain that is deleted in variant MMTVs which are implicated in lymphoma induction. TATA indicates a promoter element, GRE is the glucocorticoid responsive element, the portion conferring steroid hormone-inducible transcription. The arrow indicates the position of the poisonous sequence, the part of the *gag* gene that is difficult to clone in prokaryotic vectors.

existence of regulatory elements on the viral genome reacting with steroid hormone receptors. This was extensively documented when the MMTV LTR was molecularly cloned and proven to contain such elements [25–30] (Figure 1). The MMTV LTR is still used in assays for the interaction of receptors with DNA, even nowadays when genes encoding steroid hormone receptors have been cloned and their functional domains are dissected by mutation [31, 32].

3. MMTV has been one of the first viruses successfully used in the approach of identifying oncogenes by provirus tagging, which is the topic of this review.

3. Tumorigenesis by MMTV

MMTV usually causes adenocarcinomas of mammary epithelial tissue, which is a property unique to this virus [2]. MMTV expression and replication have also been associated with various types of other tumors, such as lymphomas and kidney carcinomas but these systems are less amenable to experimental manipulation and therefore much less understood [33, 34]. Mammary tumor-inducing virus preparations can be isolated from the milk of several high cancer incidence mouse strains like the C3H, the RIII and the DBA. In these strains, the virus is naturally transmitted from mother to offspring, causing the high spontaneous tumor incidence. Fosternursing of other mice on mothers from milk-transmitting strains results in a similar high incidence of tumors. Conversely, most high incidence strains can be cured from exogenous

MMTV by fosternursing on virus-free mothers, except for the GR. In this strain, the virus causing the high tumor incidence is present as an endogenous, germ-line transmitted provirus and is expressed in the mammary gland of all female mice [reviewed in 35].

It is unknown what the route of the virus is from the ingested milk to the mammary gland in the more mature animals. Lymphocytes serve perhaps as intermediate cells. In studying the path of infection in an animal, one could learn much from the distribution of virus receptors, but only a few retroviral receptor molecules have been identified and the MMTV receptor is not one of them. It usually takes several pregnancies and 6–12 months for tumors to develop, indicating that hormones play an important role as cofactors. In most strains, the tumors arising through virus infection and hormones are themselves independent of hormones, i.e., they grow continuously. Some other strains first develop hormone dependent tumors, that regress after pregnancy and lactation. These hormone dependent tumors arise early, and may become hormone independent after some cycles of growth and regression [reviewed in 35].

4. The molecular biology of viral tumorigenesis

Molecular hybridization techniques showed that in most strains of mice viral DNA was present in varying copy numbers, as endogenous, germ-line transmitted proviruses [21]. Tumors induced by MMTV had acquired additional MMTV sequences, as a consequence of integration of proviral DNA in the host cell genome [36, 37]. The structure of newly acquired and endogenous MMTV proviral DNA could be examined incisively with the aid of restriction enzymes and probes obtained from reverse-transcribing viral RNA [38, 39]. It was very helpful that, in the C3H strain, the endogenous proviruses had PstI restriction sites different from the milk-replicating virus [40, 41]. Using these polymorphic restriction sites in the diagnosis of virus-infected but otherwise normal mammary gland tissue, Cohen *et al.*, could establish that proviral DNA from the exogenous virus was already integrated in normal mammary cells, in copy numbers not significantly lower than in tumors [41]. Other restriction enzymes, such as EcoRI, cut only once in the provirus, generating fragments depending in size on the the actual site of integration. The absence of detectable junction fragments in infected normal glands demonstrated that no population of cells with the provirus at the same site existed [41].

The tumors, however, did contain junction restriction fragments, in stoichiometries indicating that most, if not all cells in a tumor contained a provirus at the very same location [41–47]. Thus, tumors were clonal for the proviral integration sites, and normal tissue was not. The interpretation of this experiment was that acquisition of proviral DNA is not sufficient for tumorigenic transformation, and that only a few or even one cell from an infected population grows out into a tumor [41].

Similar findings were made in other forms of viral tumorigenesis [48–50], and shared most conspicuously with viruses which, like MMTV, were unable to transform cells *in vitro*, and gave tumors after long periods of latency. In contrast, retroviruses that transform cells in culture are generally rapidly oncogenic and contain a viral oncogene, also identified by molecular approaches [1].

The clonal emergence of tumors from mass-infected populations of cells gave a plausible hypothesis to explain viral oncogenesis without viral oncogenes: proviral insertion causes a mutation in the host cell, and only mutations at sites predisposing to tumorigenic growth cause cell transformation [51]. This working hypothesis predicted that different tumors would share proviral integration sites, assuming that only a few of such cellular regions would exist.

A rigorous test of the model was possible by molecular cloning of inserted proviral DNA, thereby isolating the integration site. But before these attempts succeeded, Hayward *et al.* showed that a known cellular gene was among the targets for proviral integration in another system [52]. The majority of Bursal lymphomas induced by Avian Leukosis Virus [ALV], contained proviral DNA integrated near the c-*myc* gene and high levels of c-*myc* transcripts. The proviral insertions near c-*myc* showed for the first time that cellular progenitors of retroviral oncogenes could indeed be activated in natural tumors. In other forms of slow viral oncogenesis, proviral insertions were also found near known oncogenes, for example the ALV insertions near c-*erb*B in erythroleukemias [53].

To return to mammary tumorigenesis, no oncogene known from transforming retroviruses has ever been found to be rearranged by proviral insertion of MMTV. It was hence necessary to identify insertionally activated genes by a technique generally known as transposon tagging, originally used in Drosophila molecular genetics [54]. The principle is to search for tumors with a single or few acquired proviruses, expecting that the relevant mutation has then to be caused by the particular insertions [51, 55]. Molecular clones from the provirus linked to host cell sequences are isolated from tumor cell recombinant DNA libraries. Appropriate fragments of the host cell DNA are then used as a probe on restriction digests of other tumors. The presence of novel, tumor specific restriction fragments that also hybridize with viral probes, is taken as evidence for a common integration site. The relevant gene within the integration domain can be identified by searching for tumor-specific transcripts hybridizing with the host cell probes. Ultimately, a biological assay in which the gene of interest is introduced into appropriate target cells, can provide formal evidence that indeed an oncogene has been uncovered. The assignment to a chromosomal location can indicate whether other genes are near or even identical to the integration domain.

In MMTV-induced mouse mammary tumors, three genes, called *int*-1, *int*-2 and *int*-3 have now been identified and characterized to a variable extent, and the existence of several other genes has been inferred from less complete

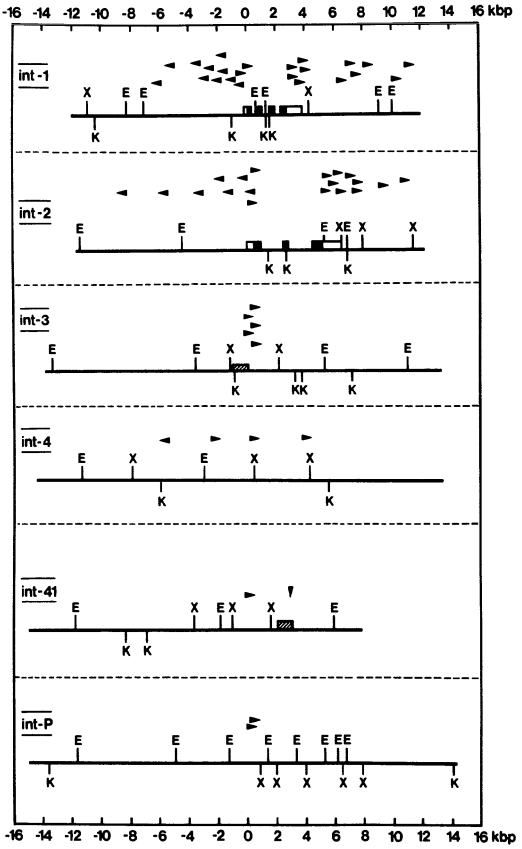


Figure 2. Physical maps of the different *int* regions, with position of some restriction sites. R:EcoRI; K:KpnI; X:XbaI. The arrows indicate the site and orientation of MMTV proviral insertion in tumors; closed blocks at *int*-1 and *int*-2 correspond to the open reading frame; open blocks are transcribed, non-coding parts. The cross-hatched blocks at *int*-3 and *int*-41 indicate the position of probe fragments that hybridize with mRNA from tumors. *int*-P is a rare common integration site from P-type mammary tumors in GR mice, recently shown to be linked to *int*-2. The maps of *int*-2 and *int*-P overlap partially.

data. The individual properties of these genes will be reviewed in the next section. Figure 2 and Table 1 summarize various data on the different *int* loci.

5. *Int*-1

The *int*-1 gene was the first cellular oncogene found by transposon tagging techniques. It was cloned by Nusse and Varmus from a mammary tumor in C3H mice bearing a single inserted MMTV copy [56]. Probes from the host cell DNA adjacent to the provirus detected insertions of MMTV in 70% of other C3H tumors, and a transcript of 2.6 kb found in tumors but not in

Table 1. Properties of different *int* genes

gene	mouse chromosome	human chromosome	transcript (kb)	protein (kD)	frequency of activation in mouse strains
int-1	15	12	2.6	41	80% C3H 70% BR6 30% GR
int-2	7	11	3.2, 3.0, 1.8, 1.4	27	65% BR6 5% C3H 20% GR
int-3	17	?	2.4	?	40% CzII 8% BR6
int-4	11	?	?	?	10% GR
int-41	?	?	5.2	?	#

The data on frequency of activation are obtained in our lab, or derived from references 78, 91 and 94.
The molecular weight of the proteins is deduced from the nucleotide sequence.
int-41 has found to be rearranged in one mammary tumor and one kidney carcinoma.

normal mammary tissue. The transcribed area was named the *int*-1 gene (for integration domain) and mapped on mouse chromosome 15 [57]. Rather surprisingly, the activated gene was situated upstream from the originally cloned provirus, at a considerable distance. This configuration proved subsequently to be the rule: many other proviruses were found to be inserted either upstream from the *int*-1 gene in the opposite transcriptional orientation, or downstream in the same orientation [57] (Figure 2).

Some proviruses were inserted within the *int*-1 gene itself, in the 3′ untranslated region [57]. In these tumors, *int*-1 transcripts were found to be longer than the usual 2.6 kb, and shown to be composed of cellular sequences covalently linked to MMTV LTR sequences. Such transcripts presumably arise by read-through transcription from *int*-1 into the U3 domain of the LTR. The absence of the regular 2.6 kb transcript in those tumors showed that the proviral activation of *int*-1 occurred *in cis*, indicating that *int*-1 is normally not transcribed in the cells forming the tumor [57].

In a few tumors, proviruses were inserted upstream in the same orientation as *int*-1. In one of these tumors, transcripts have been detected starting in the promoter of the 3′LTR of the provirus, proceeding into *int*-1. This represents a rare case of transcriptional activation by promoter insertion, which is seen predominantly in ALV integrations near c-*myc* [52, 58]. There is otherwise little clustering of MMTV insertions in different tumors; very few of the novel *int*-1 fragments are by restriction enzyme analysis identical in size [57].

The structure the *int*-1 gene has been resolved by nucleotide sequence analysis of the area hybridizing with the tumor transcripts, combined with nuclease S1 analysis and cDNA cloning to map the position of the exons [59–61]. The gene consists of four exons that together encode the *int*-1 protein. The first protein-encoding exon is preceded by TATA box. Primer

extension experiments (F. Rijsewijk, unpublished experiments) have shown that the boundary is a genuine startpoint of transcription. The S1 analysis however also indicates the presence of a longer version of the first exon, but it is yet unknown where the promoter of this transcript is [59].

The sequence around the first AUG on the *int*-1 sequence does not conform to a translational start consensus structure [62], and is soon followed by a stop codon. Theoretically, a small peptide of 10 amino acids could be synthesized. The second AUG obeys the translation start signal rules, and is followed by an open reading frame of 370 amino acids, predicting a protein of 41,185 daltons. *In vitro* translation of *int*-1 mRNA generated by SP6 transcription of cDNA yields a protein of approximately 37 kd [60, 61]. The protein-encoding domain of *int*-1 is always present in an intact configuration in tumors [59], arguing convincingly that the high frequency of insertions at *int*-1 is the consequence of selection for expression of the *int*-1 gene product and is not due to preferred integration as such.

Conspicuous properties of the *int*-1 protein, as deduced from the nucleotide sequence, are a hydrophobic leader sequence and a carboxy terminal half which is rich in cysteine residues. These are features shared with growth factors and growth factor receptors and may indicate that the *int*-1 protein functions in signalling between cells. The lack of a transmembrane domain in *int*-1 could be taken as evidence that the protein is secreted and functions as a ligand for a receptor. Assessment of the value of these predictions relies heavily on antisera to detect the actual site of the *int*-1 protein in the cell but such sera have been hard to obtain. By synthesis of short peptides and bacterial fusion proteins containing *int*-1 sequences, several antisera of variable quality have been made (A. Brown, J. Papkoff and H.E. Varmus, pers. comm. and unpublished data from our own lab). These antibodies detect several forms of the *int*-1 protein, differing in extent of glycosylation, by immunoprecipitation from cells expressing the gene. The affinity of the antibodies is rather low, and not sufficient for localization studies by *in situ* techniques. Cell fractionation studies have revealed that *int*-1 protein species enter the secretory pathway, although no direct evidence has obtained for extracellular forms of the protein (J. Papkoff and H.E. Varmus, pers. comm.).

Recent findings have shed light on the biological properties of the *int*-1 gene. Its nature as a genuine oncogene has been illustrated by an *in vitro* transformation assay and expression of the gene in normal tissue has been detected. To introduce the gene in target cells, both retroviral vectors and DNA mediated transfection have been successfully used. A cell line, called C57MG originally derived from normal mammary gland tissue of the C57/B1 mouse strain [63], becomes morphologically transformed by an *int*-1 retrovirus [64]. The cells are also suitable for a focus assay in which groups of transformed cells can be detected on monolayers of flat epithelial cells. Our lab has used another cell line, called RAC, which has been isolated originally from a mammary tumor but has lost its tumorigenicity. Transfection of these cells

with activated *int*-1 copies and co-selection for transfected cells by a dominant marker leads to morphologically transformed cells that, unlike the transformed C57MG cells, are tumorigenic in syngeneic animals [65].

These experiments demonstrate that *int*-1 is truly an oncogene and underscore the validity of the approach taken to identify oncogenes by analysis of mutation sites in tumor cells. A point of concern, nevertheless, is that the cells transformed *in vitro* by *int*-1 are very different from mammary tumor cells in which the gene is activated by MMTV [65, 66]. The latter cells have usually retained many markers of mammary epithelial tissue, detected by monoclonal antibodies to cell surface or intermediate filament proteins [67, 68]. These markers are absent on the RAC cells transfected with *int*-1. The tumors from the transformed cells can be classified as carcinosarcomas, which are otherwise only seen as rare, progressed forms of the characteristic differentiated adenocarcinomas induced by MMTV [66, 67]. It seems necessary therefore, to devise assays for the oncogenic action of *int*-1 that reflect its *in vivo* action more faithfully, for example by gene transfer into primary mammary gland cells.

What about the normal function of *int*-1? In most adult tissues, the gene is not detectably expressed, except for the testis of mature mice, presumably in post meiotic cells [69]. The size of the testis *int*-1 transcript differs slightly from the RNA seen in tumors, but it is not known whether the proteins thus made will differ. During embryogenesis of the mouse, *int*-1 is transiently expressed, between day 8 and day 13 [69]. The site of expression is in the brain, most notably in the mid and hindbrain, but not in the forebrain [69a]. By sophisticated *in situ* RNA hybridization techniques, Wilkinson *et al.* [69b] have detected *int*-1 expression in the developing neural plate and tube, especially at the site of folding of the neural tube. Although these findings do not give immediate clues, they indicate that the normal function of *int*-1 has to be sought in embryonic development of the nervous system. The restricted timing of expression may be taken as evidence for an important role in decisions made during neural differentiation.

More recently, a *Drosophila* homologue of *int*-1 has been cloned, and sequence analysis has shown that the structure of the gene is conserved to a high degree in flies [69c]. The *Drosophila int*-1 gene is expressed in most stages of development, including embryos, larvae, and pupae, but hardly detectable in adult flies. By *in situ* hybridization, a segmented pattern of expression is detectable in embryos. The gene maps at position 28A, and has been shown to be identical to the segment polarity gene *wingless*, a gene involved in pattern formation in insect development. The structure of the D*int*-1/*wingless* gene product indicates that the protein functions in morphogenesis as a signal in cell-cell interaction. These findings underscore the important function of *int*-1 in development, and represent the first example of an oncogene with known developmental mutations, allowing a systematic attack on its function.

The high degree of evolutionary conservation of *int*-1 is one of the most

striking properties of the gene. A human homologue of *int*-1 has been cloned and sequenced, and shows only four amino acid differences with the mouse gene [70, 71]. The changes are restricted to the amino terminal domain and do not affect the hydrophobic character of the leader. The gene has been mapped on chromosome 12 of man, probably at the cen-q14 region [71]. Expression or rearrangements of *int*-1 in normal human tissue or in human tumors tumors has not been found (M. van de Vijver and R. Nusse, unpublished).

6. *Int*-2

The *int*-2 gene was discovered by Peters *et al.* in essentially the same way as *int*-1, but starting from a tumor with multiple acquired proviruses, which were cloned [72]. The flanking sequences from one of these proviruses detected independent integrations in other tumors. The original virus came from a BALB/c mouse fostered on C3H mothers, whereas the other tumors were all from the BR6 strain of mice, a strain generated by crossing the C57/B1 with the RIII. The virus associated most frequently with *int*-2 insertions is therefore from the RIII strain.

The proviruses at *int*-2, with a few exceptions, point away from the gene, and are scattered over relatively large regions [73] (Figure 2). As a matter of fact, probes from the two sides of *int*-2 were originally obtained independently, from different tumors; both first indicating a common integration domain which later turned out to be closely linked [73]. A common integration from P-type mammary tumors in GR mice, first thought to be independent from the known *int* loci, has recently been found in fact to be closely linked to *int*-2 [73a, G. Peters and R. Michalides, pers. comm.], but at a distance of 20 kb from the gene itself. In between the two groups of insertions is the transcriptional unit of *int*-2, which appears to be quite complex [73, 69, 66]. At least four different transcripts are detected in tumors and in normal tissue (see below) that may arise through the use of different promoters and different polyadenylation signals (Peters and Dickson, pers. communication). These transcripts are 3.2, 3.0, 1.8 and 1.4 kb long. The gene has been mapped on mouse chromosome 7 [74].

The structure of the gene has been resolved with nucleotide sequencing and cDNA cloning but is yet incomplete due to the complex pattern of transcription [75]. The assignment of the open reading frame and the derived amino acid sequence of the protein has been less problematic, and was aided by proviral insertions close to the gene but outside of this reading frame. One such insertion is a solitary LTR, causing relatively high amounts of *int*-2 RNA starting at the viral promoter [75]. *int*-2 encodes 245 amino acids, predicting a protein of 27,000 daltons that recently has been shown to be homologous to the fibroblast growth factor family [75, 75a]. The high proportion of arginine and lysine residues, often in pairs, may be indicative of proteolytic processing of

the protein, but good antisera to assay for the *int*-2 gene product have not been developed yet, nor is a biological test available for testing its oncogenic properties. The homology to FGF nevertheless predicts some testable properties of the *int*-2 protein, such as binding to heparin.

Peters *et al.* have explored the biology of the mammary tumors in the BR6 strain to demonstrate that proviral activation of *int*-2 is an early event in the history of the tumor [76]. When hormone-dependent BR6 tumors are followed during several cycles of pregnancy, leading to cycles of growth and disappearance of the tumor, it appears that successive tumors have the same *int*-2 rearrangement [76]. The tumors can thus regress in spite of a rearranged gene, indicating that the cells with the *int*-2 activation are present in a premalignant state in the non-hormone stimulated mammary gland and need additional events to become hormone independent.

Indirect evidence for the oncogenicity of *int*-2 expression comes from the RAC mammary tumor cells [66]. This line is clonal for insertion of MMTV near *int*-2, but sublines nevertheless show dramatic differences in expression of the gene and tumorigenicity. One variant grows as differentiated polygonal cells, positive for many markers of normal mammary epithelium. These cells are tumorigenic, they give rise to adenocarcinomas and express *int*-2. In culture, polygonal cells can convert to a nontumorigenic large cuboidal phenotype. The loss of tumorigenicity is accompanied by loss of *int*-2 expression. To complicate matters, however, the cuboidal cells can transform spontaneously, but also after transfection of activated *ras* or *int*-1 [65], into highly malignant elongated cells, still negative for *int*-2 or mammary cell markers, and giving rise to undifferentiated carcinosarcomas [66].

The function of *int*-2 in normal physiology is probably in early development, even at an earlier stage than *int*-1 involvement [69]. The gene is expressed in mouse embryos at day 7½, and in several embryonic cell lines induced to differentiate into the endodermal lineage. *int*-2 is one of the few genes exclusively expressed during this type of differentiation [69], indicating a key role for the gene in events occurring during early embryogenesis. The findings on expression of the *int* genes during development have attracted wide attention from developmental biologists.

A human homologue of *int*-2 has been isolated and mapped on chromosome 11, band q13 [77]. Expression of *int*-2 in human tumors has not been reported. Restriction fragments hybridizing with *int*-2 probes are only detected in mammalian species and not in phyla where mammary tumors cannot occur.

7. *Int*-3

Gallahan and Callahan screened a number of mammary tumors in the CzII strain for MMTV insertions near *int*-1 and *int*-2, and found that the majority were negative. This CzII strain of mice is particularly suitable for studying MMTV insertions in tumors because it lacks endogenous MMTV proviral

DNA. The proviruses detected in tumors therefore all come from a congenital, milk transmitted infection. The strain has been isolated recently from the wild. The presence of identical viral restriction fragments in different tumors encouraged a search for a new common integration site. Molecular cloning and isolation of host cell probes showed indeed that five out of 16 tumors contained a provirus at a new chromosomal area, called *int*-3 [78]. An additional case of an *int*-3 insertion has been found by Peters *et al.* (pers. comm.) in one of the few BR6 tumors that were negative for *int*-1 or *int*-2. A tumor specific *int*-3 transcript of 2.4 kb is found with probes upstream from the main cluster of insertions, which also appears to be conserved among mammalian species. *int*-3 has been mapped on mouse chromosome 17 [79] and has no appreciable homology with other known genes by molecular hybridization. Unlike the proviruses near *int*-1 and *int*-2, the insertions at *int*-3 are very close to each other, some of them indistinguishable by restriction enzyme analysis. Further cloning of the area is in progress, and may well reveal more insertions at the other side of the transcriptional unit.

8. *Int*-4

The characterization of this integration area is still in its infancy. Probes from a novel common insertion site have been obtained by molecular cloning of a single acquired provirus from a tumor in Balb/c mice in which the GR virus was introduced by fosternursing. Four independent tumors showed insertions in the cloned area, called *int*-4. A transcript has hitherto not been found. *int*-4 has been mapped on chromosome 11 in the mouse (H. Roelink, J. Hilkens and R. Nusse, in preparation).

9. Mutant MMTVs in other diseases; *int*-41

The tumorigenic spectrum of MMTV is not restricted to mammary oncogenesis. The virus has also been implicated in formation of lymphomas in several strains of mice, most notably in males of the GR [34, 80, 81]. There is one report of MMTV involvement in kidney carcinomas in a Balb/c substrain developed by Claude. The virus variants of MMTV that apparently cause these diseases differ from mammary tumor inducing strains in having a different LTR. In particular, regions upstream from the promoter are changed [82–85] (Figure 1). It has been proposed that these alterations generate novel enhancers, that may then cause virus replication in cells otherwise nonpermissive for MMTV [82]. This is an attractive possibility, because it would explain both the high expression of the virus and the induction of a novel type of tumor; by *cis*-enhancement of cellular oncogenes. In one such rearranged LTR, core sequences corresponding to enhancers have indeed been found, but direct evidence for their transcriptional potency has been lacking thusfar.

There is an analogy with Murine Leukemia Virus [MLV] variants that differ in enhancer sequences in the LTR can cause a wide spectrum of diseases. By so-called mix and match experiments—exchanging parts of variant viral genomes and mapping of the disease tropism—it could be shown that indeed LTR differences can change the type of disease induced by these MLVs [86, 87]. Such a degree of sophistication cannot be attained for MMTV, due to the difficulties in cloning the virus but the variant viruses do provide interesting material to examine integration domains in other types of tumors induced by the virus.

Garcia *et al.* have found an integration domain, called *int*-41, that originally was cloned from a mammary tumor in the C3H strain [33]. No other mammary tumors contained an MMTV provirus near *int*-41, but surprisingly, one kidney carcinoma had a rearranged *int*-41 allele, probably due to an MMTV insertion [33]. In addition, probes from the region detected transcripts of various lengths, whose expression could be influenced by dexamethasone indicating that they may have arise as a consequence of an adjacent MMTV enhancer.

10. Various cases of integration domains

A good indication for new common integration sites is sometimes given by comigrating restriction fragments of MMTV, especially if common fragments are produced by more than one enzyme. Two claims for common integrations based on restriction mapping have been made [89, 90]; one such region is called *int*-H, implicated in preneoplastic lesions called hyperplastic alveolar nodules (HANs). These findings need further documentation by molecular cloning, but suggest the possible existence of novel oncogenes, the more since the known *int* genes are rarely activated in these premalignancies.

11. The frequency of activation of the *int* genes; complementation between different genes

At first sight, there are some striking differences in the frequency of rearrangements of the different *int* genes between strains of mice. *int*-1 is activated in 70–80% of C3H tumors, which have only a few *int*-2 insertions, and *int*-2 is frequently found in the BR6 strain, carrying the RIII variant of MMTV. The CzII strain is strongly associated with *int*-3, in turn rarely seen in other strains (Table 1, see also reference 91). These frequencies would suggest that there is some preference for certain *int* genes by virus variants, or mouse strains for that matter. On the other hand, these differences are by no means absolute, exemplified by the original cloning of *int*-2 from a BALB/cfC3H tumor, which, incidentally, has also been cloned by two other groups from a GR tumor [73a, 92]. The issue is also complicated by the poor characterization

of the MMTV variants, which, as outlined before, have never been cloned. Nevertheless, what mechanism could underly such preferences: It is possible that MMTV-induced mammary tumors are not all alike, and that different *int* genes act on different target cells. Another explanation could be that the MMTV enhancers activating these genes do not work equally well on different promoters, as shown by the IgG enhancer that works optimally on the IgG promoter [93].

Peters *et al.* have reported that in the BR6 strain many mammary tumors actually have MMTV insertions at both *int*-1 and *int*-2 [94]. From the stoichiometry of the restriction fragments, and from the fact that transplantation of the tumors did not result in selection for cells with one or the other gene rearranged, it appeared that the tumors are clonal for both *int*-1 and *int*-2. Thus, the genes can act within the same cell, and can perhaps cooperate in transformation. Complementation between different oncogenes has also been observed in fibroblast transformation experiments, and underscores the importance of multi-step mechanisms of oncogenesis. Tumors with a single acquired provirus would then have additional mutations not caused by insertion of viral DNA. It is however not yet clear whether MMTV-induced tumors in other strains have the same frequency of concerted activation of different genes. With probes for more genes available, these issues can now be investigated thoroughly.

A different form of complementation between the *int* genes is suggested by the behavior of early, hormone-dependent mammary tumors in the GR strain. Many of these tumors appear to be oligoclonal for insertions near the different *int* genes, and consist of balanced populations of cells with one or the other gene rearranged [95–97]. We have recently found one tumor with even three different *int* genes rearranged, co-existing during several transplant generations (unpublished). The retention of oligoclonality is highly suggestive for interdependent complementation between cells expressing various *int* genes. The later arising hormone indendent tumors are usually clonal for one of the *int* genes and are probably caused by additional mutations within the hormone-dependent populations.

12. The mechanism of activation of *int* by MMTV

How is the transcriptional activity of the *int* genes in tumors brought about? The genes are silent in normal mammary tissue, and the altered size of the transcripts in those tumors where MMTV has inserted within the transcriptional unit shows that only the *int* allele next to the provirus is transcribed [57]. The insertions have thus induced a normally silent gene to become transcribed.

Usually, the provirus is at a distance from the *int* promotor sequences, points away from the gene, and therefore has to act in an indirect manner on the transcription of the *int* genes. This situation resembles rare cases of ALV-

induced tumors where *myc* is activated by other than promoter insertions [58]. In those tumors, transcriptional activation of *myc* is attributed to the relative strong enhancer on the U3 domain of the ALV LTR [98, 99]. We have proposed, based on the distance and orientation of the MMTV proviruses at *int*-1, that the transcriptional activation of the gene is also caused by an enhancer. The restricted orientation of the MMTV provirus near the *int* genes -pointing away from the gene- could be a consequence of the inability of enhancers to act beyond the first promoter encountered, as documented for the SV40 enhancer [100]. The orientation generally seen for MMTV proviruses near the *int* genes avoids a viral promoter in between the viral enhancer and the *int* promoter.

By gene transfer experiments with recombinant MMTV LTR constructs, it has indeed been shown that the MMTV LTR has an orientation-independent cis-acting transcriptional enhancer [27–32]. This enhancer is relatively weak in most assays however, and needs dexamethasone to become active. More recently, is has been reported that another steroid hormone, progesterone, is able to activate the MMTV enhancer [101]. This finding raises the possibility that the activation of the *int* genes is likewise mediated by sex steroid hormones, an appealing suggestion in view of the role of hormones in initiation of tumorigenesis.

There is nevertheless no evidence that the transcription of *int* genes in mammary tumor cells is controlled by steroid hormones. One way of testing hormonal influences on transcription is by using cell lines from mammary tumors, cultured under defined conditions. Many such permanent mammary tumor derived cell lines are available, and the MJY and the Mm5mtc/1 have MMTV proviral insertions near *int*-1. We have measured *int*-1 transcription in these lines in the presence and absence of dexamethasone, but we found hardly detectable amounts of RNA. A more extensive analysis of these phenomena has been presented by Sonnenberg *et al.*, using the RAC cells mentioned before [66]. There are three variants of this line, with dramatic morphological and biological differences, but yet clonally derived from each other. Polygonal cells are positive for MMTV and *int*-2 expression, which is not influenced by dexamethasone. Cuboidal RAC cells have no *int*-2 expression under any circumstances whereas MMTV expression is dexamethasone-inducible.

More recently, we have made a cell line derived from a tumor with an *int*-1 insertion, called 201, that has been selected on retention of mammary epithelial markers: the line produces small polygonal cells, sometimes growing as domes. It this highly differentiated cell line, expression of *int*-1 is as high as in tumors, even in the absence of steroid hormone.

Based on these results, we have suggested that the MMTV provirus has a cell type dependent enhancer that is active in well differentiated epithelial cells. This enhancer is relatively strong and responsible for the long range *cis*-activation of the *int* genes. In other cells, the steroid hormone-dependent enhancement of MMTV would suffice for activation of the viral promoter,

but does not induce the *int* genes [66]. A further analysis of these phenomena is in progress.

13. Other oncogenes in murine mammary tumors

Besides MMTV and the *int* genes, there are two systems of experimental mammary tumorigenesis with activations of known oncogenes. Chemical carcinogens, when injected in mice or rats during the development of the mammary gland, give rise to mammary tumors later in life, after several cycles of pregnancy. In these tumors, reproducible mutations at the Ha-*ras* gene have been detected by transfection of tumor DNA and/or oligonucleotide hybridizations [102–104]. The interest in this system from the point of view of mammary tumor biology is that MNU, a carcinogen that directly acts on the DNA, is very-short lived and has to exert its action within hours after application. The latency that is apparently necessary for the tumors to develop indicates therefore that additional events must take place before tumors become manifest. The cycles of proliferation of the mammary cells during pregnancy would favor tumorigenesis by enlarging the pool of cells on which such secondary events can occur.

Similar observations have been made in another system: the generation of transgenic mice carrying a *myc* gene activated by the MMTV LTR as promoter [105–106]. These transgenic mice are excellent models for genetic predisposition to tumors, as they have an activated oncogene in all cells. Mammary tumors only develop after cycles of pregnancies and then at one site in the animal, suggesting that additional events leading to clonal outgrowth are involved.

The nature of the secondary events is unknown; activation of the *int* genes or other MMTV-related mutations have not been found in these tumors. The biology of MMTV-caused tumors is actually rather similar to the transgenic mice or the chemical carcinogenesis system. In all three models, oncogene activation is an early event; hormonal stimulations by pregnancy is essential; and clonal tumors finally arise that are themselves not dependent on hormones anymore for growth. Further progression is possible from hormone independent adenocarcinomas towards highly malignant sarcoma-type tumors, but here too, we have no knowledge of relevant oncogene activations. The latter step can be mimicked *in vitro* by transfection of activated *ras*, but also by *int*-1, into the aforementioned cuboidal RAC cells. This may indicate that oncogenes can be interchangeably involved in initiation or progression of tumorigenesis. There is yet no support for this theory from what is known of the *in vivo* course of events.

It is noteworthy that *myc*, once activated by an MMTV LTR inserted in the germ line can cause mammary tumors, but that no proviral insertions near *myc* have been found in 'natural' MMTV induced tumors despite a systematic search (unpublished results). In fact, none of the oncogenes known to us

through other means than by transposon tagging have been reported to be rearranged in virus-induced mammary tumors. Perhaps the growth advantage conferred to cells when the 'non-*int*' oncogenes are activated is not sufficient because these oncogenes are usually transcriptionally active in normal cells, and the increase upon MMTV activation would not be enough to allow selective outgrowth. For the *int* genes, completely inactive in normal cells, a small difference in level of transcription after proviral insertion would then have large consequences in cell proliferation.

At this point, it could also be mentioned that no case of transduction of a cellular oncogene by MMTV has even been observed. In other tumors, proviral insertions near oncogenes are often precursors for transduction of the cellular gene by the virus, illustrated by ALV insertions near c-*erb*B in erythroleukemias [107]. The type of insertions found in those tumors are indeed very favourable for transduction: the provirus and the adjacent c-*erb*B gene are transcribed into a hybrid mRNA that is already quite similar to a viral RNA from an oncogene carrying retrovirus [108]. Such configurations leading to hybrid mRNA are seldomly found at MMTV insertions near the *int* genes, which could explain why the gene has not been transduced.

14. Concluding remarks

MMTV has set a prime example for the proposition that retroviral integration sites can point to the existence of novel cellular oncogenes. The most absorbing task facing us now is to find out how the *int* genes work, which is still difficult in spite of the recent findings on the expression of the genes in embryogenesis and the homology between *int*-2 and FGF. From the sequence of *int*-1 we can infer that the protein is involved in signal transmission from cell to cell, because of the superficial resemblance to the cysteine-rich growth factors and their receptors, but we have no probes yet to sustain such speculations.

In natural tumorigenesis by MMTV, activation of the *int* genes is an early, perhaps the first, event, as concluded from the presence of cells with rearranged genes in hormone-dependent tumors [76, 95]. But these tumors are not very malignant; withdrawal of hormones makes them to disappear. Moreover, a superficial look at the histopathology of these early tumors shows that they are not very different from normal mammary gland tissue; the tumors are well differentiated with ductal and alveolar structures [109]. Therefore, one cannot expect that the effect of the *int* genes as such will be dramatic, and in designing gene transfer experiments one has to take these facts into account. To mention other complications for such assays: mammary tumors with activated *int* genes can also be hormone-independent, grow rapidly and kill a recipient mouse within a few weeks. These cells can thus be called malignant, but yet they do not grow in soft agar, otherwise a good parameter for cell transformation, nor are they immortal in cell culture.

Perhaps most informative with respect to the mechanism of action is the expression of *int*-1 and *int*-2 in embryogenesis. It is now clear that the identification of *int*-1 in *Drosophila* as the segment polarity gene *wingless* will lead to incisive information of its actual function in development and perhaps, by extrapolation, in tumorigenesis. We may even be dealing with a whole new class of mechanisms in oncogenesis, caused by proteins which in normal development are instrumental in differentiation during embryogenesis, working through signalling between cells. The homology between *int*-2 and FGF, and the activity of FGF-like molecules in mesoderm induction in *Xenopus* embryos point in the same direction [109a].

In studying the mechanism of action of the *int* genes in tumuorigenesis, we also wish to learn where in tumors the *int* genes are expressed. In the cell lines that our lab has derived from mammary tumors, expression of *int*-1 and *int*-2 correlates with differentiation and follows the dexamethasone-independent expression of MMTV. If one looks by immunoperoxidase techniques at the actual site of expression of MMTV in primary tumors, one sees, not surprisingly, that the viral antigens are made in the diffentiated luminal cells. Are those cells also the cells making *int* products? In the absence of good antisera we can only guess that they do. But differentiated luminal cells have lost proliferative capacity and are unlikely to become transformed. It is more plausible that the stem cells in the basal layers are the precursors of tumors [110]. I would therefore like to speculate that expression of the *int* genes generates a signal from the differentiated layer in the mammary gland towards the basal cell layer, disturbing the normal signals that the differentiated cells use to arrest proliferation of their basal precursor cells. Such a state of continued proliferation would generate the kind of hyperplasia found in the early hormone-dependent GR tumors, where it seems indeed that different cells grow in an interdependent fashion.

Needless to say, these speculations are far-fetched, but they may serve as a working hypothesis to design experiments in a system that is of general importance in the biology of epithelial tumorigenesis.

Acknowledgments

I thank Arnoud Sonnenberg for numerous discussions, people in my lab and Drs. H. Varmus, C. Dickson and G. Peters for contributions of unpublished data, and Piet Borst and Betsy Matthews for comments on the manuscript. Work in my laboratory is supported by grants from the Netherlands Cancer Foundation KWF.

References

1. Weiss R, Teich N, Varmus HE, Coffin J: The molecular biology of tumor viruses; RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

2. Hilgers J, Bentvelzen P: Interaction between viral and genetic factors in murine mammary cancer. Adv Cancer Res 26:143–195, 1979.
3. Michalides R, Van Ooyen A, Nusse R: Mouse mammary tumor virus expression and mammary tumor development. Curr Top Microbiol Immunol 106:57–78, 1983.
4. Hageman PC, Calafat J, Hilgers J: The biology of the mouse mammary tumor virus. In: Mammary tumors in the mouse, pp 391–463. Hilgers J and Sluyser M, (eds), Elsevier/ North-Holland, Amsterdam, 1981.
5. Majors JE, Varmus HE: Nucleotide sequences at host-proviral junctions for mouse mammary tumor virus. Nature 289:253–258, 1981.
6. Majors J, Varmus HE: Nucleotide sequencing of an apparent proviral copy of env mRNA defines determinants of expression of the mouse mammary tumor virus env gene. J Virol 47:495–504, 1983.
7. Groner B, Buetti E, Diggelmann H, Hynes NE: Characterization of endogenous and exogenous mouse mammary tumor virus proviral DNA with site-specific molecular clones. J Virol 36:734–745, 1980.
8. Ucker DS, Ross SR, Yamamoto KR: Mammary tumor virus DNA contains sequences required for its hormone-regulated transcription. Cell 27:257–266, 1981.
9. Fasel N, Buetti E, Firzlaff J, Pearson K, Diggelmann H: Nucleotide sequence of the 5′ noncoding region and part of the gag gene of mouse mammary tumor virus; identification of the 5′ splicing site for subgenomic mRNAs. Nucleic Acids Res 11:6943–6955, 1983.
10. Moore R, Dixon M, Smith R, Peters G, Dickson C: Complete nucleotide sequence of a milk-transmitted mouse mammary tumor virus: two frameshift suppression events are required for translation of gag and pol. J Virol 61:480–490, 1987.
11. Brookes S, Placzek M, Moore R, Dixon C, Peters G: Insertion elements and transitions in cloned mouse mammary tumour virus DNA: Further delineation of the poison sequences. Nucleic Acids Res. 14:8231–8245.
12. Buetti E, Diggelmann H: Cloned mouse mammary tumor virus DNA is biologically active in transfected mouse cells and its expression is stimulated by glucocorticoid hormones. Cell 23:335–345, 1981.
13. Diggelmann H, Vessaz AL, Buetti E: Cloned endogenous mouse mammary tumor virus DNA is biologically active in transfected mouse cells and its expression is stimulated by glucocorticoid hormones. Virology 122:332–341, 1982.
14. Salmons B, Groner B, Calberg-Bacq CM, Ponta H: Production of Mouse Mammary Tumor Virus upon transfection of a recombinant proviral DNA into cultured cells. Virology 144:101–114, 1985.
15. Dickson C, Peters G: Protein-coding potential of mouse mammary tumor virus genome RNA as examined by *in vitro* translation. J Virol 37:36–47, 1981.
16. Fasel N, Pearson K, Buetti E, Diggelmann H: The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long open reading frame and signals for hormonally regulated transcription. EMBO J 1:3–7, 1982.
17. Donehower LA, Huang AL, Hager GL: Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy. J Virol 37:226–238, 1981.
18. Donehower LA, Andre J, Berard DS, Wolford RG, Hager GL: Construction and characterization of molecular clones containing integrated mouse mammary tumor virus sequences. In: Cold Spring Harbor Symp. Quant. Biol., pp 1153–1159. Cold Spring Harbor, New York, 1980.
19. Kennedy N, Knedlitschek G, Groner B, Hynes NE, Herrlich P, Michalides R, Van Ooyen AJJ: Long terminal repeats of endogenous mouse mammary tumor virus contain a long open reading frame which extends into adjacent sequences. Nature 295:622–624, 1982.
20. Bentvelzen P, Daams JH, Hageman P, Calafat J: Genetic transmission of viruses that incite mammary tumors in mice. Proc Natl Acad Sci USA 67:377–384, 1970.
21. Varmus HE, Bishop JM, Nowinski RC, Sarkar NH: Mammary tumor virus specific nucleotide sequences in DNA of high and low incidence mouse strains. Nature New Biol 238:189–190, 1972.

22. Parks WP, Scolnick EM, Kozikowski EH: Dexamethasone stimulation of murine mammary tumor virus expression: tissue culture source of virus. Science 183:158–60, 1974.
23. Young HA, Scolnick EM, Parks WP: Glucocorticoid-receptor interaction and induction of murine mammary tumor virus. J Biol Chem 250:3337–3343, 1975.
24. Ringold GM, Yamamoto KR, Tomkins GM, Bishop JM, Varmus HE: Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action. Cell 6:299–305, 1975.
25. Buetti E, Diggelman H: Glucocorticoid regulation of mouse mammary tumor virus: Identification of a short essential DNA region. EMBO J 2:1423–1429, 1983.
26. Chandler VL, Maler BA, Yamamoto KR: DNA sequences bound specifically by glucocorticoid receptor *in vitro* render a heterologous promoter hormone responsive *in vivo*. Cell 33:489–499, 1983.
27. Hynes N, van Ooyen AJJ, Kennedy N, Herrlich P, Ponta H, Groner B: Subfragments of the large terminal repeat cause glucocorticoid-responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc Natl Acad Sci USA 80:3637–3641, 1983.
28. Majors J, Varmus HE: A small region of the mouse mammary tumor virus long terminal repeat confers glucocorticoid hormone regulation on a linked heterologous gene. Proc Natl Acad Sci USA 80:5866–5870, 1983.
29. Payvar FP, Defranco D, Firestone GL, Edgar B, Wrange O, Ockert S, Gustafsson J-A, Yamamoto KR: Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35:381–392, 1983.
30. Ucker DS, Firestone GL, Yamamoto KR: Glucocorticoids and chromosomal position modulate murine mammary tumor virus transcription by affecting efficiency of promoter utilization. Mol Cell Biol 3:551–561, 1983.
31. Godowski PJ, Rusconi S, Miesfeld R, Yamamoto KR: Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature 325:365–368, 1987.
32. Yamamoto KR: Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet 19:209–252, 1985.
33. Garcia M, Wellinger R, Vessaz A, Diggelmann H: A new site of integration for mouse mammary tumor virus proviral DNA common to BALB/cf (C3H) mammary and kidney adenocarcinomas. EMBO J 5:127–134, 1986.
34. Michalides R, Wagenaar E, Hilkens J, Hilgers J, Groner B, Hynes NE: Acquisition of proviral DNA of mouse mammary tumor virus in thymic leukemia cells from GR mice. J Virol 43:819–829, 1982.
35. Hilgers J, Sluyser M: Mammary tumors in the mouse. Elsevier/North-Holland, Amsterdam, 1981.
36. Michalides R, Vlahakis G, Schlom J: A biochemical approach to the study of the transmission of mouse mammary tumor viruses in mouse strains RIII and C3H. Int J Cancer 18:105–115, 1976.
37. Morris VL, Medeiros E, Ringold GM, Bishop JM, Varmus HE: Comparison of mouse mammary tumor virus-specific DNA in inbred, wild and Asian mice, and in tumors and normal organs from inbred mice. J Mol Biol 114:73–92, 1977.
38. Michalides R, van Nie R, Nusse R, Hynes NE, Groner B: Mammary Tumor induction loci in GR and DBAf mice contain one provirus of the Mouse Mammary Tumor Virus. Cell 23:165–173, 1981.
39. Cohen JC, Varmus HE: Endogenous mammary tumor virus DNA varies among wild mice and segregates during inbreeding. Nature 278:418–423, 1979.
40. Shank PR, Cohen JC, Varmus HE, Yamamoto KR, Ringold GM: Mapping of linear and circular forms of mouse mammary tumor virus DNA with restriction endonucleases: Evidence for a large specific deletion occurring at high frequency during circularization. Proc Natl Acad Sci USA 75:2112–2116, 1978.
41. Cohen JC, Shank PR, Morris VL, Cardiff R, Varmus HE: Integration of the DNA of

mouse mammary tumor virus in virus-infected normal and neoplastic tissues of the mouse. Cell 16:333–345, 1979.
42. Cohen JC, Varmus HE: proviruses of mouse mammary tumor virus in normal and neoplastic tissues from GR and C3Hf mouse strains. J Virol 35:298–305, 1980.
43. Groner B, Hynes N: Number and location of mouse mammary tumor virus proviral DNA in mouse DNA of normal tissue and of mammary tumors. J Virol 33:1013–1025, 1980.
44. Michalides R, Wagenaar E, Groner B, Hynes NE: Mammary tumor virus proviral DNA in normal murine tissue and nonvirally induced mammary tumors. J Virol 39:367–376, 1981.
45. Morris V, Gray DA, Jones RF, Chan ECMLee, McGrath CM: Mouse mammary tumor virus DNA sequences in tumorigenic and non-tumorigenic cells from a mammary adenocarcinoma. Virology 118:117–127, 1982.
46. Altrock BW, Cardiff RD, Puma JP, Lund JK: Detection of acquired provirus sequences in mammary tumors from low-expressor, low-risk mice. J Natl Cancer Inst 68:1037–1041, 1982.
47. Puma JP, Fanning TG, Young LJT, Cardiff RD: Identification of a unique mouse mammary tumor virus in the BALB/cNIV mouse strain. J Virol 43:158–165, 1982.
48. Steffen D, Weinberg RA: The integrated genome of murine leukemia virus. Cell 15: 1003–1010, 1978.
49. Payne GS, Courtneidge SA, Crittenden LB, Fadly AM, Bishop JM, Varmus HE: Analysis of avian leukosis virus DNA and RNA in bursal tumors: Viral gene expression is not required for maintenance of the tumor state. Cell 23:311–322, 1981.
50. Neel BG, Hayward WS, Robinson HL, Fang J, Astrin SM: Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete viral RNAs: Oncogenesis by promoter insertion. Cell 23:323–334, 1981.
51. Varmus HE: The Molecular Genetics of Cellular Oncogenes. Ann Rev Genet 18:553–612, 1984.
52. Hayward WG, Neel BE, Astrin SM: Activation of a cellular oncogene by promoter insertion in ALV induced lymphoid leukosis. Nature 290:475–480, 1981.
53. Fung YKT, Lewis WG, Crittenden LB, Kung HJ: Activation of the cellular oncogene c-*erb*B by LTR insertion: Molecular basis for induction of erythroblastosis by Avian Leukosis Virus. Cell 33:357–368, 1983.
54. Bingham PM, Levis R, Rubin GM: Cloning of DNA sequences from the white locus of D. melanogaster by a novel and general method. Cell 25:693–704, 1981.
55. Nusse R: The activation of cellular oncogenes by retroviral insertion. Trends Genet 2:244–247, 1986.
56. Nusse R, Varmus HE: Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31:99–109, 1982.
57. Nusse R, van Ooyen A, Cox D, Fung YKT, Varmus HE: Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. Nature 307:131–136, 1984.
58. Payne GS, Bishop JM, Varmus HE: Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature 295:209–213, 1982.
59. van Ooyen A, Nusse R: Structure and nucleotide sequence of the putative mammary oncogene int-1: Proviral insertions leave the protein-encoding domain intact. Cell 39: 233–240.
60. Fung YKT, Shackleford GM, Brown AMC, Sanders GS, Varmus HE: Nucleotide sequence and expression *in vitro* of cDNA derived from mRNA of *int*-1, a provirally activated mouse mammary oncogene. Mol Cell Biol 5:3337–3344, 1985.
61. Rijsewijk FAM, Van Lohuizen M, van Ooyen A, Nusse R: Construction of a retroviral cDNA version of the *int*-1 mammary oncogene and its expression *in vitro*. Nucl Acids Res 14:693–702, 1986.
62. Kozak M: Compilation and analysis of sequence upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res 12:857–872, 1984.

63. Vaidya AB, Lasfargues EY, Sheffield JB, Coutinho WG: Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. Virology 90: 12–22, 1978.
64. Brown AMC, Wildin RG, Prendergast TJ, Varmus HE: A retrovirus vector expressing the putative mammary oncogene *int*-1 causes partial transformation of a mammary epithelial cell line. Cell 46:1001–1009.
65. Rijsewijk F, Van Deemter L, Wagenaar E, Sonnenberg A, Nusse R: Transfection of the *int*-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J 6:127–131, 1987.
66. Sonnenberg A, Van Balan P, Hilgers J, Schuuring E, Nusse R: Oncogene expression during progression of mouse mammary tumor cells; activity of a proviral enhancer and the resulting expression of *int*-2 is influenced by the state of differerentiation. EMBO J. 6: 121–125, 1987.
67. Sonnenberg A, Daams J, van der Valk MA, Hilkens J, Hilgers J: Development of the mouse mammary gland: Identification of stages in the differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. J Histochem Cytochem 34:1037–1046, 1986.
68. Sonnenberg A, Daams H, Calafat J, Hilgers J: *In vitro* differentiation and progression of mouse mammary tumor cells. Cancer Res 46:5913–5922, 1986.
69. Jakobovits A, Shackleford GM, Varmus HE, Martin GR: Two proto-oncogenes implicated in mammary carcinogenesis, *int*-1 and *int*-2, are independently regulated during mouse development. Proc Natl Acad Sci USA 83:7806–7810, 1986.
69a. Shackleford GM, Varmus HE: Expression of the proto-oncogene, *int*-1, is restricted to postmeiotic male germ cells and the neural tube of midgestational embryos. Cell: 50:85–95, 1987.
69b. Wilkinson DG, Bailes JA, McMahon AP: Expression of the proto-oncogene *int*-1 is restricted to specific neural cells in the developing mouse embryo. Cell: 50:79–88, 1987.
69c. Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, Nusse R: The Drosophila homologue of the mammary oncogene *int*-1 is identical to the segment polarity gene wingless. Cell: 50:649–657, 1987.
70. Van Ooyen A, Kwee V, Nusse R: The nucleotide sequence of the human *int*-1 mammary oncogene; evolutionary conservation of coding and noncoding sequences. EMBO J 4: 2905–2909, 1985.
71. Van 't Veer LJ, Geurts van Kessel A, Van Heerikhuizen H, Van Ooyen A, Nusse R: Molecular cloning and chromosomal assignment of the human homolog of *int*-1, a mouse gene implicated in mammary tumorigenesis. Mol Cell Biol 4:2532–2534, 1984.
72. Peters G, Brookes S, Smith R, Dickson C: Tumorigenesis by Mouse Mammary Tumor Virus: Evidence for a common region for provirus integration in Mammary Tumors. Cell 33:369–377, 1983.
73. Dickson C, Smith R, Brookes S, Peters G: Tumorigenesis by mouse mammary tumor virus: Proviral activation of a cellular gene in the common integration region *int*-2. Cell 37:529–536, 1984.
73a. Schuermann M, Michalides R: A rare common integration site of proviruses of the mouse mammary tumor virus in P-type mammary tumors of mouse strain GR. Virology 156: 229–238.
74. Peters G, Kozak C, Dickson C: Mouse mammary tumor virus integration regions *int*-1 and *int*-2 map on different mouse chromosomes. Mol Cell Biol 4:375–378.
75. Moore R, Casey G, Brookes S, Dixon M, Peters G, Dickson C: Sequence, topography and protein coding potential of mouse *int*-2: A putative oncogene activated by mouse mammary tumor virus. EMBO J 5:919–924, 1986.
75a. Dickson C, Peters G: Potential oncogene product related to growth factors. Nature 326:833, 1987.
76. Peters G, Lee A, Dickson C: Activation of Cellular gene by mouse mammary tumor virus may occur early in tumor development. Nature 309:273–275, 1984.

77. Casey G, Smith R, McGillivray D, Peters G, Dickson C: Characterization and chromosome assignment of the human homolog of *int*-2, a potential proto-oncogene. Mol Cell Biol 6:502–510, 1986.
78. Gallahan D, Callahan R: Mammary tumorigenesis in feral mice: identification of a new *int* locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. J Virol 61: 66–74.
79. Gallahan D, Kozak C, Callahan R: A new common integration region (*int*-3) for mouse mammary tumor virus on mouse chromosome 17. J Virol 61:218–220, 1987.
80. Dekaban GA, Ball JK: Integration of type B retroviral DNA in virus-induced primary murine thymic lymphomas. J Virol 52:784–792, 1984.
81. Dudley J, Risser R: Amplification and novel locations of endogenous mouse mammary tumor virus genomes in mouse T-cell lymphomas. J Virol 49:92–101, 1984.
82. Michalides R, Wagenaar E, Weijers P: Rearrangement in the LTR of extra MMTV proviruses in T cell leukemias of GR result in a novel enhancer-like structure. Mol Cell Biol 5:823–830, 1985.
83. Kwon BS, Weissman SM: Mouse mammary tumor virus related sequences in mouse lymphocytes are induced by 12-o-tetradecanoyl phorbol-13-acetate. J Virol 52:1000–1004, 1984.
84. Wellinger RJ, Carcia M, Vessaz A, Diggelmann H: Exogenous mouse mammary tumor virus proviral DNA isolated from a kidney adenocarcinoma cell line contains alterations in the U3 region of the long terminal repeat. J Virol 60:1–11, 1986.
85. Dudley JP, Arfsten A, Hsu C-LL, Kozak C, Risser R: Molecular cloning and characterization of mouse mammary tumor proviruses from a T-cell lymphoma. J Virol 57:385–388, 1986.
86. Lenz J, Celander D, Crowther RL, Patarca R, Perkins D W, Haseltine WA: Determination of the leukaemogenicity of a murine retrovirus by sequences within the long terminal repeat. Nature 308:467–469, 1984.
87. DesGroseillers L, Rassart E, Jolicoeur P: Thymotropism of murine leukemia virus is conferred by its long terminal repeat. Proc Natl Acad Sci USA 80:4203–4207, 1983.
88. Etkind PR, Sarkar NH: Integration of new endogenous Mouse Mammary Tumor Virus proviral DNA at common sites in the DNA of Mammary tumors of C3Hf mice and hypomethylation of the endogenous mouse mammary tumor virus proviral DNA in C3Hf mammary tumors and spleens. J Virol 45: 114–123, 1983.
90. Gray DA, McGrath CM, Jones RF, Morris VL: A common mouse mammary tumor virus integration site in chemically induced precancerous mammary hyperplasias. Virology 148: 360–368, 1986.
91. Escot C, Hogg E, Callahan R: Mammary tumorigenesis in feral Mus cervicolor popaeus. J Virol 58:619–625, 1986.
92. Morris DW, Cardiff RD: Multistep model of mouse mammary tumor development. In: Advances in Viral Oncology, pp 123–140. Klein G, (ed), Raven Press, New York.
93. Carcia JV, Bich-Thuy L, Stafford J, Queen C: Synergism between immunoglobulin enhancers and promoters. Nature 322:383–385, 1986.
94. Peters G, Lee AE, Dickson C: Concerted activation of two potential proto-oncogenes in carcinomas induced by mouse mammary tumor virus. Nature 320:628–631, 1986.
95. Mester J, Wagenaar E, Sluyser M, Nusse R: Activation of the *int*-1 and *int*-2 mammary oncogenes in hormone-dependent and -independent mammary tumors of GR mice. J Virol 61:, (in press).
96. Gray DA, Jackson DP, Percy DH, Morris VL: Activation of *int*-1 and *int*-2 loci in GRf mammary tumors. Virology 154:271–278, 1986.
97. Macinnes JI, Chan ECML, Percy DH, Morris VL: Mammary tumors from GR mice contain more than one population of mouse mammary tumor virus infected cells. Virology 113:119–129, 1981.
98. Weber F, Schaffner W: Enhancer activity correlates with theoncogenic potential of avian retroviruses. EMBO J 4:949–956, 1985.

99. Luciw PA, Bishop JM, Varmus HE, Capecchi MR: Location and function of retroviral and SV40 sequences that enhance biochemical transformation after microinjection of DNA. Cell 33:705–716, 1983.
100. Wasylyk B, Wasylyk C, Augereau P, Chambon P: The SV40 72 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. Cell 32:503–514, 1983.
101. Cato ACB, Miksicek R, Schuetz G, Arnemann J, Beato M: The hormone regulatory element of Mouse Mammary Tumor Virus mediates progesterone induction. EMBO J 5:2237–2240, 1986.
102. Barbacid M: Mutagens, oncogens and cancer. Trends Genet 2:188–192, 1986.
103. Sukumar S, Notario V, Martin-Zanca D, Barbacid M: Induction of mammary carcinomas in rats by nitroso-methylurea involves malignant ativation of H-*ras*-1 locus by single point mutations. Nature 306:658–661, 1983.
104. Zarbl H, Sukumar S, Arthur AV, Martin-Zanca D, Barbacid M: Direct mutagenesis of Ha-*ras*-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315:382–385, 1985.
105. Stewart TA, Pattengale PK, Leder P: Spontaneous mammary adenocarcinomas in transgeneic mice that carry and express MTV/myc fusion genes. Cell 38:627–637, 1984.
106. Leder A, Pattengale PK, Kuo A, Stewart T, Leder P: Consequences of widespread deregulation of the c-*myc* gene in transgenic mice: Multiple neoplasms and normal development. Cell 45:485–495, 1986.
107. Miles BD, Robinson HL: High frequency of transduction of c-*erb*B in Avian Leukosis Virus induced erythroblastosis. J Virol 54:295–303, 1985.
108. Nilsen TW, Maroney PA, Goodwin RG, Rottman P, Crittenden L, Raines M, Kung H-J. c-*erb*B activation in ALV-induced erythroblastosis: Novel RNA processing and promoter insertion result in expression of an aminotruncated EGF receptor. Cell 41–719–726, 1985.
109. Van Nie R: Mammary tumorigenesis in the GR mouse strain. In: Mammary tumors in the mouse, pp. 201–266. Hilgers J and Sluyser M, (eds), Elsevier/North Holland, Amsterdam, 1981.
109a. Slack JMW, Darlington BG, Health JK, Godsave SF: Mesoderm induction in early Xenopus embryos by heparinbinding growth factor. Nature 326:197–200, 1987.
110. Dulbecco R Henahan M, Armstrong B: Cell types and morphogenesis in the mammary gland. Proc Natl Acad Sci USA 79:7346–7350, 1982.

15. Steroid hormone regulation of cultured breast cancer cells

Philippa D. Darbre, and Roger J.B. King

1. General introduction

Most of the information we have about the regulation of breast tumor growth relates to hormones, especially steroid hormone effects. Such actions are diverse and complex involving direct effects on the tumor cells and indirect effects via other endocrine and non-endocrine organs. Because of this complexity, it is extremely difficult to interpret *in vivo* data at the required level of cellular and molecular organization so cell culture systems have to be extensively used. Despite the general dogma that 'cancer cells have escaped from growth control', establishment of cultures from primary breast tumors has been difficult, more difficult in fact than with normal mammary cells [1]. Furthermore, the establishment of meaningful hormonal regulation of such cultures has been elusive. This has led many workers, ourselves included, to use established cell lines to answer the question of how do steroids affect mammary tumor cell growth? Cell lines clearly have the advantage of relative cellular homogeneity, reproducibility from one experiment to another, ease of experimental manipulation and an approximately defined medium in which growth can be studied. The word 'approximately' is used because, although serum free conditions have been used for looking at short to medium-term effects of steroids [2–4], serum is present in the majority of such experiments and is always used for long-term maintenance of such cultures. Despite all these advantages, it is noteworthy that until relatively recently debate continued as to whether or not steroids such as oestradiol had direct mitogenic effects on target cells [4–6]. Whilst there are undoubted indirect influences of steroid hormones, we feel that the ability to reproducibly demonstrate proliferative effects of steroids on cultured cells especially under serum-free conditions adequately justify the view that steroids do have direct effects on cell growth. However, it is equally true that many other factors also influence proliferation and by relatively simple manipulation of culture conditions such as raising the serum concentration one can accelerate growth in the absence of steroid to a stage where steroid addition has no effect [7].

On the debit side, cell lines must be acknowledged to be very abnormal because of their selection from a minority of the original population and because

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology.ISBN 978-1-4612-8975-3

of their immortality. Furthermore, the very reasons for their use as outlined above cannot detract from the fact that the complexity of *in vivo* conditions is the norm.

As a final general point, one should ask the question of what parameter of cell behavior in culture is most relevant to conditions that modulate the size of a tumor *in vivo*? Whilst it is obvious that cell proliferation is important, it is pertinent that in culture the phenomenon of density regulation ensures a limiting cell density above which proliferation is markedly reduced. Yet, in a small tumor lump, the cells are already crowded together and yet the lump can be made to grow much larger. From this type of logic we feel that our present state of biological ignorance dictates that steroid effects on all cell biological properties should be investigated.

Questions about steroid regulation of cultured breast tumor cells can essentially be broken down into two parts. Firstly, one can ask, in the short term, how do steroid hormones control tumor cell growth? In other words, how do steroids affect the rate of cell proliferation and the loss of control on growth? Secondly, the longer term question asks what renders a breast cancer cell hormone sensitive or insensitive, and why do cancer cells progress to the inevitable state of steroid hormone autonomy? Although not explicitly stated, it is generally accepted that these two questions are intimately related but this is not necessarily the case and we have addressed both these problems.

1.1 Mammary tumor cell lines

At the time we started our work no human lines were available and so we followed the initial observation of Meakin [8] by generating an androgen responsive cell line [7] from the spontaneous mouse mammary carcinoma of Shionogi DD/Sio mice [9]. This tumor (designated Shionogi 115) shows androgen-dependent growth but, after androgen withdrawal, manifests temporary regression followed by androgen-independent growth [10, 11]. The cell line we produced came from this Shionogi S115 tumor and the line was, with great ingenuity, called S115. Its general characteristics have been detailed elsewhere [11, 12] and the main features described in the older literature are given in Table 1. The overall picture is of a receptor mediated series of events which led, after a significant lag phase, to increased DNA synthesis in monolayer culture.

More recent studies have used a clone of androgen-responsive cells from this original line. These cloned S115 +A cells are now used as our *in vitro* model system for study. When these cells are maintained in tissue culture in the presence of testosterone, they exhibit cellular and molecular responses to androgen. However, long term maintenance of such cells in the absence of steroid results in the cells becoming unresponsive to androgen and such cells are then termed −A cells. Thus, these cells provide a model system for studying both short-term steroid hormone action on breast tumor cells and the

Table 1. General characteristics of the S115 mouse mammary tumor cell line.

Property	Comments
1. Receptor content.	Contain androgen and glucocorticoid receptors; estrogen and progestin receptors undetectable.
2. Cell growth.	Proliferation is stimulated by testosterone and 5α dihydrotestosterone; testosterone effective without metabolism to 5α dihydrotestosterone. Glucocorticoids inhibit log-phase growth. Pharmacological levels of estradiol, but not diethylstilboestrol, stimulate proliferation via the androgen receptor.
3. Receptor function.	Proliferative effects are receptor mediated; high concentrations of estradiol stimulate proliferation via the androgen receptor.
4. Postreceptor events.	Lag of about 8 hr between steroid addition and increased DNA synthesis. Ribosomal RNA synthesis changes at about the same time. No increase in total poly A+ mRNA or total protein synthesis occurs on steroid addition.
5. Mechanism.	Androgen accelerates proliferation by increasing the probability of overcoming a block towards the end of the Gl phase of the cell cycle.
6. Chromosome content.	Polyploid.

These early data are taken from [11, 12].

longer term problem of how breast tumor cells progress to a state of steroid hormone autonomy.

We have more limited experience with the ZR-75-1 human breast cancer cell line derived from a clinically hormone unresponsive pleural effusion from a breast cancer patient [13]. Although there is no way of being certain, the apparent anomaly of getting a hormone sensitive cell line from an unresponsive tumour is probably explained by cellular heterogeneity of the original tumour. We have used the ZR-75-1 line in preference to the more common MCF-7 line as we find the former to be more reproducible in its responsiveness to estrogens.

2. Mechanism of steroid action

2.1 Steroid regulation of S115 cell biology

S115 + A cells possess receptors for androgen (61 fmol/mg protein) and glucocorticoid (237 fmol/mg protein) but not estrogen or progestin. In accordance, growth of these cells is responsive to both testosterone and the synthetic glucocorticoid, dexamethasone but not progesterone or physiological levels of estradiol. Growth has been studied as either anchorage-dependent (monolayer culture) or anchorage-independent (suspension culture) and each can be

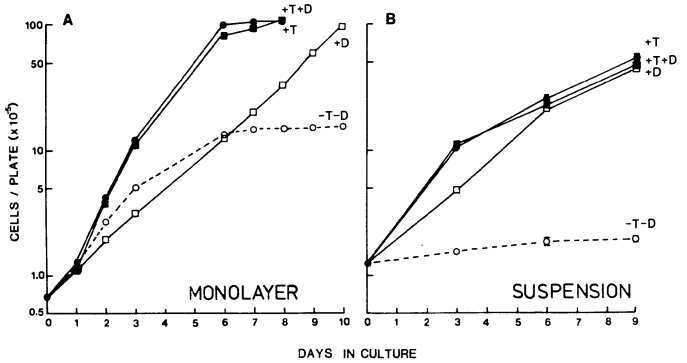


Figure 1. Effects of testosterone (T) and dexamethasone (D) on growth of S115 +A cells in monolayer (A) and suspension (B) culture. Cells were grown with 3.5×10^{-8}M testosterone (●——●), with 10^{-7}M dexamethasone (□——□), with both hormones (■——■) or without either hormone (○----○). Taken from [15].

described in terms of log-phase proliferation rate, saturation density or cell morphology.

In monolayer culture, testosterone increases both growth rate and saturation density (Figure 1A) [7, 14, 15] and in addition causes a marked change from epithelial morphology (− hormone) to fibroblastic morphology (+ hormone) (Figure 2) [16]. In the absence of hormone, there is an increased sensitivity to growth stimuli, such as serum concentration [14] and marked changes occur in the cell membrane and cytoskeletal structure. Specific focal adhesions appear between cell and substrate, there is an increase of cell surface fibronectin, and intracellular actin becomes organized into well-defined microfilament bundles [17, 18]. Dexamethasone exerts the same stimulation of saturation density (Figure 1A) and the same morphological changes (Figure 2) in monolayer as testosterone, but the effects on log-phase growth are different—it is increased by testosterone but decreased by dexamethasone (Figure 1A) [15].

In suspension culture, a dramatic difference is seen between growth in the presence and absence of steroid (Figure 1B, 2) [17]. In the absence of steroid, the cells remain viable but static, whereas in the presence of either testosterone or dexamethasone, rapid growth occurs [15]. Thus, whilst growth in monolayer is only responsive to steroid, growth in suspension is totally dependent on steroid. These same effects are also seen in agar and methylcellulose semi-solid culture systems, implying that steroids result in loss of dependence on anchorage to the substrate for growth.

Dose-response curves for the effects of testosterone and dexamethasone on log-phase growth and saturation density in monolayer and on growth in suspension, revealed maximal effects at about 10^{-8}M in each case (Figure 3) [15]. Interestingly, there is no additive effect of the two hormones in either monolayer (Figure 1A) or suspension (Figure 1B) culture.

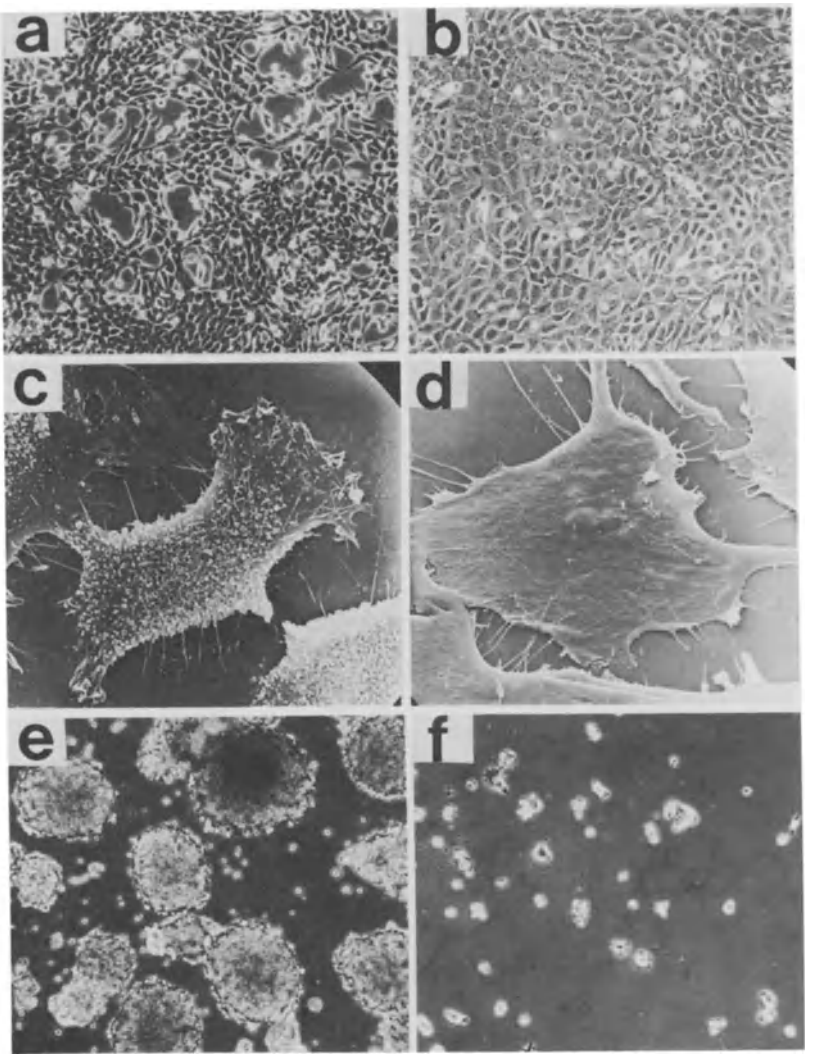


Figure 2. Photographs of S115 +A cells growing in monolayer (a-d) and suspension (e-f) culture either in the presence (a,c,e) or absence (b,d,f) of 3.5×10^{-8}M testosterone. Photographs were taken by phase contrast at mag. ×100 (a,b,e,f) or by scanning electron microscopy at mag. ×5100 (c) or mag. ×4500 (d).

The effects of steroids on the cell biology of S115 +A cells are summarised in Table 2. It is evident that steroid hormones affect many different parameters of cell growth, but not always in the same way. Thus, similar steroid effects on one parameter do not necessarily imply similar effects on all the other parameters. Another question which arises concerning steroid effects is that of the interrelationship between the different parameters. More recently, we have been able to show separation of some of the parameters. From the

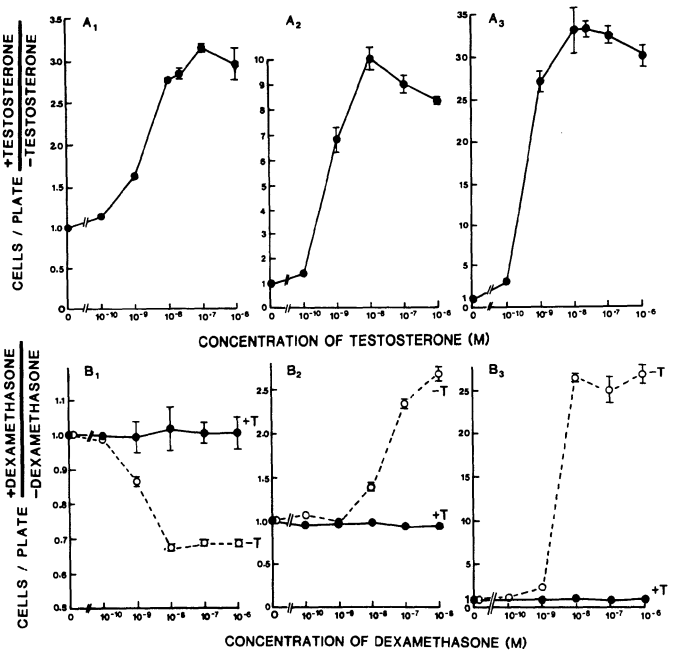


Figure 3. Effects of steroid hormone concentration on parameters involved in growth of S115 +A cells in monolayer and in suspension culture. Dose response curves are presented for testosterone (A1-3) and dexamethasone (B1-3) effects on log-phase growth in monolayer (A1, B1), on saturation density in monolayer (A2, B2) and on growth in suspension (A3, B3). Results are expressed as ratios of cell numbers per dish in the presence and absence of steroid, and effects of dexamethasone are presented in both the presence (●——●) and absence (○----○) of 3.5×10^{-8}M testosterone.

Table 2. Effects of steroid hormones on growth of S115 +A cells

Property	− Hormone	+ Testosterone	+ Dexamethasone
1. Monolayer growth			
Growth rate	+	↑	↓
Saturation density	+	↑	↑
Overall cell morphology	E	F	F
Focal adhesions	+	−	
Fibronectin	+	↓	
Microfilaments	+	−	
Sensitivity to growth stimuli	+	↓	
2. Suspension growth			
Growth rate	−	↑	↑
Maximal density	−	↑	↑
Cell morphology	−	Tightly clustered spheroids	

Code to symbols: + parameter present; − parameter negative; ↑ increased compared to no hormone; ↓ reduced compared to no hormone; E epithelial morphology; F fibroblastic morphology

androgen and glucocorticoid effects on cell biology, a separation can be seen of cell morphology/saturation density/anchorage dependence from cell proliferation rate at log phase of monolayer growth (Table 2) [15]. Using transfection experiments, it has been possible to separate the androgen-regulated stimulation of cell proliferation from the androgen-regulated cell morphology changes, although the same separation was not achieved for glucocorticoid regulation [15]. In addition, androgen- and glucocorticoid-stimulation of suspension growth were also separable [15]. A means of separating these different parameters will undoubtedly prove useful in molecular studies of steroid-regulated cell growth.

From these studies, we have developed the model that in cell biological terms, androgens convert S115 cells from a normal (− hormone) to a transformed (+ hormone) phenotype [11, 16–18]. Since these cells are tumor cells and form tumors in nude mice [19], these phenotypic alterations in culture cannot be linked directly to tumorigenesis *in vivo* but they do indicate some fundamental effects of steroids. Evidence for steroid-regulation to a transformed phenotype has been obtained now in human breast cancer cell lines and may be a more universal mechanism than thought previously [20 and see below]. Possible molecular mechanisms by which such steroid-mediated phenotypic and proliferative changes could occur are illustrated in the model in Figure 4. Under physiological conditions there is little, if any, firm evidence for non-receptor mediated pathways being important and so it can be accepted that action begins with steroid-receptor interation. Pathways of response can then be divided into three routes. Route 1 involves direct stimulation of the proliferation pathway. While DNA polymerase and nucleotide pools are increased by steroid hormones at the start of S phase of the cell cycle, these are relatively late effects of the hormone. Alterations in non-histone chromosomal proteins and enzymes like creatine kinase BB are potential candidates but most current interest centres on routes 2 and 3. Route 2 results in alteration of membrane sensitivity to external agents, and there is no doubt that steroids do alter sensitivity of cells to serum growth factors [14]. Route 3 suggests that growth factors could be produced capable of acting by autocrine and/or paracrine loops (see other chapters in this book).

2.2 Molecular markers of steroid action

Given the parallel to phenotypic change in transformation and in view of the well-documented involvement of mouse mammary tumor virus (MMTV) in the development of mammary carcinomas in the mouse, we considered it a possibility that viral elements could play a role in these steroid-regulated phenotypic changes in the S115 cell line. No virus particles have been detected in the S115 cells [17] but absence of exogenous viral units is not critical in that most inbred strains of mice contain also endogenous inherited MMTV sequences [21, 22] and it could be these latter units that are involved.

Proviral MMTV DNA contains at least three genes, *gal*, *pol* and *env* which

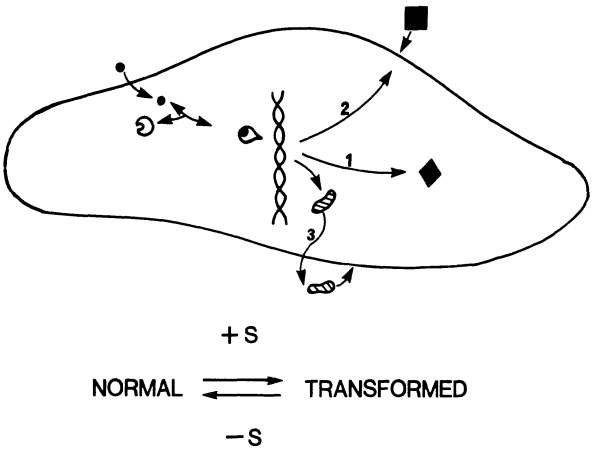


Figure 4. Possible mechanisms of steroid hormone effects on cultured cells. Steroids (S, •) by combining with their specific intracellular receptors ( ) initiate multiple events that culminate in the generation of a transformed phenotype. These processes are reversed on removal of steróid. Changes in behavior could be mediated directly (1), by altering membrane sensitivity to external agents (2), or by autocrine/paracrine loops (3).

are bounded at both ends by long terminal repeat (LTR) regions [23] (Figure 5). As yet, no oncogene has been defined in this virus and its mechanism of carcinogenesis is suggested to result from the site of viral integration and resulting altered expression of the neighboring *int* genes [24–27]. However, from sequencing studies [28–30] and *in vitro* translation [31], an open reading frame (orf) has been found within the LTR which has coding potential for additional proteins which could also be involved in some way.

S115 cells possess about four endogenous MMTV proviral units [32]. S115 cells appear to contain no additional sequences as compared to normal DNA from the mice in which the tumor arose, suggesting that integration of new exogenous units was not involved in production of this tumor. These cells appear to contain the endogenous Mtv17 unit [25], the RIII unit which has two Eco R1 sites within the env region (unpublished data) and the small 1.8 Kb LTR unit [32].

The S115 +A cells produce at least four MMTV-LTR-related RNA species [32] (Figure 6). The major RNA is about 16S in size and encodes mainly LTR sequences with a small portion of the env gene [33], which suggests it could encode the putative orf protein. Smaller amounts of the viral 35S, 24S and 20S RNAs are found also (Figure 6). However, this contrasts strongly to the pattern of MMTV RNAs found in most situations where the 16S RNA is barely detectable. Furthermore, production of these RNAs is regulated in the short-term in the cells by both androgen and glucocorticoid (Figure 6) [32, 34]. Regulation occurs within hours [34], at a time before any increase in DNA synthesis is detectable [11], thus providing a marker of an early post-receptor molecular event in steroid action on these cells. These two steroid

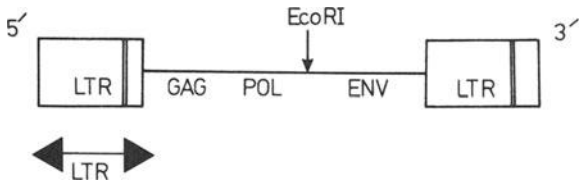


Figure 5. Structure of proviral DNA of mouse mammary tumor virus.

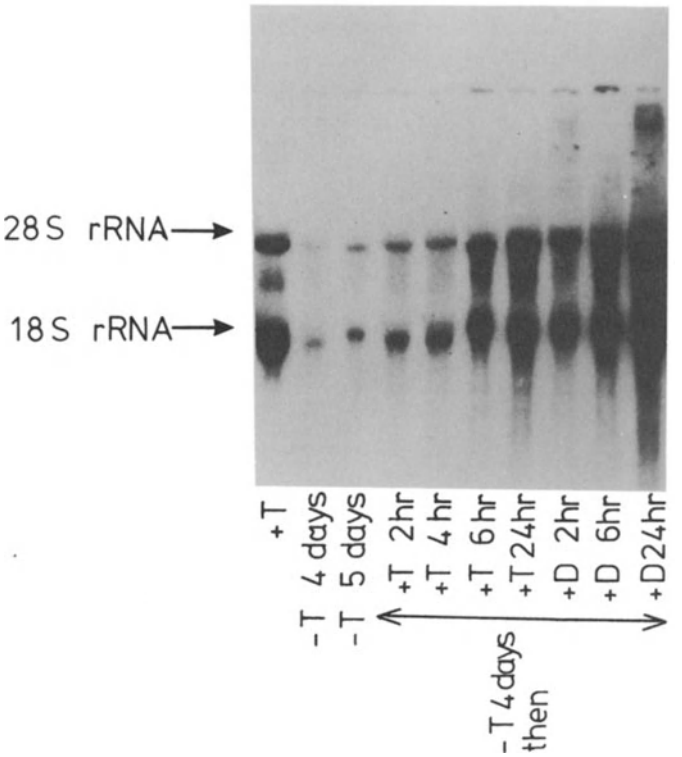


Figure 6. Regulation by both testosterone (T) and dexamethasone (D) on MMTV-LTR-related RNA in S115 +A cells. Northern blot of MMTV RNA to show short-term regulation by 3.5×10^{-8}M testosterone and 10^{-7}M dexamethasone. Cells were grown under steroid conditions for the number of days or hours as indicated. Taken from [34].

hormones act via their own receptors [34], there is no metabolic cross conversion of the steroids [35] and the effects occur at transcription, not on stabilization of m-RNA [34]. Interestingly, the effects of the two hormones are not additive [32, 36] implying a similarity of action. Yet differences also exist in that MMTV RNA appears to respond faster to glucocorticoid than to androgen and whilst dexamethasone acts directly without dependence on protein synthesis, the testosterone-induced MMTV RNA accumulation shows a

partial dependence on protein synthesis [34, 36]. Thus it remains in question whether the androgen action is direct or via intermediate proteins and could imply differences in action of the two steroids. However, the faster response of glucocorticoid could be due to lower androgen receptor levels or lower affinity of the androgen for the target site in the DNA and does not necessarily imply a separate mode of action. Similarly, a partial block of effects with cycloheximide could be explained by instability of the androgen receptor itself in the absence of protein synthesis.

The question remains as to the relationship between steroid-regulation of the cell biological parameters and of the MMTV RNAs. Since androgen and glucocorticoid have opposing effects on cell proliferation rate in monolayer (Figure 1, Table 2), yet both increase MMTV RNA accumulation in the short term (Figure 6), these parameters are clearly not linked. However, it is possible that the 16S MMTV RNA could be involved in the steroid-mediated effects on cell morphology, density regulation and/or anchorage independence, possibly by some effects on cell membrane function. At the very least, the 16S MMTV RNA provides a useful early marker of postreceptor events in steroid action in S115 cells.

2.3 Multi-hormonal regulation on MMTV-LTR: transfection studies

Glucocorticoid regulation of MMTV RNA production has been established for some time [37]. Glucocorticoid receptor binds to specific regions within the proviral LTR [38–40] and these DNA sequences show enhancer-like properties [36, 41–45]. However, regulation of MMTV RNA by androgen as well as glucocorticoid in S115 cells [32] raised the possibility of multihormonal regulation on the LTR. The reason why this had not been suspected earlier was because the appropriate receptors had never been present in the cells used [46].

We have used transfection experiments to confirm that the androgen action on MMTV RNA occurred directly through the LTR region and not via adjacent host cell DNA at the site of proviral MMTV integration [36]. The LTR of MMTV was joined to the coding sequence of genes not normally expressed in S115 cells so that expression of the foreign genes could be assayed for steroid regulation following transfection into S115 cells. Two marker genes were used: the genomic rat C3(1) gene for prostatic steroid binding protein and the β-interferon c-DNA. All 5′ sequences were eliminated so that the promoter and any upstream control elements needed for transcription were provided solely by the MMTV-LTR. These chimaeric genes were transfected into S115 cells in the pSV2gpt transfection vector (Figure 7) and their steroid regulation studied in clones of stably transfected cells. Expression of the C3(1) gene and β-interferon was regulated by both androgen and glucocorticoid but not by oestrogen or progesterone (Figure 8) [36]. Since S115 cells possess only receptors for androgen and glucocorticoid but not estrogen or progesterone, this hormonal regulation follows the receptor status of the

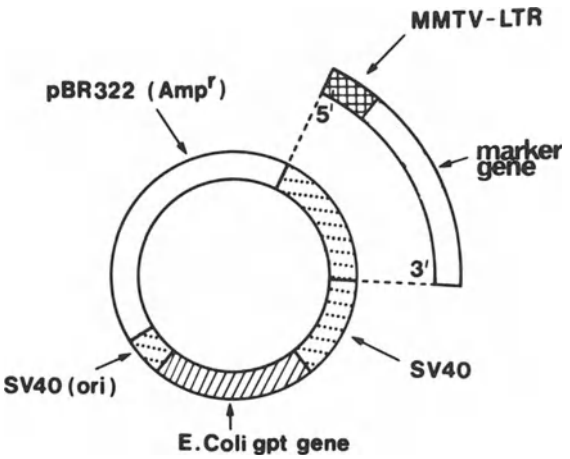


Figure 7. Construction of transfection vectors used to study steroid hormone regulation of the LTR of mouse mammary tumor virus in S115 cells. The LTR was linked to a marker coding sequence (either the rat C3(1) gene or human β-interferon) and the chimaeric gene inserted into the pSV2gpt vector [60].

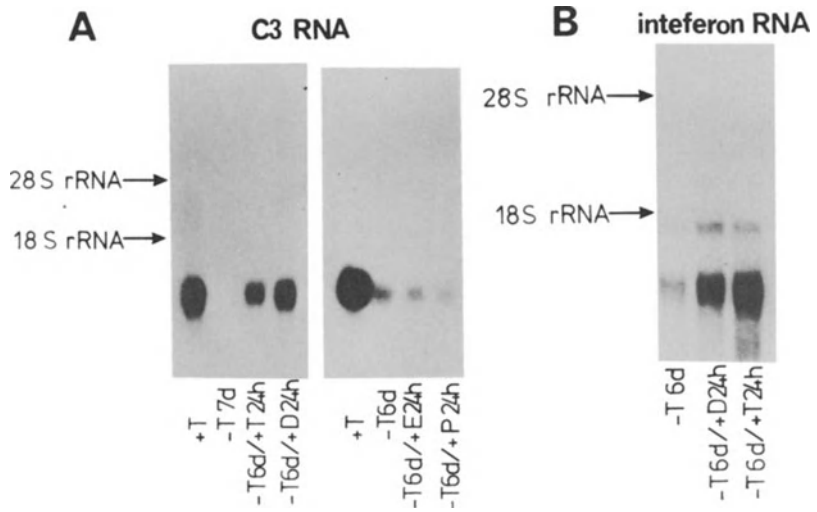


Figure 8. Steroid hormone regulation of expression of the transfected rat C3(1) gene (A) and human β-interferon gene (B) in S115 +A cells. Northern blots of C3 and interferon RNA from two clones of transfected cells to show regulation by testosterone (T), dexamethasone (D), estrogen (E) and progesterone (P). (d—days; h—hours). Taken from [36].

cells, suggesting that receptor status or even receptor levels are critical in determining the hormonal regulation of genes in a cell. This is further substantiated by the demonstration that regulatory elements in DNA for different steroid hormones share some structural similarities [47, 48].

The steroid regulatory effects on these transfected genes were specific and not merely a reflection of altered total RNA levels, were via transcriptional changes and at least partially independent of simultaneous protein synthesis [36]. The faster response of the transfected genes to glucocorticoid as compared to androgen and the partial inhibition of androgen but not glucocorticoid action by cycloheximide are the same problems encountered with the endogenous MMTV RNA and have been discussed earlier. Thus, it is a real probability that androgen and glucocorticoid act both at a similar place and by a similar mechanism directly on the LTR of MMTV. Data from transfection assays using cells with the relevant receptors have shown that the LTR can respond directly also to progesterone [49, 50].

Models of the mechanism of carcinogenesis by MMTV stress the importance of the regulatory regions of the LTR in that they could control expression levels of adjacent oncogenes [43, 51–54] or other cellular genes [55, 56] but the biological relevance to tumor production of a region sensitive to only glucocorticoids is unclear. However, new biological importance is revealed by regulation on the LTR of androgen in cells derived from an androgen-regulated tumor and of progesterone in pregnancy-dependent mouse mammary carcinomas.

2.4 Enhancer-like activity of MMTV-LTR

DNA sequences, termed enhancer elements, located upstream of the promoter and transcription start site have been shown to regulate transcription of several genes [57, 58]. Such enhancer sequences are able to stimulate gene transcription from long distances and appear to be very flexible in their requirements for spacing and orientation relative to the start site of transcription. It is now accepted that steroid hormones can exert their effects through such enhancer like elements and such DNA sequences, called hormone response elements (HRE), have been characterised in the LTR of MMTV [41–45]. However, it is becoming evident that hormonal regulation through these HRE in the LTR may not be confined only to the coding regions controlled by the LTR promoter but that other adjacent promoters and/or enhancers can also be affected [43, 59]. Such effects became evident to us when regulation of the cotransfected gpt gene was assayed in the clones of transfected cells used for the previous studies of multihormonal regulation on the LTR.

Since in the vectors used for our transfection studies, transcriptional regulation for the *gpt* gene is provided by SV40 sequences (Figure 7) [60], no hormonal regulation of the gpt transcripts would be expected. However, we found that the *gpt* RNA was also regulated by both androgen and glucocorticoid (Figure 9) [36]. The regulation is less than for the C3 or interferon RNA (Figure 8) but is nonetheless present.

To demonstrate that hormonal regulation on the *gpt* gene was solely due to DNA sequences of the LTR in the vector and not merely an internal function

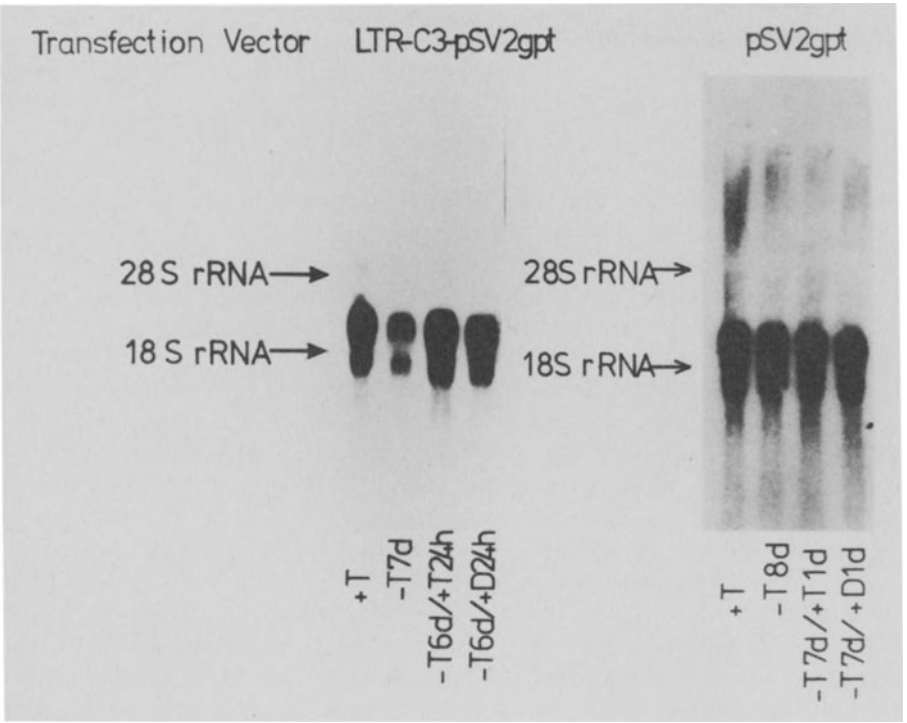


Figure 9. Steroid hormone regulation of expression of the co-transfected gpt gene in S115 +A cells. Northern blots of gpt RNA from cells transfected with vector pSV2gpt DNA with or without an inserted chimaeric LTR-C3 sequence to show regulation by testosterone (T) and dexamethasone (D) (d—days; h—hours). Taken from [36].

of S115 cells, vector pSV2*gpt* DNA was transfected into cells alone without any inserted chimaeric gene. The cells then produced *gpt* RNA which was no longer affected by hormonal manipulation (Figure 9) [36]. The lack of hormonal control could not be explained by the cells having become unresponsive to steroids since both cell proliferation and MMTV-LTR RNA were still regulated by hormones [36].

Such hormonal regulation of the *gpt* transcripts suggests that the LTR can confer hormonal control not only on its own promoter (regulating C3 or interferon RNA) but also on the neighboring *gpt* gene. Since SV40 promoter and enhancer sequences are present in the vector to control transcription of the *gpt* gene, this suggests that hormonal effects through the LTR must be able to operate over and above not only the adjacent viral promoter but also the viral enhancer sequences. Thus, insertion of an LTR in the genome could have far-reaching effects on hormonal regulation of neighboring genes by means of a type of enhancer insertion mechanism, although it remains to be seen how far through the genome such effects might operate.

3. Mechanism of loss of steroid sensitivity

3.1 Loss of steroid sensitivity: introduction

Manipulation of the steroid environment affects breast tumor growth in many species [61, 62] but only on a temporary basis, and even in rodents, where effects are greatest, it never eliminates the tumor completely [63]. In man, 30% of breast cancers regress under endocrine therapy, but regression is invariably of a temporary nature, to be replaced by growth of hormone-independent tumors and metastatic disease. This progression to steroid hormone inmsensitivity is a major clinical problem.

A recent approach to breast cancer management has been to select hormone-sensitive tumors by measuring their oestrogen and progesterone receptor levels [64]. Implicit in this, is the assumption that loss of receptor is a fundamental event in the progression from a responsive to an unresponsive state. However, the presence of receptors alone is clearly not the whole story because not all receptor-positive tumors respond to endocrine therapy.

It is now accepted that the origin of mammary tumors is monoclonal [65], but tumors themselves are composed of very mixed populations of cells. Steroid sensitivity/insensitivity is only one of many phenotypes which diverge during tumor progression. Cells from individual rodent mammary tumors have been shown to differ not only in hormone receptor content [66] and hormone-dependent growth [67, 68], but also in immunological properties [69], transplantability [70], metastatic capability [71], tumorigenicity [72], drug resistance [73], growth rate [74], karyotype [74, 75], MMTV expression [70] and MMTV proviral copies [76]. The mechanism by which such diversity arises could be genotypic or phenotypic. Evidence for a genotypic mechanism, involving mutation followed by cell selection, has been implied from karyotype studies [74, 75, 77] and from changes in exogenous integrated MMTV [76]. A phenotypic mechanism would involve stable alterations in the program of gene expression without mutation and could result from either selection of phenotypically altered cells (e.g., hormone-unresponsive cells growing selectively under low hormone conditions) or universal change throughout the whole cell population. Evidence for phenotypic mechanisms is much more recent [77–79].

Experiments designed to elucidate the origin of such heterogeneity are fraught with difficulties and the best way in which meaningful data can be obtained is to study divergence of cloned cells in tissue culture. We have used cloned S115 +A cells as our *in vitro* model. These cells were cloned from a tumor which progressed *in vivo* from a steroid sensitive to insensitive state but also mimicked this progression *in vitro* (see 'general introduction').

3.2 Loss of steroid sensitivity: cell biology

For analysis of loss of steroid sensitivity, cloned S115 +A cells were kept long-term as stock monolayer cultures in the absence of any steroid. To study

recovery of responsiveness, steroid was added back to the medium of some stock cells. Such cells were then assayed for steroid sensitivity at various time intervals by removing an aliquot of cells from stock cultures and carrying out short-term growth curves in the presence and absence of steroid. Analyses of these growth curves up to and including confluence have enabled us to study the time course of loss of steroid sensitivity in the S115 cells in terms of proliferative, saturation density and morphological responses in monolayer [78] and in terms of responses in suspension growth [15]. Taken all together, the data demonstrate that in a cloned hormone-responsive cell line, the transition to steroid autonomy can involve a rapid, ordered series of phenotypic changes.

Proliferative response to androgen in monolayer was calculated with respect to the doubling time of the cells in the short-term presence and absence of testosterone (Figure 10A). Stock androgen-responsive +A cells grow much faster in the presence than in the absence of testosterone (week 0, Figure 10A). After 2 weeks of androgen withdrawal the rate of cell growth dropped but especially in the short-term presence of testosterone. However, after 3 weeks of androgen withdrawal, the rate of growth increased in the short-term absence of testosterone, such that after 4 weeks of androgen deprivation the stock cells grew faster without than with testosterone. Thereafter, the rate of cell growth recovered both with and without testosterone, until after 9 weeks of androgen deprivation, the cells had lost all proliferative response to androgen and were growing both with or without steroid at the same rate as the +A cells with steroid.

Loss of androgen sensitivity in monolayer culture was also studied in terms of morphological and saturation density responses. These features took about twice as long to be lost as the proliferative response. Cell morphology was examined by phase-contrast microscopy and cell numbers per dish at confluence were plotted for all short-term growth assays. Observed changes in cell morphology and saturation density of the cells occurred simultaneously, and could be broadly divided into four stages: a) Stock androgen-responsive +A cells grew to much higher saturation densities in the presence than in the absence of testosterone (week 0, Figure 10B). In the presence of testosterone, the cells at confluence were of rounded fibroblastic morphology (Figure 2), forming an uneven spread on the dish, with dense piles of cells in some areas of the dish and empty patches in between. Removal of testosterone resulted in a change to very flattened epithelial morphology (Figure 2), and increased anchorage dependence which prevented the cells piling up on top of each other, and this is reflected in the much lower cell number at saturation density. b) After 2 weeks of androgen deprivation in stock cultures, the saturation density in the presence of testosterone dropped (Figure 10B). At this stage, the cells lost their ability to revert to the fibroblastic morphology after 1 week in the presence of testosterone. c) After 3–14 weeks of androgen withdrawal in stock cultures, the cells now grew to lower saturation density in the presence of testosterone than in its absence (Figure 10B). The stock androgen-depleted cells maintained an epithelial morphology through-

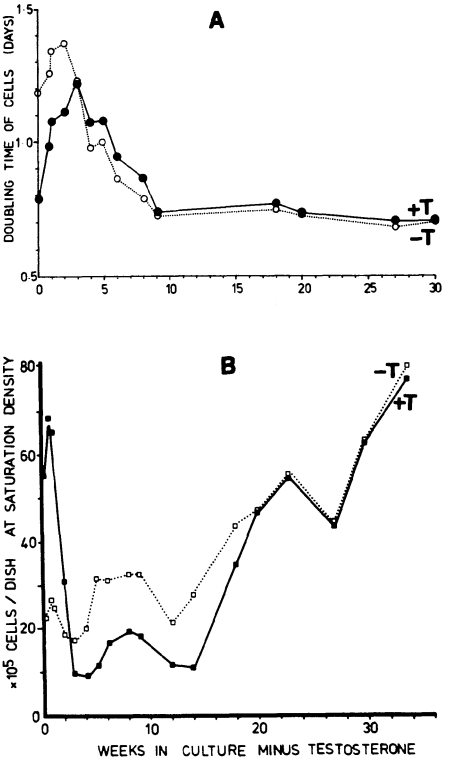


Figure 10. Loss of proliferative (A) and saturation density (B) responses in monolayer culture after long-term withdrawal of steroid in stock S115 cell cultures. Changes in rate of cell proliferation are expressed as cell doubling times in the short-term presence (●——●) or absence (○----○) of 3.5×10^{-8}M testosterone. Changes in saturation density are expressed as the greatest cell numbers per dish attained in the short-term presence (■——■) or absence (□----□) of 3.5×10^{-8} testosterone.

out but changed from a circular to a more elongated form. The elongated cells packed together more closely into an even monolayer than did the circular cells and grew to higher saturation densities. After 1 week in the presence of testosterone, the cells were of the circular epithelial form and grew to lower saturation density than in the absence of testosterone where all cells were of the elongated form. d) After even longer periods of androgen withdrawal in stock cultures, saturation density of the cells both in the presence and absence of testosterone increased, such that after 20 weeks of androgen deprivation, the cells had lost all androgen responsiveness in terms of saturation density (Figure 10B). No further morphological changes were observed. The cells grew in elongated form in well-packed even monolayer under all androgen conditions.

Loss of androgen sensitivity in terms of suspension growth was studied both for proliferative and maximal density responses (Figure 11) [15] and

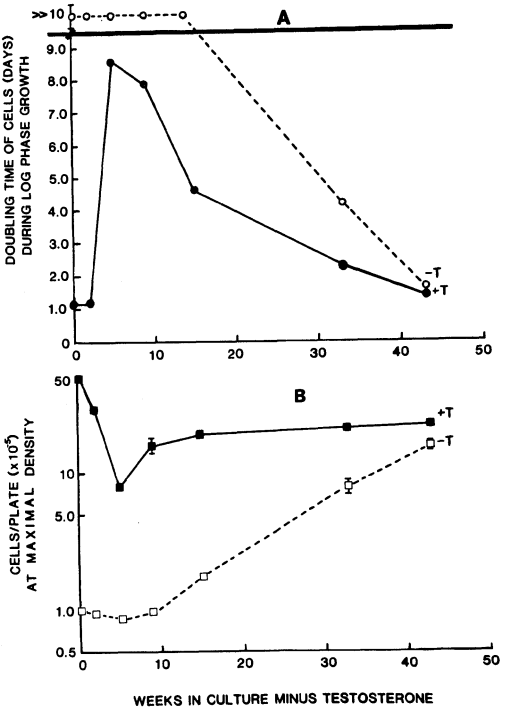


Figure 11. Loss of proliferative (A) and maximal density (B) responses in suspension culture after long-term withdrawal of steroid in stock S115 cell cultures. Changes in the rate of cell proliferation are expressed as cell doubling times in the short-term presence (●——●) or absence (○----○) of 3.5×10^{-8}M testosterone. Changes in maximal density are expressed as the greatest cell numbers per dish attained in the short-term presence (■——■) or absence (□----□) of 3.5×10^{-8}M testosterone.

similar trends were seen in both cases. Stock androgen-responsive +A cells grow in suspension only in the presence of steroid (week 0, Figures 11A and 11B). Following initial androgen deprivation, both proliferation and maximal density dropped in the short-term presence of steroid but recovered several weeks later. At the same time as this recovery, the cells began to grow in suspension without steroid and progressed to a state of steroid autonomy, growing well both with or without steroid but only after 42 weeks of androgen deprivation.

Although cell numbers suggested the growth of the −A cells in suspension to be indistinguishable from that of +A cells in the presence of steroid, behavior of these cells in suspension was different. Phase contrast photographs of the −A cells in suspension (Figure 12) showed that although spheroids were formed in the presence of testosterone, the cells held together much less tightly (Figure 12A) than in the +A androgen-responsive colonies (c.f. Figure 2). Without hormone, the −A cells held together even more loosely (Figure 12B). This phenomenon of loss of cell contact in the −A spheroids is seen

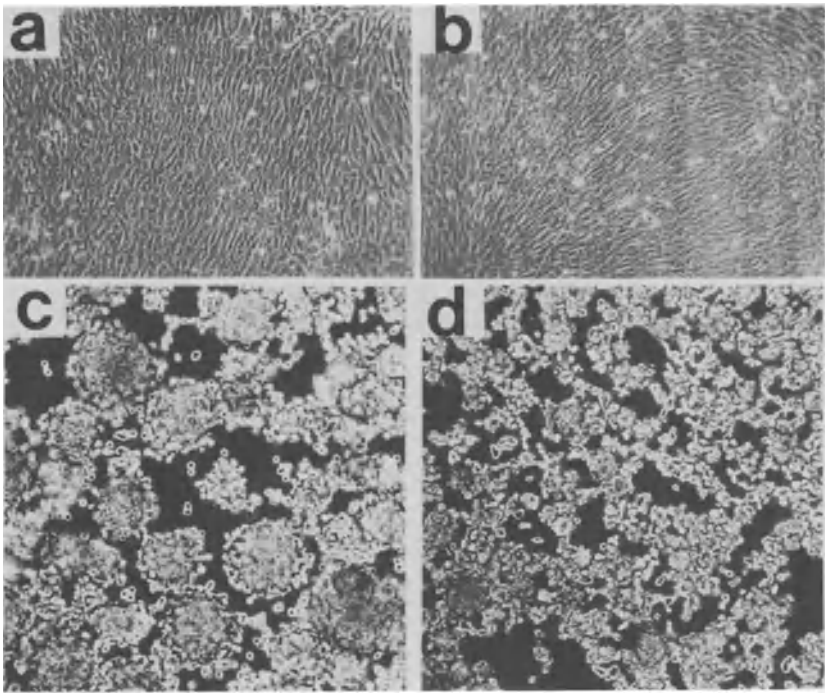


Figure 12. Photographs of steroid unresponsive S115 −A cells growing in monolayer (a,b) and suspension (c,d) either in the presence (a,c) or absence (b,d) of 3.5×10^{-8}M testosterone. Photographs were taken by phase contrast microscopy at magnification ×125 (a,b) or ×100 (c,d).

even more clearly in histological sections (Figure 13) and suggests that loss of steroid sensitivity in these cells could involve a loss of cell-cell interactions.

Attempts to recover androgen sensitivity were made on several occasions [78]. In principle, processes were reversible only for a specific period of time and the longer the cells had been maintained without steroid, the longer it took to reverse the process. Fully unresponsive (−A) cells kept in androgen-supplemented medium for 42 weeks never reattained any hint of responsiveness.

Since S115 +A cells are evidently sensitive not only to androgens but also to glucocorticoids (see earlier), the question of interaction between steroids during loss of sensitivity has arisen. Both normal and tumor mammary cells have complex endocrine requirements, yet until recently responsiveness had only ever been assessed in any one system in terms of response to one steroid. Recent data with S115 +A cells has demonstrated that they can be protected against any loss of response to either androgen or glucocorticoid with either steroid alone (Figure 14). Androgen protects against loss of glucocorticoid sensitivity and glucocorticoid protects against loss of androgen sensitivity [118].

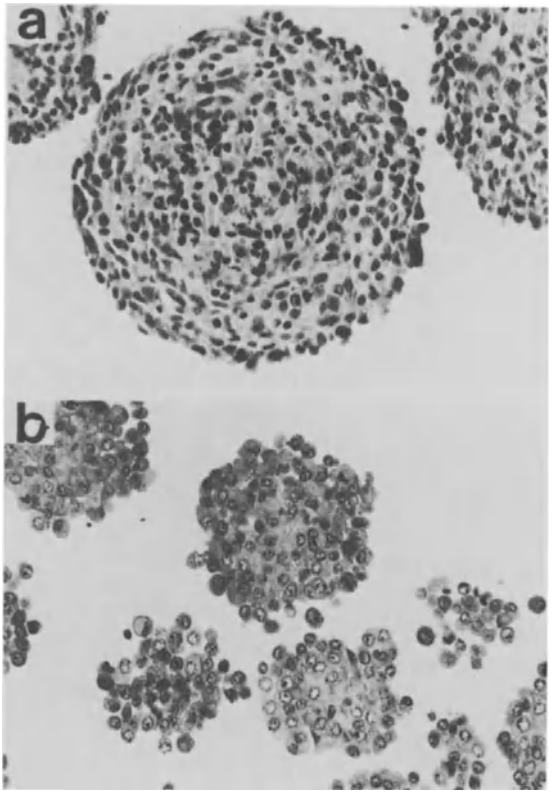


Figure 13. Histological sections of the balls of androgen responsive S115 +A (a) and androgen unresponsive S115 −A (b) cells growing in suspension culture in the presence of 3.5×10^{-8}M testosterone. Sections were stained with haematoxylin and eosin, and photographed at magnification ×500.

3.3 Loss of steroid sensitivity: molecular markers and role of DNA methylation

For reasons outlined earlier, we have been interested in the role of MMTV in steroid regulation of S115 cells, and in this context we have used the hormone-regulated production of MMTV RNA as a molecular marker of steroid action. Proviral MMTV sequences are present in the DNA of S115 cells and no gross genotypic change accompanies the transition from hormone-responsive (+A) to unresponsive (−A) state [32]. However, RNA from MMTV is present only in +A cells and not in −A cells [32]. A time course of loss of this RNA from +A cells during androgen withdrawal revealed loss of RNA after 6 days and complete disappearance by 9 weeks [78]. Recovery of this RNA by readdition of androgen could be achieved but only for a specific period of time (Figure 15) [78].

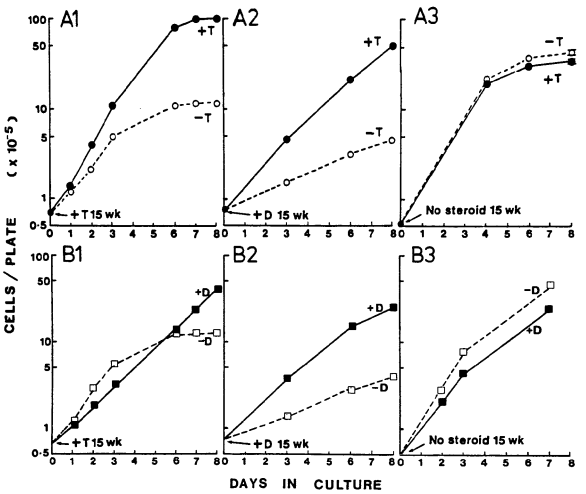


Figure 14. Androgen protects against loss of glucocorticoid sensitivity and glucocorticoid protects against loss of androgen sensitivity. Growth curves in monolayer culture are shown of S115 cells grown in the short-term presence or absence of 3.5×10^{-8}M testosterone (A1-3) or 10^{-7}M dexamethasone (B1-3) following prior treatment for 15 weeks with testosterone alone (A1, B1), dexamethasone alone (A2, B2) or neither steroid (A3, B3).

As was seen for the cell biology, glucocorticoid also interacts in regulation of MMTV-LTR-related RNAs in these cells. Dexamethasone does not increase MMTV RNA production in +A cells grown already with testosterone, and cannot stimulate any RNA production in −A cells. The only time when dexamethasone affects MMTV RNA levels is following limited-term androgen withdrawal. We have thus concluded that either androgen or glucocorticoid alone is sufficient to prevent loss of MMTV RNA, and either steroid alone will result in recovery of the RNA following total steroid deprivation for a limited period. Once fully unresponsive, the RNA cannot be recovered with either steroid.

Interestingly, the loss of MMTV RNA following total steroid withdrawal is accompanied by increased methylation of proviral copies in the DNA [32, 78]. Methylation studies have utilized cleavage of MMTV-LTR sequences with the isoschizomeric restriction enzymes HpaII and MspI, which cut unmethylated DNA at the same sites but differ in their specificity to cytosine methylation [80]. Since androgen-maintained +A cells show different patterns with HpaII and MspI (Figure 16), the LTR sequences of S115 +A cells are methylated already to some extent. Major increases in methylation at HpaII-sensitive sites were obvious after 35 weeks of androgen deprivation but subtle changes began much earlier (Figure 16) [78].

This increase in methylation occurs late in the sequence of events, after loss of all other detectable responses to androgen but interestingly, at a time when reversal ceases to be possible. Since it occurs many weeks after MMTV RNA production has been lost, it cannot be involved in initial loss of the

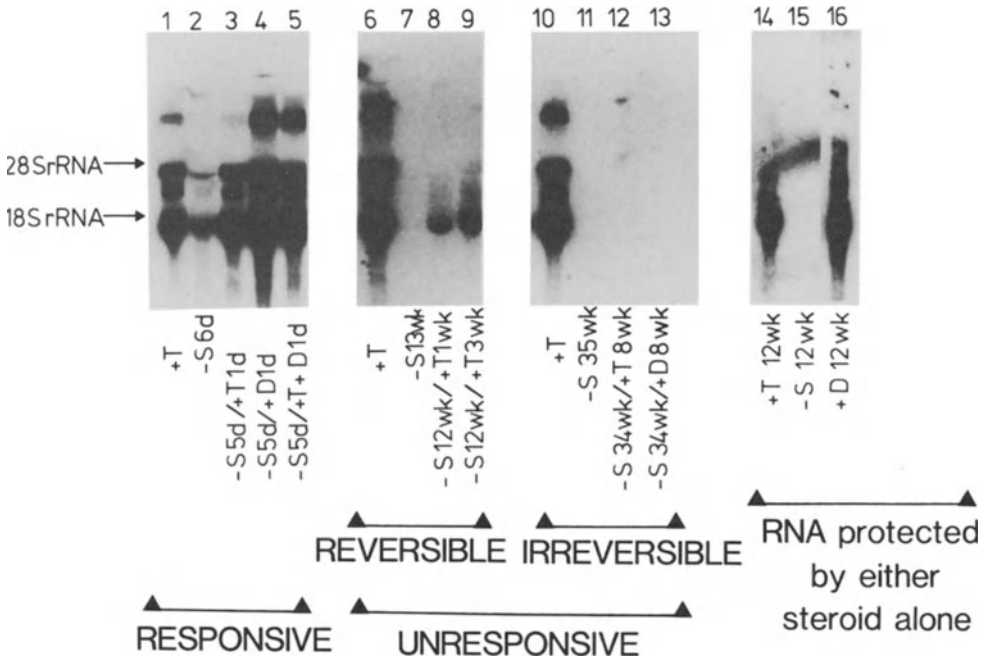


Figure 15. MMTV-LTR RNA as a molecular marker of loss of steroid sensitivity in S115 +A cells. Northern blots of MMTV-LTR RNA following treatment of S115 cells with 3.5×10^{-8}M testosterone (+T), 10^{-7}M dexamethasone (+D) or neither steroid (−S) for the number of days (d) or weeks (wk) indicated. MMTV RNA regulation by steroid can be classified as responsive (1-5), response lost upon removal of steroid but can be recovered by readdition of steroid (6-9), or irreversible unresponsive (10-13). Loss of MMTV RNA can be protected by either androgen (14) or glucocorticoid (16) alone.

RNA. However, methylation could be involved in the final irreversible steps towards steroid insensitivity. Whether it is a direct ultimate control mechanism or merely a consequence of long-term lack of expression remains unclear. DNA methylation has been implicated in eucaryotic gene expression regulation [81–84], in viral regulation [85], in control of MMTV expression [86], in tumorigenesis in mice [87–89] and in human cancer [90–93] as well as in tumor progression to steroid insensitivity [78, 94]. It is noteworthy that changes in methylation are associated with changes in glucocorticoid sensitivity of both the metallothionein-I gene [95] and lymphoma cells [94, 96] and with activation of the estrogen sensitive vitellogenin gene [97]. It may be, however, that it is the site and not the extent of methylation which is important. We have been unable to detect gross methylation of cytosine residues in S115 −A cells by high pressure liquid chromatography methods (A. Darbre and P. Darbre, unpublished results) and evidently, all genes in the −A cells are not switched off, since if they were the cells would no longer grow. Recent genomic sequencing methods suggest that specifically methyla-

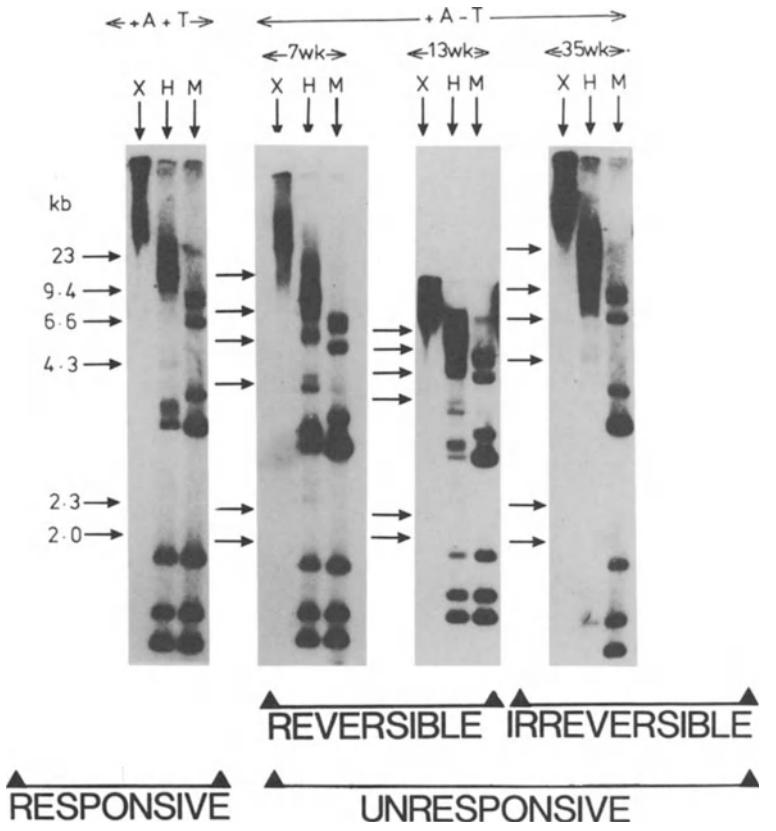


Figure 16. Methylation of MMTV-LTR sequences in the DNA of cloned S115 +A cells following androgen withdrawal (−T) for the number of weeks (wk) indicated. Restriction endonuclease analysis of DNA either undigested (X) or digested with HpaII (H) or MspI(M). Molecular weight standards were provided by a Hind III digest of bacteriophage λ DNA and their positions are indicated by horizontal arrows. Major increases in methylation are obvious when the other parameters have become steroid unresponsive and irreversible, although subtle changes do occur earlier.

tion of cytosine residues at receptor binding sites in the DNA could provide an answer [97].

3.4 Loss of steroid sensitivity: role of receptors

The majority of steroid hormone effects are mediated by intracellular receptor proteins. However, transition from steroid-sensitive to insensitive state is not always associated with loss of receptor. Examples of receptor positive but steroid insensitive cells have been described for glucocorticoid-lymphoma [98, 100], androgen-skin [101, 102], estrogen-mammary gland [103] and

estrogen-mammary tumor [104, 105] systems. In some of these models, the ineffectiveness of the steroid receptor complex is due to abnormal receptors with defects distal to the initial steroid binding step [98, 99, 106] but two examples have been published where the receptors are apparently normal [103, 107]. The loss of endogenous hormone sensitive parameters in S115 +A cells grown in the prolonged absence of steroid also occurs without loss of receptor or receptor function. There is no significant loss of androgen receptor number, as measured by a steroid binding assay, no changed nuclear transfer [12] and no loss in function of any of the machinery for steroid responsiveness as measured using transfection as a biological assay [119].

In recent experiments, the steroid-inducible chimaeric LTR-C3 gene (see Figure 7) was transfected into steroid-insensitive −A cells and full inducibility of that gene was found with both androgen and glucocorticoid (Figure 17). Thus, although all known endogenous inducible parameters had been lost in these cells [78], steroid sensitivity of an exogenous parameter introduced into the cells demonstrated that the whole machinery for steroid responsiveness, including receptor, is still fully functional. Interestingly, steroid sensitivity of the transfected gene was only protected against loss of response whilst steroid was present and removal of steroid ultimately resulted also in loss of the exogenous inducible parameter.

We have thus concluded that loss of steroid sensitivity occurs solely upon removal of steroid, can be prevented by either androgen or glucocorticoid alone, and occurs in the face of fully functional receptors. At a DNA level, both androgen and glucocorticoid receptor complexes regulate transcription directly via the LTR, indicating that loss of sensitivity to these genes probably occurs by changes in the DNA rather than by other regulatory proteins. A model which accommodates all these observations is given in Figure 18. The presence of either androgen or glucocorticoid steroid receptor complex protects the hormone response element of DNA against inactivation. Whether this is due to hormone effects on inactivating proteins or simply to protection of susceptible DNA sites by the steroid receptor complex, remains unknown but it is interesting to note that glucocorticoid receptor can protect against *in vitro* methylation of the hormone response element in the MMTV-LTR [48].

4. Relevance to human cell lines

Many of the steroid mediated events described for the S115 cells can also be seen in human breast cancer cell lines. Estradiol alters log-phase proliferation, density regulation and suspension growth (Figure 19) of ZR-75-1 cells [108]. We have previously argued that the proliferation seen in the absence of added estrogen was not due to residual estrogen in the serum [108] a result that was confirmed under serum-free conditions [4]. This led to the hypothesis that steroids were permissive agents modulating a basic growth rate [11, 61].

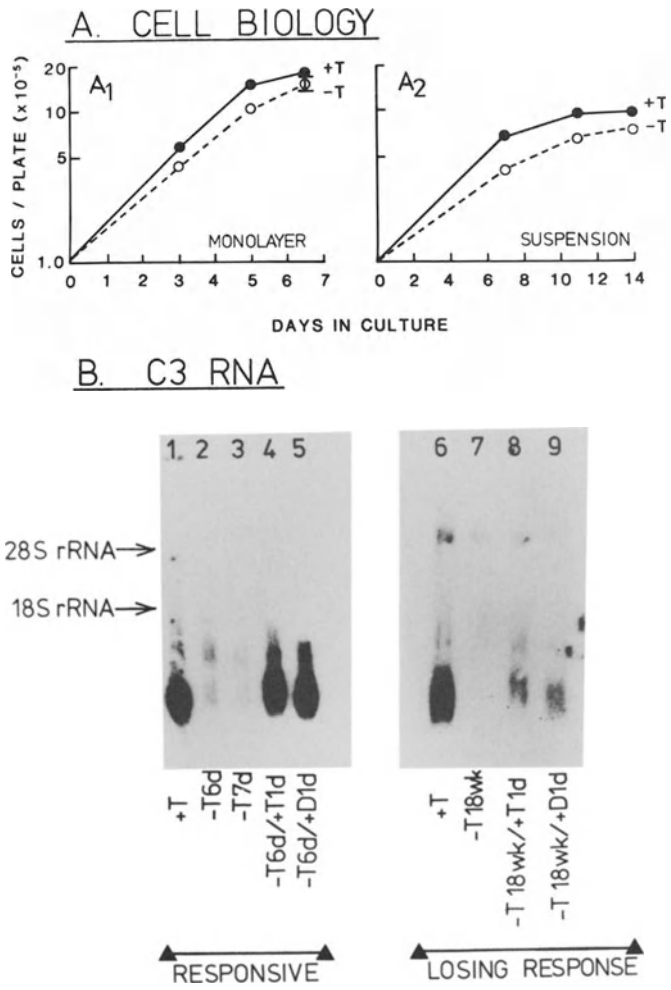


Figure 17. Androgen and glucocorticoid receptors remain functional in unresponsive S115 −A cells. S115 +A cells were deprived of androgen for 43 weeks, transfected with the vector containing the LTR-C3 chimaeric gene (Figure 7) and mycophenolic acid resistant clones of cells studied. Cell growth in both monolayer (A1) and suspension (A2) culture remained steroid insensitive whereas the transfected LTR-C3 gene was regulated by both testosterone (T) and dexamethasone (D) (B1-5), (d = days). Long term growth of this same clone without steroid resulted in loss of sensitivity of the C3 RNA (B6-9), (wk = weeks).

That view needs to be modified in the light of the discovery that phenol red in the culture medium acts as an estrogen [109]. In the absence of phenol red as well as steroid, ZR-75-1 cells exhibit much less proliferation either in monolayer or suspension culture (Figure 19).

The question of whether estradiol is switching on/off cell functions or simply modulating an existing reaction(s) is an important one in the context of

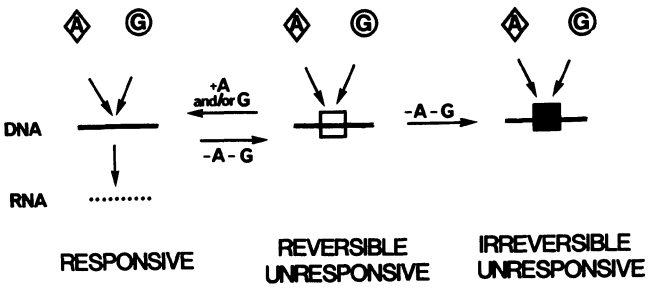


Figure 18. Model for the mechanism of loss of steroid sensitivity. Either androgen (A) or glucocorticoid (G) can influence sensitivity of a gene through their specific receptors (◊, ○). Initial changes in transcriptional activity are reversible (□) with either steroid alone, but later changes become irreversible (■).

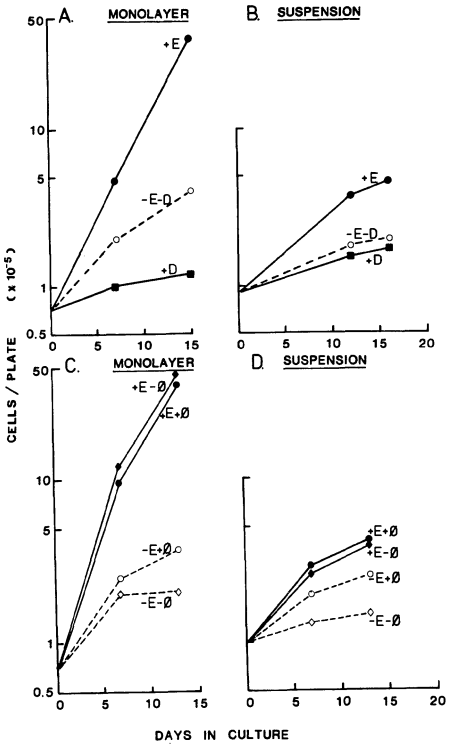


Figure 19. Effects of estradiol and dexamethasone on growth of ZR-75-1 cells in monolayer (A) and suspension (B) culture in the presence of phenol red. Steroid-regulated growth in the absence of phenol red is given for monolayer (C) and suspension (D) culture. Cells were grown with 10^{-8}M estradiol (E), 10^{-7}M dexamethasone (D) with or without phenol red (ϕ).

growth factor production. If modulation is occurring then estradiol-induced changes in cell behaviour could occur via quantitative differences whereas a switch mechanism would imply qualitative changes. This has potential clinical consequences but at the present time there are insufficient data on which to base conclusions.

Estradiol also increases serum sensitivity of ZR-75-1 cells (Table 3) and changes the morphology of both ZR-75-1 (unpublished observations) and MCF-7 [20, 110] cell lines. It is therefore evident that the transforming model of steroid action based on the mouse S115 cell data (Figure 4) also has application to the human cell lines. The most evident difference between the mouse and human lines in the context of that model is in the degree of morphological change engendered by steroid, with the human 'lines' exhibiting more subtle changes than the S115 cells. The former requires electron microscopic studies to identify the increased number of microvilli and more rounded appearance of the cells in contrast to the epithelial-fibroblastic transition seen with S115 cells under the light microscope. These species-related morphological differences could be causally related to the 16S MMTV-related mRNA in the mouse cells. This would be compatible with the conclusion about the function of this mRNA derived from other experiments (see above). It is equally possible that there are no fundamental differences between the mouse and human responses to sex steroid; the 16S mRNA seen in S115 cells could simply be a steroid-sensitive marker unrelated to growth changes and the morphological differences reflect quantitative rather than qualitative changes possibly due to differences in ploidy between the S115 and ZR-75-1/MCF-7 cell lines.

Whatever the explanation for the different degree of morphological response to steroids between mouse and human breast tumor cell lines, we are impressed by the similarity in steroid-induced changes in log-phase growth, density regulation and anchorage independence. It suggests to us that the factors mediating these changes may be common to both species.

Table 3. Effects of steroid hormones on growth of ZR-75-1 cells

Property	− Hormone*	+ Estradiol	+ Dexamethasone
1. Monolayer growth			
Growth rate	+	↑	↓
Saturation density	+	↑	↓
Cell morphology	E	E	E
2. Suspension growth			
Growth rate	+	↑	↓
Maximal density	+	↑	↓

Code to symbols: + growth; ↑ increased growth compared to no hormone; ↓ reduced growth compared to no hormone; E epithelial morphology

*Results presented are in phenol red-containing medium. In the absence of phenol red and steroid, there remains minimal growth but less than when phenol red is present. In the absence of phenol red, glucocorticoid is weakly stimulatory.

A second difference between the two cell lines is seen in the glucocorticoid responses. Whilst dexamethasone is inhibitory for all cell responses of the ZR-75-1 cells (Figure 19 and Table 3), it inhibits only log-phase proliferation of the mouse cells and increases saturation density and growth in suspension cultures (Figure 1 and Table 2).

It will be important to establish whether loss of steroid sensitivity resulting from steroid deprivation is a feature also of the human cell lines. Fragmentary data suggest that this may indeed be the case. As little as six days estradiol deprivation markedly depressed the proliferative responses of ZR-75-1 cells (Figure 20). Analogous effects have been observed with the CAMA-1 [111] and MCF-7 [112] human breast cancer cell lines. In both the latter cases, loss of proliferative effect of estradiol occurred in the face of normal induction of progesterone receptor by estradiol. These data point to selective desensitization of estradiol sensitive genes. Furthermore they indicate that, as with the S115 cells, loss of response does not involve loss of receptor function.

5. Clinical relevance

The valid point has frequently been made that cell lines, by the very fact that they are immortal, are abnormal even when data derived therefrom relate to cancer. Whilst accepting that point, we subscribe to the view that cell lines can generate clues as to the situation pertaining in the more complex *in vivo* situation. The types of information generated in the experiments described here have little if any relevance to carcinogenesis but are important in considering the growth of established tumors. From a clinical standpoint, the two questions of how do steroids regulate growth and what seemingly inevitable event(s) causes the progression from responsive to unresponsive state have become even more related than in a scientific context. The pertinent question here becomes how important is loss of estradiol receptor or its functionality in the generation of unresponsive human breast tumor. Based on the type of experiments described in this article, we feel that a new model should be considered, namely that loss of receptor function is consequent to other earlier more important changes. One reason behind that suggestion is the ability to generate an unresponsive state without changing receptor functionality. A different line of reasoning leads to the same conclusion. It is becoming increasingly evident that some steroid effects on breast tumor behavior are mediated by growth factors (see other articles in this book). If the steroid unresponsive state is consequential to loss of response to steroid, it follows that the 'growth factor' effects should be lost which is patently not the case. If anything, unresponsive tumors are more active than their responsive counterparts [113, 114]. This increased cellular activity cannot be explained on the basis of steroids switching off a negative regulator because, in any model based primarily on loss of receptor or response to receptor, the bio-

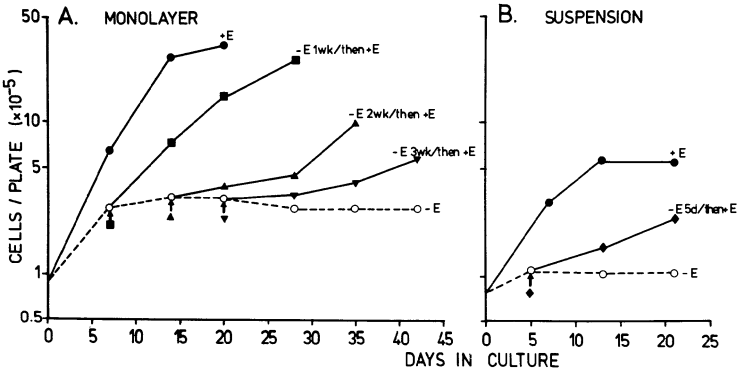


Figure 20. Loss of estradiol sensitivity in ZR-75-1 cells. Effect of estradiol deprivation for various lengths of time on the subsequent growth in the presence of 10^{-8}M estradiol in monolayer (A) and suspension (B) culture. Experiments were carried out in dextran-charcoal-stripped foetal calf serum but in the presence of phenol red. Cells were grown in monolayer culture without steroid for 0 days (●), 7 days (■), 14 days (▲) or 20 days (▼) or in suspension culture for 0 days (●) or 5 days (◆) before readdition of estradiol. Control cells were grown continuously without steroid (○----○).

logical consequences would be the same as removing steroid—a less active cell. Furthermore, it is already evident that at least two growth promoting factors, IGFl and TGFα are stimulated by estradiol [115, 116].

We feel that the data are more easily accommodated by a model in which the prime event is a change in growth regulation unrelated to receptor loss. The four basic tenets of our hypothesis are: 1) hormone withdrawal promotes the hormone insensitive state; 2) the presence of one class of steroid plus its receptor, can protect against loss of response to a different class of steroid; 3) the unresponsive state can be subdivided into reversible and irreversible categories; 4) loss of oestradiol receptor is a late and possibly inconsequential event in the progression. Does this model have any relevance to the clinical situation? A unitary hypothesis to explain all types of breast cancer is not tenable but our model is compatible with some of the existing data.

One of the major enigmas about breast cancer is why the incidence should be so high in postmenopausal women whose ovarian steroid levels are relatively low? Could it be that the hormone deprivation accompanying the climacteric helps to generate more aggressive unresponsive tumors? In a similar vein, the temporary decline in incidence known as the 'Clemeson Hook' that occurs about the time of the menopause [117] might be related to a temporary transition from responsive to slower growing unresponsive but reversible tumors? It has long been a puzzle as to why estradiol receptor should predict for response to all types of endocrine therapy. Could it be explained on the basis of one class of steroid receptor complex protecting against loss of sensitivity to other hormone classes?

The subdivision of unresponsive tumors into reversible and irreversible categories is compatible with the data on receptor phenotype and response to

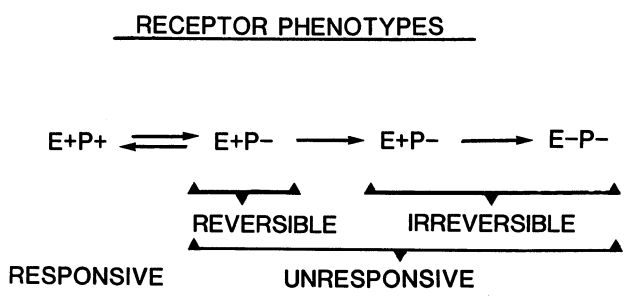


Figure 21. Model, based on Figure 18, for estradiol (E) and progesterone (P) receptor phenotypes of human breast tumors. Loss of estradiol receptor may be a very late and possibly inconsequential event in progression to the unresponsive state.

hormone therapy. Adaptation of our progression model to the estradiol receptor (ER) and progesterone receptor (PR) data would suggest the sequence depicted in Figure 21. The heterogeneous response of ER+PR− tumors to hormone treatment has usually been explained as the basis of low endogenous estrogen and therefore undetectable levels of PR in the 25% of tumors of this phenotype that respond to treatment; the unresponsive group are thought to have a post-receptor defect in the response machinery [105]. Our hypothesis would suggest that this defect occurs at the site of receptor-DNA interaction, the steroid response element. Our explanation for the ER+PR− but responsive tumors would be that regeneration of the responsive state has occurred whereas the conventional explanation is that they always have been responsive.

In none of our experiments has progression to a receptor negative state been seen. However, it could be that an analogous but much slower inactivation of the receptor gene itself occurs in the breast tumors. These speculations about clinical breast cancer are largely unsubstantiated but they provide a basis on which future experimentation can and will be based.

References

1. Smith HS, Wolman SR, Hackett AJ: The biology of breast cancer at the cellular level. Biochim Biophys Acta 738:103–123, 1984.
2. Allegra JC, Lippman ME: Growth of a human breast cancer cell line in serum-free hormone-supplemented medium. Cancer Res 38:3823–3829, 1978.
3. Barnes D, Sato G: Growth of a human mammary tumour cell line in a serum-free medium. Nature 281:388–389, 1979.
4. Darbre PD, Curtis S, King RJB: Effects of estradiol and tamoxifen on human breast cancer cells in serum-free culture. Cancer Res. 44:2790–2793, 1984.
5. Sonnenschein C, Soto AM: But are estrogens per se growth-promoting hormones? J Nat Cancer Inst 64:211–215, 1980.
6. Sirbasku DA, Ikeda T, Danielpour D: Endocrine and autocrine estromedins for mammary and pituitary cells. In: Mediators in cell growth and differentiation, Ford RJ and Maizel AL, (eds), pp 213–232. Raven Press: New York, 1985.

7. Smith JA, King RJB: Effects of steroids on growth of an androgen-dependent mouse mammary carcinoma in cell culture. Exp Cell Res 73:351–359, 1972.
8. Meakin JW: Cell biology of a transplantable androgen-dependent mammary tumor in the mouse. Proceedings of the Third International Congress of Endocrinology. In: Excerpta Medica International Congress Series 157, 165 (abstract).
9. Minesita T, Yamaguchi K: An androgen-dependent mouse mammary tumor. Cancer Res 25:1168–1175, 1965.
10. Matsumoto K, Sato B, Kitamura Y: Roles of androgen and its receptors in mouse mammary tumor. In: Hormonal Regulation of Mammary Tumors. Vol. 1: Steroid Hormones, Leung BS (ed), pp 216–244. Eden Press: Montreal, Canada, 1982.
11. King RJB, Cambray GJ, Jagus-Smith R, Robinson JH, Smith JA: Steroid hormones and the control of tumor growth: studies on androgen-responsive tumor cells in culture. In: Receptors and Mechanism, of Steroid Hormones Pasqualini JR (ed), pp 215–261. Marcel Dekker: New York, 1976.
12. King RJB: Studies on the regulation of cell proliferation in culture by steroids. In: Hormones and Cell Regulation, Vol. 2, Dumont J and Nunez J (eds), pp 15–36. Elsevier North Holland Biomedical Press: Amsterdam, 1978.
13. Engel LW, Young NA, Tralka TS, Lippman ME, O'Brian SJ, Joyce MJ: Establishment and characterization of three new continues cell lines derived from human breast carcinomas. Cancer Res 38:3352–3364, 1978.
14. Yates J, King RJB: Multiple sensitivities of mammary tumor cells in culture. Cancer Res 38:4135–4137, 1978.
15. Darbre PD, King RJB: Differential effects of steroid hormones on parameters of cell growth. Cancer Res 47:2937–2944, 1987.
16. Yates J, King RJB: Correlation of growth properties and morphology with hormone responsiveness of mammary tumor cells in culture. Cancer Res 41:258–262, 1981.
17. Yates J, Couchman JR, King RJB: Androgen effects on growth, morphology, and sensitivity of S115 mouse mammary tumor cells in culture. In: Hormones and Cancer King RJB, Iacobelli S, Lindner HR and Lippman ME (eds), Vol. 14, pp 31–39. Raven Press: New York, 1980.
18. Couchman JR, Yates J, King RJB, Badley RA: Changes in microfilament and focal adhesion distribution with loss of androgen responsiveness in cultured mammary tumor cells. Cancer Res 41:263–269, 1981.
19. Yates J, King RJB: Lack of correlation between transformed characteristics in culture and tumorigenicity of mouse mammary tumor cells. Eur J Cancer Clin Oncol 18:399–403, 1982.
20. Sapino A, Pietribiasi F, Bussolati G, Marchisio PC: Estrogen- and tamoxifen-induced rearrangement of cytoskeletal and adhesion structures in breast cancer MCF7 cells. Cancer Res 46:2526–2531, 1986.
21. Traina VL, Taylor BA, Cohen JC: Genetic mapping of endogenous mouse mammary tumor viruses: locus characterization, segregation and chromosomal distribution. J Virol 40:735–744, 1981.
22. Peters G, Placzek M, Brookes S, Kozak C, Smith R, Dickson C. Characterization, chromosome assignment and segregation analysis of endogenous proviral units of mouse mammary tumor virus. J Virol 59:535–544, 1986.
23. Ponta M, Gunzburg WH, Salmons B, Groner B, Herrlich P: Mouse mammary tumor virus: a proviral gene contributes to the understanding of eukaryotic gene expression and mammary tumorigenesis. J Gen Virol 66:931–943, 1985.
24. Nusse R, Varmus HE: Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31:99–109, 1982.
25. Peters G, Brookes S, Smith R, Dickson C: Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. Cell 33:369–377, 1983.
26. Brown AMC, Wildin RS, Prendergast TJ, Varmus HE: A retrovirus vector expressing the putative mammary oncogene *int*-I causes partial transformation of a mammary epithelial

cell line. Cell 46:1001–1009, 1986.
27. Garcia M, Wellinger R, Vessaz A, Diggelmann H: A new site of integration for mouse mammary tumor virus proviral DNA common to BALB/cf(C3H) mammary and kidney adenocarcinomas. EMBO J 5:127–134, 1986.
28. Donehower LA, Huang AL, Hager GL: Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy J Virol 37:226–238, 1981.
29. Fasel N, Pearson K, Buetti E, Diggelmann H: The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long coding sequence and signals for hormonally regulated transcription. EMBO J 1:3–7, 1982.
30. Donehower LA, Fleurdelys B, Hager GL: Further evidence for the protein coding potential of the mouse mammary tumor virus long terminal repeat: nucleotide sequence of an endogenous proviral long terminal repeat. J Virol 45:941–949, 1983.
31. Dickson C, Smith R, Peters G: *In vitro* synthesis of polypeptides encoded by the long terminal repeat region of mouse mammary tumour virus DNA. Nature 291:511–513, 1981.
32. Darbre P, Dickson C, Peters G, Page M, Curtis S, King RJB: Androgen regulation of cell proliferation and expression of viral sequences in mouse mammary tumor cells. Nature 303:431–433, 1983.
33. Darbre PD, Curtis SA, King RJB: Cellular and molecular events regulated by androgens in the S115 mouse mammary tumor cell line. In: Progress in Cancer Research and Therapy, Bresciani F, King RJB, Lippman ME, Namer M and Raynaud JP (eds), vol. 31, pp 261–268, Raven Press: New York, 1984.
34. Darbre PD, Moriarty A, Curtis SA, King RJB: Androgen regulates MMTV RNA in the short-term in S115 mouse mammary tumour cells. J Steroid Biochem 23:379–384, 1985.
35. Gordon J, Smith JA, King RJB: Metabolism and binding of androgens by mouse mammary tumour cells in culture. Mol Cell Endocrinol 1:259–270, 1974.
36. Darbre P, Page M, King RJB: Androgen regulation by the long terminal repeat of mouse mammary tumor virus. Mol Cell Biol 6:2847–2854, 1986.
37. Ringold GM: Regulation of mouse mammary tumor virus gene expression by glucocorticoid hormones. Current Topics Microbiol Immun 106:79–103, 1983.
38. Payvar F, DeFranco D, Firestone GL, Edgar B, Wrange O, Okret S, Gustafsson JA, Yamamoto KR: Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35:381–392, 1983.
39. Pfahl M, McGinnis D, Hendricks M, Groner B, Hynes NE: Correlation of glucocorticoid receptor binding sites on MMTV proviral DNA with hormone inducible transcription. Science 222:1341–1343, 1983.
40. Scheidereit C, Geisse S, Westphal HM, Beato M: The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. Nature 304:749–752, 1983.
41. Chandler VL, Maler BA, Yamamoto KR: DNA sequences bound specifically by glucocorticoid receptor *in vitro* render a heterologous promoter hormone responsive *in vivo*. Cell 33:489–499, 1983.
42. Parker M: Enhancer elements activated by steroid hormones? Nature 304:687–688, 1983.
43. Ostrowski MC, Huang AL, Kessel M, Wolford RG, Hager GL: Modulation of enhancer activity by the hormone responsive regulatory element from mouse mammary tumor virus. EMBO J. 3, 1891–1899, 1984.
44. Ponta H, Kennedy N, Skroch P, Hynes NE, Groner B: Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc Natl Acad Sci USA 82:1020–1024, 1985.
45. Overhauser J, Fan H: Generation of glucocorticoid-responsive Moloney murine leukemia virus by insertion of regulatory sequences from murine mammary tumor virus into the long terminal repeat. J Virol 54:133–144, 1985.
46. Young HA, Scolnick EM, Parks WP: Glucocorticoid-receptor interaction and induction of murine mammary tumor virus. J Biol Chem 250:3337–3343, 1975.
47. von der Ahe D, Janich S, Scheidereit C, Renkawitz R, Schutz G, Beato M: Glucocorticoid

and progesterone receptors bind to the same sites in two hormonally regulated promoters. Nature 313:706–709, 1985.
48. von der Ahe D, Renoir JM, Buchou T, Baulieu EE, Beato M: Receptors for glucocorticosteroid and progesterone recognize distinct features of a DNA regulatory element. Proc Natl Acad Sci USA 83:2817–2821, 1986.
49. Cato ACB, Miksicek R, Schutz G, Arnemann J, Beato M: The hormone regulatory element of mouse mammary tumor virus mediates progesterone induction. EMBO J 5: 2237–2240, 1986.
50. Cato ACB, Henderson D, Ponta H: The hormone response element of the mouse mammary tumor virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region. EMBO J 6:363–368, 1987.
51. Jakobovits EB, Majors JE, Varmus HE: Hormonal regulation of the Rous sarcoma virus Src gene via a heterologous promoter defines a threshold dose for cellular transformation. Cell 38:757–765, 1984.
52. Papkoff J, Ringold GM: Use of the mouse mammary tumor virus long terminal repeat to promote steroid-inducible expression of v-*mos*. J Virol 52:420–430, 1984.
53. Stewart TA, Pattengale PK, Leder P: Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/*myc* fusion genes. Cell 38:627–637, 1984.
54. Jaggi R, Salmons B, Muellener D, Groner B: The v-*mos* and H-*ras* oncogene expression represses glucocorticoid hormone-dependent transcription from the mouse mammary tumor virus LTR. EMBO J 5:2609–2616, 1986.
55. Dickson C, Smith R, Brooks S, Peters G: Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. Cell 37:529–536, 1984.
56. Nusse R, van Ooyen A, Cox D, Fung YKT, Vamus H: Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature 307:131–136, 1984.
57. Khoury G, Gruss P: Enhancer elements. Cell 33:313–314, 1983.
58. Kolata G: New clues to gene regulation. Science 224:588–589, 1984.
59. Hynes N, van Ooyen AJJ, Kennedy N, Herrlich P, Ponta H: Groner B: Subfragments of the large terminal repeat cause glucocorticoid-responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc Natl Acad Sci USA 80:3637–3641, 1983.
60. Mulligan RC, Berg P: Expression of a bacterial gene in mammalian cells. Science 209:1422–1427, 1980.
61. King RJB: How important are steroids in regulating the growth of mammary tumors? Biochem Actions Hormones 6:247–265, 1979.
62. Leung BS (ed) In: Hormonal Regulation of Mammary Tumors, Vols. I and II. Eden Press: Montreal, Canada, 1982.
63. Foulds L: In: Neoplastic Development, Vol. I, pp 54–56. Academic Press: New York, 1969.
64. DeSombre ER, Jensen EV: Clinical usefulness of steroid receptor determinations in breast cancer. In: Hormonal Regulation of Mammary Tumors, Vol. 1, Leung BS (ed), pp 155–182. Eden Press: Montreal, Canada. 1982.
65. Dexter DL, Calabresi P: Intraneoplastic diversity. Biochim Biophys Acta 695:97–112, 1982.
66. Sluyser M: Hormone receptors in mouse mammary tumors. In: Mammary Tumors in the Mouse, Hilgers J and Sluyser M (eds), pp 267–299. Elsevier North Holland Biomedical: Amsterdam. 1981.
67. Desmond WJ, Wolbers SJ, Sato G: Cloned mouse mammary cell lines requiring androgens for growth in culture. Cell 8:79–86, 1976.
68. Stanley ER, Palmer RE, Sohn U: Development of methods for the quantitative *in vitro* analysis of androgen-dependent and autonomous Shionogi carcinoma 115 cells. Cell 10:35–44, 1977.
69. Kerbel RS: Implications of immunological heterogeneity of tumors. Nature 280:358–360, 1979.

70. Sluyser M, DeGoeij CCJ, Evers SG: Changes in sensitivity to cyclophosphamide of mouse mammary tumors during serial transplantation. J Natl Cancer Inst 66:327–330, 1981.
71. Fidler IJ, Hart IR: The origin of metastatic heterogeneity in tumors. Eur J Cancer 17:487–494, 1981.
72. Soule HD, Maloney T, McGrath CM: Phenotypic variance among cells isolated from spontaneous mouse mammary tumors in primary suspension culture. Cancer Res 41:1154–1164, 1981.
73. Heppner GH, Dexter DL, DeNucci T, Miller FR, Calabresi P: Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. Cancer Res 38:3758–3763, 1978.
74. Dexter DL, Kowalski HM, Blazar BA, Fligiel Z, Vogel R, Heppner G: Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res 38:3174–3181, 1978.
75. Hager JC, Fligiel S, Stanley W, Richardson AM, Heppner GH: Characterization of a variant-producing tumor cell line from a heterogeneous strain BALB/cfC3H mouse mammary tumor. Cancer Res 41:1293–1300, 1981.
76. Michalides R, Wagenaar E, Sluyser M: Mammary tumor virus DNA as a marker for genotypic variance within hormone-responsive GR mouse mammary tumors. Cancer Res 42:1154–1158, 1982.
77. Kim U: Factors influencing the generation of phenotypic heterogeneity in mammary tumors. In: Biological Responses in Cancer, Mihich E (ed), Vol. 4, pp 91–124. Plenum Press: New York, 1985.
78. Darbre P, King RJB: Progression to steroid autonomy in S115 mouse mammary tumor cells: role of DNA methylation. J Cell Biol 99:1410–1415, 1984.
79. Nicholson GL: The evolution of phenotypic diversity in metastatic tumor cells. In: Biological Responses in Cancer, Mihich E, (ed), Vol. 4, pp 71–89. Plenum Press: New York, 1985.
80. Waalwijk C, Flavell RA: DNA methylation at a CCGG sequence in the large intron of the rabbit β-globin gene: tissue specific variations. Nucleic Acids Res 5:4631–4641, 1978.
81. Razin A, Riggs AD: DNA methylation and gene function. Science 210:604–610, 1980.
82. Felsenfeld G, McGhee J: Methylation and gene control. Nature 296:602–603, 1982.
83. Doerfler W: DNA methylation and gene activity. Ann Rev Biochem 52:93–124, 1983.
84. Bird AP: DNA methylation—how important in gene control? Nature 307:503–504, 1984.
85. Doerfler W: DNA methylation: role in viral transformation and persistence. Adv Viral Oncol 4:217–247, 1984.
86. Cohen JC: Methylation of milk-born and genetically transmitted mouse mammary tumor virus proviral DNA. Cell 19:653–662, 1980.
87. Breznik T, Cohen JC: Altered methylation of endogenous viral promoter sequences during mammary carcinogenesis. Nature 295:255–257, 1982.
88. Drohan WN, Benado LE, Graham DE, Smith GH: Mouse mammary tumor virus proviral sequences congenital to C3H/Sm mice are differentially hypomethylated in chemically induced, virus-induced and spontaneous mammary tumors. J Virol 43:876–884, 1982.
89. Fanning TG, Vassos AB, Cardiff RD: Methylation and amplification of mouse mammary tumor virus DNA in normal, premalignant, and malignant cells of GR/A mice. J Virol 41:1007–1013, 1982.
90. Diala ES, Cheah MSC, Rowitch D, Hoffman RM: The extent of DNA methylation in human tumor cells. J Natl Cancer Inst 71:755–764, 1983.
91. Feinberg AP, Vogelstein B: Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 301:89–92, 1983.
92. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, Enrlich M: The 5-methyl-cytosine content of DNA from human tumors. Nucleic Acids Res 11: 6883–6894, 1983.
93. Jones PA: DNA methylation and cancer. Cancer Res 46:461–466, 1986.
94. Gasson JC, Ryden T, Bourgeois S: Role of de novo DNA methylation in the glucocorticoid resistance of a T-lymphoid cell line. Nature 302:621–623, 1983.

95. Compere SJ, Palmiter RD: DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. Cell 25:233–240, 1981.
96. Gasson JC, Bourgeois S: A new determinant of glucocorticoid sensitivity in lymphoid cell lines. J Cell Biol 96:409–415, 1983.
97. Saluz HP, Jiricny J, Jost JP: Genomic sequencing reveals a positive correlation between the kinetics of strand-specific DNA demethylation of the overlapping estradiol/glucocorticoid-receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proc Natl Acad Sci USA 83:7167–7171, 1986.
98. Miesfeld R, Okret S, Wikstrom AC, Wrange O, Gustafsson JA, Yamamoto KR: Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. Nature 312:779–781, 1984.
99. Westphal HM, Mugele K, Beato M, Gehring U: Immunochemical characterization of wild-type and variant glucocorticoid receptors by monoclonal antibodies. EMBO J 3:1493–1498, 1984.
100. Gehring U: Genetics of glucocorticoid receptors. Mol Cell Endocrinol 48:89–96, 1986.
101. George FW, Wilson JD: Hormonal control of sexual development. Vitam Horm 43:145–196, 1986.
102. Kontula KK, Janne OA, Bardin CW: Intracellular hormone receptor defects and disease. In: The Receptors, Conn PM, (ed), Vol. IV, pp 37–74. Academic Press, New York, 1986.
103. Gaubert CM, Carriero R, Shyamala G: Relationships between mammary estrogen receptor and estrogenic sensitivity. Molecular properties of cytoplasmic receptor and its binding to deoxyribonucleic acid. Endocrinology 118:1504–1512, 1986.
104. Nawata H, Chong MT, Bronzert D, Lippman ME: Estradiol-independent growth of a subline of MCF-7 human breast cancer cells in culture. J Biol Chem 256:6895–6902, 1981.
105. Sedlacek SM, Horwitz KB: The role of progestins and progesterone receptors in the treatment of breast cancer. Steroids 44:467–484, 1984.
106. Danielsen M, Northrop JP, Ringold GM: The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. EMBO J 5:2513–2522, 1986.
107. Sibley CH, Yamamoto KR: Mouse lymphoma cells: mechanisms of resistance to glucocorticoids. In: Glucocorticoid Hormone Action, Baxter JD and Rousseau GG (eds), Monographs in Endocrinology 12:357–376. Springer Verlag: New York, 1979.
108. Darbre P, Yates J, Curtis S, King RJB: Effect of estradiol on human breast cancer cells in culture. Cancer Res. 43, 349–354, 1983.
109. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS: Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500, 1986.
110. Vic P, Vignon F, Deroc D, Rochefort H: Effect of estradiol on the ultrastructure of the MCF, human breast cancer cells in culture. Cancer Res 42:667–673, 1982.
111. Leung BS, Qureshi S, Leung J: Response to estrogen by the human mammary carcinoma cell line CAMA 1. Cancer Res 42:5060–5066, 1982.
112. Katzenellenbogen BS, Kendra KL, Berthois Y: Proliferation of estrogen-responsive MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. J Cell Biochem Suppl 11A: p 131, 1987.
113. King RJB, Smith JA, Steggles AW: Oestrogen-binding and the hormone responsiveness of tumors. Steroidologia 1:73–88, 1970.
114. Hahnel R: Steroid receptor status, tumor growth and prognosis. In New Aspects of Breast Cancer, Stoll BA (ed), Vol. 5, pp 107–143: William Heinemann Medical Books: London, 1982.
115. Bates S, McManaway M, Lippman ME, Dickson RB: Characterization of Estrogen Responsive Transforming activity in Human Breast Cancer Cell Lines. Cancer Res 46:1707–1713, 1986.
116. Huff KK, Kanabbe C, Kaufman D, Lindsay R, Lippman ME, Dickson RB: Multihormonal Regulation of Insulin-like Growth Factor-I-Related Proteins in MCF7—A Human Breast

Cancer Cells. Molecular Endocrinology, in-press, 1988.
117. Farewell VT: Statistical methods and mathematical models for research in breast disease. In: Commentaries on Research in Breast Disease, ed. Bulbrook RD and Taylor DJ (eds), Vol. 1, pp 226–229. Alan R. Liss: New York, 1979.
118. Darbre P and King RJB: Interaction of different steroid hormones during progression of tumor cells to steroid autonomy. Int J Cancer 40:802–806, 1987.
119. Darbre P and King RJB: Progression to steroid insensitivity can occur irrespective of the presence of functional steroid receptors. Cell 51:521–528, 1987.

16. The role of epidermal growth factor in normal and neoplastic growth of mouse mammary epithelial cells

Takami Oka, Osamu Tsutsumi, Hirohisa Kurachi, and Shigeru Okamoto

1. Introduction

Epidermal growth factor (EGF) was discovered by Cohen in 1962 [1] as the principal in extracts of male mouse submandibular gland which produced acceleration of incisor eruption and eyelid opening when administered to newborn mice. It has since been purified to homogeneity and sequenced [2]. Mouse EGF is a single-chain polypeptide (mol. wt. 6045) containing 53 amino acid residues and 3 disulfide linkages. EGF is also present in biological fluids such as plasma, saliva, milk, urine, amniotic fluid, sweat, pancreatic juice, and cerebrospinal fluid [3–5]. EGF exhibits diverse biological actions, influencing proliferation, differentiation, and functional activities of various types of mammalian cells [3–6]. EGF has also been a useful tool for the analysis of receptor hormone interactions and receptor mediated endocytosis of polypeptide hormones. Several excellent reviews of the chemistry and biology of EGF have been published [3–7].

In recent years studies on the biological action of EGF have attracted greater interest, partly because EGF has been implicated in the processes of neoplastic transformation. EGF stimulates proliferation of various tumor cells in culture [3–7]. Several types of tumor cells, including breast cancer cells, possess EGF receptors [6–10]. It has also been shown that EGF enhances the induction of mouse skin tumor by methycholanthrene and potentiates the viral transformation of rat ovarian granulosa cells [12]. In addition, EGF has been shown to have a strong amino acid sequence homology with transforming growth factor [13]. More recently, it was reported that the amino acid sequence of EGF receptor is similar to that of the product of an oncogene, *erb* B, avian erythroblastosis virus [14]. Although these important findings stimulated a wide range of research activities, the physiological role of EGF remains unclear.

Our interest in EGF stems from our study elucidating the regulatory mechanisms involved in the growth and differentiation of the mammary gland. In the past several years we have been focusing our studies on the role of EGF in the development of the mouse mammary gland during pregnancy. These studies have provided several lines of evidence suggesting that

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

EGF serves as a physiological stimulus for the development of the mammary gland. More recently we have extended these studies to assess the role of EGF in mouse mammary tumorigenesis. In this chapter, we will review the experimental results which suggest the involvement of EGF in normal development of the mammary gland during pregnancy and spontaneous mammary tumorigenesis in female mice.

2. EGF and the development of mouse mammary gland during pregnancy

2.1 Growth of mammary gland during pregnancy

The mammary gland in virgin mice is largely made up of adipose tissue and contains a relatively small number of epithelial cells which are developmentally dormant. A marked growth of mammary tissue occurs following the onset of pregnancy, culminating in the production of milk after parturition. During the first stage of pregnancy, ductal growth occurs predominantly, producing a further branching of the ducts and formation of the end buds. During the middle and latter stages of pregnancy, mammary epithelial cells continue to proliferate to form the lobulo-alveolar structure while their differentiated function is suppressed. As parturition approaches, the alveolar epithelial cells gradually stop dividing and demonstrate secretory activity which reaches its maximum during lactation.

A number of studies have indicated that growth of the mammary gland during pregnancy is under hormonal control. Earlier work has implicated ovarian steroid hormones, estrogens and progesterone, prolactin, growth hormone, placental lactogen, glucocorticoids and thyroxine in growth and development of the mammary gland during pregnancy [15–17]. However, these hormones, which have been thought to promote growth of the mammary gland *in vivo*, are largely ineffective in stimulating mammary epithelial cell proliferation *in vitro*. Thus, the mitogenic stimulus of mammary epithelial cells during pregnancy remains unclear.

2.2 EGF concentration of submandibular gland and plasma during various reproduction stages

The mouse submandibular gland produces large amounts of EGF [4, 5]. The EGF concentration in the submandibular gland of virgin mice (2 to 3 months old, C3H/HeN) was on average 37.3 ± 4.2 ng/mg wet weight (S.E.M.) (Figure 1) [18]. During pregnancy, the concentration of EGF in the gland increased significantly over virgin levels. The average values were 284.7 ± 42.1 ng/mg wet weight at early pregnancy (day 1–9), 185.5 ± 22.4 ng/mg wet weight at mid-pregnancy (days 10–15) and 210 ± 16.1 ng/mg wet weight at late pregnancy (days 16–20). These high levels persisted during lactation and after its cessation.

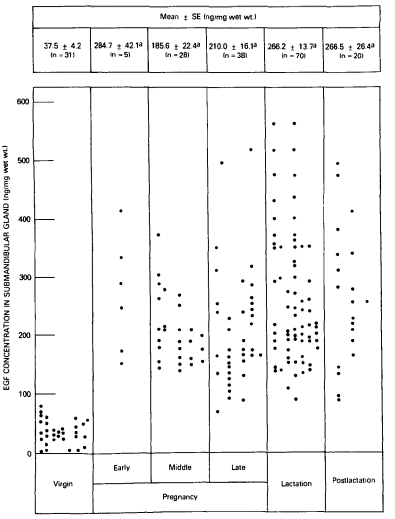


Figure 1. EGF concentration in submandibular glands of female mice at various reproductive stages. The amount of EGF was determined by radioimmunoassay. The number of points in each column represents the number of mice used (data from Kurachi and Oka, 1985).

In mice, EGF concentrations in plasma and serum were essentially the same and no evidence for the release of EGF from blood elements was found (19). Plasma EGF levels undergo circadian periodicity; being much higher at 24 hr–8 hr than at 12 hr–20 hr at all reproductive stages examined [18]. The plasma EGF at 24.00 hr were 0.18 ± 0.11 ng/ml for virgin mice, whereas the EGF concentrations in the plasma of pregnant, lactating and post-lactational mice were increased to 0.87 ± 0.20 ng/ml, 2.33 ± 0.89 ng/ml and 1.20 ± 0.46 ng/ml, respectively. The levels of plasma EGF in sialoadenectomized pregnant and lactating mice were below the level of detection by the assay used, i.e., less than 0.1 ng/ml. These results suggest that the submandibular gland may be the major source of plasma EGF in female mice. Recently several studies also showed that removal of submandibular glands decreased plasma levels of EGF in mice [20, 21], although it was reported earlier that sialoadenectomy had little effect on plasma levels of EGF [22]. The reason for this discrepancy is not known at present.

The above results indicate that the level of plasma EGF parallels the submandibular EGF level. There are indications that EGF is excreted in an exocrine fashion from the gland [23], and is resorbed from the digestive tract into the circulatory system [24]. However, earlier studies [22] have suggested that the production and release of EGF in the submandibular gland is not always correlated. It is possible that additional factors which control the release of EGF from the submandibular gland, participate in parallel changes in the submandibular and plasma EGF following gestation.

The physiological control mechanism of the synthesis and secretion of EGF during pregnancy remains to be clarified. Earlier studies have shown that the

concentration of EGF in the submandibular gland is increased by androgens and progestins [22, 25]. Because the circulating levels of these steroid hormones increase during pregnancy [26], it is conceivable that the rise in submandibular EGF during gestation is caused by androgen and/or progesterone. However, it is not clear whether the stimulatory effect of the steroid hormones persists long enough to account for the sustained increase of EGF in the submandibular glands of lactating and post-lactational mice, since the circulating levels of progesterone and testosterone decrease to low levels in these mice.

2.3 Effects of EGF on mamary epithelium in culture

One of the unique features of the mammary gland is that it is feasible to induce growth and differentiation of mammary epithelium *in vitro* in the presence of appropriate stimuli. For example, the combination of insulin, glucocorticoids and prolactin stimulates milk protein gene expression in the mammary explant culture [17]. More recently, mammary epithelial cell culture has been developed using non-plastic substrates such as collagen-coated dishes, collagen gels, and extracellular matrix [27]. These *in vitro* systems have been extremely useful in delineating the role of hormones and their mechanisms of action in the regulation of mammary gland development.

Several investigators have shown that EGF stimulates proliferation and morphological development of mouse mammary epithelial cells in *in vitro* systems such as mammary tissue explants, whole gland and cell culture [28–31]. In a primary mouse mammary epithelial cell culture using collagen as a substrate, the combination of insulin, cortisol and prolactin stimulates production of milk proteins, casein and α-lactalbumin [32, 33]. The addition of EGF at 50 ng/ml increased the total number of epithelial cells by 30–40% and thymidine incorporation into DNA approximately five-fold. In contrast, EGF inhibited hormonal induction of casein and α-lactalbumin in those cells by about 45% and 55%, respectively and decreased the translatable casein mRNA activity by 55%. The effect of EGF to stimulate mammary cell proliferation and to inhibit casein production was apparent at 0.1 ng/ml and was maximal at 50–100 ng/ml. The ED_{50} value was about 2–3 ng/ml, which is in the range of physiological concentrations of circulating EGF [19] (Figure 2). These effects of EGF are specific, since other growth factors such as fibroblast growth factor, multiplication stimulating activity, nerve growth factor, and platelet-derived growth factor were ineffective. Recently it was reported that EGF inhibits the induction of both κ casein polypeptides and κ casein mRNA sequences in mammary explants from rats and mice cultured in the presence of insulin, aldosterone, corticosterone and prolactin [34]. However, when prolactin was omitted, EGF increased the level of α casein mRNA in pregnant mouse mammary explants, but not in rats [34]. The bifunctional effect of EGF was also manifested in the synthesis of total casein and α-lactalbumin in cultured mammary tissue.

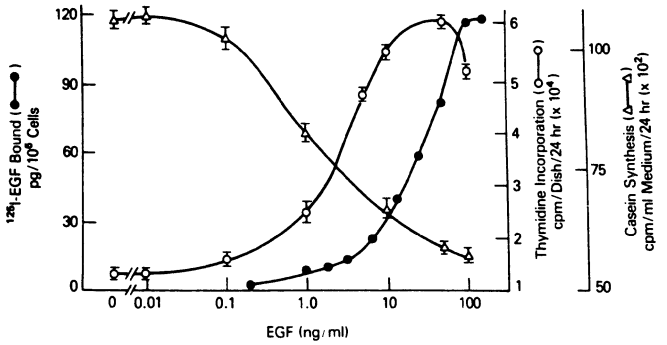


Figure 2. Effect of EGF concentration on ^{125}I-EGF binding, DNA synthesis, and casein synthesis in mouse mammary epithelium in culture. The amount of ^{125}I-EGF binding to mammary cells was determined at zero time before plating. The synthesis of DNA and of casein was measured in mammary cells cultured for 4 days in medium containing insulin, cortisol, prolactin, and the indicated concentrations of EGF. Each value represents the means ± SEM of 5-8 determinations (data from Taketani and Oka, 1983b).

During pregnancy, mammary epithelial cells undergo massive proliferation, whereas their differentiated function is suppressed. Progesterone [16, 17] and cAMP [35], which inhibit the hormonal induction of milk protein synthesis *in vitro* have been considered putative suppressive factors for the differentiation of mammary epithelium during gestation. However, the finding of the dual abilities of EGF to stimulate mammary cell proliferation and to inhibit milk protein synthesis raises the possibility that it plays a role in the development of the mammary gland during pregnancy.

12-0-tetradecanoylphorbol 13-acetate (TPA) is a potent tumor promoter and shares several biological activities of EGF [36]. In a primary mammary cell, TPA also mimicked the effect of EGF by stimulating cell proliferation and inhibiting the synthesis of milk proteins casein and α lactalbumin [37]. The ability of various TPA analogs to stimulate DNA synthesis and inhibit casein synthesis correlated with their potency as tumor promoters. In addition, both TPA and EGF decreased the specific prolactin binding of mammary cells by 50%.

2.4 EGF receptors in mouse mammary epithelium

Biological actions of EGF are thought to be mediated through specific high-affinity receptors on the plasma membrane [38]. FGF receptor is a glycoprotein with a molecular weight of 170,000. Binding of EGF to its specific receptor triggers a cascade of events, one of the earliest being the activation of tyrosine kinase activity. Normal mouse mammary epithelial cells in a primary cell culture possess the capacity to bind EGF in a specific and saturable manner [33]. Scatchard plot analysis showed a curvilinear pattern which suggests the presence of two classes of receptors with a high and a low affinity: Kd values = 1×10^{-10} M and 3.6×10^{-9} M respectively. The oc-

cupancy of EGF receptors for a half-maximal stimulation of DNA synthesis or inhibition of casein production was 10% and 6% of total receptors, respectively [33] (Figure 2).

TPA stimulates DNA synthesis synergistically with EGF in cultured mammary cells and increases the amount of EGF binding to mammary cells [33]. It is not known whether TPA increases the number and/or affinity of EGF receptors. One possible explanation is that TPA prevents the down-regulation of EGF receptors which occurs as a result of internalization and lysosomal degradation of the hormone-receptor complex [39].

More recently, using membrane preparation of the mouse mammary gland, the presence of specific high affinity receptors for EGF has been demonstrated [40]. In addition, the level of EGF receptors in mammary tissue was found to change at various physiological states: EGF receptor levels are relatively low in the glands of virgin mice, increase during pregnancy, reaching a peak on day 10 of gestation, and decrease to low levels during lactation. These changes are consistent with the possible involvement of EGF in the development of mammary gland during pregnancy.

2.5 The effect of sialoadenectomy on mammary gland development during pregnancy

As described above, a large amount of EGF is synthesized and secreted by the submandibular gland. After sialoadenectomy, plasma EGF decreases rapidly to undetectable levels, suggesting that the submandibular gland is a major source of circulating EGF. In order to assess the possible physiological role of EGF in the development of the mammary gland during pregnancy, sialoadenectomy (bilateral removal of the submandibular gland) of virgin mice was performed to examine its subsequent effect on mammary gland development during pregnancy and lactation [41].

Sialoadenectomized virgin mice showed no apparent signs of abnormality as judged by their eating and drinking habits, weight gain, and general and mating behavior. A substantial number of sialoadenectomized mice completed normal pregnancy and delivered approximately 6 pups per mother. However, the mammary gland of lactating sialoadenectomized mice was smaller in size and produced less milk compared with those of sham-operated mice (Table 1). The decreased growth and milk-producing capacity of the mammary gland of sialoadenectomized mice were also evident during pregnancy. Mammary explants from those mice synthesized less casein in response to lactogenic stimuli, insulin, cortisol and prolactin in an organ culture system [41].

The major consequence of reduced growth of the mammary gland in sialoadenectomized mice is that a substantial number of pups born to and nursed by sialoadenectomized mothers die within 5 days of birth (Figure 3). This did not occur among pups born to normal or parotidectomized mice. Crossfoster nursing experiments indicated that the increased mortality of pups was due

Table 1. Comparison of mammary gland weight and milk production of normal and sialoadenectomized lactating mice.

Mice (n)	Days of lactation	Body wt (g)	Mammary gland wt (g)	Milk volume (ml)
Normal				
(7)	5	30.5 ± 0.94	3.35 ± 0.21	0.35 ± 0.04
(8)	10	33.0 ± 0.41	4.31 ± 0.14	0.67 ± 0.06
(11)	15	34.0 ± 0.53	5.32 ± 0.17	0.72 ± 0.06
Sialoadenectomized				
(10)	5	26.8 ± 0.73	2.53 ± 0.21	0.20 ± 0.02
(11)	10	29.0 ± 0.39	3.49 ± 0.14	0.52 ± 0.03
(10)	15	30.1 ± 0.90	4.45 ± 0.37	0.55 ± 0.05

Significant differences ($P < 0.01$) were found between normal and sialoadenectomized mice in all parameters by analysis of variance. Sialoadenectomy was performed pregestationally (data from Okamoto and Oka, 1984).

to the inability of sialoadenectomized mothers to nourish the normal-sized litters. Analysis of the milk protein produced by normal and sialoadenectomized mothers during lactation revealed no significant differences in those identifiable species of milk proteins. When EGF was administered daily at a dose of 5 μg into sialoadenectomized pregnant mice, the survival rate of the pups increased to normal levels. It was also found that EGF-treated sialoadenectomized mothers had mammary tissue of normal size and weight and produced similar amounts of milk comparable to those of normal mothers. These results are consistent with the view that EGF derived from the submandibular gland plays a critical physiological role in the development of mammary gland during pregnancy.

The series of experimental results presented above led us to propose that EGF is an important growth factor for the development of mammary gland

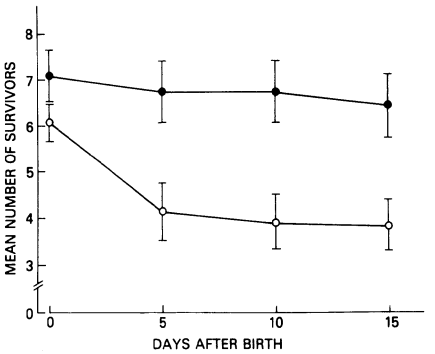


Figure 3. The mean number of surviving pups born to and nursed by (•) control (n = 11) or (○) sialoadenectomized (n = 14) mothers during lactation (data from Okamoto and Oka, 1984).

during pregnancy. It is noteworthy, however, that in spite of EGF deficiency, the mammary gland of sialoadenctomized mice exhibits a considerable degree of development during pregnancy, which suggests that there are other growth factors which stimulate growth of the mammary gland during gestation.

3. EGF and spontaneous mammary tumorigenesis

3.1 Spontaneous mouse mammary tumors

Certain inbred strains of mice such as C3H, A, and DBA have a high incidence of spontaneous mammary tumors. The mouse mammary tumor system is one of the most extensively investigated models, spanning a 50-year period. The etiology of mammary tumors is complex, involving viral, genetic and humoral factors, and these aspects of mammary tumors have been reviewed [16, 42–45]. It is generally thought that a virus in the milk of the mother is vertically transmitted to the daughter. The virus modifies the properties of the mammary cells so that under the proper hormonal stimuli and genetic predisposition, some mammary cells develop into tumor cells [45]. Mammary tumors appear during the latter part of life and thus are thought to be non-hormone-dependent. The growth factor for mouse mammary tumors has not been identified.

3.2 EGF concentrations at various ages

In female mice the concentration of EGF in the submandibular gland was low, 0.4–1.0 ng/mg wet weight, during the first 6 weeks of age, but increasing to 46 ng/mg wet weight by 8 weeks of age. It remained relatively constant up to 24 weeks of age but, after 30 weeks of age, the EGF concentrations in the gland began to increase again, reaching 400 ng/mg wet weight at 40–48 weeks of age [46] (Figure 4).

The level of plasma EGF at 37–47 weeks of age was significantly elevated when compared to that of 9–12 week old female mice: more than 50% of the former group had plasma EGF at levels higher than 0.1 ng/ml, whereas only 5% of the latter group had detectable levels (Table 2). Sialoadenectomy of aged mice reduced circulating EGF to undetectable levels. A large increase in the submandibular gland EGF concentration occurring in female mice between 30 and 40 weeks of age coincided with the time of decreasing ovarian function, suggesting that ovarian dysfunction is a cause of the greater increase in the submandibular gland EGF concentration in aged female mice. This hypothesis is supported by the finding that the submandibular EGF concentration of 12-week-old female mice increased to 400 ng/mg wet weight 4 weeks after ovariectomy and this increase was sustained for the next 4 weeks [46]. These levels were very similar to those of mice at 40–48 weeks of age. The increase in submandibular EGF in ovariectomized mice was suppressed

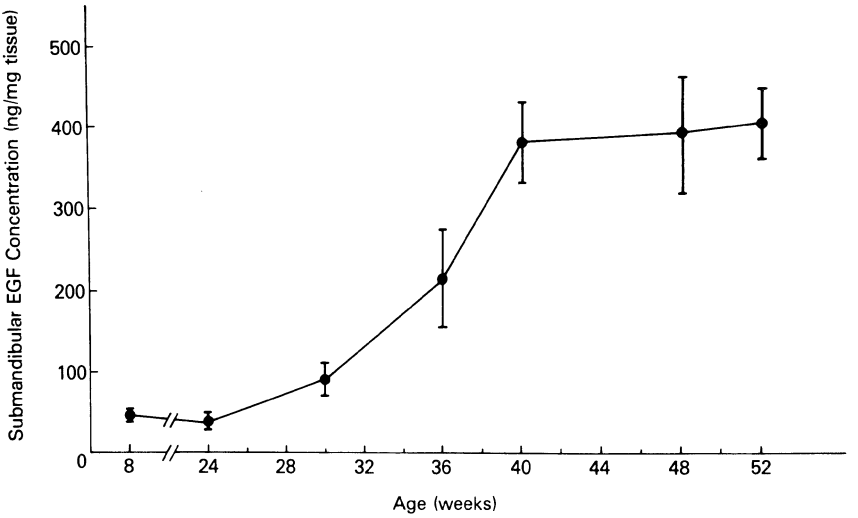


Figure 4. EGF concentration in submandibular glands of virgin mice at various ages. Each point represents the mean ± SEM of 4-6 mice (data from Kurachi and Oka, 1986).

by the administration of estriadol (1 μg/mouse/day) but not progesterone (1 mg/mouse/day).

Immunohistological studies have shown that EGF is produced and stored in granular convoluted tubular (GCT) cells of the mouse submandibular gland [4]. Our histological studies indicated the GCT cells were more abundant in the glands of 40-week-old females and ovariectomized 12-week-old mice than in those of 12-week-old female mice and estrogentreated ovariectomized mice (Figure 5). These findings are consistent with the data obtained by radioimmunoassay of EGF in the gland. Furthermore, they suggest that the number of GCT cells in the gland is a major determinant of its EGF content.

3.3 Involvement of EGF in spontaneous mammary tumorigenesis

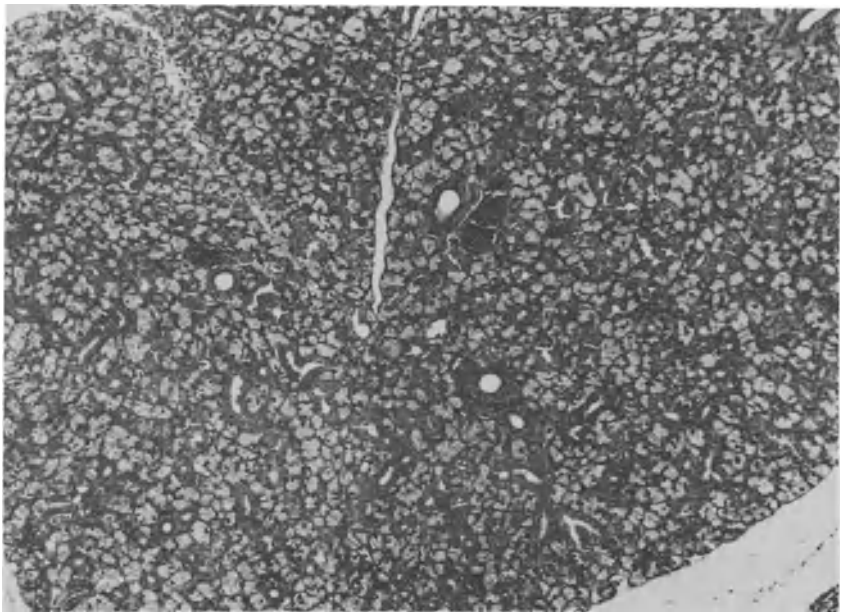
IN the C3H strain, female mice have a high incidence of spontaneous mammary tumors during the latter part of life [45]. As shown in Figure 6,

Table 2. EGF levels in plasma of normal young and old virgin female mice.

Age	n	Plasma EGF % detected (> 0.1 ng/ml)
9–12 weeks	20	5
37–47 weeks	11	54.5*

*$P < 0.01$ by Chi-square test.

A.



B.

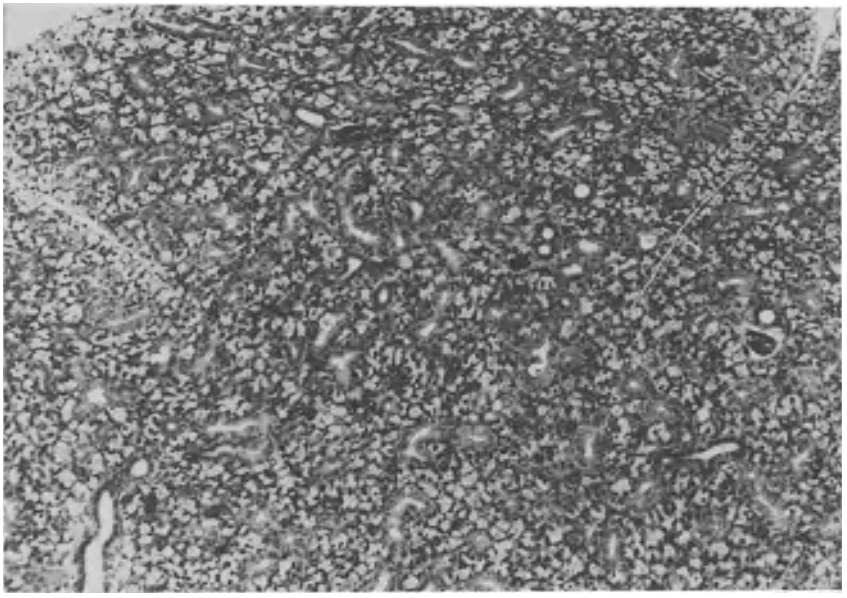
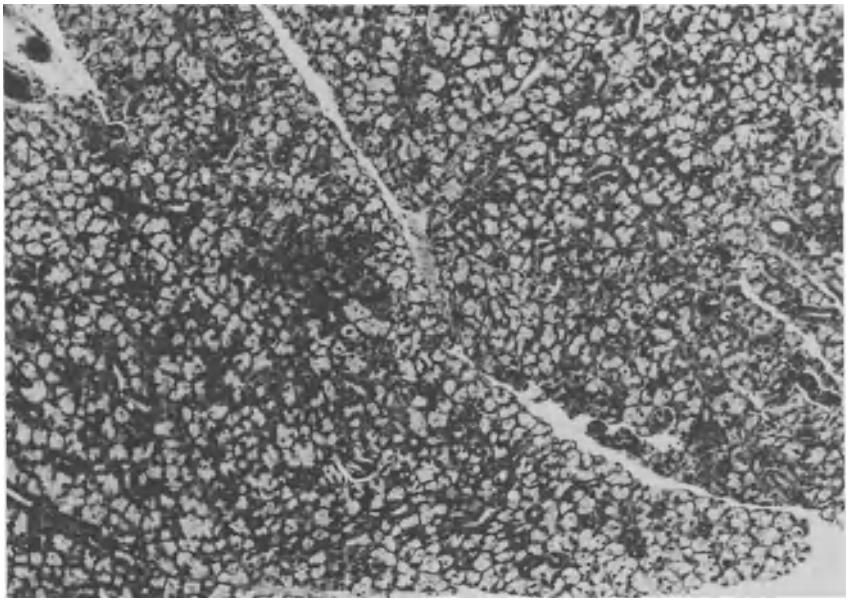


Figure 5. Histological sections of submandibular gland in 40-week-old female (A), ovariectomized, 12-week-old female (B), and 12-week-old control (C).

C. 

mammary tumors in normal C3H/HeN females first appear at 31 weeks of age and thereafter the incidence of tumor increases with age [47]. The most rapid increase occurs between 42 and 46 weeks of age. The tumor incidence reaches a plateau of 62.5% at 52 weeks of age. This value is in accordance with that reported previously [42–45].

The temporal pattern of mammary tumor incidence closely follows the increase in the EGF concentration of submandibular gland that occurs after 30 weeks of age (see Figures 4 and 6). In order to examine a possible role of the submandibular gland EGF in mouse mammary tumorigenesis, virgin females were sialoadenectomized at 14 or 22 weeks of age. In these animals the incidence of mammary tumor was greatly reduced to approximately 13% at 52 weeks of age (Figure 6). Furthermore, the latency period of tumor incidence in sialoadenecotmized mice was prolonged as much as 14 weeks when compared with that of normal females. Mammary tumor incidence in sialoadenectomized mice increases to 28% at 66 weeks of age, suggesting that tumors may grow more slowly in these animals.

Administration of EGF (5 μg per mouse) to 14-week-old sialoadenectomized mice every other day up to the age of 52 weeks increased the incidence of mammary tumor to 33.3% [47]. It may be possible to enhance the effect of EGF on mammary tumor incidence by more frequent injections of EGF.

Histological examination of mammary tumors found in normal female mice indicated that mammary tumor cells are composed of small cuboidal

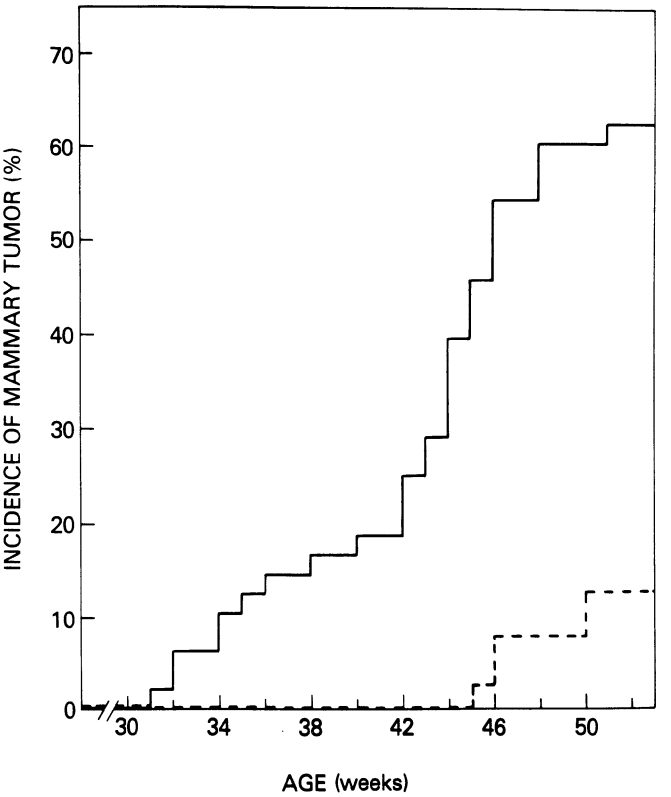
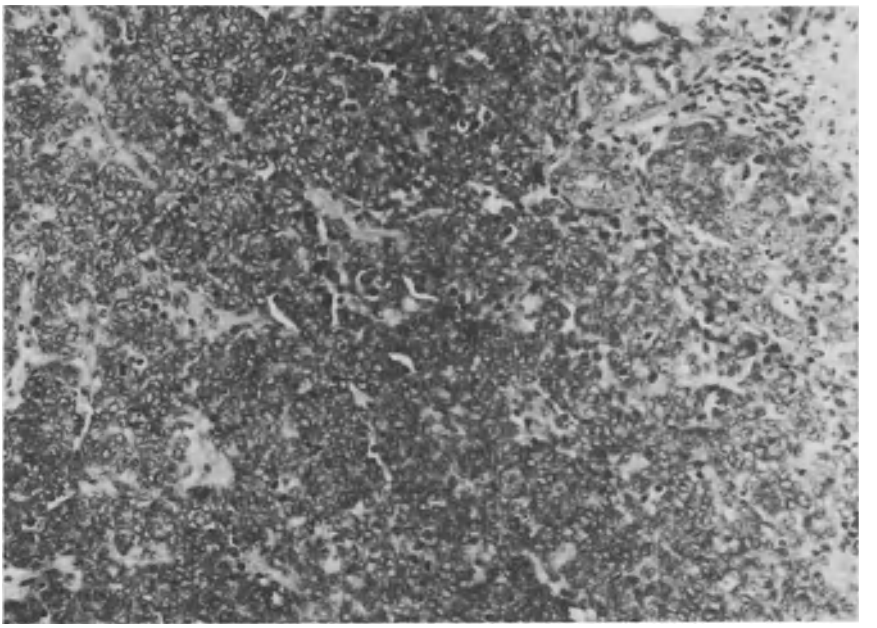
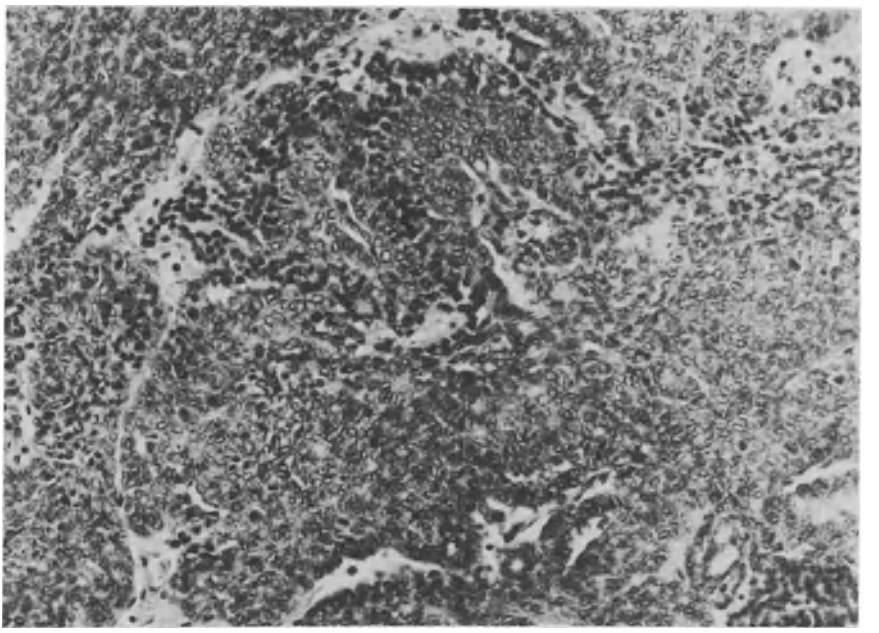


Figure 6. Mammary tumor incidence in normal and sialoadenectomized virgin mice at various ages. Normal (——, n = 48); sialoadenectomized mice (----, n = 39). (Data from Kurachi, Okamoto and Oka, 1985.)

epithelial cells and they form a highly distorted glandular pattern (Figure 7A). These histological features resemble type B adenocarcinoma, which is commonly found in mammary tumors of C3H mice [45]. The appearance of the tumors found in sialoadenectomized mice showed some difference in the structural pattern and cytological features, having a more organized pattern of ductal and glandular structure (Figure 7B). It is possible that mammary tumors in sialoadenectomized and control mice are different in terms of origin, cell type, etiological agents, and their dependency on EGF. The data presented above show that sialoadenectomy markedly decreases the mammary tumor incidence and delays the time of tumor onset. The question as to whether a low incidence of mammary tumor in sialoadenectomized mice is due to a decrease in the number of transformed cells or it reflects slower growth of the same number of transformed cells in the absence of EGF remains to be answered. Since EGF stimulates proliferation of normal and neoplastic mammary cells in culture [28–33, 48], it is possible that EGF serves as a promoter of mammary tumors.



A.



B

Figure 7. Histological sections of mammary tumors in control (A) and sialoadenectomized mice (B) (data from Kurachi, Okamoto and Oka, 1985.)

3.4 EGF and mammary tumor growth

The above results indicate that long-term sialoadenectomy greatly reduces the incidence of mouse mammary tumor and suggest that EGF is important for mammary tumorigenesis. We also examined the short-term effects of sialoadenectomy on the growth of tumors [47]. As shown in Figure 8, mammary tumors in the sham-operated mice grew extensively until the animals died on 58th day after tumor occurrence. By contrast, sialoadenectomy of tumor-bearing mice rapidly produced sustained inhibition of tumor growth. However, when EGF was administered daily (5 μg/mouse) to tumor-bearing sialoadenectomized mice, the tumors quickly resumed growth at a rate similar to that in the control group. Our preliminary experiments indicate that removal of the parotid glands, another salivary organ, is ineffective in inhibiting tumor growth.

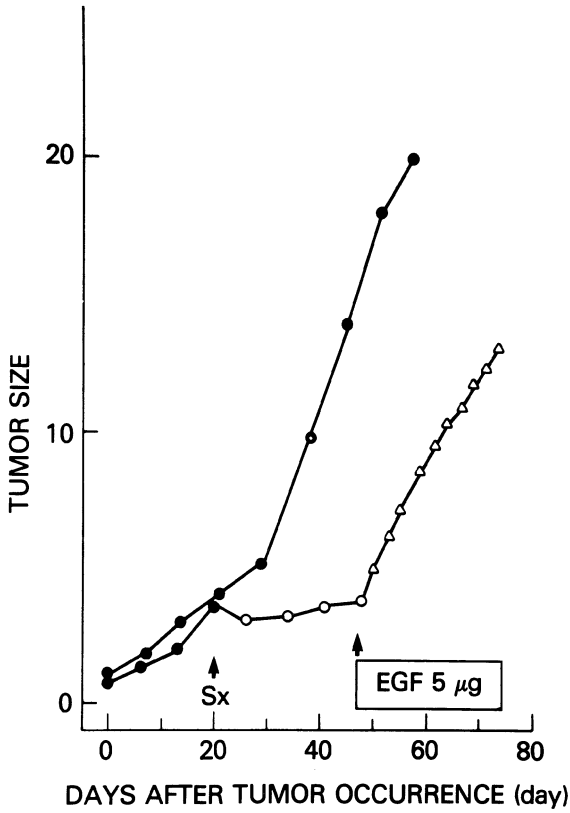


Figure 8. The effect of sialoadenectomy and EGF treatment on mammary tumor growth. Tumor-bearing mice were sham-operated or sialoadenectomized (Sx), or they were sialoadenectomized and then treated with EGF at the time indicated by arrows. The tumor growth was examined daily: •, control; ○, sialoadenectomized; △, sialoadenectomized and EGF-treated mice. (data from Kurachi, Okamoto and Oka, 1985).

In addition to sialoadenectomy, the administration of anti-EGF antiserum (prepared in rabbits) inhibited the growth of mammary tumors (Figure 9). However, the effect of anti-EGF was not long lasting; usually within 10 days of initial injection, mammary tumors began to grow as fast as those in the normal mice injected with normal rabbit serum. Although the effect of anti-EGF serum could be restored for a period of time by increasing the dose, eventually the amount of antiserum that could be injected into mice posed practical limitations. The diminution in the effectiveness of anti-EGF antibody was probably due to the host immune response to the rabbit serum. This problem might be circumvented by raising anti-EGF antibodies which are less antigenic to mice or by applying active immunization by heterologous EGF.

Anti-EGF-antiserum given together with sialoadenectomy produced no greater inhibition of mammary tumor growth than did sialoadenectomy alone, suggesting that sialoadenectomy is sufficient to reduce the level of host EGF needed for mammary tumor growth.

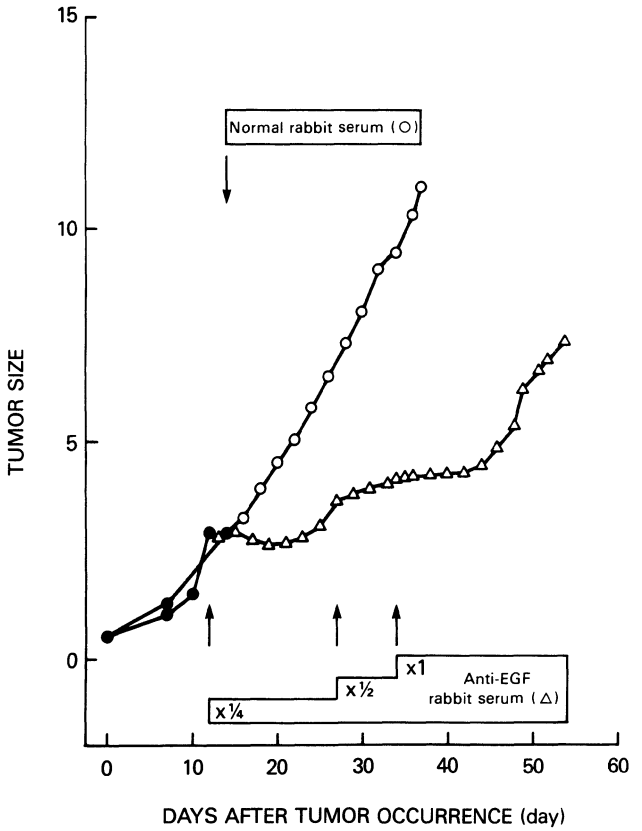


Figure 9. The effect of anti-EGF serum on mammary tumor growth. Normal rabbit serum (○), anti-EGF serum (△), Arrows indicate the time of injection of antiserum.

The results presented above indicate that EGF is important to the growth of mouse mammary tumor and that the tumor growth can be inhibited by reducing the availability of EGF by sialoadenectomy or treatment with anti-EGF antiserum. As a result of sialoadenectomy and anti-EGF treatment, the survival time of tumor-bearing mice was increased from 40.1 ± 3.7 days to 95.5 ± 9.1 days and 59.2 ± 5.8 days, respectively (Kurachi & Oka, unpublished). These results suggest a possible means of preventing and treating mammary tumors.

3.5 EGF and mammary tumor transplant in nude mice

The nude mouse has been an important tool in biomedical research because of its unique immunological property of accepting grafts of various tissue transplants, including tumors [49]. As an extension of our study of assessing the role of EGF in mammary tumor growth, we examined the involvement of EGF in transplantation of mouse mammary tumor in nude mice.

In female nude mice, EGF was present at a mean concentration of 42.8 ± 16.9 (SD) ng/mg wet weight in the submandibular gland and 0.28 ± 0.13 ng/ml in the plasma at 7 weeks of age. These levels are comparable to those of normal female mice. Sialoadenectomy decreased circulating EGF to undetectable levels (<0.1 ng/ml), suggesting that the submandibular gland is a major source of circulating EGF in nude mice [50].

The possible role of EGF in transplantation and growth of mouse mammary tumors in nude mice was examined by use of sialoadenectomy, anti-EGF treatment, and EGF replacement therapy. As shown in Table 3, the success rate of transplantation of a spontaneous mouse mammary tumor into nude mice was 55% in sham-operated female mice, whereas the remaining 45% of the tumors failed to implant and became remnant. This value is in agreement with those reported earlier in studies of various types of tumor

Table 3. Effect of sialoadenectomy, treatment with EGF antiserum and replacement of EGF on implantation of mouse mammary tumor into nude mice.

Mice	N	Treatment	Implantation (%)
Sham-operated	24	None	55
Sham-operated	10	EGF	90
Sialoadenectomized	12	None	17
Sialoadenectomized	8	EGF	88
Sialoadenectomized	8	Normal rabbit serum	25
Sialoadenectomized	8	EGF antiserum	0

Daily EGF treatment was given s.c. to sham-operated or sialoadenectomized female nude mice at a doze of 5 μg per mouse for 3 weeks starting immediately after C3H/HeN mammary tumor transplantation. Anti-EGF antiserum and normal rabbit serum were given to sialoadenectomized mice at a dose of 100 μl per mouse for 3 weeks immediately after tumor inoculation (data from Tsutsumi, Tsutsumi, Oka, 1987).

transplants in nude mice [49]. The success rate of mammary tumor implantation was increased to 90% by daily treatment of nude mice with EGF (5 μg/mouse) [50].

The success rate of mammary tumor implantations in sialoadenectomized nude mice was only 17%: the majority of tumor transplants failed to survive. Daily administration of EGF (5 μg/mouse) to sialoadenectomized mice increased the success rate of implantation to 88%. On the other hand, daily administration of anti-EGF antiserum (100 μl/mouse) to sialoadenectomized mice totally prevented the implantation of mammary tumor transplants. Administration of normal rabbit serum had no effect on the rate of tumor implantation.

Mouse mammary tumors successfully implanted in nude mice started to grow 3 weeks after inoculation; the growth rate was relatively constant with doubling of tumor size in 2.4 days. Both sialoadenectomy and daily anti-EGF treatment of nude mice decreased the rate of tumor growth by 50%. Daily treatment of sialoadenectomized mice with anti-EGF almost completely inhibited tumor growth for the first 3 days and thereafter the tumors grew slowly with a doubling time of 8 days [50].

The results presented above suggest that EGF in nude mice plays an important role in both implantation and growth of mouse mammary tumor transplants. These results are also consistent with our previous findings that EGF serves as a physiological agent for growth of mouse mammary tumors.

It is noteworthy that anti-EGF administered to sialoadenectomized mice completely prevented tumor implantation in nude mice. This finding raises the possibility that there may be some other sources of EGF, in addition to the submandibular gland. Several investigators have reported that EGF-like polypeptides are produced by breast cancer cells [51, 52]. It is possible that locally produced EGF also plays a role in the implantation and growth of the mammary tumor cells. Finally, the present study suggests that sialoadenectomy and anti-EGF treatment of nude mice may be useful in screening the EGF-dependency of various types of tumors.

4. Concluding remarks

The biological action of EGF has been studied extensively in a number of experimental systems while physiological studies to assess the function of EGF in the body have received less attention. In this chapter we have reviewed the physiological function of EGF in normal and neoplastic growth of mammary epithelial cells in mice. EGF has been shown to be present not only in mice and rats but also in humans, and exhibits phylogenetic structural conservation and biological cross-reactivity [3–5]. These features of EGF suggest that it has important physiological functions. The mouse system has proved to be an excellent model in which to examine the role of EGF in the body because the submandibular gland serves as a major source of circulating

EGF and also because large amounts of pure mouse EGF and anti-EGF serum are comercially available. Thus it is possible to induce EGF deficiency in mice by means of sialoadenectomy (and anti-EGF antibody treatment) and to institute EGF replacement therapy in order to evaluate the function of EGF in the body. These approaches, which have been used successfully in the classical studies of endocrinology, have been very useful in our study when combined with measurement of EGF levels by radio-immunoassay. In addition, we employed mammary cells cultures to study the biological action of EGF directly. At present, similar experimental approaches may not be fully applicable to the rat, humans and other systems, since the major site of EGF production and origin of circulating EGF have not been identified in these species. Nevertheless, the experimental data obtained in the mouse system may provide useful information for understanding the physiological role of EGF in normal and neoplastic growth of mammalian cells. The scope of this chapter has been limited to the physiological and biological action of EGF in the mouse mammary system. Thus, other important aspects of EGF in human breast cancer, the biochemistry and molecular biology of EGF receptors and the study of its mechanism of action have, unfortunately, not been covered. The reader is referred to several excellent articles in this volume and elsewhere for an up-to-date development and in-depth discussion of these areas of EGF research [53–56].

References

1. Cohen S: Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. J Biol Chem 237:1555–1562, 1962.
2. Savage CR, Jr Inagami T, Cohen S: The primary structure of epidermal growth factor. J Biol Chem 247:7612–7621, 1972.
3. Cohen S, Savage CR, Jr: Recent studies on the chemistry and biology of epidermal growth factor. Recent Progress in Horm Res 30:551–575, 1974.
4. Carpenter G: The regulation of cell proliferation: advances in the biology and mechanism of action of epidermal growth factor. J Invest Dermatol 71:283–287.
5. Barka T: Biologically active polypeptides in submandibular glands. J Histochem Cytochem 28:836–859, 1980.
6. Cohen S: The epidermal growth factor. Cancer 51:1787–1791, 1983.
7. Schlessinger J, Schreiber AB, Levi A, Lax I, Liberman T, Yarden Y: Regulation of cell proliferation by epidermal growth factor. CRC Critical Reviews in Biochemistry 14:93–111, 1983.
8. Fitzpatrick SL, Lachance MP, Schultz GS: Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. Cancer Res 44:3442–3447, 1984.
9. Sainsbury JRC, Farndon JR, Sherbet GV, Harris AL: Epidermal growth factor receptors and oestrogen receptors in human breast cancer. Lancet i:364–366, 1985.
10. Roos V, Fabbro D, Kung W, Costa SD, Eppenberger U: Correlation between hormone dependency and the regulation of epidermal growth factor receptor by tumor promoters in human mammary carcinoma cells. Proc Natl Acad Sci USA 83:991–995, 1986.
11. Rose SP, Stahn R, Passovoy DS, Herschman H: Epidermal growth factor enhancement of skin tumor induction in mice. Experientia 32:913–915, 1976.
12. Harrison J, Auersperg N: Epidermal growth factor enhances viral transformation of

granulosa cells. Science 213:218–219, 1981.
13. Marquardt H, Hunkapiller MW, Hood LE, Twardzik DR, DeLarco JE, Stephenson JR, Todaro GJ: Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor. Proc Natl Acad Sci USA 80:4684–4688, 1983.
14. Downward J, Yarden Y, Mayers E, Secrase G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. Nature 307:521–527, 1984.
15. Nandi S: Endocrine control of mammary gland development and function in the C3H/HeCrgl. J Natl Cancer Inst 21: 1039–1063, 1958.
16. Banerjee MR: Responses of mammary cells to hormones. Int Rev Cytal 47, 1–97, 1976.
17. Topper YJ, Freeman CS: Multiple hormone interactions in the developmental biology of the mammary gland. Physiol Rev 60:1049–1106, 1980.
18. Kurachi H, Oka T: Changes in epidermal growth factor concentrations of submandibular gland, plasma and urine of normal and sialoadenectomized female mice during various reproductive stages. J Endocrinol 106:197–202, 1985.
19. Peeheentupa J, Laksmanan J, Hoath SB, Beri U, Kim H, Macaso T, Fisher DA: Epidermal growth factor measurements in mouse plasma: Method, ontogeny and sex difference. Am J Physiol 248:E391–396, 1985.
20. Perheentupa J, Laksmanan J, Fisher DA: Epidermal growth factor in mouse urine: non-blood origin and increase by sialoadenectomy and T4 therapy. Acta Endocrinol 108:428–432, 1985.
21. Tsutsumi O, Kurachi H, Oka T: A physiological role of epidermal growth factor in male reproductive function. Science 232:975–977, 1986.
22. Byyny RL, Orth DN, Cohen S: Epidermal growth factor: effects of androgens and adrenergic agents. Endocrinology 95, 776–782, 1974.
23. Murphy RA, Watson AY, Metz J, Forssman WG: The mouse submandibular gland: an exocrine organ for growth factor. J Histochem Cytochem 28:890–902, 1980.
24. Thornburg W, Matrisian L, Magun B, Koldovsky O: Gastrointestinal absorption of epidermal growth factor in suckling rats. Am J Physiol 246:G80–85, 1984.
25. Bullock L, Barthe PL, Mowszowicz I, Orth DN, Bardin CW: The effects of progestins on submaxillary gland epidermal growth factor: demonstration of androgenic, synandrogenic and anti-androgenic action. Endocrinology 97:189–195, 1975.
26. Barkley MS, Geschwind II, Bradford GE: The gestational pattern of estradiol, testosterone and progesterone secretion in selected strais of mice. Biol Reprod 20:733–738, 1979.
27. Emerman JT, Enami J, Pitelka DR, Nandi S: Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes. Proc Natl Acad Sci USA 74:4466–4470, 1977.
28. Turkington RW: The role of epithelial growth factor in mammary gland development *in vitro*. Exp Cell Res 57:79–85, 1969.
29. Tonelli OJ, Sorof S: Epidermal growth factor reqirement for development of cultured mammary gland. Nature 285, 250–252 1980.
30. Yang J, Guzman R, Richards J, Imagawa N, McCormick K, Nandi S: Growth factor and cyclic nucleotide-induced proliferation of normal and malignant mammary epithelial cells in primary culture. Endocrinology 107, 35–41, 1980.
31. Richard J, Guzman R, Konrad M, Yang J, Nandi S: Growth of mouse mammary gland end-buds cultured in a collagen gel matrix. Exp Cell Res 141:433–439, 1982.
32. Taketani Y, Oka T: Epidermal growth factor stimulates cell proliferation and inhibits functional differentiation of mouse mammary epithelial cells in culture. Endocrinology 113:871–877, 1983a.
33. Taketani Y, Oka T: Biological action of epidermal growth factor and its functional receptors in normal mammary epithelial cells. Proc Natl Acad Sci USA 80:2647–2650, 1983b.
34. Vonderhaar BK, Nakhasi HL: Bifunctional activity of epidermal growth factor on α- and κ-casein gene expression in rodent mammary glands *in vitro*. Endocrinology 119:1178–1184, 1986.

35. Perry JW, Oka T: Cyclic-AMP as a negative regulator of hormonally induced lactogenesis in mouse mammary gland organ culture. Proc Natl Acad Sci USA 77:2093–2097, 1980.
36. Boutwell RK: The function and mechanism of promoters of carcinogenesis. CRC Crit Rev Toxicol 2:419–443, 1974.
37. Taketani Y, Oka T: Tumor promoter 12-0-tetradecanoylphorbol 13-acetate, like epidermal growth factor, stimulates cell proliferation and inhibits differentiation of mouse mammary epithelial cells in culture. Proc Natl Acad Sci USA 80:1646–1649, 1983.
38. Schechter Y, Hernaez L, Cuatrecasas P: Epidermal growth factor: Biological activity requires persistent occupation of high-affinity cell surface receptors. Proc Natl Acad Sci USA 75:5788–5791, 1978.
39. Gordon P, Carpenter JL, Cohen S, Orchi L: Epidermal growth factor: Morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. Proc Natl Acad Sci USA 75:5025–5029, 1978.
40. Ederg M, Pang K, Larson L, Colosi T, Nandi S: Epidermal growth factor receptor levels in mouse mammary glands in various physiological states. Endocrinology 117:405–411, 1985.
41. Okamoto S, Oka T: Evidence for physiological function of epidermal growth factor: pregestational sialoadenectomy of mice decreases milk production and increases offspring mortality during lactation period. Proc Natl Acad Sci USA 81:6059–6063, 1984.
42. Nandi S, McGrath CS: Mammary neoplasia in mice. Adv Cancer Res 17:353–414, 1973.
43. Bern HA, Nandi S: Recent studies of the hormonal influence in mouse mammary tumorigenesis. Prog Exp Tumor Res 2:91–145, 1961.
44. Abraham S, Bartley JC. In: Hormones and Cancer McKerns K, (ed), pp 29–74, Academic Press, New York, 1974.
45. Medina D: Mammary tumors. In: The Mouse in Biomedical Research, Foster HL, Small JD, and Fox JG (eds), Vol. 4, pp 373–396: New York, Academic Press, 1982.
46. Kurachi H, Oka T: Regulation of the level of epidermal growth factor by oestrogen in the submandibular gland of female mice. J Endocrinol 109:221–225, 1986.
47. Kurachi H, Okamoto S, Oka T: Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorigenesis. Proc Natl Acad Sci USA 82:5940–5943, 1985.
48. Turkington RW: Stimulation of mammary carcinoma cell proliferation to epidermal growth factor *in vitro*. Cancer Res 29:1457–1458, 1969.
49. Rygaard J: The nude mouse—background, some achievements, and implications. In: The Nude Mouse in Experimental and Clinical Research Fogle J and Giovanella BC, (eds), pp 95–109, New York: Academic Press, 1978.
50. Tsutsumi O, Tsutsumi A, Oka T: Importance of epidermal growth factor in implantation and growth of mouse mammary tumor in female nude mice. Cancer Res: (Submitted), 1987.
51. Salomon DS, Zwiebel JA, Bano M, Losoczy I, Fehnel P, Kidwell WR: Presence of transforming growth factors in human breast cancer cells. Cancer Res 44:4069–4099, 1984.
52. Dickson RB, Huff KK, Spencer EM, Lippman ME: Induction of epidermal growth factor-related polypeptides by 17 β estradiol in MCF-7 human breast cancer cells. Endocrinology 118:138–142, 1985.
53. James R, Bradshaw RA: Polypeptide growth factors. Ann Rev Biochem 53:259–292, 1984.
54. Scott J, Urdea M, Quiroga M: Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins. Science 221:236–240, 1983.
55. Hunter T: The epidermal growth factor receptor gene and its product. Nature 311:414–416, 1984.
56. Sporn MB, Roberts AB: Autocrine growth factor and cancer. Nature 313:745–747, 1985.

17. Tumor-associated growth factors in malignant rodent and human mammary epithelial cells

David S. Salomon, and William R. Kidwell

1. Introduction

Before considering the potential role of tumor-derived growth factors in regulating the proliferation of rodent and human mammary epithelial cells, it is important to emphasize several important aspects concerning the *in vivo* biology of the normal mammary gland. First, the mammary gland is not a static organ but is relatively dynamic. It periodically undergoes cycles of growth, development, differentiation, and regression. These cyclical events, which occur primarily during pregnancy and lactation, are in large part controlled by a complex interplay of various polypeptide and steroid hormones [1]. Super-imposed upon this endocrine background are proliferative and differentiation controls which might be exerted by several peptide growth factors. These growth factors may originate from tissues outside of the mammary gland in an endocrine fashion or they may be derived locally from within the gland as products of discrete cell types acting as either paracrine or autocrine factors [2–6]. This raises a second important point, namely that the mammary gland is not homogeneous but is a heterogenous tissue in which the mammary epithelial and myoepithelial cells are embedded within an adipose and mesenchymal stroma and separated from this compartment by a basal lamina or basement membrane. Survival and growth of mammary epithelial cells *in vitro* and *in vivo* is dependent upon the synthesis and maintenance of an intact basement membrane which is a specialized form of the extracellular matrix consisting of type IV collagen, laminin, and proteoglycans [7]. This structure is normally synthesized by the myoepithelial cells within the mammary gland [8]. The proliferation of mammary epithelial cells *in vitro* in response to specific growth factors can be modifed depending on the composition of the extracellular matrix upon which these cells rest [8, 9]. Conversely, certain growth factors may modulate the production of various extracellular matrix proteins by the mammary epithelial cells which, in turn, may control cell shape, substrate adherence, and the ability of these cells to respond to other growth factors and hormones. *In vivo*, this may have important implications as to how various cell types within the mammary gland interact to regulate cell proliferation and differentiation. In this scheme, the basement

M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology. ISBN 978-1-4612-8975-3

membrane performs an active and not a passive role as demonstrated by the ability of mammary epithelial cells to elaborate specific milk proteins when propagated on an appropriate extracellular matrix substrate [10, 11]. Finally, ductal and alveolar epithelial cells, basal or myoepithelial cells, and stromal fibroblasts may exhibit different requirements for various growth factors or hormones [2, 12, 13]. These requirements may fluctuate either coordinately or independently as a function of the pathophysiological state of the mammary gland. In addition, various mammotrophic hormones such as estrogens and prolactin may promote the growth and/or differentiation of mammary epithelial cells by indirectly or directly regulating the production of growth factors or receptors for growth factors in mammary epithelial cells, adjacent myoepithelial or stromal cells, or in more distant hormone-responsive target tissues [3–5, 14].

1.1 Growth factors, oncogenes and cell growth

The current interest in growth factors and their receptors stems from the pivotal role that these activities may perform in the etiology or progression of several disease states. Specifically, it is becoming apparent that the abnormal expression of specific growth factors or their cell surface receptors may be involved at certain stages in the initiation and development of a variety of malignancies, including breast cancer [15, 16]. One important phenotypic characteristic of all tumor cells is their ability to proliferate indefinitely in an unrestrained fashion. Whereas nontransformed epithelial cells and fibroblasts generally exhibit a strong dependency upon externally derived growth factors for growth regulation, transformed cells show a partial or complete relaxation in their growth factor requirements for anchorage-dependent proliferation [15–18]. This reduction in growth regulatory constraints may eventually lead to the acquisition of anchorage-independent growth, an *in vitro* property which shows a strong correlation with tumorigenic potential *in vivo* [19]. The progression of a normal cell to a fully malignant metastatic cell *in vivo* is a continuous multistep process [20]. During this conversion, changes in the responsiveness of cells to specific growth factors and in the interaction of cells with each other and surrounding extracellular matrix components within the basement membrane or stroma can ultimately lead to aberrations in cell proliferation, cell motility, and cell differentiation, which will have an eventual impact upon tumorigenicity and metastasis [21, 22]. One consequence of these changes is that tumor cells may eventually become totally independent of exogenous, host-derived growth factors for their proliferation because of the constitutive production of a spectrum of endogenous growth factors, such as TGFs, EGF, PDGF, IGF-I, IGF-II, and FGF, which may be involved in the autocrine growth of these cells [15, 16, 23]. In addition to elevated production and secretion of growth factors by tumor cells, overexpression of normal or aberrant cell surface receptors for growth factors may equally contribute to the enhanced growth potential of these transformed cells by sensitizing them to lower concentrations of growth factors. In conjunction

with these results is the recent knowledge that certain viral oncogenes or their cellular counterparts, the proto-oncogenes, can code for growth factors, growth factor receptors, or cell cycle-related proteins which may be involved in the intracellular signal transduction pathway for growth factors [15, 16, 23–26]. The activation by point mutation or inappropriate overexpression of specific oncogenes or proto-oncogenes may confer growth factor autonomy upon cells or may quantitatively or qualitatively change the responsiveness or sensitivity of cells to specific sets of growth factors [27–29]. For example, it is known that epithelial cells probably require the concerted action of several growth factors for their progression through the cell cycle. Moreover, the phenotypic transformation of normal fibroblasts *in vitro* is also dependent upon the coordinated effects of several growth factors, including TGFα, TGFβ, FGF, IGF-I, and PDGF [15, 16]. This situation is analogous to the *in vitro* transformation of primary embryonic or neonatal rodent fibroblasts by transfected retroviral oncogenes or activated cellular proto-oncogenes. In this case, expression of at least two functionally distinct oncogenes such as *ras* and *myc* is required to complete the transformation process *in vitro* and to initiate tumorigenesis *in vivo* [30]. The expression of multiple oncogenes to promote transformation is therefore probably a reflection of the interaction of proto-oncogene proteins that occurs during normal cell proliferation.

With respect to breast cancer, the possible function, distribution, and relationships of various growth factors and oncogenes is just starting to be defined [4–6, 31–52]. A number of peptide growth factors have been identified that can stimulate the proliferation of normal and neoplastic rodent and human mammary epithelial cells. In some cases, novel growth factors have been isolated and characterized from mammary tumors or breast cancer cell lines which strongly implicates these activities in the autocrine or paracrine growth of mammary tumor cells. However, there is little evidence to date to unequivocally state that any of these tumor-derived growth factors are obligatory and solely responsible for the maintenance of tumor cell growth. Nevertheless, the development of antibodies and pharmacological antagonists against these growth factors or their receptors should quickly resolve these questions. Our collective approach to this problem has been severalfold: 1) to identify those known growth factors which can regulate the proliferation of normal and neoplastic mammary epithelial cells; 2) to characterize novel growth factors from and/or for neoplastic mammary epithelial cells which may be involved in driving their proliferation and which may be crucial for maintaining their transformed phenotype; 3) to ellucidate the mechanism(s) by which these potential mitogens may regulate mammary epithelial cell proliferation; and 4) to compare the relative dependency for growth and survival of normal and malignant mammary epithelial cells on these growth factors. Toward this end, we would like to summarize in this review the biochemical and biological characteristics of four growth factors which we have identified in rodent and human breast cancer cell lines and in the extracts prepared from rodent and human mammary tumors. The reader is referred to other publications for data relating to the presence of additional growth factors from these

sources [31–52]. The four growth factors are collagen synthesis stimulating factor (CSSF), mammary-derived growth factor I (MDGF-I), mammary-derived growth factor II (MDGF-II), and transforming growth factor alpha (TGFα), an epidermal growth factor (EGF)-related activity [36, 37, 43, 44, 46, 47]. MDGF-II is related to TGFα, while MDGF-I and CSSF are unique activities. It should be stressed at the outset that a majority of these growth factors are not unique to mammary tumor cells, since they are found in large quantities in human milk, suggesting that normal mammary epithelial cells may have the capacity at certain stages in the differentiation of the mammary gland to elaborate and secrete these growth factors [4–6, 47, 53].

2. TGFα and MDGF-II

TGFs represent a large family of acid and heat-stable polypeptides or peptides which have been suggested to control the autocrine growth of tumor cells [23, 54, 55]. TGFs reversibly confer upon normal nontransformed cells several properties which are associated with the transformed phenotype, namely anchorage-independent growth (AIG) and a loss of contact inhibition of growth. TGFs consist of at least two functionally and structurally distinct classes of factors, TGFα and TGFβ. TGFα (M_r 5,400) is a potent mitogen which is functionally related to EGF and which binds to the EGF receptor [16, 54, 56]. The genes for rat and human TGFα have been cloned and the size of the mRNA (approximately 4.8 kb) for TGFα indicates that low molecular weight TGFα is probably derived from larger precursor polypeptides [57, 58]. These larger precursor molecules have been identified in a variety of cell lines and from human urine [36, 37, 41, 49–63]. They are biologically and immunologically active. Messenger RNA species for TGFα, which are capable of hybridizing to specific TGFα cDNA probes, have also been identified in a number of human and rodent tumor cell lines [42, 44, 57, 58]. TGFα and β cooperate with each other and with other growth factors, such as PDGF and IGF-I, to induce the AIG of some nontransformed rodent fibroblast cell lines. TGFα has been identified in normal and malignant rodent and human mammary epithelial cells [36–45]. Production of this class of growth factors is controlled by estrogens, suggesting that the growth-promoting effects of this hormone may be mediated in part by TGFα. The recent demonstration that enhanced synthesis and secretion of TGFα by rat fibroblasts which have been transfected with a human TGFα cDNA expression vector can lead to the induction of tumor formation in nude mice lends support to the thesis of TGFα being an autocrine growth factor [64].

2.1 Rat mammary tumors

EGF is broad spectrum mitogen for mesenchymal and epithelial cells [65]. EGF can stimulate the proliferation of primary cultures of rodent and human

mammary epithelial cells and to a lesser extent the growth of some human breast cancer cell lines [3, 5]. EGF is also capable of stimulating the lobuloalveolar development of mouse mammary gland explants *in vitro* and may be involved in the development and growth of certain spontaneous mouse mammary tumors *in vivo* [2, 66–68]. We initially observed that the growth and survival of primary cultures of rat or mouse mammary epithelial cells was dependent upon the presence of EGF and a glucocorticoid in a serum-free, hormone-defined medium [8, 9]. Under these growth conditions, the proliferation of rat mammary epithelial cells could be modulated not only by the presence of EGF and dexamethasone but also by the composition of the extracellular substratum. For example, on type I stromal collagen or tissue culture plastic, EGF and dexamethasone are normally required for mammary epithelial cell growth, while on type IV basement membrane collagen, mammary epithelial cells no longer required these two agents for proliferation. The refractoriness of the cells to EGF and dexamethasone on type IV collagen and their dependency on these two agents for growth on type I collagen or plastic was due to the ability of EGF to differentially enhance the endogenous synthesis of type IV collagen on foreign substrates (i.e., type I collagen and plastic) but not on type IV collagen. It was found that these effects were due to the ability of EGF to stimulate type IV collagen synthesis, while dexamethasone was found to attenuate the turnover of type IV collagen by inhibiting the appearance of a type IV-specific collagenase.

In contrast to normal rat mammary epithelial cells, primary cultures of mammary epithelial cells obtained from primary dimethylbenze-alpha-anthracene (DMBA)- or nitrosomethylurea (NMU)-induced rat mammary tumors were found to be unresponsive to EGF both with respect to the stimulation of growth and to the enhancement of type IV collagen production [36, 46]. In addition, these tumor cells possessed fewer EGF receptors than normal rat mammary epithelial cells (2.5×10^4 versus 8.4×10^4 sites/cell), whereas transplantable DMBA and NMU tumor cells totally lack EGF receptors. These results suggested that the tumor cells might be elaborating EGF or an EGF-like activity such as TGFα which would reduce or totally eliminate their dependency upon exogenous EGF. A TGFα activity, which we had designated as mammary tumor factor (MTF), was identified in the conditioned medium (CM) of cultured tumor cells and in the acid-ethanol extracts prepared directly from primary DMBA- or NMU-induced rat mammary adenocarcinomas [36]. MTF activity was initially detected by its ability to compete with EGF for binding to EGF receptors in a radioreceptor assay (RRA) and by its ability to stimulate the AIG of normal rat kidney (NRK) cells in soft agar as colonies. MTF could also enhance the anchorage-dependent growth of NRK cells, BALB/c 3T3 cells, chick embryo fibroblasts, and normal rat mammary epithelial cells. MTF, however, was unable to stimulate the proliferation of primary cultures of DMBA-induced rat mammary tumor cells.

The biological activity(ies) associated with MTF was found to be stable to

acid treatment, like EGF or TGFα, but was inactivated by heat denaturation and reduction. Following gel filtration chromatography under neutral and isotonic conditions, two major species of MTF were observed, one at 68,000 (pI 5.2) and a smaller 6,000 M_r species. EGF receptor-competing activity and NRK colony growth-promoting activity were associated with both species. At the present time, it is still unclear whether the larger species represents a binding protein associated with, or a precursor for, the smaller peptide. The low molecular weight species appears to be identical to TGFα, since immunoreactive TGFα can be detected in these crude tumor extracts and the 6,000 M_r species can compete with authentic TGFα for binding to anti-TGFα antibodies. Moreover, a TGFα 4.8-kb mRNA species can be detected in the poly(A)$^+$ RNA population from these tumors following Northern blot analysis and hybridization of the nitrocellulose filters with a mixture of ^{32}P nick-translated TGFα mouse and human cDNA probes [69].

Figure 1 shows the relative amounts of EGF receptor-competing activity in crude tissue extracts prepared from the primary DMBA- and NMU-induced rat mammary adenocarcinomas and from transplantable DMBA-I and NMU-II rat mammary tumors. Little or no EGF receptor-competing activity could be detected in the transplantable tumors, whereas extracts prepared from the primary DMBA- and NMU-induced rat mammary tumors contain substantial amounts of EGF receptor-competing activity. The primary chemically induced tumors represent well-differentiated, estrogen- and prolactin-dependent adenocarcinomas, while their transplantable counterparts are hormone-independent undifferentiated carcinomas. [70, 71]. Figure 2 illustrates the amount of NRK colony-stimulating activity and immunoreactive TGFα which

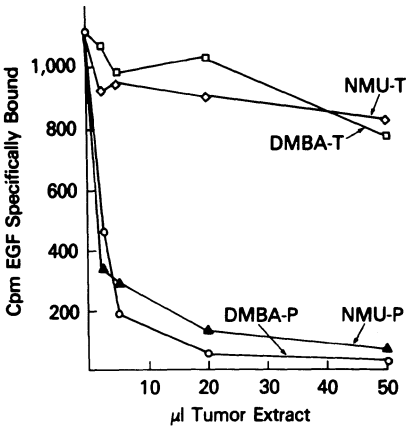


Figure 1. Presence of EGF receptor-competing activity in acid-ethanol extracts from primary and transplantable rat mammary tumors. Various amounts of acid-ethanol extracts prepared from primary DMBA-induced (○), primary NMU-induced (▲), transplantable (T) DMBA-induced (□), or transplantable (T) NMU-induced (◊) rat mammary tumors were incubated with ^{125}I-EGF (0.5 ng/ml) in an EGF RRA.

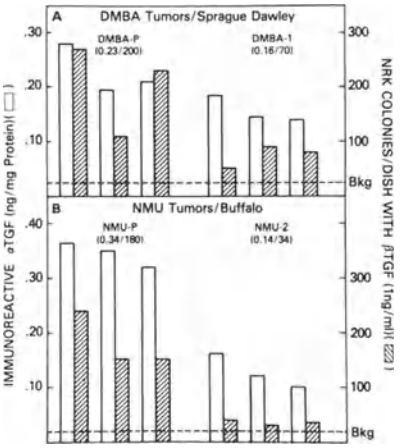


Figure 2. Presence of NRK soft agar growth-promoting activity and immunoreactive TGFα in primary and transplantable rat mammary tumors. Individual tumors were homogenized in 0.4 N HCl, centrifuged at 10,000 rpm for 30 min at 4°C, and absorbed to an activated Waters Sep-Pak C18 cartridge. Columns were washed with 4% acetic acid and eluted with absolute methanol containing 0.2% TFA. Following evaporation to dryness, samples were assayed in a NRK soft agar assay and in a TGFα RIA (Biotope Inc.). Each bar represents an individual tumor. Values in parentheses represent the average amounts of immunoreactive TGFα per milligram protein and the average number of NRK colonies for each of the four groups of tumors. NRK soft agar assays were routinely run in the presence of TGFβ (1 ng/ml) to score for endogenous TGFα.

could be detected in three individual tumors from each of the four types of tumors. The results demonstrate that the primary DMBA- and NMU-induced rat mammary tumors possess, on the average, higher levels of immunoreactive and biologically active TGFα than the transplantable DMBA-I and NMU-II tumors. In addition, the transplantable tumors either lack or possess low levels of expression of TGFα mRNA. The presence of higher levels of MTF or TGFα in the primary tumors suggests that the presence of this growth factor may be indicative of the degree of cellular differentiation in these rodent tumors or the hormone dependency of these tumors. This may be the case, since ovariectomy of Sprague-Dawley rats bearing primary DMBA-induced tumors results in the rapid decline in TGFα mRNA [69]. This response can be observed within 6 hr following ovariectomy and is specific for TGFα mRNA in the tumors. These results suggest that estrogens may be involved in inducing the expression of TGFα and that this growth factor may be one of several growth factors being elaborated by these tumor cells which are under estrogenic control. It should also be mentioned that the primary DMBA- or NMU-induced rat mammary tumors have either an elevated expression of the c-Harvey (Ha)-*ras* proto-oncogene (primary DMBA-induced tumors) or a point-mutated (position 12) activated form of the Ha-*ras* gene (primary NMU-induced tumors) (Table 1) [31, 33, 72]. In contrast, the transplantable DMBA-I and NMU-II tumors possess a lower level of expression of the Ha-

Table 1. Phenotype of rat mammary tumors

Tumor type	Collagen produced		ER	EGFR	TGFα	CSSF	p21$^{Ha\text{-}ras}$
	Type IV	Type I					
Adenocarcinoma (DMBA-induced)	++[b]	−	+	+	++[b]	++	+++ (elevated)
Adenocarcinoma (NMU-induced)	++[b]	−	+	+	+++[b]	+++	+ (point mutated)
Carcinoma[a] (DMBA-I)	−	+	−	−	±	±	−
Carcinoma[a] (NMU-II)	−	+	−	−	±	±	−

[a]Transplantable estrogen-independent tumors established from primary DMBA- or NMU-induced adenocarcinomas.

[b]Type IV collagen and TGFα mRNA species also detected [69].

The type of collagen produced by the tumors was determined by labeling the collagen in cell culture, extracting the collagen, and identifying the type by comparing its electrophoretic mobility to that of standards or by determining the species of collagen directly in tumor cells by indirect immunofluorescence with monospecific polyclonal antisera to the two collagen types. The relative amounts of biologically active TGFα were determined in the acid-ethanol extracts prepared from these tumors by assay on NRK cells in soft agar or by assay for competition with ^{125}I-EGF in a RRA [36]. EGF receptor expression was measured by determining the binding of ^{125}I-EGF to cultured tumor cells, to whole cell suspensions, or to isolated tumor cell membranes. The relative levels of p21$^{Ha\text{-}ras}$ were determined by Western blot analysis using Y-259 rat monoclonal anti-p21^{ras} antibody, and activated point-mutated p21^{ras} was detected by transfection into NIH/3T3 cells [33, 72].

ras proto-oncogene. Therefore, the production of TGFα and possibly other growth factors in these primary tumors (e.g., CSSF) may also be linked to or controlled by the state or level of *ras* gene expression [73]. In this respect, we have demonstrated that normal mouse mammary epithelial cells, which have been transfected and transformed with an activated human c-Ha-*ras* proto-oncogene, synthesize and secrete or possess higher levels of TGFα and TGFα mRNA than their normal nontransformed counterparts [42]. These results may be mechanistically important since activation or overexpression of the *ras* gene can abbrogate the growth requirements for exogenous EGF [42, 74–76]. This may also have significance for human breast cancer, as a majority of primary human breast tumors have been shown to express elevated levels of p21^{ras} and to possess high levels of expression of the c-Ha-*ras* proto-oncogene [32–34].

2.2 Human breast cancer cell lines and primary human breast tumors

We next turned our attention toward a well-characterized human breast cancer cell line, MCF-7, to determine whether comparable TGF activities could be detected from a human mammary tumor cell. The acid-treated con-

centrated CM from MCF-7 cells was able to stimulate the AIG of NRK cells in soft agar and to compete with ^{125}I-EGF for binding to EGF receptors in a RRA [37, 39, 41]. By comparison of the competition curves for the CM samples and for unlabeled authentic EGF in inhibiting ^{125}I-EGF binding in the RRA, the MCF-7 tumor cells were secreting approximately 100 ng EGF equivalent units per liter of CM obtained from 72-hr harvests [37]. Following gel filtration high pressure liquid chromatography (HPLC) under low salt and neutral pH conditions, a 30,000 M_r species can be detected which possesses both NRK colony-stimulating and EGF receptor-competing activities [41]. A comparable 30,000 M_r species could also be detected when CM was obtained from [^{35}S]cysteine-labeled MCF-7 cells and immunoprecipitated with a monospecific polyclonal goat anti-human TGFα antibody. Isoelectric focusing demonstrated that this TGFα species had a pI of 4.0. Biological activity associated with this species could be destroyed by reduction but was stable to acid and heat treatment [37]. Several clonal lines of the parental MCF-7 cell line were also examined and found to possess varying levels of TGFα activity in their CM. However, no clear correlations could be observed between the number of detectable EGF receptors on the individual clones (range 3 to 9 × 10^3 receptor sites/cell) and the amount of TGFα activity in the CM from these clones. Furthermore, the relative amount of total TGFs (α and β) associated with the CM from these various clones showed no correlation with the intrinsic ability of these cells to grow as colonies in soft agar. These results suggested that the original MCF-7 parental cell line contains a heterogenous population of tumor cells, some of which may be relatively high or low producers of either TGFα and/or TGFβ.

These TGF activities were not restricted to a single human breast cancer cell line. Using a highly sensitive and specific radioimmunoassay (RIA) for TGFα, we were able to detect and quantitate immunoreactive TGFα in the CM prepared from three additional human breast cancer cell lines and in the tissue extracts prepared from normal, benign, and malignant breast biopsies [41, 43]. Table 2 demonstrates the relative levels of RIA detectable TGFα in the concentrated CM from the breast cancer cell lines MCF-7, ZR-75-1, T47-D, and MDA-MB-231 [43]. The first three cell lines are estrogen receptor positive and estrogen responsive with respect to growth, while the MDA-MB-231 cells are estrogen receptor negative and nonresponsive to estrogens. In untreated cultures, the levels of TGFα in the CM ranged from 35 to 0.5 ng/10^8 cells. The MDA-MB-231 cells consistently had higher levels of biologically active and immunoreactive TGFα than the other human breast cancer cell lines. When MCF-7 or ZR-75-1 cells were grown in the presence of 17β-estradiol (10^{-8} M) for 48 hr, there was generally a 2- to 3-fold increase in the levels of TGFα found in the CM compared with untreated cultures. These results were confirmed by Northern blot analysis of poly(A)$^+$ RNA isolated from the breast cancer cell lines and from MCF-7 cells grown in nude mice as xenografts. Following electrophoresis on denaturing agarose gels, the poly(A)$^+$ RNA on nitrocellulose filters was hybridized to a ^{32}P nick-translated

human TGFα cDNA insert and subsequently washed under stringent conditions. As illustrated in Figure 3, a prominent 4.8-kb TGFα mRNA species could be detected in the MCF-7, T47-D, and MDA-MB-231 tumor cells. In addition, a minor 1.6-kb mRNA species was also detected. Equivalent amounts of poly(A)$^+$ RNA were loaded onto the gels in this experiment. MDA-MB-231 cells had the highest level of TGFα mRNA followed in order by MCF-7 and T47-D, respectively (Table 2). These results are in reasonable

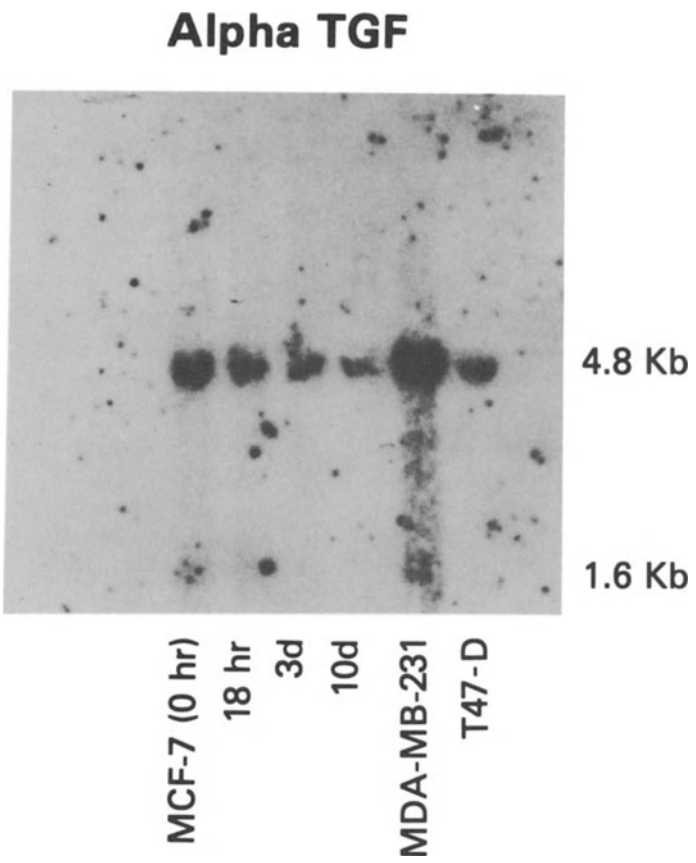


Figure 3. Presence of TGFα mRNA in human breast cancer cell lines and in MCF-7 tumor xenografts as determined by Northern blot analysis. Poly(A)$^+$ RNA was isolated from confluent cultures of MDA-MB-231 and T47-D cells or from MCF-7 tumors. MCF-7 xenografts were propagated in nude mice which were maintained on estrogen pellets (10 mg/pellet) for 3 weeks. At this time, some tumors (0 hr) were excised and extracted for RNA, and the estrogen pellets were removed from the remaining mice. Animals were sacrificed at varying times following the removal of the E_2 pellets, and poly(A)$^+$ RNA was isolated from the tumors. Equivalent amounts of poly(A)$^+$ RNA (10 μg/lane) were electrophoresed in 1% denaturing agarose gels, transferred to a nitrocellulose filter, and hybridized to a ^{32}P nick-translated full-length human TGFα cDNA insert which contains the coding sequence for the entire TGFα precursor. The filter was washed under stringent conditions.

Table 2. Immunoreactive TGFα in the conditioned medium from human breast cancer cell lines and expression of TGFα mRNA

Cell line	TGFα ng/10^8 cells (%)	Relative basal level of TGFα mRNA
MCF-7	18 (100)	++
MCF-7 + E_2 (10^{-8}M)	40 (222)	
ZR-75-1	0.5 (100)	±
ZR-75-1 + E_2 (10^{-8}M)	1.4 (280)	
T47-D	4.6 (100)	+
T47-D + E_2 (10^{-8}M)	6.5 (141)	
MDA-MB-231	35	+++

Cells were grown for 48 hr in the absence or presence of 17β-estradiol (E_2, 10^{-8}M) in serum-free medium. Conditioned medium was harvested and processed for analysis in a TGFα RIA following concentration on a Water's Sep-Pak C18 cartridge. The average amount of TGFα in the conditioned medium was normalized to ng/10^8 cells. The numbers in parentheses represent the percent increase in detectable TGFα in the conditioned medium obtained from E_2-treated cells compared with control cells (100%). Data reproduced from Perroteau *et al.* (1986). Relative basal levels of TGFα mRNA were determined by examination of the intensity of the 4.8-kb mRNA species which was detected in the cell lines following Northern blot analysis (c.f., Figure 3) of poly(A)$^+$ RNA and hybridization with a full-length ^{32}P nick-translated human TGFα cDNA insert.

agreement with the levels of immunoreactive TGFα which could be detected in the CM from these cell lines (Table 2). Moreover, the data demonstrate that there is a time-dependent decline in the level of TGFα mRNA expression *in vivo* in MCF-7 tumors obtained from nude mice at various times following removal of exogenous supportive estrogen pellets. After 10 days of removal of the estrogen source, there is an approximately 3-fold decrease in the level of TGFα mRNA in these tumors. This response is specific for TGFα mRNA, since there is no change in the level of actin mRNA (data not shown). At this time, there was only a 20–30% decrease in tumor growth rate. Collectively, these results illustrate that estrogens can regulate TGFα mRNA expression and TGFα production in estrogen-responsive human breast cancer cells *in vitro* and *in vivo*.

The presence of immunoreactive TGFα and TGFα mRNA is not restricted to cultured human breast cancer cells. Twenty-two primary human breast carcinomas, one benign fibrocystic lesion, two fibroadenomas, and one normal breast biopsy were analyzed in the TGFα RIA for immunoreactive TGFα [43]. An additional 15 primary human breast tumors were also analyzed by Northern blot analysis of poly (A)$^+$ RNA for TGFα mRNA using a human TGFα riboprobe [44]. The levels of TGFα in the tumor tissues and normal tissue extracts ranged from 1.5 to 6 ng/mg protein. In approximately 50% of the primary breast tumors, the levels of immunoreactive TGFα were greater than 2.5 ng/mg protein, which exceeds the level found in the single normal breast tissue examined. Moreover, in the 15 tumors analyzed by Northern

blot analysis for the presence of TGFα mRNA, 53% of the tumors within this group possessed a demonstrable 4.8-kb TGFα mRNA species. Moreover, in this latter TGFα mRNA-positive group of tumors, 75% of these tumors were estrogen and progesterone receptor positive. In contrast, in the TGFα mRNA-negative population, the majority of these tumors were estrogen and progesterone receptor negative. A larger number of tumor samples will, however, have to be analyzed to ascertain whether this association of TGFα and estrogen and progesterone receptor status is statistically significant. Nevertheless, the data clearly demonstrate that TGFα is not entirely restricted to human breast cancer cells, since we were able to detect immunoreative TGFα in the extracts of a reductive mammoplasty biopsy [43]. In addition, the concentrated CM obtained from primary cultures of human mammary epithelial cells and an established cell line of human mammary epithelial cells was found to contain immunoreactive TGFα [5]. These results suggest that TGFα may be synthesized by normal mammary epithelial cells which are rapidly proliferating and that this growth factor may be synthesized and secreted by mammary epithelial cells at specific times during the development of the mammary gland, possibly during lactation [53].

2.3 Human milk

During the course of these studies of human breast tumor-associated TGFs, it became apparent that the MCF-7 cells, either in culture or propagated as tumors in nude mice, were a relatively poor source for obtaining sufficient quantities of these human TGFα activities for further purification. Furthermore, the recovery of such activity from the breast cancer cell lines or from the primary tumor extracts was generally low. In searching for an alternate source, we screened crude preparations of human milk for these activities, since human milk is known to contain a variety of hormones and other growth factors such as EGF [6]. Bioactivity in human milk samples were scored by use of the TGFα RIA and by appropriate bioassays. Since EGF is present in crude milk preparations, the EGF RRA could only be used on semipurified samples [53]. In our initial studies, we found that a majority (18 of 20) of dilipidated human milk samples obtained from individual donors contained varying levels of TGFα activity. In some of the milk preparations, the TGFα activity was equivalent to those levels detected in the CM samples from the breast cancer cell lines or associated with the human mammary tumor extracts. The bulk of TGFα activity was found to reside in the whey fraction in milk, since removal of the caseins by isoelectric precipitation failed to eliminate this activity.

Since EGF (pI 4.5) is found in human milk at fairly high concentrations (approximately 50 ng/ml) and since it is one of the major mitogens associated with milk [77], it was necessary to further purify and characterize these milk-associated TGFα activities. Large volumes of delipidated milk were, therefore, fractionated by isoelectric focusing (IEF) and monitored for TGFα

activity. Following IEF, three major TGFα species were detected with pIs of 4.0, 6.0, and 7.0 (Table 3). Comparable isoelectric variants of TGFα were also observed in the acid-ethanol extracts prepared from primary human breast tumors. However, the recovery of TGFα activity of these three species from three of four human breast tumor samples was generally low. We have concentrated our efforts on characterizing the pI 4.0 TGFα from human milk for two reasons: 1) this activity appears to be identical to the TGFα species associated with the CM from MCF-7 cells and from the human breast tumors with respect to its isoelectric point and 2) the relative degree of purification of the species at this stage, since only 5% of the total protein is in this region of the gradient. We have designated the pI 4.0 TGFα as mammary-derived growth factor II (MDGF-II). As demonstrated in Table 3, all three milk TGFα species were able to stimulate NRK colony growth in soft agar, and all three species scored positively to varying degrees in the EGF RRA. In addition, all three fractions contained immunoreactive TGFα. However, the bulk of immunoreactive TGFα was found in the pI 6.5 to 7.0 region of the IEF gradient. It is clear that a portion of the immunoreactive TGFα in the pI 7.0 fraction is biologically inert and that the majority of bioactive TGFα is associated with the more acidic fractions. The relative proportion of these three TGFα species varied in milk preparations obtained from different donors. None of these three TGFα species contained significant amounts of immunoreactive EGF as determined by RIA analysis. The majority of EGF in the milk samples was found to focus at pH 4.4 to 4.5.

MDGF-II has an apparent M_r of 6,000 following gel filtration HPLC under low salt eluting conditions. However, in a high salt buffer at neutral pH, the majority of TGFα activity elutes with a M_r of approximately 17,000. Following reverse phase HPLC on a C_{18} column, the 17,000 M_r species elutes as a single peak which possesses both EGF receptor-competing and NRK colony growth-promoting activities. Both of these activities are destroyed by prior reduction

Table 3. TGFα species in human milk

		Bioactivity or immunoreactivity/mg protein		
pI	M_r	NRK colony-forming units[a]	ng EGF equivalent units[b]	ng TGFα[c]
4.0	14,000–17,000 (MDGF-II)	4600	5.6	3.2
6.0	N.D.	2600	4.4	8.4
7.0	N.D.	780	0.4	16.0

N.D., not determined; bioactivity determined in [a]NRK soft agar assay and in [b]EGF RRA using fixed mambranes from A431 human epidermoid carcinoma cells. [c]Immunoreactivity determined in a RIA with a polyclonal rabbit anti-rat TGFα antibody (Biotope Inc., Bellevue, WA). No immunoreactive EGF was detected in any of these fractions using a polyclonal rabbit anti-human EGF antibody.

but not by acid or heat treatment. Figure 4 demonstrates that, at this stage of purification, the MDGF-II is almost homogeneous, since a major solitary band is observed on a 15% SDS polyacrylamide gel following reduction and denaturation. This 17,000 M_r protein band, when excised and eluted from a nonreducing gel, possessed NRK colony growth-promoting activity. MDGF-II, therefore, differs from EGF by its isoelectric point and by its apparent M_r. We can estimate that 1 litre of human milk contains approximately 10 to 15 μg of MDGF-II. Crude human milk preparations were then probed by

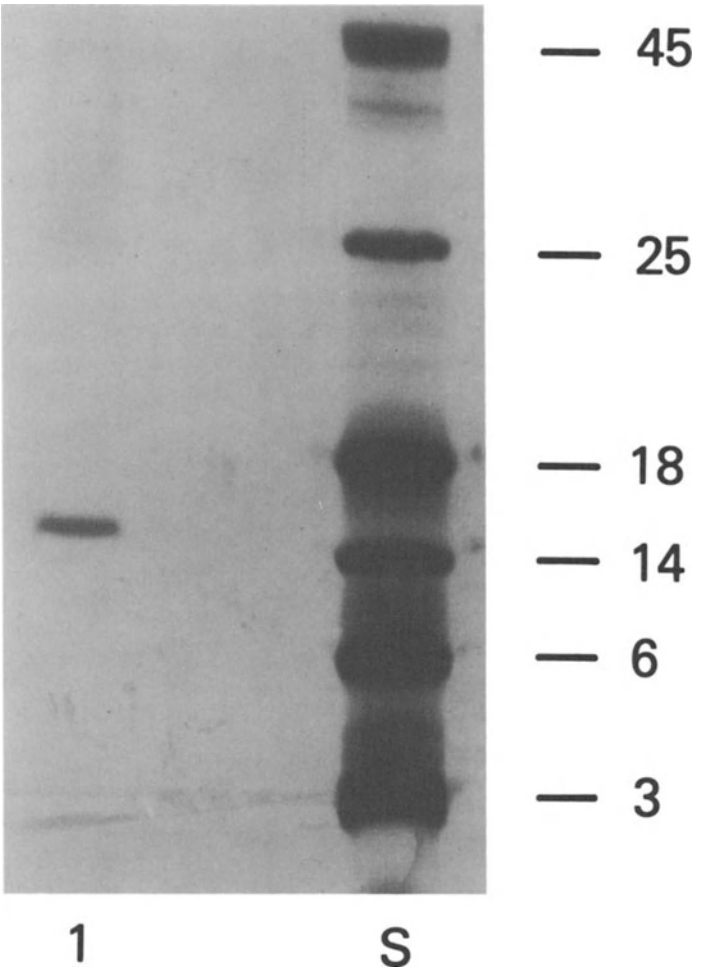


Figure 4. SDS-polyacrylamide gel electrophoresis of reverse-phase HPLC purified MDGF-II. Fractions from a C18 μ Bondapak reverse-phase HPLC which contained NRK soft agar growth-promoting activity and EGF receptor-competing activity were pooled, concentrated, and run on a 15% SDS gel following reduction and denaturation (lane 1). Standard proteins (lane S) were 10 μg of ovalbumin, 45K; α-chymotrypsinogen, 25K; β-lactoglobulin, 18K; lysozyme, 14K; bovine trypsin inhibitor, 6K; and insulin (α and β chains), 3K. Gel was silver stained.

Western blot analysis to determine the identify of the total TGFα-related species from this source. Acid-ethanol extracted or acid-treated human milk preparations were absorbed to formalin-fixed A431 cells to bioaffinity purify any EGF-like activities. The A431 cells were then washed with acid to remove any EGF-like material which was bound to the high numbers of EGF receptors on these cells. The eluted material was then concentrated, denatured, reduced, and electrophoresed on 15 to 20% gradient SDS polyacrylamide gels. The gels were then analyzed by Western blot analysis for TGFα proteins. Figure 5B demonstrates the presence of a major immunoreactive 14,000 to 16,000 M_r species and a minor 25,000 M_r species in these milk samples which can be detected on the nitrocellulose blots with a monospecific polyclonal rabbit anti-human TGFα antiserum. Control, preimmune rabbit serum failed to identify these bands (Figure 5A). It is not known whether these high M_r TGFα species in crude human milk are identical or related to the three previously described TGFα species (Table 3). Nevertheless, the results demonstrate that multiple high M_r TGFα species with different isoelectric points are present in human milk and that some of these species are very similar to the TGFα activities found in the CM of the human breast cancer cell lines and in the acid-ethanol extracts prepared from primary human mammary tumors [37, 41]. MDGF-II is apparently related to authentic TGFα, since it is able to inhibit the binding of ^{125}I-EGF to A431 cell membranes in a RRA. Moreover, in the presence of a mouse monoclonal antibody against the EGF receptor, approximately 80% of the NRK colony-stimulating activity in the MDGF-II is inhibited, while inclusion of a polyclonal rabbit anti-human EGF antiserum in this assay with MDGF-II had no effect. These results demonstrate that interaction of MDGF-II through EGF receptors on a responsive cell is necessary for the manifestation of its biological activity and that this growth factor is immunologically unrelated to human EGF. The ability of MDGF-II to stimulate AIG is not restricted to NRK fibroblasts. For example, purified MDGF-II was able to enhance the growth of mouse mammary tumor cells and a clone of MCF-7 cells in soft agar as colonies. In summary, MDGF-II and additional high M_r TGFα activities have been identified and purified to varying extents from human milk. Comparable species are also present in human mammary tumors. These activities are apparently biochemically and immunologically distinct from human EGF. It is not known whether comparable high M_r activities are elaborated by normal human mammary epithelial cells and, if so, whether their synthesis and/or secretion can be modulated by mammotrophic hormones. Moreover, the presence of multiple TGFα species with different M_rs and isoelectric points suggests that they may represent larger precursor molecules for TGFα in much the same manner as mouse EGF is processed from a larger prepro molecule [78]. In fact, high M_r TGFα species have been detected in human urine and pleural effusions from cancer patients and in the CM from several human and rodent tumor cell lines [45, 59–63]. These species, like the milk-associated TGFα activities, are biologically and immunologically active.

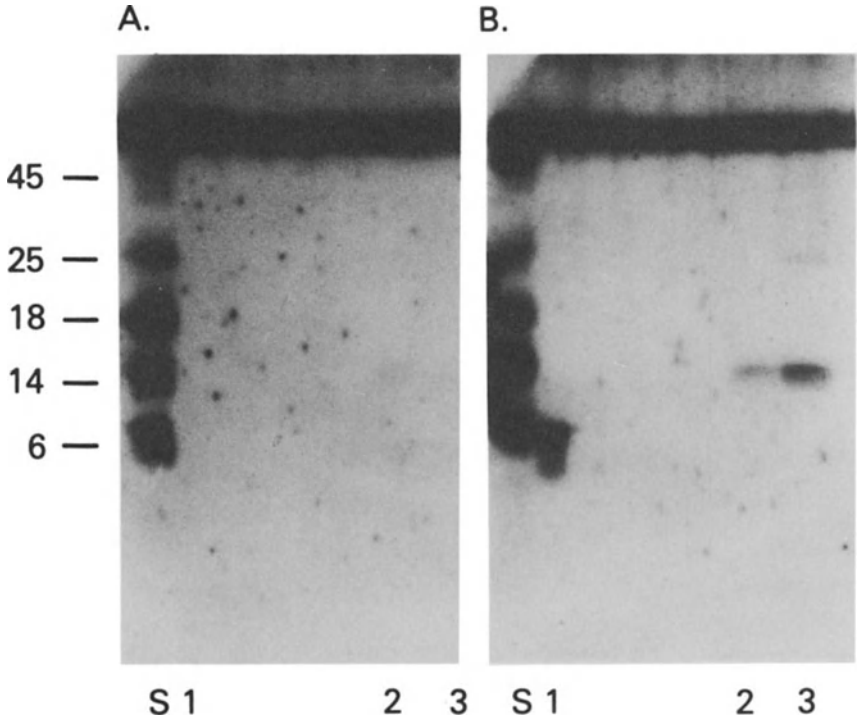


Figure 5. Western blot analysis for TGFα in human milk. Human milk samples (50 ml) were either dialyzed against 1% acetic acid for 48 hr at 4°C or acid-ethanol extracted. TGFα-like activities which will bind to EGF receptors were affinity purified by repeated cycling over formalin-fixed A431 cells. EGF receptor-bound material was eluted with 0.1% acetic acid, lyophilized, and electrophoresed on 15-20% gradient SDS polyacrylamide gels following reduction and denaturation. Samples were transferred to nitrocellulose paper, blocked with a 3% bovine serum albumin solution at 4°C for 12 hr, and reacted with a 1:100 dilution of pre-immune rabbit serum (panel A) or with a 1:100 dilution of rabbit anti-human TGFα antiserum (panel B) for 3 hr at 4°C. After extensive washing, the filters were incubated for 1 hr at 4°C with ^{125}I donkey anti-rabbit antiserum (2×10^5 cpm/ml), washed, air dried, and exposed at −70°C to X-ray film for 4 days. Lanes: S, ^{14}C-labeled protein standards; lane 1, 25 ng low M_r chemically synthesized TGFα; lane 2, acid-dialyzed human milk sample; and lane 3, acid-ethanol extracted human milk sample.

3. CSSF and MDGF-I

As was previously mentioned, there is an intimate interaction between mammary epithelial cells and the surrounding extracellular matrix. Growth and survival of normal mammary epithelial cells and differentiated mammary tumor cells is dependent upon the presence of an intact basement membrane *in vitro* and *in vivo* [8, 9, 79]. Breakdown of the basement membrane normally occurs during involution of the mammary gland following lactation [80]. However, unscheduled disruption of this structure in a well-differentiated tumor may lead to metastasis [22]. Therefore, growth factors that can regulate

the synthesis of components associated with the basement membrane may have physiological and pathological significance in the normal development of the gland and during tumor progression. A number of growth factors, such as EGF and TGFα, can differentially stimulate type IV collagen production in rodent and human mammary epithelial cells. In fact, this function is central in the ability of these agents to function as mitogens for normal mammary epithelial cells and for some mammary tumor cells [8]. CSSF and MDGF-I are two novel growth factors that have been isolated from rat and human mammary tumor cells, respectively [46, 47]. CSSF and MDGF-I can differentially stimulate the synthesis of various components associated with the basement membrane. These two growth factors functioning individually or in concert with other growth factors, such as TGFα, which are also produced by mammary epithelial cells, may be important in regulating mammary epithelial cell growth.

3.1 CSSF

Initial studies demonstrated that EGF could produce a 2- to 3-fold differential increase in collagen production in normal rat mammary epithelial cells but not in mammary epithelial cells cultured from primary DMBA- or NMU-induced rat mammary tumors. The lack of responsiveness of the primary tumor cells to exogenous EGF with respect to the stimulation of collagen production is due in part to the elaboration of CSSF [46]. CSSF was found to be present in the acid-ethanol extracts prepared from primary DMBA- and NMU-induced rat mammary adenocarcinomas. CSSF was found to differentially stimulate collagen synthesis by 2- to 10-fold in normal rat mammary epithelial cells, NRK cells, and BALB/c 3T3 cells. Pulse-chase experiments demonstrated that CSSF was not affecting the turnover of type IV collagen in normal rat mammary epithelial cells or the turnover of type I collagen in the case of NRK cells. CSSF was ineffective in stimulating type IV collagen production in the DMBA or NMU tumor cells in primary culture. CSSF has been purified from primary DMBA- and NMU-induced rat mammary tumors by preparative gel filtration chromatography, CM-cellulose ion exchange chromatography, and isoelectric focusing. CSSF is a 68,000 M_r protein which is acid stable. Biological activity could be destroyed by heating or by pepsin treatment. CSSF has a pI of 5.9 following isoelectric focusing. The pI 5.9 fraction, when run on a 7.5% SDS polyacrylamide gel, exhibits a M_r of approximately 68,000. The biological activity could be directly recovered from this region of the gel which represents approximately 0.0001% of the total tumor cell protein. CSSF is active in the 10 to 20 ng/ml range and on a molar basis is 3- to 5-fold more potent than either EGF or TGFα in differentially stimulating collagen synthesis.

The distribution of CSSF in various types of rat mammary tumors and other rat tissues has been investigated. CSSF activity could not be detected in proliferating rat mammary glands or in rat liver. However, normal bovine

mammary tissue obtained from estrogen- and progesterone-primed cows contained considerable amounts of CSSF activity, suggesting that a similar hormone priming in rodents may induce CSSF production in these animals. CSSF activity was highest in the primary DMBA- and NMU-induced rat mammary tumors, while little or no activity could be detected in the transplantable DMBA and NMU tumors. These data were in agreement with the lack of a 68,000 M_r protein which could be detected in the transplantable tumors. The relative abundance of this protein correlated with the amount of CSSF activity detected in the crude acid-ethanol tumor extracts. The presence of CSSF activity was found to correlate with the degree of tumor differentiation and with the presence of a basement membrane (Table 1). In addition, the primary tumors synthesize basement membrane type IV collagen, while the transplantable tumors elaborate stromal type I collagen. It is, therefore, possible that the growth of these primary tumors is regulated in part by CSSF and by TGFα which function by stimulating basement membrane production. Conversely, the transplantable tumors which fail to elaborate these two growth factors also lack a basement membrane. More importantly, these transplantable tumors readily metastasize. Further studies are necessary to ascertain whether this loss in growth factor production (i.e., CSSF and TGFα) is involved in the progressive acquisition of the metastatic phenotype in these tumors or is just epiphenomenon.

3.2 MDGF-I

MDGF-I is the human counterpart of CSSF [47]. Like CSSF, MDGF-I can differentially stimulate the production of type IV collagen in normal rodent and human mammary epithelial cells and type I collagen in NRK cells. MDGF-I was initially identified and purified from human milk, but an identical growth factor is also found in primary human mammary tumors. MDGF-I was purified to homogeneity from 5 liters of human milk by isoelectric focusing, HPLC gel filtration and SDS gel electrophoresis. MDGF-I has a M_r of 62,000 and an isoelectric point of 4.8. The biological activity of MDGF-I is heat stable and insensitive to disulfide reduction. These latter two properties are in contrast to CSSF. MDGF-I or CSSF are both capable of stimulating the anchorage-dependent growth of rodent and human mammary epithelial cells but not of rodent fibroblasts. Neither growth factor is able to enhance the AIG of NRK cells in soft agar, suggesting that they are unrelated to the TGFs. Moreover, each activity does not inhibit the binding of EGF to its receptor, suggesting that a unique receptor exists for these two growth factors. Since approximately 23 μg of MDGF-I could be recovered from 1 litre of human milk, sufficient quantities of growth factor were available for radioiodination. High affinity receptor (K_d, 2×10^{-10} M) for MDGF-I exist on mouse and human mammary epithelial cells, on NRK cells, and in isolated membranes obtained from human A431 epidermoid carcinoma cells. ^{125}I-MDGF-I binding was not inhibited by EGF or a variety of other growth

factors, suggesting that these receptors were specific for MDGF-I. Saturation of specific binding of ^{125}I-MDGF-I occurs between 5 to 10 ng/ml, which is in agreement with the amount of growth factor that is optimal for stimulating cell proliferation or collagen production. MDGF-I is not unique to human mammary tumor cells, as demonstrated by its initial identification in human milk. In addition, normal human mammary epithelial cells in primary culture have a comparable activity associated with their CM. This activity competes with ^{125}I-MDGF-I for binding to receptors on these cells and on NRK cells. However, human mammary tumor cells produce approximately 3- to 5-fold more MDGF-I than the normal cells.

The hallmark property of MDGF-I is its ability to differentially stimulate the production of collagen in responsive cell types. Like CSSF, this effect is primarily due to a selective increase in collagen synthesis rather than to a decrease in collagen turnover. Furthermore, in NRK cells which fail to respond mitogenically to this growth factor yet show an increase in collagen production, this effect on collagen synthesis is due to an ehancement in the level of expression of the type I collagen mRNA. Whether a similar mechanism is operative in mammary epithelial cells *in vitro* is not yet known. There is evidence to suggest that the levels of type IV collagen mRNA expression in primary DMBA- and NMU-induced rat mammary tumors is directly coupled to the growth rate of these tumors, suggesting that estrogens might regulate the production of CSSF in these tumors or the response of these tumor cells to CSSF. This may also be the case for MDGF-I, since the growth-promoting effects of MDGF-I on mouse mammary epithelial cells were found to be negligible when the cells were isolated from ovariectomized mice. However, if ovariectomized animals were given estrogen replacement, then the growth response to MDGF-I could be restored *in vitro* using mammary epithelial cells obtained from these mice.

We have previously demonstrated that the growth response of mammary epithelial cells *in vitro* to various mitogens, such as EGF, could be modulated by the composition of extracellular substrate [8, 9]. A similar situation exists for MDGF-I. For example, when normal mouse mammary epithelial cells are grown on type IV collagen, MDGF-I was incapable of differentially stimulating the synthesis of type IV collagen in these cells. However, when cells were maintained on type I collagen or plastic, MDGF-I produced a 4-fold differential increase in type IV collagen synthesis. These results suggest that the basement membrane may negatively regulate the response of mammary epithelial cells to various mitogens. One possible mechanism by which this might occur is through the regulation of receptor expression or processing on mammary epithelial cells by the extracellular matrix. Such a situation has been demonstrated for EGF receptor down-modulation in mouse mammary epithelial cells [81]. Collectively, these data support the hypothesis that 1) collagen production is tightly coupled to the mitogenic response of mammary epithelial cells to various growth factors, including MDGF-I, and 2) the magnitude of the mitogenic response to specific growth factors through receptor

modulation is controlled by the composition of the extracellular matrix in which the mammary epithelial cells are situated. This cross-talk between the mammary epithelial cells and the surrounding extracellular matrix may have important implications with respect to the normal growth and differentiation of mammary epithelial cells and the pathogenesis and progression of breast tumors *in vivo*. The synthesis and turnover of the basement membrane is crucial and apparently rate-limiting for the growth of normal mammary epithelial cells in response to various hormonal stimuli *in vivo*. Factors such as MDGF-I or TGFα, which are produced within the gland in response to specific hormones such as estrogen and which can regulate the synthesis of components associated with basement membrane, would therefore perform an essential function in modulating the proliferation of malignant and normal mammary epithelial cells.

4. Conclusions

It is apparent from this brief review that normal and malignant rodent and human mammary epithelial cells contain and secrete a variety of chemically and biologically distinct growth factors. The presence in human milk of comparable growth factors suggests that these activities may be produced by normal mammary epithelial cells and that they may have some function in the developing neonate. Primary cultures of human mammary epithelial cells do secrete biologically active TGFα and MDGF-I. However, it is not yet known as to which cell type within these cultures or within the mammary gland synthesize these two activities. At the present, we assume that the ductal or alveolar epithelial cells are elaborating these two growth factors and that the myoepithelial cells which are the major producers of type IV collagen are potential target cells for TGFα and MDGF-I. In addition, the ability of certain mammotrophic hormones such as estrogens to regulate the production or sensitivity of cells to TGFα and MDGF-I suggests that these growth factors may perform a normal physiological function during the growth and development of the mammary gland. This may be the case, since estrogen and progesterone can regulate the appearance of an EGF-like growth factor *in vivo* in normal mouse mammary tissue and estrogens can control the level of TGFα mRNA expression in human breast tumor xenografts [44, 67]. In addition, EGF or EGF-like growth factors such as TGFα have been demonstrated to regulate the proliferation and differentiation of primary mammary epithelial cell cultures and of mouse mammary explants *in vitro* [2, 3, 5, 66, 67]. The coordinated effects of several locally synthesized or systemically derived growth factors are therefore probably required for regulating these various physiological responses *in vivo*. In this respect, growth factors or growth inhibitors not reviewed in this article, such as IGF-I, IGF-II, PDGF, FGF, TGFβ, mammary growth inhibitor, human mammary tumor growth factor,

and a 52-kDa protein, have all been shown to be associated with either normal and/or malignant mammary epithelial cells [4, 36–52].

Overexpression of these growth factors or their receptors or loss of hormonal control of their production could have important pathophysiological consequences, especially during the development and progression of rodent and human mammary tumors. For example, the presence of CSSF and TGFα in well-differentiated, hormone-dependent, rat mammary adenocarcinomas and their apparent absence in more aggressive, metastatic, hormone-independent rat mammary carcinomas is noteworthy in this respect. One might expect to find a whole spectrum of malignancies ranging from primary hormone-dependent tumors to metastatic hormone-independent variants which express different growth factors, receptors for growth factors, and various oncogenes. For example, several laboratories have now demonstrated that there is a significant inverse relationship between the presence of estrogen receptors and the level of EGF receptors [82–87]. Specifically, human breast tumors or human breast cancer cell lines which are expressing high levels of EGF receptors were generally found to be estorgen receptor negative. Those primary human tumors which were estrogen receptor negative and which contained high levels of EGF receptors were found to be less well differentiated and highly metastatic [84, 85]. Since the EGF receptor is now known to be the product of the c-*erbB* proto-oncogene and since TGFα interacts with this receptor system, these results become even more significant [15, 16]. Overexpression, amplification, and/or rearrangement of c-*erbB* has in fact been demonstrated to occur in some human breast cancer cell lines [88]. Moreover, a recently discovered oncogene *neu* or c-*erbB-2* encodes an EGF receptor-related cell surface glycoprotein which possesses a tyrosine kinase activity like the EGF receptor [89, 90]. Although the endogenous ligand which utilizes this potential receptor has yet to be identified, amplification of this oncogene has been detected in a subset of human breast tumors [35]. In a retrospective study, approximately 28% of the primary human breast tumors tested contained one to twenty extra copies of this gene. This study showed that there was a strong prognostic correlation between amplification of the *neu* oncogene and the frequency of breast cancer recurrence. In addition to c-*erbB* and *neu*, there is also evidence that overexpression of or deletions within the c-Ha-*ras*-1 proto-oncogene are present in some human breast tumors and in primary DMBA-induced rat mammary adenocarcinomas [32–34]. Moreover, primary NMU-induced rat mammary adenocarcinomas contain a point mutated form of c-H-*ras* [72]. One consequence of activation or overexpression of the *ras* oncogene or proto-oncogene is the enhanced synthesis and secretion of TGFα, which has been demonstrated in rodent fibroblasts and in mouse and rat mammary epithelial cells and the concommitant loss in the growth responsiveness of these cells to exogenous EGF [17, 33, 36, 42, 72–76]. These data imply that there may be a relationship in mammary tumor cells between the pattern of oncogene expression and the production of or response to particular growth factors in certain types of human breast tumors. How-

ever, a number of questions remained unanswered. These questions or relationships include the sites of synthesis or expression of different growth factors or growth factor receptors by subpopulations of cells within any given breast tumor, the pathology of a particular breast lesion, and the activation, overexpression, or rearrangement of various oncogenes within these tumors. Are these growth factors functioning as true autocrine or paracrine activities in regulating the proliferation of mammary tumor cells or possibly surrounding host cells? The use of highly specific polyclonal or monoclonal antibodies against defined growth factors or growth factor receptors for immunocytochemical localization and the application of appropriate growth factor or growth factor receptor cDNA probes or riboprobes to *in situ* hybridization will help address these questions [25]. If these immunological reagents can inhibit the growth of these tumor cells, this would provide strong evidence for an autocrine role of specific growth factors in regulating tumor cell proliferation [25]. There is evidence that this may be the case for some types of tumor cells. For example, monoclonal antibodies against the EGF receptor or against the *neu* oncogene protein have been shown to arrest the *in vitro* and *in vivo* growth of tumor cells which are expressing these two proteins [91, 92]. Pharmacological antagonists that bind to specific growth factor receptors but which lack any biological activity may also prove to be effective in controlling breast tumor cell growth.

In summary, it should be apparent that mammary epithelial cell proliferation, differentiation, and basement membrane synthesis are tightly coupled and coordinated processes. Several growth factors participate in regulating these events. Production of these growth factors is under the control of certain mammotrophic hormones, suggesting that the biological responses that are induced by these hormones may be mediated in part by these locally derived activities.

References

1. Topper YJ, Freeman CS: Multiple hormone interactions in the developmental biology of the mammary gland. Physiol Rev 60: 1049–1106, 1980.
2. Oka T, Yoshimura M: Paracrine regulation of mammary gland growth. Clin Endocrin Metab 15: 79–97, 1986.
3. Salomon DS, Bano M, Kidwell WR: Polypeptide growth factors and the growth of mammary epithelial cells. In: Breast Cancer: Origins, Detection and Treatment, Rich MA, Hager JC and Papadimitriou JT (eds). Martinus Nijhoff, Inc., Boston, 1986, pp 42–56, 1986.
4. Salomon DS, Perroteau I, Kidwell WR: Tumor-derived growth factors in rodent and human mammary carcinoma cells. In: Contribution to Oncology: Endocrine Therapy of Breast Cancer, Eppenberger U, Fabbro D and Schafer P (eds). Karger, Basel, Switzerland, Vol. 23, pp 5–16, 1986.
5. Kidwell WR, Salomon DS, Mohanam S: Production of growth factors by normal human mammary cells in culture. In: Effects of Human Milk on the Recipient Infant, Goldman A, Atkinson S and Hanson LA (eds.) Plenum Press, New York, in press.
6. Kidwell WR, Bano M, Burdette K, Losonczy I, Salomon DS: Mammary-derived growth

factors in human milk. In: Human Lactation: Milk Components and Methodologies, Jansen RG and Neville MC (eds). Plenum Pub. Co., New York, 1985, pp 209–219.
7. Kleinman HK, Klebe RJ, Martin GR: Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 88:473–485, 1981.
8. Kidwell WR, Bano M, Zweibel J, Salomon DS: Growth of mammary epithelial cells on collagen substratum in serum-free medium. In: Cell Culture Methods for Molecular and Cell Biology. Preparation of Media, Supplements and Substrata for Serum-Free Animal Cell Culture, Barnes DW, Sirbasku DA and Sato GH (eds). Alan R. Liss, Inc., New York, Vol. 2, pp 105–125, 1984.
9. Salomon DS, Liotta LA, Kidwell WR: Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. Proc Natl Acad Sci USA 78:382–386, 1981.
10. Lee EY-H, Parry G, Bissell M: Modulation of secreted proteins of mouse mammary epithelial cells by collagenous substrata. J Cell Biol 98: 146–155, 1985.
11. Lee EY-H, Lee W-H, Kaetzel CS, Parry G, Bissel M: Interaction of mouse mammary epithelial cells with collagen substrata: regulation of casein gene expression and secretion. Proc Natl Acad Sci USA 82:1419– 1423, 1985.
12. Smith JA, Winslow DP, Rudland PS: Different growth factors stimulate cell division of rat mammary epithelial, myoepithelial, and stromal cell lines in culture. J Cell Physiol 119:320–326, 1984.
13. Hirayun A, Yoshida Y, Sato M, Tominaga T, Mitsui H: Isolation of two syngeneic cell lines from a rat mammary carcinoma: growth factor production by neoplastic epithelial cells. J Natl Cancer Inst 75:471–482, 1985.
14. Lippman ME, Dickson RB, Bates S, Knabbe C, Huff K, Swain S, McManaway M, Bronzert D, Kasid A, Gelman EP: Autocrine and paracrine growth regulation of human breast cancer. Breast Cancer Res Treat 7:59–70, 1986.
15. Salomon DS, Perroteau I: Growth factors in cancer and their relationship to oncogenes. Cancer Invest 4:43–60, 1986.
16. Goustin AS, Leof EB, Shipley GD, Moses HL: Growth factors and cancer. Cancer Res 46:1015–1029, 1986.
17. Kaplan PL, Ozanne P: Cellular responsiveness to growth factors correlates with a cell's ability to express the transformed phenotype. Cell 33: 931–938, 1983.
18. Peréz-Rodriguez R, Chambard JC, Van Obberghen-Schilling E, Franchi A, Pousségur J: Emergence of hamster fibroblast tumors in nude mice—evidence for *in vivo* selection leading to loss of growth factor requirement. J Cell Physiol 109:397–406, 1981.
19. Stanbridge EJ, Der CJ, Doersen C-J, Nishimi RY, Peehl DM, Weissman BE, Wilkinson JE: Human cell hybrids: analysis of transformation and tumorigenicity. Science 215:252–259, 1982.
20. Weinstein IB, Horowitz AD, Fisher P, Irunovic V, Gattoni-Celli S, Kirschmeier P: Mechanisms of multistage carcinogenesis and their relevance to tumor cell heterogeneity. In: Tumor Cell Heterogeneity, Origin and Implications, Owens AH, Coffey DS and Baylin SB (eds). Academic Press, New York, 1982, pp 261–283.
21. Vlodavsky I, Lui GM, Gospodarowicz D: Morphological appearance, growth behavior and migratory activity of human tumor cells maintained on extracellular matrix versus plastic. Cell 19:607–616, 1980.
22. Liotta LA: Tumor invasion and metastases—role of the extracellular matrix. Cancer Res 46:1–7, 1986.
23. Sporn MB, Roberts AB: Autocrine growth factors and cancer. Nature 313:745–747, 1985.
24. Heldin CH, Westermark B: Growth factors: mechanism of action and relation to oncogenes. Cell 37:9–20, 1984.
25. Salomon DS, Perroteau I: Oncological aspects of growth factors. Ann Rep Med Chem 21:159–168, 1986.
26. Green S, Chambon P: A superfamily of potentially oncogenic hormone receptors. Nature 324:615–616, 1986.

27. Bishop JM: Viral oncogenes. Cell 42:23–38, 1985.
28. Weinberg RA: The action of oncogenes in the cytoplasm and nucleus. Science 230:770–776, 1985.
29. Chung LC, Silnutzer J, Pipas JM, Barnes DW: Selection of transformed cells in serum-free medium, *In Vitro* Cell Dev Biol 21:707–712, 1985.
30. Land H, Parador LF, Weinberg RA: Cellular oncogenes and multistep carcinogenesis. Science 222:771–778, 1983.
31. Pulciani S, Santos E, Long LK, Sorrentino V, Barbacid M: *ras* gene amplification and malignant transformation. Mol Cell Biol 5:2836–2841, 1985.
32. Horan-Hand P, Thor A, Wunderlich D, Muraro R, Caruso A, Schlom J: Monoclonal antibodies of predefined specificity detect activated *ras* gene expression in human mammary carcinomas. Proc Natl Acad Sci USA 81:5227–5231, 1984.
33. De Bortoli ME, Abou-Issa H, Haley BE, Cho-Chung YS: Amplified expression of p21 *ras* protein in hormone-dependent mammary carcinomas of humans and rodents. Biochem Biophys Res Commun 127:699–706, 1985.
34. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R: Loss of a c-Ha-*ras*-1 allele and aggressive human primary breast carcinomas. Cancer Res 46:4776–4781, 1986.
35. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–182, 1987.
36. Zweibel JA, Davis MR, Kohn E, Salomon DS, Kidwell WR: Anchorage-independent growth-conferring factor production by rat mammary tumor cells. Cancer Res 42:5117–5125, 1982.
37. Salomon DS, Zwiebel JA, Bano M, Losonczy I, Fehnel P, Kidwell WR: Presence of transforming growth factors in human breast cancer cells. Cancer Res 44:4069–4077, 1984.
38. Eckert K, Lübbe L, Schön R, Grosse R: Demonstration of transforming growth factor activity in mammary epithelial tissues. Biochem Int 11:441–451, 1985.
39. Dickson RB, Huff KK, Spencer EM, Lippman ME: Induction of epidermal growth factor-related polypeptides by 17β-estradiol in human breast cancer cells. Endocrinol 118:138–142, 1985.
40. Dickson RB, McManaway ME, Lippman ME: Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. Science 232:1540–1543, 1986.
41. Bates SE, McManaway ME, Lippman ME, Dickson RB: Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer Res 46:1707–1713, 1986.
42. Salomon DS, Perroteau I, Kidwell WR, Tam J, Derynck R: Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha-transforming growth factors in *ras*-transformed mouse mammary epithelial cells. J Cell Physiol, 130:397–409, 1987.
43. Perroteau I, Salomon DS, De Bortoli M, Kidwell W, Hazarika P, Pardue R, Dedman J, Tam J: Immunological detection and quantitation of alpha transforming growth factor in human breast carcinoma cells. Breast Cancer Res Treat 7:201–210, 1986.
44. Salomon DS, Kidwell WR, Liu S, Kim N, Callahan R, Theillet C, Lidereau R, Derynck R: Presence of alpha TGF mRNA in human breast cancer cell lines and in human breast carcinomas. Breast Cancer Res Treat 8:106 (abstr. 109), 1986.
45. Hanauske A-R, Arteaga CL, Clark GM, Buchok J, Hazarika P, Pardue RL, Von Hoff DD: Comparison of three independent assays for α-transforming growth factor activity in effusions from breast cancer patients. Breast Cancer Res Treat 8:86 (abstr. 32), 1986.
46. Bano M, Zweibel JA, Salomon DS, Kidwell WR: Detection and partial characterization of collagen synthesis stimulating activities in rat mammary adenocarcinomas. J Biol Chem 258:2729–2735, 1983.
47. Bano M, Salomon DS, Kidwell WR: Purification of a mammary-derived growth factor from human milk and human mammary tumors. J Biol Chem 260:5745–5752, 1985.
48. Rozengurt E, Sinnett-Smith J, Papadimitriou J-T: Production of PDGF-like growth factor by

breast cancer cell lines. Int J Cancer 36:247–252, 1985.
49. Garcia M, Capony F, Derocq D, Simon D, Pau B, Rochefort H: Characterization of monoclonal antibodies to estrogen-regulated Mr 52,000 glycoprotein and their use in MCF-7 cells. Cancer Res 45:709–716, 1985.
50. Garcia M, Salazar-Retanu G, Richer G, Domergue J, Capony F, Pujol H, Laffargue F, Pau B, Rochefort H: Immunohistochemical detection of estrogen regulated 52,000 mol wgt protein in primary breast cancer but not in normal breast or uterus. J Clin Endocrinol Metab 59:564–566, 1984.
51. Bohmer FD, Lehman W, Eberhart H, Langen P, Grosse R: Purification of a growth inhibitor for Ehrlich ascites mammary carcinoma cells from bovine mammary gland. Exp Cell Res 150:466–476, 1984.
52. Rowe JM, Kasper S, Shiu RP, Friesen HG: Purification and characterization of a human mammary tumor-derived growth factor. Cancer Res 46:1408–1412, 1986.
53. Zwiebel JA, Bano M, Nexö E, Salomon DS, Kidwell WR: Partial purification of transforming growth factors from human milk, Cancer Res 46:933–939, 1986.
54. Roberts AB, Sporn MB: Transforming growth factors. Cancer Surv 4:684–705, 1985.
55. Sporn MB, Roberts AB, Wakefield LM, Assoian RK: Transforming growth factor-β: biological function and chemical structure. Science 233:532–534, 1986.
56. Massagué J: Epidermal growth factor-like transforming growth factor II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J Biol Chem 258:13614–13620, 1983.
57. Lee DC, Rose TM, Webb NR, Todaro GJ: Cloning and sequence analysis of cDNA for rat transforming growth factor-α. Nature 313:489–491, 1985.
58. Derynck R, Roberts AB, Eaton DH, Winlker ME, Goeddel DV: Human transforming growth factor-α: precursor sequence, gene structure and heterologous expression. In: Growth Factors and Transformation, Feramisco J, Ozanne B and Stiles CA (eds). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1985, Vol. III, pp 79–86.
59. Dart LL, Smith DM, Meyers CA, Sporn MB, Frolik CA: Transforming growth factors from a human tumor cell: characterization of transforming growth factor β and identification of a high molecular weight transforming growth factor α. Biochemistry 24:5925–5931, 1985.
60. Kim MK, Warren TC, Kimball ES: Purification and characterization of a low molecular weight transforming growth factor from urine of melanoma patients. J Biol Chem 260:9237–9243, 1985.
61. Linsley PS, Hargreaves WR, Twardzik DR, Todaro GJ: Detection of larger polypeptides structurally and functionally related to type I transforming growth factor. Proc Natl Acad Sci USA 82:356–360, 1985.
62. Ignotz RA, Kelly B, Davis RJ, Massagué J: Biologically active precursor for transforming growth factor type α, released by retrovirally transformed cells. Proc Natl Acad Sci USA 83:6307–6311, 1986.
63. Stromberg K, Hudgins WR: Urinary transforming growth factors in neoplasia: separation of ^{125}I-labeled transforming growth factor-α from epidermal growth factor in human urine. Cancer Res 46:6004–6010, 1986.
64. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R: Expression in rat fibroblasts of a human transforming growth factor-α cDNA results in transformation. Cell 46:301–309, 1986.
65. Carpenter G: Epidermal growth factor. In: Handbook of Experimental Pharmacology. Tissue Growth Factors, Baserga R (ed). Springer-Verlag, New York, 1981, Vol. 57, pp 89–132.
66. Tonelli OJ, Sorof S: Epidermal growth factor requirement for development of cultured mammary gland. Nature 285:250–252, 1980.
67. Vonderhaar BK: Hormones and growth factors in mammary gland development. In: Control of Cell Growth and Proliferation Veneziale CM (ed). Van Nostrand Reinhold Co., New York, 1984, pp 11–33.
68. Kurachi H, Okamoto S, Oka T: Evidence for the involvement of the submandibular gland

epidermal growth factor in mouse mammary tumorigenesis. Proc Natl Acad Sci USA 82: 5940–5943, 1985.
69. Liu S, Kidwell WR, Derynck R, Tam J, Salomon DS: Transforming growth factor alpha production by normal and neoplastic mammary epithelium. J Cell Biol 103:443a (abstr. 1649), 1986.
70. Jordan VC: Laboratory models of hormone-dependent cancer. Clin Oncol 1:21–70, 1982.
71. Rose DP, Gottardis M: Interactions between estrogen, prolactin and growth hormone on the growth of *N*-nitrosomethylurea-induced rat mammry tumors. Anticancer Res 5:397–402, 1985.
72. Sukumar S, Notario V, Martin-Zanca D, Barbacid M: Induction of mammary carcinomas in rats by nitroso-methyl-urea involves the malignant activation of the Ha-*ras*-1 locus by single point mutations. Nature 306:658–660, 1983.
73. Spandidos DA: Mechanism of carcinogenesis: the role of oncogenes, transcriptional enhancers and growth factors. Anticancer Res 5:485–498, 1985.
74. Kamata T, Feramisco JR: Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of *ras* oncogene proteins. Nature 310:147–150, 1984.
75. Mulcahy LS, Smith MR, Stacey DW: Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH/3T3 cells. Nature 313:241–243, 1985.
76. Weissman B, Aaronson SA: Members of the *src* and *ras* oncogene families supplant the epidermal growth factor requirement of BALB/MK-2 keratinocytes and induce distinct alteration in their terminal differentiation. Mol Cell Biol 5:3386–3396, 1985.
77. Jansson L, Karlson FA, Westermark B: Mitogenic activity and epidermal growth factor content in human milk. Acta Paediatr Scand 74:250–253, 1985.
78. Scott J, Urdea M, Quiroga R, Sanchez-Percador N, Fong M, Selby M, Rutter WJ, Bell GI: Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins. Science 221:236–240, 1983.
79. Lewko WL, Liotta LA, Wicha MS, Vonderhaar BK, Kidwell WR: Sensitivity of *N*-nitrosomethylurea-induced rat mammary tumors to *cis*-hydroxyproline, an inhibitor of collagen production. Cancer Res 41:2855–2862. 1981.
80. Wicha MS, Liotta LA, Vonderhaar BK, Kidwell WR: Effects of inhibition of basement membrane collagen deposition on rat mammary gland development. Dev Biol 80:253–266, 1980.
81. Kidwell WR, Mohanam S, Salomon DS: Extracellular matrix effects on growth factor receptors. Cancer Res 27:223 (abstr. 883), 1986.
82. Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS: Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen and progesterone receptor levels. Cancer Res 44:3448–3453, 1984.
83. Peréz R, Pascual M, Macias A, Lage A: Epidermal growth factor receptors in human breast cancer. Breast Cancer Res Treat 4:189–193, 1984.
84. Sainsbury JRC, Farndon JR, Harris AL, Sherbert GV: Epidermal growth factor receptors of human breast cancers. Br J Surg 72:186–188, 1985.
85. Sainsbury JRC, Sherbert GV, Farndon JR, Harris AL: Epidermal growth factor receptors and oestrogen receptors in human breast cancer. Lancet 2:364–366, 1985.
86. Roos W, Fabbro D, Küng W, Costa SD, Eppenberger U: Correlation between hormone dependency and the regulation of epidermal growth factor receptor by tumor promoters in human mammary carcinoma cells. Proc Natl Acad Sci USA 83:991–995, 1986.
87. Skoog L, Macias A, Azavedo E, Lombardero J, Klintenberg C: Receptors for EGF and oestradiol and thymidine kinase activity in different histological subgroups of human mammary carcinomas. Br J Cancer 54:271–276, 1986.
88. Filmus J, Pollak MN, Cailleau R, Buick RN: MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem Biophys Res Commun 128:898–905, 1985.
89. Schecter AL, Stern DF, Vaidynathan L, Decker SJ, Drebin JA, Greene MI, Weinberg RA: The *neu* oncogene: an *erb*-B-related gene encoding a 185,000-Mr tumor antigen. Nature

312:514–516, 1984.
90. Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, Seeburg PH, Liberman TA, Schlessinger J, Francke U, Levinson A, Ullrich A: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230:1132–1139, 1985.
91. Masui H, Moroyama T, Mendelsohn J: Mechanism of antitumor activity in mice for anti-epidermal growth factor receptor monoclonal antibodies with different isotypes. Cancer Res 46:5592–5598, 1986.
92. Drebin JA, Link VC, Weinberg RA, Greene MI: Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. Proc Natl Acad Sci USA 83:9129–9133, 1986.

Index