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Alison L. Allan *Editor*

Cancer Stem Cells in Solid Tumors

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Series Editor
Kursad Turksen, Ph.D.
kturksen@ohri.ca

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Editor

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Editor
Alison L. Allan
Depts. of Oncology and Anat. & Cell Biology
Schulich School of Med. and Dent.
University of Western Ontario
London, Ontario, Canada
alison.allan@lhsc.on.ca

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Preface

Recently, there has been increasing support for the "cancer stem cell" hypothesis, which postulates that cancer arises from a subpopulation of tumor-initiating cells or cancer stem cells (CSCs). There are currently two conflicting views that attempt to explain tumor formation. The classical stochastic model suggests that every cell within a tumor is a potential tumor-initiator, but that entry into the cell cycle is governed by a low probability of stochastic mutations. According to this model, it would be impossible to tell which cell initiated the tumor since each cell has an equal ability to be malignant. By contrast, the hierarchy theory (upon which the CSC hypothesis is based) proposes that only a subset of cells within a tumor is capable of initiating tumor growth, but that these cells all do so at a high frequency. According to this theory, it should be possible to identify and target the cells responsible for tumor initiation and progression because not all cells have the same phenotypic and functional characteristics.

While the idea of CSCs has been around for more than 100 years, evidence from the hematology field has now demonstrated the critical role of stem cells in hematological malignancies and suggested that these same mechanisms could also be central to the initiation, progression, and treatment of solid cancers. Indeed, several pivotal studies have recently provided compelling evidence that these cells do exist in solid tumors of many types including breast, brain, colorectal, pancreas, prostate, melanoma, lung, ovarian, liver, and head and neck cancer. Furthermore, clinical and experimental studies have demonstrated that CSCs exhibit many classical properties of normal stem cells, including a high self-renewal capacity and the ability to generate heterogeneous lineages; the requirement for a specific "niche"/microenvironment to grow; and an increased capacity for self-protection against harsh environments, toxins, and drugs.

This multi-authored volume focuses specifically on the role of CSCs in solid cancers. The authors are all active investigators with research programs related to oncology and/or stem cell biology, and are leaders in their field. Part I (Chap. 1) serves to introduce the concept of CSCs vs. normal stem cells, including a historical perspective and the contributing lessons from leukemia. Part II (Chaps. 2–11) describes the identification and role of CSCs in various forms of solid cancer,

organized according to disease site. Part III (Chaps. 12–14) elaborates on molecular pathways that are involved in driving CSC function, with a particular focus on the convergence of embryonic and tumorigenic signaling pathways. Part IV (Chaps. 15–18) describes available model systems and modalities for studying CSC biology and therapeutic development, including in vitro and in vivo model systems and assays and imaging modalities. Part V (Chaps. 19–23) discusses the importance of CSCs for cancer management and treatment, including implications for prognosis, prediction, and treatment resistance. Finally, Part VI (Chap. 24) provides the concluding thoughts for the book, including consideration of the controversy surrounding the CSC hypothesis. The editor and the authors hope that this work will provide a comprehensive overview of this evolving and important field.

London, ON Alison L. Allan
Canada

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Contents

Contributors

Laurie Ailles Ontario Cancer Institute, Campbell Family Institute of Cancer Research; and Departments of Medical Biophysics, University of Toronto, Toronto, ON, Canada

Alison L. Allan Departments of Oncology and Anatomy & Cell Biology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada

London Regional Cancer Program, London Health Sciences Centre, London, ON, Canada

Viviana Anelli IFOM, The FIRC Institute of Molecular Oncology, Milan, Italy

Itzhak Avital National Cancer Institute (NIH), Surgery Branch, Bethesda, MD, USA

Curt Balch Medical Sciences Program, Indiana University, Bloomington, IN, USA

Indiana University Simon Cancer Center, Indianapolis, IN, USA

Mauro Biffoni Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy

Jenny E. Chu Department of Anatomy & Cell Biology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada

Robert B. Clarke Breast Biology Group, School of Cancer and Enabling Sciences, Paterson Institute for Cancer Research, University of Manchester, Manchester, UK

Christopher R. Cogle Department of Medicine, Division of Hematology/ Oncology, University of Florida, Gainesville, FL, USA

Program in Stem Cell Biology and Regenerative Medicine, University of Florida, Gainesville, FL, USA

Anne T. Collins Cancer Research Unit, Department of Biology, University of York, York, UK

Alysha K. Croker Department of Anatomy & Cell Biology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada

Samantha J. Davis Department of Otolaryngology-Head and Neck Surgery, University of Michigan, Ann Arbor, MI, USA

Maximilian Diehn Stanford Cancer Center, CA, USA

Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford, CA, USA

Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA, USA

Albert D. Donnenberg Department of Medicine, Division of Hematology/Oncology, University of Pittsburgh Cancer Institute and University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Vera S. Donnenberg Department of Cardiovascular Surgery, Division of Hematology/Oncology, University of Pittsburgh Cancer Institute and University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Jorge Dorado Clinical Research Programme, Stem Cells & Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

Eros Fabrizi Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy

Fang Fang Medical Sciences Program, Indiana University, Bloomington, IN, USA

Gillian Farnie Cancer Stem Cell Research, University of Manchester, School of Cancer and Enabling Sciences, Paterson Institute for Cancer Research, Manchester, UK

Paula Foster Robarts Research Institute, London, ON, Canada

Department of Medical Biophysics, University of Western Ontario, London, ON, Canada

Fiona M. Frame Cancer Research Unit, Department of Biology, University of York, York, UK

Jonas Fuxe Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden

Olga Gaidarenko Section of Molecular Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA, USA

Craig Gedye Ontario Cancer Institute, Campbell Family Institute of Cancer Research, Toronto, ON, Canada

Christopher Heeschen Clinical Research Programme, Stem Cells & Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

David A. Hess Department of Physiology & Pharmacology, The University of Western Ontario, London, ON, Canada

Vascular Biology Group, Krembil Centre for Stem Cell Biology, Robarts Research Institute, London, ON, Canada

Richard P. Hill Ontario Cancer Institute, Campbell Family Institute of Cancer Research, Departments of Medical Biophysics and Radiation Oncology, University of Toronto, Toronto, ON, Canada

Sacha J. Howell Department of Medical Oncology, The Christie NHS Foundation Trust, University of Manchester, Manchester, UK

Michael Jewer Department of Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada

Feng Jiang Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA

Ping Jin Cell Processing Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Jeanne Kowalski Winship Cancer Institute, Emory University, Atlanta, GA, USA

Paula Kroon Cancer Research Unit, Department of Biology, University of York, York, UK

Russell C. Langan Surgery Branch, National Cancer Institute, National Institute of Health, Bethesda, MD, USA

Cleo Y-F Lee Stanford Cancer Center, Stanford University School of Medicine, Stanford, CA, USA

Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA, USA

Meng Li Medical Sciences Program, Indiana University, Bloomington, IN, USA

Qiuzhen Liu Infectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Norman J. Maitland Cancer Research Unit, Department of Biology, University of York, York, UK

Francesco M. Marincola Infectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

William H. Matsui The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Marina C. Mione IFOM, The FIRC Institute of Molecular Oncology, Milan, Italy

Kenneth P. Nephew Medical Sciences Program, Indiana University, Bloomington, IN, USA

Indiana University Simon Cancer Center, Indianapolis, IN, USA

Ciara S. O'Brien Department of Medical Oncology, The Christie NHS Foundation Trust, University of Manchester, Manchester, UK

Davide Pellacani Cancer Research Unit, Department of Biology, University of York, York, UK

Jay M. Pilrose Medical Sciences Program, Indiana University, Bloomington, IN, USA

Lynne-Marie Postovit Department of Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada

Mark E.P. Prince Department of Otolaryngology-Head and Neck Surgery, University of Michigan, Ann Arbor, MI, USA

Daniela Quail Department of Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada

Zeshaan A. Rasheed The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Lucia Ricci-Vitiani Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy

Marianna Sabatino Cell Processing Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Cristina Santoriello IFOM, The FIRC Institute of Molecular Oncology, Milan, Italy

Alicia G. Serrano Clinical Research Programme, Stem Cells & Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

Jun Shen Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA

Sheila K. Singh McMaster Stem Cell and Cancer Research Institute, McMaster University, Hamilton, ON, Canada

Departments of Surgery, Biochemistry & Biomedical Sciences, and Neuroscience, Faculty of Health Sciences, McMaster University, Hamilton, ON, Canada

David F. Stroncek Cell Processing Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Meghan Taylor Department of Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada

Chitra Venugopal McMaster Stem Cell and Cancer Research Institute, McMaster University, Hamilton, ON, Canada

Ena Wang Infectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Xin Wang McMaster Stem Cell and Cancer Research Institute, McMaster University, Hamilton, ON, Canada

Pamela M. Willan Cancer Stem Cell Research, University of Manchester, School of Cancer and Enabling Sciences, Paterson Institute for Cancer Research, Manchester, UK

Yang Xu Section of Molecular Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA, USA

Ludovic Zimmerlin Department of Cardiovascular Surgery, Division of Hematology/Oncology, University of Pittsburgh Cancer Institute Pittsburgh, Pittsburgh, PA, USA

Part I
Introduction to Cancer Stem Cells

Chapter 1
Cancer Stem Cells: Historical Perspectives and Lessons from Leukemia

Christopher R. Cogle

Abstract Cancer has a long history rooted in developmental biology. Early scientists regarded cancer as remnant embryonal tissues waiting to be provoked into a malignant state. Whereas this embryonal rest theory fits well with certain childhood cancers like teratocarcinomas, acquired cancers in adulthood require more explanation. Because of early advances in hematology and immunology, investigations of hematologic malignancies like leukemias have benefited from translated technology. Seminal discoveries in leukemia stem cell biology are reviewed in this chapter. Some of these discoveries translate to novel opportunities for improved diagnostics and therapeutics. Importantly, several lessons in the leukemia stem cell experience are applicable to ongoing cancer stem cell investigations. These lessons are discussed relative to leukemia stem cells and with an eye toward defining and testing cancer stem cells in solid tumors.

Abbreviations

ABC	ATP binding cassette
ABL	Ableson
ALDH	Aldehyde dehydrogenase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia

C.R. Cogle (✉)
Department of Medicine, Division of Hematology/Oncology,
University of Florida, Gainesville, FL, USA

Program in Stem Cell Biology and Regenerative Medicine, University of Florida,
Gainesville, FL, USA
e-mail: c@ufl.edu

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ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
CD	Cluster of differentiation
CML	Chronic myeloid leukemia
ENL	Eleven nineteen leukemia
FISH	Fluorescent in situ hybridization
MDR	Multi-drug resistance
MLL	Mixed lineage leukemia
MOZ	Monocytic leukemia zinc finger protein
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
NOG	Non-obese diabetic/severe combined immunodeficiency/IL2 receptor γ-null
PCR	Polymerase chain reaction
TIF2	Transcriptional intermediary factor 2

1.1 Historical Postulates for the Stem Cell Basis of Cancer

Today, cancer stem cells are defined as "a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" [1]. However, this idea that primitive cells can lead to cancer is not new.

The earliest reports of a cancer stem cell hypothesis appeared in the 1800s. Similarities between teratocarcinomas and the developing embryo led biologists to postulate that cancers arise from embryonic remnants in adults [2]. Certainly, the existence of teratocarcinomas which contain cells of all three germ layers and afflict young adults along midline migration pathways between gonads to brain endorses this embryonal rest theory. Subsequent investigators further developed this theory and suggested that adult tissues may contain embryonic remnants that are normally dormant but can become cancerous if provoked [3–5].

Whereas the embryonal rest hypothesis may explain teratocarcinomas, which primarily arise in children, the hypothesis requires more elaboration to understand the genesis of acquired cancers, which arise in adulthood and not necessarily along the midline. Given evidence for tissue-resident stem and progenitor cells in the adult, it is possible that these normally self-renewing and multi-lineage differentiating stem cells may be provoked by carcinogens to acquire hallmark properties of cancer, including evasion of apoptosis, growth factor independence, self-renewal, tissue invasion, and sustained angiogenesis. Hematologic malignancies, which usually arise in the seventh and eighth decades of life and which coincide with normal hematopoietic stem and progenitor cells, provide a clear opportunity to define adult cancer stem cells [6].

1.2 History of Leukemia Stem Cells

The first reports of leukemia stem cells were in the 1930s when Furth and Kahn transplanted leukemia from one mouse to another via a single undifferentiated leukemia cell [7]. These experiments demonstrated that a self-renewing malignant hematopoietic stem cell was present; however, without the ability to characterize source cells or define progeny, no definite comment could be made about a hierarchy of malignant stem cells which exhibit the two cardinal features of stem cells: self-renewal and multi-lineage differentiation. Defining leukemia stem cells would come decades later, after advancements in immunology and cell sorting techniques.

The first detailed investigation for leukemia stem cells came in the 1990s out of John Dick's laboratory [8, 9]. Taking cues from normal hematopoietic stem cell biology, these investigators identified a subpopulation of $CD34^{+}CD38^{-}$ human acute myeloid leukemia (AML) cells that propagated colonies in culture and recapitulated human leukemia in immunocompromised mice. Using limiting dilution xenotransplant experiments, AML stem cells were estimated to exist at a frequency of 1 in 250,000 $CD34^{+}CD38^{-}$ AML cells. In contrast, when these investigators xenografted more committed leukemia cells expressing a $CD38^{+}$ phenotype, they were unable to recapitulate AML. Together, these experiments showed that AML stem cells were present, prospectively identifiable, and rare. Moreover, an AML hierarchy was apparent, with AML stem cells giving rise to terminally differentiated yet malignant progeny.

Studies subsequent to these seminal discoveries have shed new light on leukemia stem cells and serve as important lessons for the field of cancer stem cell biology.

1.3 Lesson: Normal Stem Cells Aren't Always the Origin

The fact that AML stem cells can be enriched using the same selection strategy as normal hematopoietic stem cells (e.g., immunosorting for $CD34^{+}CD38^{-}$) suggests that leukemia stem cells may be a malignant transformation of normal stem cells. However, follow-up experiments of AML stem cells found that they do not express CD90 (Thy1), in contrast to normal hematopoietic stem cells, which do express Thy1 [10]. This finding begged the question of whether malignant transformation of normal hematopoietic stem cells results in loss of Thy1 expression, or whether hematopoietic progenitors lacking Thy1 are the target of malignant transformation into leukemia stem cells. The answer depends on the type of leukemia.

In leukemias that harbor the fusion oncogene *BCR-ABL* (which can be found in patients with chronic myeloid leukemia [CML], acute lymphoblastic leukemia [ALL] and AML with translocation of chromosomes 9 and 22), the cancer-initiating cell is believed to be at the level of the hematopoietic stem cell or higher. Forced expression of *BCR-ABL* in hematopoietic progenitor cells resulted in a proliferation of leukemia cells; however, the transformed hematopoietic progenitors could not

self-renew and recapitulate disease [11]. In other types of leukemia, hematopoietic progenitors may serve as the origin for transformation. For example, forced expression of oncogene fusions such as *MLL-ENL* or *MOZ-TIF2*, which can be found in patients with AML, endow hematopoietic progenitor cells with the ability to self-renew and differentiate [11, 12]. Together, these results show the heterogeneity of leukemia origin and may explain the heterogeneity in clinical behavior.

In context to cancer stem cells in solid tumors, the hunt for the source should not be restricted to the organ-resident stem cell. Candidates for oncogenic transformation should also include more committed tissue progenitor and differentiated cells, especially in epithelial situations where field cancerization and dysplasia can be found.

1.4 Lesson: Don't Underestimate the Microenvironment

In early leukemia stem cell experiments, when investigators replaced the severe combined immunodeficiency (SCID) mouse with the more immunocompromised non-obese diabetic (NOD)/*scid* strain, xenotransplanted human AML $CD34^{+}CD38^{-}$ cells more readily repopulated secondary mice, thus demonstrating in vivo self-renewal typical of stem cells. Use of even more immunodeficient mice, such as NOD/*scid*/IL2R-$\gamma^{-/-}$ (NOG) mice [13], resulted in even higher engraftment levels of human AML cells [14]. Moreover, in these NOG mice, consistent AML engraftment can be found in secondary and tertiary xenograft recipients. Interestingly, female NOG mice are more tolerant of AML stem cell engraftment than male mice [15]. Taken together, these data implicate the host microenvironment as a key factor in determining the presence and frequency of cancer stem cells. Careful consideration and scrutiny should be applied to the model system used to detect, quantify, and characterize putative cancer stem cells. Discoveries from one lab may not replicate in another lab simply due to differences in host model and/or manipulations of the host model. For example, conditioning transplant recipients with ionizing irradiation or antibodies to immune cells may enhance the gain when reading out putative cancer stem cell engraftment.

Although differences in the host microenvironment may complicate consensus on the definition of cancer stem cells, these differences may also be explored as opportunities to discover which situations support cancer survival. Once defined, these host microenvironmental factors may then be targeted as novel therapeutic strategies. For example, blood vessels in the bone marrow microenvironment are important for leukemia stem cell survival and proliferation [16–18]. Targeting these blood vessels in the microenvironment causes regression of leukemia and may be a promising therapeutic for patients with this cancer [19, 20]. As another example, given evidence of robust AML engraftment in severely deficient animals, host immune response to leukemia stem cells is likely important. In fact, leukemia stem cells were shown to over-express CD47, a surface protein that inhibits macrophage recognition [21]. Clinically, patients whose leukemia cells expressed high levels of CD47 had inferior outcomes after chemotherapy, which suggests the importance of

macrophage immunosurveillance in leukemia [22]. Modulating host immune response to overcome leukemia's evasion may therefore represent a novel potential therapeutic strategy.

1.5 Lesson: Surface Molecules Aren't Just Markers

Immunophenotyping is a common method for identifying and selecting cancer stem cells after advancements in immunology and cell sorting technology (e.g., flow cytometry, magnetic separation). Increasingly, investigators have used the term "marker" to describe a unique surface molecule or constellation of surface molecules on putative cancer stem cells. However, the term "marker" is a restrictive term that disregards the molecule's biological function.

As an example, the normal hematopoietic stem cell expresses CD44 receptors, which tether it to stromal adhesion molecules like hyaluronic acid, osteopontin, collagens, and matrix metalloproteinases. Leukemia stem cells also express CD44 isoforms [23]. Recognizing that CD44 is more than a "marker" of leukemia stem cells, investigators have blocked CD44 stroma binding and found impairments in leukemogenesis. When *BCR-ABL* leukemia CD44 receptors were mutated, leukemia proliferation was inhibited. Furthermore, the application of blocking antibodies to CD44 inhibits leukemia stem cell engraftment [24].

1.6 Lesson: There May Be More Than One Cancer Stem Cell Population

Clear evidence shows that leukemia stem cells can be found in the $CD34^{+}CD38^{-}$ subpopulation of leukemic bone marrow. However, there is also evidence that leukemia stem cells can be found in the $CD34^{-}$ subpopulation [25–27]. Whether leukemia stem cells lose CD34 expression after oncogenic transformation or whether CD34-negative leukemia stem cells represent transformation of a very primitive bone marrow–derived stem cell is yet to be defined.

Leukemia stem cells have also been defined by their functional characteristics. For example, aldehyde dehydrogenase (ALDH) is important for eliminating intracellular toxins. Normal hematopoietic stem cells are known to have higher levels of this enzyme and can thereby be prospectively identified based on functional ALDH activity [28]. Taking cues from normal stem cell biology, leukemia investigators have reported enrichment of leukemia stem cells by selecting leukemic bone marrow cells with high ALDH activity [29]. Another functional assay exploits the drug efflux capacity of stem cells. In normal stem cell biology, side-population cells, defined by their ability to efflux the DNA-binding dye Hoechst 33342, have shown self-renewal and multi-lineage differentiation [30, 31]. Following suit, leukemia

investigators have also identified a small subpopulation of leukemia stem cells that reside within this side-population of leukemic bone marrow [32, 33].

At face value, these multiple and overlapping reports may suggest contradictions. But it is more likely that there are different leukemia stem cell populations for different types of leukemias. In addition, it has yet to be determined whether there are multiple leukemia stem cells within each patient's leukemia.

1.7 Lesson: Treatment Failure May Be Due to Cancer Stem Cell Resistance

The identification of self-renewing leukemia stem cells that reside in protective microenvironments suggests that these cells may be sources of primary refractory and relapsed disease. If so, then these leukemia stem cells must be less sensitive to conventional therapies than their differentiated progeny.

Given the important role of multiple drug resistance (MDR) transporters in stem cells (a family of at least 48 human ATP binding cassette [ABC] transporters discovered to date), this mechanism has been suggested as cause for leukemia stem cell resistance to conventional chemotherapies [34]. In younger patients with AML, MDR1 is less frequent, which may explain better responses to therapy [35]. Administration of MDR inhibitors as adjuvant therapy does bring about improvements in remission rates [35, 36]. However, it is not clear whether the more effective response rates are due to MDR inhibition in leukemia stem cells and increased sensitivity to chemotherapy, or increases in circulating chemotherapy levels due to altered chemotherapy metabolism related to side effects of the MDR inhibitor.

For patients with CML, the *BCR-ABL* fusion oncogene can be targeted with the tyrosine kinase inhibitor, imatinib. Imatinib directly targets the *BCR-ABL*–encoded tyrosine kinase activity in CML leading to decreased proliferation of myeloid progenitors. However, despite cytogenetic responses measured by fluorescent in situ hybridization (FISH), molecular eradication of the disease measured by more sensitive quantitative polymerase chain reaction (PCR) is difficult to achieve and the current standard of care is to keep patients on imatinib indefinitely or until disease relapse or progression. The persistence of CML despite tyrosine kinase inhibitor therapy within imatinib is a result of resistance by quiescent CML stem cells [37]. Several strategies are now being developed to target resistant CML-initiating cells.

1.8 Conclusions

Traced back far enough, the roots of cancer can be found in developmental biology. From the embryonal rest theory, more detailed investigations of cancer have uncovered rare cancer stem cells with the potency to self-renew and differentiate.

Because of advances in normal hematopoietic stem cell biology and immunology, significant progress has been made in defining leukemia stem cells. Translating technology from the normal to malignant setting has illuminated mechanisms of leukemogenesis, resistance to treatment, and relapse. This enlightened understanding empowers physician scientists to move beyond brute force cytotoxicity and closer to strategic strikes.

Several lessons stand out from the leukemia stem cell experience that are relevant to most cancer stem cell investigations. These lessons all have in common the central idea that cancer is a heterogeneous mixture of primitive and differentiated cells that each has multidirectional relationships with each other and the host microenvironment. The idea that multiple subpopulations enriching for cancer stem cells are supported by many microenvironmental interactions is more likely than the concept of one cancer stem cell dependent on only one pathway. Certainly, it is easier to present and think about cancer stem cell data in one dimension, but creating new therapies and optimizing old ones will require us to broaden our scientific considerations.

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Part II
Cancer Stem Cells in Solid Tumors

Chapter 2
Cancer Stem Cells in Breast Cancer

Jenny E. Chu and Alison L. Allan

Abstract Breast cancer is one of the leading causes of cancer-related deaths among women worldwide. While it is highly treatable during the primary stages, the disease is often lethal if it successfully metastasizes. Breast cancer stem cells (CSCs) show distinct similarities to normal breast stem cells, have been shown to be the driving force behind primary tumorigenesis, and are postulated to be the cells responsible for metastasis. Many groups have used the $CD44^{+}CD24^{-}$ and/or $ALDH^{+}$ phenotype for breast CSC isolation; however, this definition does not apply to all breast cancers and needs further refining. As CSCs have been shown to be therapy resistant, identification of additional markers will aid in the isolation of a pure CSC population, which can then be used to elucidate effective treatments. This chapter will discuss normal breast stem cells, breast CSC identification, the relationship between normal mammary stem cells and breast CSCs, and the clinical implications of the CSC population in breast cancer.

Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ALDH	Aldehyde dehydrogenase

A.L. Allan (✉)
Departments of Oncology and Anatomy & Cell Biology,
Schulich School of Medicine and Dentistry, University of Western Ontario,
London, ON, Canada

London Regional Cancer Program, London Health Sciences Centre,
London, ON, Canada
e-mail: alison.allan@lhsc.on.ca

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BCRP1	Breast cancer resistance protein 1
BMP	Bone morphogenic protein
BRCA1	Breast cancer susceptibility gene
CD	Cluster of differentiation
CSC	Cancer stem cell
CXCR4	Chemokine receptor 4
DCIS	Ductal carcinoma in situ
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ESA	Epithelial specific antigen
HA	Hyaluronic acid
HER2	Human epidermal growth factor receptor 2
HSC	Hematopoietic stem cell
IHC	Immunohistochemistry
LCIS	Lobular carcinoma in situ
Lin	Lineage
MaSC	Mammary epithelial stem cell
MDR1	Multi drug resistance pump 1
MMTV	Mouse mammary tumor virus
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NOD/SCID	Non-obese diabetic/severe combined immune deficiency
PR	Progesterone receptor
RA	Retinoic acid
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SDF	Stromal derived factor
TGF-β	Transforming growth factor beta
T-IC	Tumor-initiating cells

2.1 Breast Cancer

2.1.1 Statistics

Excluding nonmelanoma skin cancers, breast cancer is the most frequently diagnosed cancer and the second highest cause of cancer-related deaths among both Canadian and American women [1, 2]. On a global scale, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of total cancer cases and 14% of cancer deaths [3].

2.1.2 Initiation and Disease Progression

Breast cancer originates from the transformation of breast epithelial cells found either lining the milk ducts or in the milk-producing lobules of the breast. Lobules and ducts are formed from three lineages of cells in two layers: the myoepithelial layer is common to both structures and forms the basal layer, while ductal epithelial cells line the ducts and alveolar epithelial cells synthesize the milk within the lobules [4, 5]. While still confined within the duct or lobule of origin, breast tumors are classified as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), respectively. When breast cancers are diagnosed in the in situ stage, treatments are highly effective (DCIS) if even necessary (LCIS) [6–8]. Prognosis worsens when the tumor invades adjacent tissues and gains the potential to metastasize. Metastatic disease is the aspect of breast cancer that is responsible for the majority of breast cancer-related deaths.

Breast cancer tumors exhibit two levels of heterogeneity: different tumor subtypes [9, 10] and functional differences at the cellular level within the tumor [11, 12]. Among patients and even among different tumors within the same patient, breast tumor subtype can vary in many ways: through histopathology (i.e., where the tumor is located and the type of cellular morphology), molecular pathology (ER/PR/HER2 status and other cellular markers), and through variability of genetic composition and expression (loss or gain of chromosomal material, oncogene expression, or mutation carriers) [9]. Through the use of gene expression analysis, six breast tumor subtypes have been identified, each having different characteristics and prognosis. These include two unique luminal subtypes (A and B); basal-like; HER2-overexpressing; normal breast-like; and the most recently identified, claudin-low subtype [13–15].

Cell populations that make up individual tumors are not homogenous, but are in fact functionally heterogeneous. The two categories consist of the tumor-initiating cells (T-ICs), capable of tumor propagation and maintenance due to their ability to self-renew, and terminally differentiated cells that are not capable of producing large amounts of progeny and are not capable of tumor propagation [12, 16, 17]. These observed levels of heterogeneity are accounted for by the cancer stem cell (CSC) hypothesis, which postulates that cancers are hierarchically organized stemming from progenitor cells, or CSCs [18]. The hierarchal nature of the tumors mirrors that of the normal breast tissue for which a normal mammary epithelial stem cell (MaSC) has recently putatively been identified in human and murine tissues.

2.2 Normal Breast Organization and Mammary Stem Cells

Recent studies point strongly to the existence of both murine and human MaSCs. Indeed, the dynamic nature of breast development throughout life dictates the need for some type of long-lived progenitor capable of multiple types of differentiation with a large capacity for cellular proliferation. The breast undergoes restructuring

involving proliferation, remodeling, and differentiation in response to hormonal changes during embryogenesis, puberty and pregnancy [4]. A stem cell (defined as a cell capable of unlimited self-renewal and possessing the ability to produce at least one kind of differentiated progeny [19]) is likely the driving force behind this continual remodeling. Unlike pluripotent embryonic stem cells that are able to give rise to all cells of the body, these tissue-specific stem cells are multipotent – they are restricted to producing cells found within the breast tissue.

2.2.1 Support for Normal Murine Mammary Stem Cells

The first evidence of a potential mammary stem cell was observed by Deome et al. [20]. In their transplantation experiments, a sample of normal mammary tissue was implanted into a cleared mammary fat pad, resulting in outgrowths with normal mammary gland appearance. Further transplantation experiments demonstrated that single cells are capable of re-creating the entire heterogeneity of a mammary gland [21]. Single cell implantation experiments using sorted cells have verified that murine cells depleted of hematopoietic cells (Lin^-) and expressing CD29 and/or CD49f in combination with CD24 are capable of self-renewal and differentiation into the breast cell lineages, forming a functional mammary gland [22, 23].

2.2.2 Support for Normal Human Mammary Stem Cells

An exact identification of a human mammary epithelial stem cell has yet to be solidified, but many groups have identified putative mammary epithelial progenitor cells. Technical challenges have arisen due to the complex nature of the hormonal requirements for MaSC differentiation and also for a suitable environment to support growth [24]. Work with human breast stem cells builds on the foundations of experiments investigating the murine population. Work by Kuperwasser et al. [24] has resulted in the development of a humanized murine fat pad that more accurately represents the human breast stroma. They demonstrated that fat pad injection with a mixture of irradiated and nonirradiated human mammary epithelial cells allows for the successful engraftment of the stromal cells and for the creation of a humanized environment [24, 25]. More recently, a new model has been described by Eirew et al. [26], whereby fibroblast and putative mammary stem cells are engrafted in a collagen plug under the murine kidney capsule. The outgrowths observed recapitulate the hierarchal nature of the normal human mammary gland. Through the use of these assays, $CD49f^{hi}EpCAM^-$ has been established as the fraction containing the human breast stem cell population [26, 27]. To complement these cell surface markers, a functional marker, aldehyde dehydrogenase 1A1 ($ALDH^+$) (Fig. 2.1) has been established as a functional marker for mammary stem cells [28] among others [29].

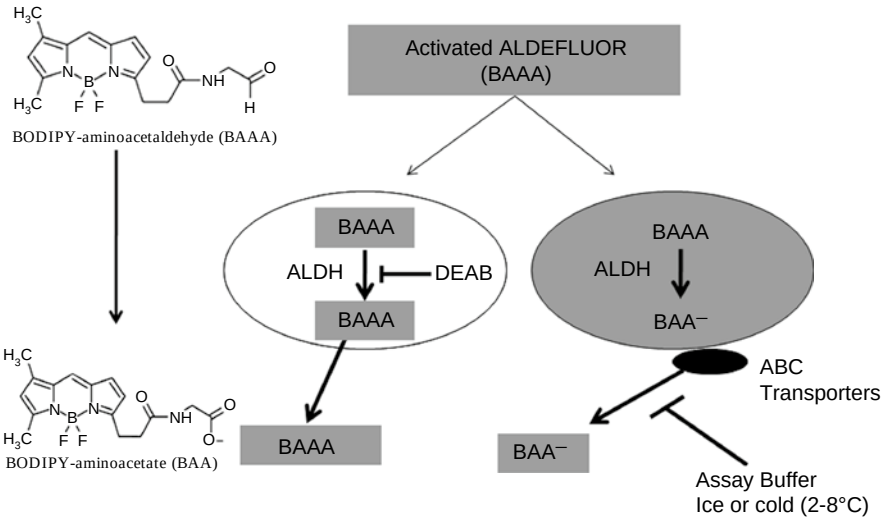

Fig. 2.1 The Aldefluor® assay. The Aldefluor® assay is a fluorometric assay that detects the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) (StemCell Technologies, Vancouver, BC, Canada). Cells are incubated with the intrinsically fluorescent ALDH substrate, BODIPY-aminoacetaldehyde (BAAA). BAAA is a neutral molecule and enters the cell through passive diffusion, where it is then converted into BAA^- by ALDH and is unable to leave the cell due to its negative charge. The active removal of BAA^- by ATP Binding Cassettes is quenched through the use of the assay buffer and through incubation of cells between 2 and 8°C. The resulting fluorescence of the cells is then assessed by flow cytometry, providing single cell analysis of ALDH activity. As a negative control, the activity of ALDH is quenched by the addition of diethylaminobenzaldehyde (DEAB), and the fluorescence of these cells is assessed by flow cytometry. The population observed in the DEAB sample is used to create the gate for the $ALDH^+$ cells, whereby cells are only included if they demonstrate higher levels of fluorescence compared to the DEAB sample. Adapted from StemCell Technologies (www.stemcell.com)

2.3 Identification of Cancer Stem Cells in Breast Cancer

The first identification of a CSC in solid tumors came from the work of Al-Hajj et al. [30] using cells isolated from pleural effusions and primary tumors of breast cancer patients. Cells with an $ESA^+CD44^+CD24^{-/low}Lin^-$ phenotype were capable of forming tumors in numbers as low as 100 when injected into the mammary fat pad of nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice, while tens of thousands of cells from other populations were nontumorigenic. Further work by Ginestier et al. [28] identified a small subset of $CD44^+CD24^-$ cells which were $ALDH^+$ and were able to initiate tumor formation in NOD/SCID mice with as few as 20 cells injected. These cells recreated the heterogeneity of the initial tumor, exhibiting nontumorigenic populations in addition to the tumorigenic cells. This recapitulation could be repeated upon serial passaging in naïve NOD/SCID mice, demonstrating both differentiation and self-renewal potential [28]. The presence of ALDH expressing cells in tumors has been correlated with poor prognosis in breast

cancer patients [28, 31, 32]. Additionally, the $CD44^{+}CD24^{-}$ population appears to be enriched in basal-like tumors (ER, PR, HER2 negative) and in BRCA1 tumors [33], both of which have been associated with poor patient prognosis [34, 35]. The presence of a CSC population has also been verified in breast cancer cell lines and primary tumor samples [36].

Due to the functional stem cell-like characteristics of these cells, the term "cancer stem cell" is a fitting descriptor. However, it does not mean that these cells are indeed stem cells re-wired, although they may be. A consensus on the definition of CSCs was created by the leaders in the field to be "a cell within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" [37]. It is hypothesized that CSCs arise either from a normal tissue stem cell that has acquired mutations that make it tumorigenic or from a more differentiated progenitor or mature cell that has dedifferentiated and acquired the ability to self-renew in addition to the tumorigenic mutations. While the described phenotype is not an absolute definition of the breast CSC population, it provides a basis for further work.

2.4 Markers Used to Identify CSCs

In order to elucidate the functions and the populations of CSCs within solid tumors, the phenotypic definition of a CSC must first be established. Selectable markers are either found on the cell surface or confer functional properties that are characteristics of normal stem cells that have extended to malignant stem cell populations. As previously mentioned, the current definition of a breast CSC is $CD44^{+}CD24^{-}$ and/or $ALDH^{+}$. In the following section, these markers and other putative CSC markers will be discussed.

2.4.1 CD44

CD44 is a multifunctional cell membrane protein that plays a role in both cell–cell and cell–extracellular matrix (ECM) interactions primarily through the binding of hyaluronan (HA). Other ligands of CD44 include collagen, fibronectin, fibrinogen, laminin, chondroitin sulfate, mucosal vascular addressin, serglycin, osteopontin, class II major histocompatability complex invariant chain, L-selectin, and E-selectin [38, 39]. As CD44 is widely expressed throughout the body, and its ligands are common, the successful binding of CD44 to its ligands often depends on an external stimulus. Alternative splicing and protein glycosylation gives rise to multiple CD44 isoforms that differ in size (85–230 kDa), functionality, and tissue localization [39, 40].

2.4.1.1 Function in Normal Tissue

Work by Lesley et al. [41] has identified three states of CD44: active, inducible, and inactive. The activity is dictated by the glycosylation status of the protein: the active form is least glycosylated and constitutively binds HA; inducible CD44 is moderately glycosylated and requires activation by monoclonal antibodies, cytokines, growth factors, or phorbol ester; and inactive CD44, the most glycosylated, is unable to bind HA (reviewed by Naor et al. [38]). Adding additional variability, the types of glycosylation may vary from isoform to isoform, using side chains such as heparin sulfate and chondroitin sulfate, resulting not only in variations of molecular weight but also in differentially charged environments that affect CD44 function [42].

The human CD44 gene consists of 19 exons, the first 5 of which are constant [39]. The middle 9 exons (v2–v10) are variable regions which may be removed depending on the variant expressed. The next three exons (16–18) are constant, and the last two exons (19 and 20) are variable. Exons 1–17 encode the extracellular domain of the protein, while 18 encodes the transmembrane domain, and 19 and 20 encode the cytoplasmic tail [43]. Individual cells are capable of altering the splicing of CD44, allowing for much diversity. The standard form, CD44s, is the smallest of the isoforms (37 kDa unglycosylated; 80–100 kDa when glycosylated [42]), and was first identified on hematopoietic cells [44] and is therefore additionally termed hematopoietic CD44, or CD44H [38]. Further research has highlighted CD44s expression in a variety of tissues including the epidermis, liver, pancreas, lung, and central nervous system. The distribution of variant CD44 (CD44v) isoforms is much more restricted and apparently tissue specific (reviewed by Sneath [42]). Nomenclature for CD44v isoforms depends on the variant expressed. A CD44v expressing only variant exon 6 would be called CD44v6.

CD44 is involved in cell–ECM and cell–cell interactions. In cell–ECM interactions, CD44 functions through the binding of its previously mentioned ligands, which may facilitate cellular functions such as adhesion and migration. Additionally, CD44 binding of HA causes the internalization of the CD44–HA complex and the lysosomally facilitated degradation of HA [45]. In cell–cell interactions, CD44 allows for the aggregation of cells through the binding of exogenous or endogenous HA [42]. CD44s has also been implicated in the lymph node homing and activation of lymphocytes through its binding of mucosal addressin. The standard and variant forms of CD44 are also involved in myelopoiesis and lymphopoiesis, angiogenesis, chemokine and growth factor presentation, and growth and apoptosis signaling [39, 42, 46].

In normal breast tissue, expression of CD44s and CD44v has been observed by immunohistochemistry (IHC) to be in the myoepithelial layer, while the remaining epithelial cells are $CD44^-$ [47–50]. Normal breast stromal elements have been observed to express only CD44s [47]. These IHC observations also apply to clinical tumor specimens, as high levels of mainly CD44v have been observed. The correlation between CD44 expression and patient prognosis varies from study to study, likely due to differences in technique, isoform, and the breast cancer population studied (reviewed by Herrera-Gayol and Jothy [51]).

2.4.1.2 First Implications in Cancer and Potential Role in CSCs

CD44 was first implicated in cancer when a nonmetastatic cell line acquired metastatic potential upon transfection with CD44v4-v7, a variant previously found to be expressed by a metastatic rat pancreatic adenocarcinoma. Studies have demonstrated that CD44s is involved in breast cancer cell adhesion, motility, and invasion; whereas CD44v6 is involved solely in cell motility [52]. CD44 most likely acts in tumorigenesis by allowing for more efficient colony formation through increased adhesion to its multitude of ligands in the surrounding environment, its ability to aggregate cells, its induction of cellular growth signals via intracellular signaling partners, and by facilitating the degradation of the surrounding ECM and basal lamina, allowing a path for cellular migration and tumor expansion (reviewed in [42, 51]). Notably, CD44 has been shown to interact with matrix metalloproteinases, activating them and attaching them to the cell surface of tumor cells, thus enabling efficient tumor cell invasion through collagen IV [53, 54]. It is also thought that CD44 plays a distinct role in tumor metastasis; however, the absolute mechanism remains elusive due to the many isoforms and variable functions in different environments [53]. A possible component is revealed through the observation that CD44v4 has been shown to mediate breast cancer transendothelial metastasis through its binding to E-selectin [54]. Contradicting studies show that the presence of CD44s reduced metastasis, potentially explained through the masking of HA from other receptors [55].

The function of CD44 in breast CSCs has yet to be fully elucidated; however, it is likely that the molecule plays a role in enabling CSCs to be the metastasis-initiating cells observed by Croker et al. [56] and Charafe-Jauffret et al. [31, 57]. Recent evidence has shown that CD44 plays a role in protection against apoptosis [58], an important characteristic for a tumor-initiating and metastasis-initiating cell. Additionally, CD44's dual ability for cell–cell and cell–ECM adherence could confer an advantage for CSCs as they travel through the bloodstream and arrive at and enter their secondary site [53]. Within the last few years, much work has been done on the HA–CD44 interaction, revealing that it promotes growth through an EGFR-MAP/ERK (MEK)–dependent mechanism in head and neck cancer [59], and through a HER2-β-catenin–dependent manner in ovarian cancer [60]. In breast and ovarian cancers, the HA–CD44 interaction has been shown to activate transcription of Nanog (an embryonic stem cell transcription factor) transcription, which proceeds to activate Rex1, SOX2, and Multi-drug resistance pump 1 (MDR1) [61], all stem cell-related products. These responses to HA-CD44 binding may provide insight into the observed properties of breast CSCs, especially with regard to their therapy resistance.

There is no distinct rule regarding CD44 isoforms and functions within cancer. In some cases, CD44 variants are involved in promoting malignancy, while in others it is the standard form [62]. A further exception to the rule is the observation that CD44 can in fact act as a metastasis suppressor, holding the tumor within the primary site [55, 63]. Diaz and colleagues suggest that the expression of CD44s in node-negative invasive cancer may be associated with increased disease-free survival [64].

Further studies must be done to investigate the functional aspects of CD44 expression in CSC populations through transfection experiments introducing CD44 into non-CSC populations, and more relevantly, through knockdown experiments looking at loss of function due to downregulated CD44 expression.

2.4.2 CD24

Like CD44, CD24 is a glycosylated cellular adhesion molecule, with a weight ranging from 30 to 70 kDa depending on the glycosylation present [65]. It was first described as a B-cell surface protein, but has since been found to be expressed by other hematopoietic cells, the developing brain and pancreas, as well as by a large number of epithelial cells such as keratinocytes and renal tubular cells [65, 66]. Of particular interest, CD24 is emerging as a marker of malignant cells either due to its expression or lack thereof.

2.4.2.1 Functions in Normal Tissue

CD24 has been putatively implicated in B-cell maturation and the determination of T and B lymphoid progenitors to survive and proliferate. It has additionally been defined as an important T-cell co-stimulatory molecule, although the exact mechanism remains to be elucidated [66]. The CD24-bound oligosaccharides act as a ligand for P-selectin, a cell adhesion molecule expressed by activated blood vessel endothelial cells and activated platelets. This interaction may facilitate tumor passage through the blood stream, and has been shown to mediate breast cancer cell rolling on P-selectin through the blood stream [67].

2.4.2.2 Implications in Cancer and Potential Role in CSCs

A study investigating tumor invasiveness found that downregulation of CD24 correlated with increased invasion in mammary cancer cell lines; however, a study in a glioma mouse model demonstrated opposite results [66]. These studies have been mirrored by many contradicting studies demonstrating that the presence of CD24 both enhances [65] and inhibits breast cancer cell invasion and metastasis (reviewed by Giatromanolaki et al. [36]). Additionally, work by Schabath et al. [68] demonstrated that low CD24 expression might enhance the growth ability and metastatic potential of breast tumor cells, as CD24 closely regulates the CXCR4 response. This would suggest that the low level of CD24 expression in the CSC population increases the metastatic potential of these cells. Interestingly, Rappa and Lorico [69] noted that within the breast cancer MA-11 cell line, tumorigenicity did not differ between sorted $CD44^{+}CD24^{-}$ and $CD44^{+}CD24^{high}$ populations, and that both populations were capable of producing cells with heterogeneous CD24 expression.

Whether or not CD24 is simply a marker of CSCs or actually plays a functional role in CSC cell behavior has yet to be established. However, the molecule plays a role in many functions that may influence tumorigenicity, and the functionality of this molecule in CSCs requires further study.

2.4.3 Lineage Markers

In the original identification of the breast CSC, cells positive for lineage markers CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b were discarded during flow cytometry in order to exclude normal human leukocytes, endothelial cells, mesothelial cells, and fibroblasts from the population being analyzed [30]. Work by Sheridan et al. has highlighted that CD10 is expressed on several breast cancer cell lines, and that perhaps CD10 should be excluded from the lineage criteria, as it has been defined as a marker of basal cells and might provide a further subdivision for the breast CSC population [70, 71].

2.4.4 Additional Cell Surface Markers

While the $CD44^{+}CD24^{-}$ selection criterion appears to enrich the tumor-initiating capability of breast cancer cells, it is not a definitive identification of these cells, nor does it apply to all breast cancers. Thus, other groups have been investigating other potential markers to further narrow down the CSC phenotype.

As discussed previously, the mouse mammary stem cell markers have been established as $Lin^{-}CD29^{hi}CD49f^{hi}$ (α6-integrin) and human mammary stem cells putatively identified as $CD49f^{hi}EpCAM^{-}$. It is notable that a subpopulation in the human breast cancer line MCF-7 was recently identified as overexpressing α6-integrin. These cells were capable of propagation as mammospheres, resisted pro-apoptotic agents and exhibited increased tumorigenicity when compared to the whole population, and as few as 1,000 cells were capable of tumor formation. Furthermore, knockdown of α6-integrin caused the loss of mammosphere capability and tumorigenicity [72].

In mouse models, CD29 and CD61 have been highlighted as potential proteins active in driving luminal cell fate. Within the $CD24^{+}$ population, CD29 differentiates between luminal committed ($CD29^{low}$) and mammary stem cells ($CD29^{high}$) [23]. The addition of CD61 allows for further division of the luminal committed cells into progenitors ($CD61^{+}$) and mature differentiated cells ($CD61^{-}$) [73]. Recent work in a mouse model of luminal breast cancer (MMTV-WNT1) demonstrated that the selection of the $CD61^{+}$ population resulted in a much more tumorigenic population when compared to the $CD61^{-}$ population [74].

Most recently, Meyer et al. [75] isolated a tumorigenic subset of $CD44^{+}$ cells from ER-negative breast cancers and found that $CD49f^{hi}CD133/2^{hi}$ cells exhibited

xenograft-initiating capability, whereas the CD49f$^{neg/low}$CD133/2$^{neg/low}$ population did not. They noted that while this new population enriched for xenograft initiation in mouse mammary fat pads, capability varied between their samples. Additionally, other markers established as CSC markers for other cancers, such as CD133 (a marker for colon and brain cancer initiating cells [76, 77]), may be good candidates for further refining the breast CSC phenotype.

Although knowledge translation from murine models and from other cancers to breast cancer is anything but direct, results from these highlighted surface markers merit more investigation into their application on the human breast cancer front. Furthermore, the lack of identified markers for the human mammary gland stem cell highlights the need for more research and standardized assays in this area.

2.4.5 ALDH

A hallmark of cancer cells is the genomic instability that allows for the accrual of the multiple mutations necessary for a cell to become tumorigenic [78]. The additional selection criterion afforded by the Aldefluor® assay (Fig. 2.1) provides quantitative analysis of ALDH functionality within CSCs, and this is emerging as an important tool in the study of normal stem cells and CSCs. ALDH activity has been shown to be a functional marker of stem cells. As a result, it might be a common property of CSC populations across all subtypes of the cancer in question (unlike the CD44$^+$CD24$^-$ phenotype). Interestingly, work by Ginestier et al. demonstrated that CD44$^+$CD24$^-$Lin$^-$Aldefluor$^-$ cells were nontumorigenic [28], suggesting that the CD44$^+$CD24$^-$Lin$^-$ phenotype is itself heterogeneous and does not contain strictly CSCs.

The aldehyde dehydrogenases are a large family of enzymes responsible for the oxidation of aldehydes into their corresponding carboxylic acids in a NAD(P)$^+$-dependent manner [79]. Different subfamilies are responsible for many functions in the body such as facilitation of retinoic acid biosynthesis, metabolizing cyclophosphamides and its derivatives, and clearing toxic byproducts of reactive oxygen species [29, 80].

High ALDH activity has been used to isolate a variety of normal stem cells, most notably human hematopoietic (HSCs) [81, 82] and murine neural stem cells [83]. Additionally, ALDH activity has been reported to identify leukemic stem cells [84, 85], head and neck CSCs [86], colon CSCs [87], and normal and malignant breast epithelial stem cells [28]. Consequently, ALDH is emerging as an important marker of both normal and malignant stem cell populations. Gene expression studies in HSCs and IHC staining of normal and malignant breast tissue reveal that ALDH 1A1 is likely the isoform responsible for the observed ALDH activity within these stem cell populations [80].

In addition to the conferred resistance to cyclophosphamide and its derivatives, ALDH is responsible for the metabolism of retinal to retinoic acid (RA) [88, 89], and therefore plays an important role in cellular differentiation during development

[90, 91] and in stem cell self-protection from intracellular aldehydes for the duration of an organism's life [29]. The formed RA can proceed to interact with nuclear retinoic acid receptors (RAR) and retinoid X receptors (RXR). RA–RAR interactions cause downstream effects on histone deacetylases, which control the epigenetic regulation of gene expression [92]. It is thought that this ALDH-dependent gene regulation and drug resistance play a role in creating the CSC phenotype.

2.5 Comparison of Breast CSCs and Normal Mammary Stem Cells

Although CSCs may arise from a normal tissue stem cell that has undergone cancerous mutations, CSCs may also arise from a more differentiated progenitor that has acquired self-renewal capabilities. Putative pathways involved in mammary stem cell self-renewal include LIF, Hedgehog, Wnt, Notch, TGFβ, EGF, Prl/GH, and ER/PR (reviewed by Kalirai and Clarke [5]). Similarly, Notch, HOXB4, Wnt, and bone morphogenetic protein (BMP) signaling pathways are identified pathways regulating HSC self-renewal [90]. Notably, Notch has been identified as being upregulated in $CD44^{+}$ populations of both normal and malignant breast cells [93], which may translate into an upregulation in the $CD44^{+}CD24^{-}$ CSC population. Additionally, $CD44^{+}CD49f^{hi}CD133/2^{hi}$ cells demonstrated upregulation of Sox2, Bmi-1, and Nanog (transcription factors known to play key roles in the stem cell self-renewal process) [75]. Unfortunately, due to the complex nature of stem cell self-renewal, it is unlikely that a single pathway will be shown to be responsible for CSC self-renewal.

2.6 The Role of CSCs in Metastasis

Breast cancer is a highly treatable disease if caught in the primary stage; however, once the disease metastasizes, patient prognosis becomes much worse [94, 95]. The stepwise process of metastasis is well established, whereby cells must first escape from the primary tumor into the bloodstream and/or the lymphatic system via intravasation. Once in the circulation, the cells must survive until they reach a secondary site where they arrest and enter the tissue (extravasate). Tumor cells able to initiate and maintain colony growth in the secondary sites form micrometastases, which, following angiogenesis, progress to macrometastases [94, 96, 97]. Although tumor cells may readily escape the primary tumor and enter circulation, production of sustainable metastatic lesions is a highly inefficient process (reviewed by Hunter et al. [98]). This was exemplified by an in vivo videomicroscopy study by Luzzi et al. which reported that only 0.02% of melanoma cells injected to target the liver could successfully complete the metastatic cascade [99]. Interestingly, this paper highlighted that not all metastatic stages are equally inefficient: the main inefficiencies occur during the initiation and maintenance of the metastatic lesions once tumor cells have reached

the secondary site. This observed inefficiency may be accounted for by the rarity of the CSC population and the lack of a conducive microenvironment for secondary growth. In an eloquent review, Croker and Allan [100] summarize that breast CSCs would be an ideal metastasis initiating cell, as they exhibit unlimited self-renewal, require a specific microenvironment to inhabit, use the SDF-1/CXCR4 axis to migrate, resist apoptosis, and are inherently resistant to many drugs.

Breast CSCs have been shown to demonstrate an increased metastatic propensity in vitro [56, 71, 101], in vivo [56, 57, 102], and in clinical observations [31, 103]. Although the mechanisms by which this occurs have yet to be identified, there are many theories about how CSCs contribute to breast cancer metastasis. The most common site of breast cancer metastasis is the bone, but metastatic lesions are also found in the lymph nodes, liver, lungs, and brain. Interestingly, both HA and osteopontin, common ligands for CD44, are expressed in the bone and other common sites of breast cancer metastasis [104], suggesting a possible adhesive interaction for circulating tumor cell arrest. Experimentally, CD44 has been shown to mediate the attachment of metastatic breast cancer cells to human bone marrow endothelial cells [105]. Additionally, breast cancer cell lines exhibit different levels of CXCR4, which appears to correlate with CSC proportions and the propensity to metastasize [56, 106]. Similar observations have been made in pancreatic cancer, where, within the identified $CD133^+$ CSC population, there existed two populations of CXCR4 expression, and only the $CXCR4^+$ population was capable of metastasizing [107]. Although the mechanisms have not yet been elucidated, there is much evidence to suggest that CSCs are not only tumor-initiating cells but also metastasis-initiating cells. This area requires further investigation, as it might reveal novel targets for therapy.

2.7 Breast CSCs and Therapy Resistance

Recent studies have indicated that breast CSCs [108] and other tumorigenic stem cells demonstrate resistance to chemotherapy and radiation therapy [4, 109, 110]. A study in human leukemia revealed that CSCs are often quiescent, and remain in the G_0 phase, conferring resistance to many chemotherapy agents as they often target actively replicating cells [111]. Clinical observations have noted an increase in $CD44^+CD24^-$ breast cancer cells after neoadjuvant chemotherapy treatment, indicating they may be resistant to therapy [112]. Possible mechanisms for this include the expression of cell surface pumps, including ABCG2/BCRP1, capable of expelling chemotherapeutic drugs [113]. Interestingly, this same pump has been found to be highly expressed in normal hematopoietic stem cells [114]. Additionally, the presence and activity of ALDH allows CSCs to metabolize cytotoxics such as cyclophosphamide [29]. Other factors potentially prolonging the lifespan of CSCs include the increased expression of anti-apoptotic molecules such as BCL2 and survivin [115, 116].

There is evidence in glioma and leukemic stem cell populations that cell cycle checkpoints and DNA repair mechanisms play a role in both radiation and

chemotherapy resistance, and that these mechanisms may apply to breast CSCs [117–119]. Further, the observed radiotherapy resistance of CSCs may be due to the decreased levels of pro-oxidants in the $CD44^{+}CD24^{-}$ population [120] or through Wnt/β-catenin pathway signaling [121].

These innate therapy resistance mechanisms make breast CSCs a difficult target to treat; however, their defined characteristics may provide the basis for new therapies. For example, deregulated pathways in breast cancer offer potential treatment options. However, the exact pathways responsible for the self-renewal of these cells have yet to be firmly established, and when they are, it is likely that they will heavily overlap with those used by normal stem cells, thus providing a barrier to treatment. Preclinical and Phase I clinical trials are underway targeting hedgehog, Notch, Akt, and CXCR1 [17]. Currently, high throughput screening is being used on cells sorted for CSC phenotypes, looking for small molecules, siRNA or lentiviral shRNA that target the CSC population. The effects of therapy may be analyzed in many ways including through changes in cellular growth [122], spheroid formation [123], migration [124], or through pathway-specific flow cytometry [125]. Until the biology of CSC therapy resistance is thoroughly understood, high throughput screening may provide the best hope of finding new therapies to target the CSC population.

2.8 Conclusions and Future Perspectives

While large steps have been made toward the absolute identification of the breast CSC, the definition still requires further refining. The $CD44^{+}CD24^{-}$and/or $ALDH^{+}$ phenotype has allowed for the establishment of the presence of a CSC population; indeed, gene expression profiling based on stem and differentiated cell markers indicates that the $CD44^{+}$ population is more stem-like and that the $CD24^{+}$ population is more differentiated [101, 126]. Unfortunately, due to the vast heterogeneity observed between breast cancers, this phenotype does not extend to all cases, thus further markers need to be established.

CSCs exist both in primary tumors and in metastatic lesions where they appear to play a role in the initiation and maintenance of both tumors. When an unambiguous definition of the CSC phenotype is elucidated, further research should be done to define the role of the CSCs in metastasis, and to identify unique therapy targets, either based on cell surface markers or based on a functional target. Before work targeting CSCs can move forward, it is essential that the functional and cell surface characterization of CSCs is completed. Once a pure population is identified, scientists will then be able to generate novel treatment strategies that aim to eradicate the cells postulated to be responsible for tumor initiation, recurrence, and metastasis.

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Chapter 3
Cancer Stem Cells in Brain Cancer

Xin Wang, Chitra Venugopal, and Sheila K. Singh

Abstract Several lines of evidence suggest that brain tumors arise from the transformation of a normal neural stem cell (NSC) or progenitor cell, which relies on the recognition of the many functional and genetic similarities shared by somatic stem cells and cancer cells. A minority population of human brain tumor initiating cells (BTICs) was identified through application of stem cell assays to brain tumors, and only these cells are able to recapitulate the patient tumor phenotype in an immunodeficient mouse model. Although the molecular mechanisms that regulate BTICs are still poorly understood, many of the signaling pathways that are active during development may be implicated as targets for transformation. BTICs have important implications for treatment, as these cells may evade current chemotherapy and radiotherapy. Further understanding of the role of BTICs in brain tumorigenesis may yield novel therapeutic agents aimed at targeting these rare cancer stem cells.

Abbreviations

AGAP2	GTPase-activating protein for ARF1 and ARF5
APC	Adenomatous polyposis coli
bFGF	Basic fibroblast growth factor

S.K. Singh (✉)
McMaster Stem Cell and Cancer Research Institute, McMaster University,
Hamilton, ON, Canada

Departments of Surgery, Biochemistry & Biomedical Sciences,
and Neuroscience, Faculty of Health Sciences, McMaster University, Hamilton, ON, Canada
e-mail: ssingh@mcmaster.ca

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BMP	Bone morphogenic protein
BTIC	Brain tumor initiating cell
BTSC	Brain tumor stem cell
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase inhibitor
CENTG1	Centaurin, gamma1
CNS	Central nervous system
CSC	Cancer stem cell
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma multiforme
HSC	Hematopoietic stem cell
LIF	Leukemia inhibitory factor
LSC	Leukemic stem cells
MDM2	Murine double minute 2
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
NSC	Neural stem cell
PDGFRα	Platelet-derived growth factor receptor alpha
PTCH	Patched
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma
SFM	Serum-free media
Shh	Sonic hedgehog
STAT3	Signal transducer and activator of transcription 3
TSM	Tumor sphere media

3.1 Brain Tumor Initiating Cells: The Starting Line

3.1.1 Lessons from Leukemia

Brain tumors are typically comprised of morphologically diverse cells that express a variety of neural lineage markers. It is recognized that tumors with vastly different histology have a different prognosis, but often brain tumors that share similar morphology and phenotype can have a very different prognosis and response to treatment. The cancer stem cell (CSC) hypothesis [1], based on work in leukemia [2] and breast cancer [3], suggests that not all the cells in the tumor have the same ability to proliferate and maintain the growth of the tumor. Only a relatively small fraction of cells in the tumor, termed CSCs, possess an ability to extensively proliferate and self-renew. Most of the other tumor cells lose the ability to proliferate and self-renew and instead differentiate into tumor cells that become the phenotypic signature of the tumor.

The CSC hypothesis is a variation on a theme first introduced more than 150 years ago by the pathologists Rudolph Virchow and Julius Cohnheim, both of whom observed histological similarities between primitive tumors such as teratocarcinomas

and the developing fetus [4–6]. They postulated that cancer arises from the activation of dormant embryonic rests, or tissue remnants [5]. The tools to explore the heterogeneous potential of cancer cells to self-renew emerged a century later, when Till, McCulloch and colleagues made the essential discovery that bone marrow contained single cells that could give rise to myeloerythroid colonies in the spleen. These colonies were clonal and self-renewing as well as radioprotected, and could reconstitute lethally irradiated mice [7–10]. The researchers applied their spleen colony-forming unit assay to myeloblastic leukemia, and isolated proliferative blast cells that were capable of self-renewal and abnormal patterns of differentiation [11–16]. These methods were adapted to allow for the assay of clonogenicity of human neoplastic cells in myelomonocytic leukemia [17], and later in solid cancers such as ovarian cancer [18]. With the advent of multiparameter fluorescent activated cell sorting and monoclonal antibodies, the purification of hematopoietic stem cells (HSCs) and their leukemic counterparts could be achieved [19–25] with prospective cell sorting combined with established in vitro clonogenic assays. To truly test the hypothesis that cancers arise from the clonal expansion of a single transformed stem cell, a functional in vivo xenotransplantation model was required to definitively identify the neoplastic clone exclusively capable of indefinite self-renewal in vivo. A remarkable series of experiments carried out by Dick and colleagues led to the identification and purification of leukemic stem cells (LSCs) capable of repopulating NOD/SCID (non-obese diabetic severe combined immunodeficient) mice [2, 26], laying the groundwork for the application of the CSC hypothesis to a broad range of cancers.

3.1.2 Lessons from Neural Stem Cells

Stem cells are functionally defined as self-renewing cells that exhibit multilineage differentiation [1, 27, 28]. Somatic stem cells are thought to self-renew to generate all the mature cell types of a particular tissue through proliferative expansion of progenitor cells followed by differentiation into mature cell types. The discovery that multipotential, self-renewing neural stem cells (NSCs) exist throughout life in the adult mammalian brain has only re-emerged in the recent past [29–31], reflecting a rediscovery of 1960s evidence that suggested that neurogenesis was occurring in the adult brain [32]. When multipotent NSCs were isolated from the mammalian neuroaxis more than a decade ago, culture conditions were developed that allowed embryonic Epidermal Growth Factor (EGF) responsive cells to proliferate as floating spheres (neurospheres), which could be easily manipulated for subsequent passage and differentiation [33]. Serum-free media (SFM) allowed for the maintenance of an undifferentiated state, and the addition of saturating concentrations of basic Fibroblast Growth Factor (bFGF) and EGF (20 ng/mL) induced the proliferation of multipotent, self-renewing, and expandable NSCs [34, 35]. This neurosphere culture system and analysis to identify NSCs has permitted in vitro characterization of these cells, but in a retrospective fashion, as the multipotential floating clusters of cells are inferred to have been derived from clonal expansion of a single NSC. Prospective study of this cell has been previously limited by lack of cell surface

markers necessary for its isolation, until recent reports of NSC enrichment using antibodies to the cell surface protein CD133 [36]. Uchida et al. [37] selected hybridomas that produced monoclonal antibodies against clonogenic NSCs from human fetal brain. They sought monoclonal antibodies that cleanly separated human fetal brain into neurosphere-forming and non–neurosphere-forming fractions. They found that CD133 enriched highly for clonogenic human NSCs, in vitro and in vivo, identifying 95% of all neurosphere-forming cells that represented 1–6% of total fetal brain cells.

Normal $CD133^+$ human fetal brain cells not only efficiently form neurospheres in vitro but also demonstrate the key stem cell properties of self-renewal and multilineage differentiation, and are capable of seamless lifelong engraftment and multilineage contribution to the mouse brain [37]. These findings represented the first evidence that the in vitro neurosphere-forming cell, when prospectively isolated, bore key stem cell properties both in vitro and in vivo. The discovery of brain tumor initiating cells (BTICs) is largely accredited to the groundwork laid by haematopoiesis research and prospective studies of NSCs.

3.2 Parallels Between Development and Cancer: Self-Renewal

3.2.1 Molecular Basis for Stem Cell Self-Renewal

The molecular mechanisms that regulate normal stem cell self-renewal are still poorly understood, despite recent advances in the characterization of this defining stem cell property [1, 38, 39]. Self-renewal is defined as the ability of the parental cell to generate an identical daughter cell, and a second daughter cell of the same or different phenotype, depending on requirements of the microenvironment. By perpetuating themselves in this manner, stem cells give rise to a hierarchy of cell lineages that make up an organ or tissue, and can be heterogeneous for self-renewal ability. The factors that maintain the relative balance between self-renewal and differentiation are likely dysregulated in cancer, and many of the key signaling pathways that are active during development (such as Shh [Sonic Hedgehog], Wnt, and Notch) are also implicated as targets for transformation [40, 41]. Both normal and CSCs have shown upregulation or activation of candidate genes involved in self-renewal and proliferation (many of them originally identified as oncogenes), including Shh [42], Wnt [43, 44], Notch [45], cyclin E [46], Hox A and B group genes [47–52], leukemia inhibitory factor (LIF), Signal transducer and activator of transcription 3 (STAT3), bone morphogenic protein 2 (BMP2) [47], Bmi1 [53, 54], and Nanog [55]. Whereas self-renewal can be dependent on extrinsic factors such as cytokines, elements of the previously mentioned signaling pathways, and cell–cell interactions [39]; intrinsic transcriptional determinants such as Oct-4 and Nanog in embryonic stem cells [56] also underlie self-renewal ability in vitro and in vivo.

The concept of the CSC arose from the observation of striking similarities between the self-renewal mechanisms of stem cells and cancer cells [1, 57].

Since normal somatic stem cells must self-renew and maintain a relative balance between self-renewal and differentiation, cancer can be conceptualized as a disease of unregulated self-renewal [1]. NSCs possess self-renewal machinery that is primed and could be harnessed to create a cancer cell, and their longevity targets them for the accumulation of genetic mutations. For these reasons, we believe that NSCs represent strong candidates for the cell of origin of brain tumors [58]. Therefore, NSCs and likely also their closely related, rapidly proliferating downstream progenitors should be further investigated as possible targets of transformation in the development of brain tumors.

3.2.2 Do Brain Tumors Arise from a Transformed Neural Stem Cell: What's the Evidence?

The traditional hypothesis has been that brain tumors arise from the dedifferentiation of a mature brain cell in response to genetic alterations. This hypothesis prevailed because it was felt that the postnatal brain possessed no proliferating cell populations. It has also been considered for some time that brain tumors may arise from a transformation event in a resident immature brain cell. With the discovery of adult NSCs in the early 1990s [35, 59, 60], it became conceivable that a normal NSC or progenitor cell that resides in the brain may be the target for transformation leading to a brain tumor.

Several lines of evidence suggest that brain tumors arise from the transformation of a normal NSC or progenitor cell, all of which rely on the recognition of the many functional and genetic similarities shared by somatic stem cells and cancer cells [57]. Histological studies of brain tumors note the absence of expression of differentiated cell markers in morphologically primitive tumors, as well as the presence of immunostaining for nestin [61, 62], a marker of neural precursor cells [63]. Brain tumors can be very heterogeneous, being comprised of cells expressing phenotypes of more than one neural lineage, implicating a multipotential cell of origin. By investigating the mechanisms underlying gliomagenesis, Holland and colleagues have found that undifferentiated neural precursor cells may be more sensitive to transformation than differentiated cells [64, 65]. Although brain tumors may arise from a dedifferentiated cell that has accumulated a series of oncogenic mutations, an NSC may be seen as a more permissive and likely compartment for transformation, since it already has the self-renewal machinery primed and it has a long lifespan favoring the accumulation of mutations. A progenitor cell is also a possible target if the genetic alteration allows it to reacquire the ability to self-renew. Presumably, a mutational event occurring in a progenitor is not as dangerous as in a stem cell, as this cell normally has limited self-renewal ability and it quickly becomes clonally exhausted as it generates differentiated cells. Whether the transforming event of a brain tumor occurs in an NSC, or in a more differentiated cell type that has re-acquired stem cell characteristics remains to be proven.

3.3 Discovery of Brain Tumor Initiating Cells

The concept of a CSC suggests that tumors are organized into a hierarchy with distinct clonal populations of cells having different proliferative potentials [57]. It is therefore crucial to characterize the existence of these cells through demonstrating properties of proliferation, self-renewal, as well as differentiation in vitro. Furthermore, as the only true measures of a CSC are their capacity to generate an exact copy of the tumor from which they were derived, in vivo validation is thus paramount [1, 57, 66]. Using assays developed by Uchida and Buck to purify NSCs through neurospheres, Singh et al. [67] reported the identification and purification of a cell from primary human brain tumors of different phenotypes that had a marked capacity for proliferation, self-renewal, and differentiation (Fig. 3.1). This cell represented a minority of the tumor cell population and was identified by expression of the cell surface marker CD133. This $CD133^{+}$ cell, which was termed the brain tumor stem cell (BTSC), lacked the expression of neural differentiation markers and was necessary for the proliferation and self-renewal of the tumor in culture. This cell was also capable of differentiating in vitro into cell phenotypes identical to the tumor in situ. Irrespective of the tumor pathology, the marker phenotype of the BTSC was similar to that of normal NSCs, in that it expressed CD133 and nestin. This suggests that brain tumors can be generated from BTSCs that share a very

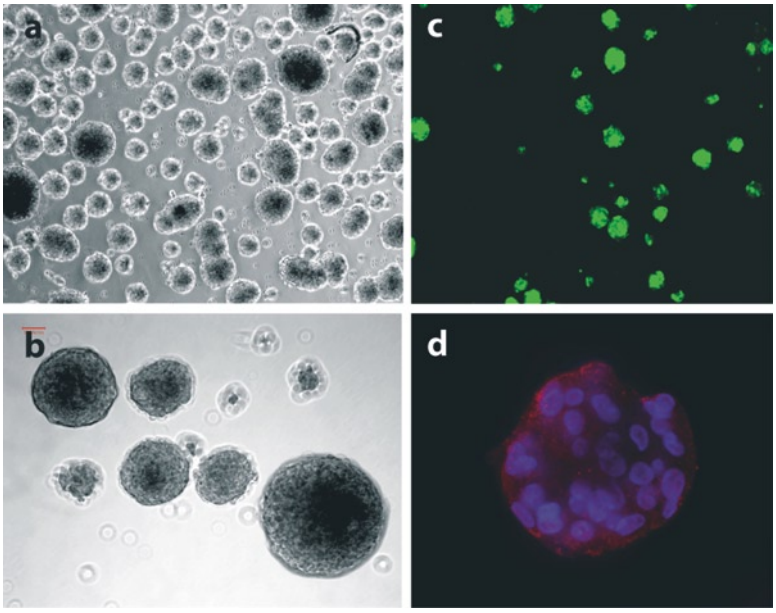

Fig. 3.1 BTICs in culture. (**a**) Microphotograph (4×) showing high density neurospheres in culture. (**b**) Microphotograph (10×) showing neurospheres in culture. (**c**) Neurospheres (4×) transfected with a vector expressing GFP. Magnified neurospheres. (**d**) Confocal image of neurosphere stained with nuclear stain DAPI (*blue*) and nestin (*red*)

similar phenotype. The discovery of a CSC in human adult gliomas extends the definition of the BTSC to describe a class of cells that may drive tumorigenesis in an increasing number of brain tumors, both pediatric and adult.

As the true validation of CSCs rests in the establishment of an in vivo recapitulation of a tumor's original patient phenotype, Singh et al. [68] developed a xenograft assay to identify human BTICs that had the capacity to initiate tumors in vivo. Corresponding to the in vitro data, only the $CD133^+$ brain tumor fraction contained cells that were capable of tumor initiation in NOD/SCID mouse brains. Injection of as few as 100 $CD133^+$ live cells produced a tumor that was serially transplantable and was a phenocopy of the patient's original tumor, whereas injection of 10^5 live $CD133^-$ cells engrafted but did not cause a tumor. Together, these data indicate that the $CD133^+$ human brain tumor cell fraction from adult and pediatric tumors of different types contain BTICs which exclusively initiate tumor formation in immunodeficient mice. Thus, BTICs possess all the key properties ascribed to a stem cell. These findings support the application of principles of leukemogenesis to solid tumors: namely, the principle that only a small subset of CSCs is enriched for clonogenic capacity, and that these cells alone are capable of tumor propagation. This work was corroborated by the results of several labs that found similar findings in various brain tumor models [69, 70]. Hemmati and colleagues applied the neurosphere assay to several childhood brain tumors including medulloblastoma and glioma, and found that these stem-like cells expressed high levels of stem cell genes such as CD133, Sox2, Musashi-1, and Bmi1 [70]. Yuan et al. [71] characterized CSC populations from adult glioblastoma, and Kelly et al. [72] identified GBM BTIC populations that proliferate independently of exogenous mitogens.

Since the discovery of BTICs, much work has been done to characterize these cells. Vescovi et al. [69] continued this work by characterizing BTICs through isolating clonogenic, neurosphere-forming progenitors from adult human glioblastoma multiforme (GBM). There is now strong evidence that the same key mechanisms that control the activity of normal neural progenitors are altered in brain tumors. Pathways that regulate neural stem-cell proliferation and cell-fate commitments such as Wnt-beta-catenin, Sonic hedgehog, Notch, and Bmi1 are aberrantly expressed in brain malignancies [69]. More recently, Phosphatase and Tensin Homolog (PTEN) deficiency has been documented as a potential molecular marker for self-renewing, tumor-initiating cells in glioblastoma [73]. The search is ongoing for novel BTIC markers that can further purify these populations.

3.4 The Search for BTIC Markers

CD133, or human prominin-1, is a 120 kDa, five-transmembrane cell surface protein of unknown function originally shown to be a HSC marker, and is specifically associated with plasma membrane protrusions in embryonic, but not adult epithelia [74–76]. There are two isoforms that may be generally co-expressed; prominin-1 (AC133-1) mRNA is more prominent in fetal brain and adult skeletal muscle,

whereas prominin-2 (AC133-2) is more strongly expressed in HSC populations in the bone marrow, fetal liver, and peripheral blood [77]. Both isoforms have been more recently found to define a broad population of stem cells, including mesenchymal progenitors [78], endothelial precursors [79], placenta and trophoblast [80], adult renal progenitor cells [81], umbilical cord blood stem cells [82], developing spermatozoa in the testis [83], prostatic epithelial stem cells [84], and normal human NSCs [36, 85, 86]. In addition, studies using a novel epitope (alphaE2), instead of the glycosylation-dependent epitope AC133-1, have demonstrated that human prominin-1 is present in several adult epithelial tissues, including adult kidney and mammary gland ducts, and that only AC133 is downregulated upon cell differentiation. AC133 was also detected in several kidney carcinomas, indicating its potential utility for investigating solid cancers [74].

Lenkiewicz et al. [87] applied culture conditions and assays originally used to characterize normal NSCs in vitro [34, 88] to a variety of pediatric and adult brain tumors. BTICs were exclusively isolated by fluorescence activated cell sorting for the neural precursor cell surface marker CD133 [76, 77]. Only the $CD133^+$ brain tumor fraction contains cells that are capable of sphere formation and sustained self-renewal in vitro, as well as tumor initiation in NOD/SCID mouse brains. Therefore, $CD133^+$ BTICs satisfy the definition of a CSC in that they are able to generate a replica of the patient's tumor and they exhibit self-renewal ability both in vitro and in vivo through serial retransplantation [1, 26]. This formally established that only a rare subset of brain tumor cells with stem cell properties are tumor-initiating.

The limitations of CD133 as a single marker to identify a stem cell population from heterogeneous brain tumors became apparent when subsequent studies showed that $CD133^-$ cells derived from GBM sphere cultures were also capable of tumor initiation. However, these studies often employed long-term cell culture, and expression levels of CD133 vary with media conditions, duration in culture, and degree of hypoxia. Recently, GBM cells negative for staining with the anti-CD133 antibody AC133 have been shown to express a truncated variant of the CD133 protein [89]. It was also recently shown that some PTEN-deficient GBM tumors produce both $CD133^+$ and $CD133^-$ self-renewing tumor initiating cell types that constitute a lineage hierarchy. The authors suggest that the capacities for self-renewal and tumor initiation in GBM need not be restricted to a uniform population of stem-like cells, but can be shared by a lineage of self-renewing cell types expressing a range of markers of forebrain lineage [73]. Clearly, reliance on a single, technically contentious stem cell marker to prospectively define a BTIC population is limiting, and further specific and selective BTIC markers must be sought.

Stage-specific embryonic antigen 1 (SSEA1, also known as CD15 or Lewis X) was first identified in neural progenitors in the embryonic nervous system [90] and has also been applied to GBM sphere cultures as a putative marker of BTICs. $CD15^+$ GBM cells fulfill the functional criteria for BTICs: they are highly tumorigenic in vivo, can give rise to both $CD15^+$ and $CD15^-$ cells, thereby establishing a cellular hierarchy, and have self-renewal and multilineage differentiation potential. Most $CD133^+$ tumor cells were also $CD15^+$, suggesting that CD15 may enrich further for

BTICs in human GBMs [91]. Since its initial identification, CD15 has also been characterized in medulloblastomas; $CD15^+$ cells have a unique expression profile with increased proliferation and decreased tendency to undergo apoptosis and differentiation [92, 93].

Other putative BTIC candidate markers include the RNA binding protein Mushashi-1 and the transcription factor Sox2. Musashi-1 is an evolutionally conserved marker for central nervous system (CNS) progenitor cells including NSCs [94], and was later shown to also be expressed in tumor spheres [70]. Sox2 is a key transcription factor that maintains the proliferation of NSCs, inhibits neuronal fate commitment, and may also represent glial tumor precursor cells [95].

3.5 Molecular Genetics of Brain Tumors: Disruption of Signaling Pathways Regulating Growth and Development May Predispose to BTIC Generation

Brain tumors are comprised of cells that can resemble any of the normal neural cell lineages that compose the brain: astrocytes, neurons, oligodendrocytes, and ependymal cells. The tumors that recapitulate these lineages include GBM, medulloblastoma, oligodendroglioma, and ependymoma (Fig. 3.2). Our understanding of the genetic and epigenetic pathogenic events of these tumors has advanced considerably toward a molecular reclassification of brain tumors that will transform clinical medicine [96].

GBM, the most frequent brain tumor, is a highly malignant astrocytic tumor that usually occurs in the cerebral hemispheres of adults, and can occur in young children and infants as well. Its growth is rapid and infiltrative, and diagnostic pathological features include nuclear pleomorphism, microvascular proliferation, and necrosis. Many genes involved in control of proliferation, cell cycle and apoptosis have been implicated in its pathogenesis, including epidermal growth factor receptor (EGFR), p53, murine double minute 2 (MDM2), PTEN, and platelet-derived growth factor receptor (PDGFR) [97]. Interestingly, these genes are distinctly dysregulated, depending on whether the glioblastoma arises de novo (primary GBM) or from a pre-existing lower grade glioma (secondary GBM) [98]. Since gliomagenesis and progression from low- to high-grade gliomas can be seen as a process of multistep carcinogenesis in secondary gliomas, certain genetic alterations involved in both low- and high-grade gliomas (such as loss of p53 or NF1) can be seen as tumor initiating events [99]. Later events in gliomagenesis, such as CDK4 amplification or loss of retinoblastoma (RB) gene expression, could be part of a tumor progression pathway. In primary GBM, several different mechanisms disrupt the RB and p53 tumor suppressor gene pathways, respectively, with loss of the genes that encode INK4A and ARF [100]. Also in support of the multistep carcinogenesis model of gliomagenesis is the fact that any of these mutations created singly in astrocytoma mouse models (p53 loss, PDGFRα overexpression) are insufficient to

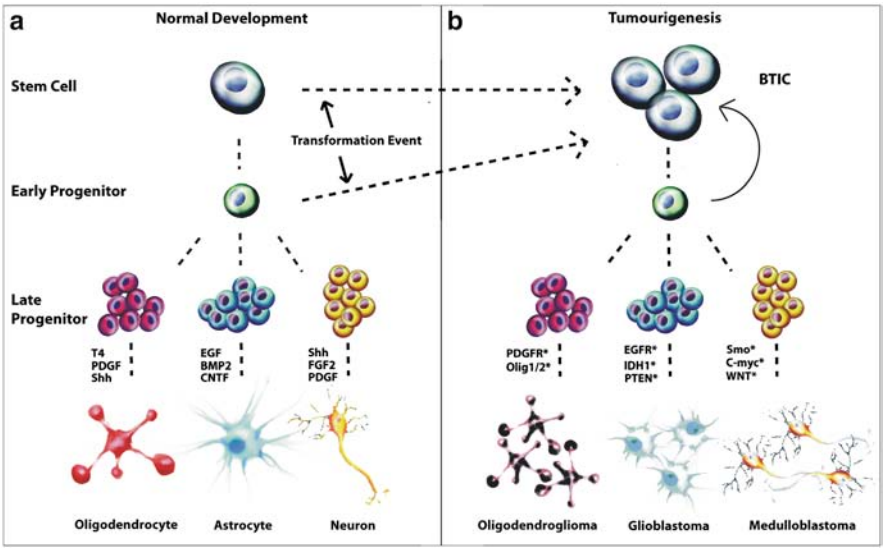

Fig. 3.2 Comparison between the role of stem cells in normal development and tumorigenesis. (**a**) Neural stem cells (NSC) give rise to early and late progenitors and depending on its niche and extrinsic factors may differentiate into any of the three neural lineages, oligodendrocyte, astrocyte, and neuron. (**b**) In a tumorigenesis model, cancer stem cells may arise from the transformation of normal NSC or an early progenitor. With accumulation of genetic changes, enhanced self-renewal and proliferation is seen. Many genetic alterations such as copy number changes, overexpression or deletion, as indicated by the *asterisk*, are observed and ultimately contribute to tumor formation

incur tumor growth [101]. It is therefore likely that multiple genetic or epigenetic events accumulate in target cells and cooperatively induce transformation.

GBM is the first cancer with comprehensive genomic profiles mapped by The Cancer Genome Atlas (TCGA) project. It was found that GBM alterations tend to occur within specific functional modules, and that two of the largest modules involve signaling via Rb, p53, PI3K, and receptor protein kinases. New candidate drivers were also identified in GBM, including AGAP2/CENTG1(GTPase-activating protein for ARF1 and ARF5/Centaurin, gamma1), a putative oncogene and activator of the PI3K pathway, as well as three additional significantly altered modules including one involved in microtubule organization [102].

Medulloblastoma is a malignant embryonal tumor of the cerebellum that manifests largely in children, and has a dominant pattern of neuronal differentiation. Many developmental signaling pathways, such as Shh and Wnt, have been implicated in its pathogenesis [1, 97]. Mutations in the Shh pathway, which regulates the growth of normal NSCs and cerebellar granule cell precursors, have been shown to convey predisposition to medulloblastomas in both mice and humans [103–106]. The Wnt pathway, critical for self-renewal of hematopoietic, epithelial, and likely NSCs [43], is also activated in a subset of medulloblastomas that harbor mutations

in β-catenin, axin, or APC (adenomatous polyposis coli) [107]. Another gene recently shown to be critical for maintenance of self-renewal of NSCs, HSCs, and LSCs is Bmi1 [53, 54], which is overexpressed in human medulloblastomas in conjunction with the Shh pathway receptor Patched (PTCH) [108]. Downstream events of the mutations in these self-renewal pathways in medulloblastoma may lead to repression of RB and p53, potentially disturbing the balance of proliferation and differentiation in cerebellar precursor cells to incur tumorigenesis. We have also shown that Bmi1 plays an important role in BTIC-driven tumorigenesis in human medulloblastoma [109].

Oligodendrogliomas and oligoastrocytomas are diffusely infiltrating tumors occurring predominantly in adults and are composed of cells morphologically resembling oligodendrocytes and astrocytes. They can be induced experimentally with chemical carcinogens such as ethynitrosourea, and often bear loss of heterozygosity on chromosome 19q [98]. The basic helix-loop-helix transcription factor Olig2, which is involved in oligodendroglial specification [110] is expressed highly in oligodendrogliomas and oligoastrocytomas, and may serve as a tumor biomarker or play a pivotal role in tumor development [111, 112]. It was shown that p21(WAF1/CIP1) is directly repressed by Olig2 in neural progenitors and gliomas, indicating that Olig2-regulated lineage-restricted pathway is critical for proliferation of normal and tumorigenic CNS stem cells [113]. Ependymomas are slowly growing, insidious tumors thought to arise from the ependymal lining of the cerebral ventricles and spinal canal of children and adults, and can also occur as an anaplastic variant [97]. Genetic pathways underlying ependymoma pathogenesis remain elusive, and the only consistent cytogenetic event occurring in this tumor is loss of chromosome 22, and potential NF2 mutations [114]. CSCs which were isolated from ependymomas, showed a radial glia phenotype and produced tumors when orthotopically transplanted in mice. Thus, restricted populations of radial glia cells can be candidate stem cells of the different subgroups of ependymoma, and they support a general hypothesis that subgroups of the same histologic tumor type are produced by different populations of progenitor cells in the source tissue [115]. New insights into the causes and potential therapeutics of brain tumors have arisen from recognized defects in signaling pathways that govern cell growth, differentiation, and death in normal brain development [97]. If brain tumors represent development gone awry, the underpinnings of brain tumorigenesis may lie in normal neurogenesis.

3.6 Controversies in BTIC Identification and Propagation

3.6.1 Divergence in BTIC Culture Methods

The neurosphere assay and culture conditions originally described by Reynolds and Weiss [34] represent the gold standard method for induction and maintenance of multipotent, self-renewing, and expandable stem cells from both normal and

cancerous neural tissue. Drawbacks to the neurosphere assay exist, not the least of which is the heterogeneity of the clone as demonstrated by recent adult mammalian NSC transcriptome analysis [116]. Neurospheres of different passages have a surprisingly high number of differentially expressed genes (>380), which may reflect either differing composition of the parental cell and cell types within each sphere or changes in gene expression induced by continual passaging of spheres in culture. Resolution to this heterogeneity was sought by the development of an adherent BTIC culture methodology [117], which offered a more homogeneous and stable BTIC population that could be efficiently subjected to chemical and genetic screening. Advantages and pitfalls of both BTIC culture methodologies were vigorously debated in the literature [117, 118], and in the meantime, a novel culture method for BTICs that utilized SFM without growth factors emerged as another alternative means for long-term BTIC passage, independent of exogenous mitogens such as EGF or bFGF [72]. As BTIC culture methods diverge, it has become clear that there is no standardized protocol for propagation of these cells [119], and comparison of experimental results must be contextualized within these variation in methodology.

3.6.2 Prospective Identification of the BTIC: Caveats of Cell Surface Markers

The BTIC was originally prospectively identified by cell sorting for the NSC cell surface marker CD133. Subsequently, other cell surface markers, such as CD15, have been found to enrich for BTIC activity in both human [120] and mouse models [93]. However, loss or change of cell surface marker expression can be assumed in a rapidly evolving CSC population, in which environmental conditions may dynamically alter the presence of receptors on cells in constant flux. For example, expression of CD133 on BTICs in culture can be altered by hypoxia [121], the use of trypsin for tissue digestion [122, 123], targeting of glycosylated epitopes [124], and mitochondrial dysfunction/bioenergetic stress induced by long-term culture [125]. Therefore, reliance on any single cell surface marker to stably specify the BTIC state should be avoided, as BTIC populations may undergo dynamic changes in their cell surface receptor topography over time and passage. Future prospective identification of BTIC populations should incorporate the study of signaling pathways that are activated within these cells during brain tumor evolution, through methods such as intracellular phospho-flow cytometry.

3.7 BTIC Targeting: Implications for Therapy

The identification of BTICs has important implications for understanding the molecular mechanisms of brain tumorigenesis, since current molecular pathological analyses of global tumor cell populations may not be sufficient to determine the key molecular alterations in rare tumor stem cells. The presence of a BTIC will also

have important implications for understanding brain tumor dissemination if these are the cells that migrate and establish CNS metastasis. The functional analysis of the BTIC may also provide a novel means for testing of new treatment strategies that focus on the eradication of the tumor maintaining BTIC. The fact that we are able to differentiate BTICs into cells that express more mature markers may lead to the development of differentiation therapy. BMPs have been used to block tumor growth leading to a reduction in proliferation and increased expression of markers of neural differentiation with a concomitant decrease in the pool of CD133+ stem cells [126]. In fact, current work has been done to target the stem-like cell population in GBM both in vitro and in vivo [127].

Conventional chemotherapy and radiotherapy have been key treatment modalities and have largely remained stagnant in our arsenal. Patients treated with these therapies often receive moderate benefit in the short term, but eventually relapse in their primary cancer or develop local metastases or infiltrative disease. This clinical observation, unfortunately all too familiar to physicians, may be explained by the existence of CSCs that are chemo- and radioresistant [128–130]. Rich and colleagues reported that $CD133^{+}$ glioma stem cells preferentially activate DNA damage checkpoints in response to radiation, and repair radiation-induced DNA damage more effectively than $CD133^{-}$ cells [129]. This suggests that the $CD133^{+}$ glioma stem cells are radioresistant and may be the cause of tumor recurrence post radiation. Immunohistochemical staining of GBM cell lines has revealed co-staining for multidrug resistance (MDR1) and CD133, suggesting that these cells are also chemoresistant [128]. New therapies must target these rare stem cells in order to overcome conventional treatment barriers.

Purification of BTICs implies that a hierarchy may exist in the tumor cell population, as not all tumor cells are capable of maintaining the tumor in culture or immunodeficient mice. This apparent hierarchy may be functionally elucidated as more surface markers for NSCs emerge and further tumor subpopulations identified. It is important to note that cell sorting using surface markers may only represent a snapshot in time for these rare cells, thus functional characterization of these stem cells is paramount. Future investigations of the BTIC may clarify whether the BTIC sits at the top of a lineage hierarchy, or further down as a lineage-restricted progenitor. Finally, as growing evidence indicates that normal stem cells and CSCs share similar phenotypic and functional properties, studies of stem cells found in brain tumors may shed further light on the biology of normal NSCs. With the ultimate aim of finding novel targeted therapeutic agents against the BTIC, understanding its regulation and molecular phenotype will bring us one step closer to this goal.

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Chapter 4
Cancer Stem Cells in Colorectal Cancer

Mauro Biffoni, Eros Fabrizi, and Lucia Ricci-Vitiani

Abstract Colorectal cancer (CRC) is the third most common form of cancer and the second cause of cancer-related death in the Western world. Despite advances in diagnosis, surgery, and new targeted agents for CRC, only a modest improvement in mortality has occurred for advanced disease. A growing body of evidence supports the idea that human cancers arise from a rare population of cells with stem cell-like properties which would be the pathological counterpart of the normal epithelial stem cell. These "cancer stem cells" (CSCs), firstly identified in hematologic malignancies, have been recently isolated in several solid tumors including CRC. The hypothesis that only a subset of cells drives tumor formation in CRC raises questions as to whether current therapies are able to efficiently eradicate the CSC population. This chapter will discuss different aspects of stem cell biology in the context of CRC that may contribute to understanding the mechanisms responsible for tumor development and therapy resistance.

Abbreviations

ALDH1	Aldehyde dehydrogenase 1
APC	Adenomatous polyposis coli
Ascl2	Achaete scute-like 2
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
CBC	Crypt base columnar cells

L. Ricci-Vitiani (✉)
Department of Hematology, Oncology and Molecular Medicine,
Istituto Superiore di Sanità, Rome, Italy
e-mail: lriccivitiani@yahoo.it

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CD	Cluster of differentiation
CK20	Cytokeratin 20
COX	Cytochrome C oxidase
CRC	Colorectal cancer
CSC	Cancer stem cell
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
EphB	Ephrin B receptor
ESA	Epithelial surface antigen
FAP	Familial adenomatous polyposis
GFP	Green fluorescent protein
HNPCC	Hereditary non-polyposis colon cancer
IL-4	Interleukin-4
JPS	Juvenile polyposis syndrome
MLH1	MutL homolog 1
mPAS	Mild periodic acid-Schiff reagent staining
Msi-1	Musashi-1
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
OAT	*O*-acetyl transferase
OLFM4	Olfactomedin-4
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol biphosphate
PIP_3	Phosphatidylinositol triphosphate
PTEN	Phosphatase and tensin homolog
RBP-J	Recombination signal-binding protein 1 for J-kappa
SC	Stem cell
VEGF	Vascular endothelial growth factor

4.1 From Normal Crypt Organization to Colorectal Cancer Development

4.1.1 The Intestinal Epithelium

The mammalian intestinal tube consists of the small intestine (duodenum, jejunum, and ileum) and the large intestine or colon, and is lined by a single layer of epithelial cells called the mucosa. The intestinal epithelium has a well-defined architecture where proliferation and differentiation take place along organized structures. The simple columnar epithelium of the small intestine is folded to form a number of flask-shaped mucosal invaginations known as crypts of Lieberkühn and finger-shaped luminal protrusions termed *villi*. These structures generate a large surface

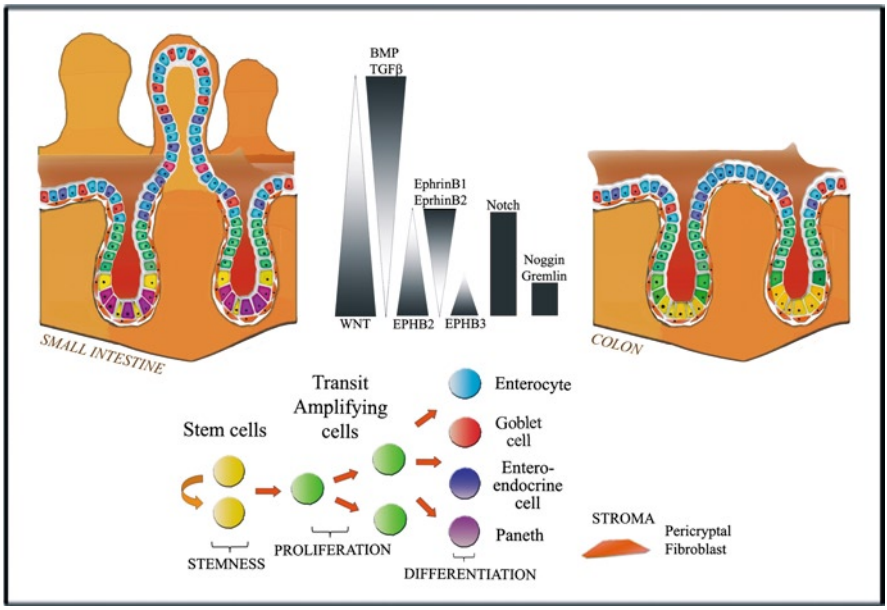

Fig. 4.1 Morphological unit of the small (*left*) and large (*right*) intestine epithelium. Few stem cells located at the base of the crypt, interspersed with Paneth cells in the small intestine, produce transit amplifying cells which migrate through the crypt walls and generate the large number of differentiated enterocytes and goblet cells lining the intestinal lumen and, in the small intestine, the *villi*. Different signaling pathways (*middle panel*) are modulated and play a major role during this course which is schematically summarized at the *bottom* of the figure

area, allowing efficient absorption of nutrients from the intestinal lumen [1]. The colon lacks *villi* and has a flat surface mucosa [2]. The crypt is the proliferative compartment of the intestinal epithelium, every crypt has a monoclonal origin and multipotent stem cells warrant the turnover of the epithelial cells (Fig. 4.1). Multipotent stem cells slowly proliferate, undergoing asymmetric division such that they generate daughter cells with different fates: one remains quiescent as a stem cell, whereas the other expands to produce a progeny of cells committed to differentiate. Epithelial cells produced in the lower cryptal region migrate up the crypt onto an adjacent *villus* in coherent columns, where they perform their function before being shed into the lumen. In the colon, cells migrate to the intercrypt surface at the top of the colonic crypt. In both the small intestine and colon, cells differentiate into three functional epithelial lineages: the predominant enterocyte with absorptive function, enteroendocrine cells specialized in secretion of peptide hormones, and the mucous-secreting goblet cells. A fourth differentiated type, the Paneth cell, is functionally similar to a neutrophil, resides at the bottom of crypt, and secretes antimicrobial agents [3–6].

Cells of the enterocyte lineage divide several more times as they migrate up the crypts, and as they migrate onto the *villi*, differentiate further into the highly polarized mature

absorptive cells that express all the transport proteins and enzymes characteristic of those cells. In the crypt–*villus* units of the small intestine, both absorptive and goblet cells migrate outwards with a turnover time of about 3 days, whereas Paneth cells migrate inwards and their turnover time is about 15 days.

4.1.2 Morphogenetic Pathways in Intestinal Development and Homeostasis

The gastrointestinal tract is one of the most dynamically self-renewing tissues in the adult mammal. The epithelium undergoes virtually complete self-renewal every 2–7 days. The homeostasis of the intestine depends on a fine-tuned interaction of epithelial cells and underlying mesenchymal cells, and is coordinated by a relatively small number of highly evolutionary conserved signaling pathways whose deregulation may lead to pathological conditions, including cancer [7]. The molecular definition of these pathways has received a great impulse by studies aimed at defining the genetic background of familial syndromes, which account for 5–10% of colorectal carcinoma cases, such as Familial Adenomatous Polyposis (FAP), Juvenile Polyposis Syndrome (JPS), and Cowden Syndrome [8].

FAP is an autosomal dominantly inherited disease characterized by the development of multiple bowel adenomas in the second and third decades of life. Although these benign tumors are not individually life threatening, their large number virtually guarantees that some will progress to an invasive lesion if patients do not undergo a prophylactic colectomy [8]. In FAP, approximately 80% of patients display truncating mutations of the *APC* (adenomatous polyposis coli) gene, a critical component of the Wnt pathway. *APC* encodes a protein that is part of a complex that binds β-catenin, targeting it for degradation. In the absence of binding and degradation by this complex, β-catenin translocates to the nucleus and activates multiple transcription factors responsible for proliferation, differentiation, migration, and apoptosis, including cyclin D1 and c-myc.

There is strong genetic evidence that the components of the so-called “canonical” Wnt pathway play a critical role in the regulation of proliferation in the stem cell compartment of the intestinal crypt. Progenitors at the bottom of the intestinal crypt accumulate nuclear β-catenin [9]. Moreover, mice bearing loss-of-function mutations in key players of the transcriptional program controlled by Wnt (i.e., β-catenin, Tcf-4) or mice with over-expression of Wnt inhibitors such as Dickkopf fail to develop colonic crypts due to a complete loss of proliferation in the crypt compartment. This supports the hypothesis that the Wnt signaling pathway is a dominant force in controlling the proliferative activity in the intestinal crypt [10–12].

The identification of many different Wnt target genes indicates how the Wnt signaling pathway is involved in the crypt stem cell compartment regulation, and

also shows that Wnt signaling has different effects in different cell types, depending on their localization along the crypt axis. Among Wnt targets, B subclass ephrins and their tyrosine kinase receptors have been recently shown to coordinate migration and proliferation in the intestinal stem cell niche [13]. These receptors allow the correct positioning of epithelial cells in a Wnt gradient along the crypt–*villus* axis as well as the positioning of the Paneth cells at the bottom of the crypt [14].

The Wnt cascade interplays with the Notch pathway to maintain undifferentiated, proliferating cells in normal crypts and adenomas [15]. Notch signaling is known to control cell fate decisions in the development of many tissues. The *Notch* genes encode single pass transmembrane receptors that interact with transmembrane ligands on adjacent cells. Engagement of the receptor by its ligands Delta or Jagged induce its proteolytic cleavage by γ-secretase. A cleaved fragment of Notch (NCID) translocates into the nucleus and acts as a transcription factor after dimerization with the DNA binding protein RBP-J/CSL. The best characterized Notch target genes are the bHLH hairy/enhancer of split (Hes) transcription regulators, which in turn activate factors involved in the control of proliferation and differentiation [16]. Knock-down of RBP-J or Hes-1 as well as treatment with γ-secretase inhibitors leads to an increased number of secretory epithelial cells [15, 17, 18]. Moreover, inducible gut-specific *Notch*-mutant mice have shown that Notch is important for maintaining the proliferative crypt compartment [19], confirming a role of Notch signaling in triggering proliferation of crypt progenitor cells in the transit-amplifying units. Conversely, a regulated reduction of Notch signaling in cooperation with the activation of specific bHLH factors repressed by Notch induces specific differentiation into the intestinal epithelial lineages.

Another important regulator of the intestinal homeostasis is the phosphatidylinositol 3-kinase (PI3K), a major player of the PTEN-PI3K-Akt pathway. PI3K is composed by the p110 catalytic subunit and the regulatory subunit p85. Upon binding, p110 is activated and phosphorylates its substrate leading to the activation of the kinase Akt by PDK1. The major negative regulator of the PI3K pathway is the phosphatase PTEN, which inhibits Akt function through the reconversion of phosphatidylinositol triphosphate (PIP_3) to phosphatidylinositol biphosphate (PIP_2) [20]. PI3K is activated in many different human tumors, including 40% of CRC [21], probably related to its important role in promoting cell survival and proliferation in cooperation with the Wnt pathway. Indeed, one of the targets of Akt phosphorylation is β-catenin at Ser552; thus Akt may induce nuclear accumulation of β-catenin and enhance its transcriptional activity. PTEN mutations are responsible for 80% of cases of Cowden disease, characterized by hamartomatous intestinal polyps with epithelial and stromal involvement. The relationship between PTEN inactivation and intestinal polyposis has been recently elucidated in a model of PTEN-deficient mice that showed an excess of intestinal stem cells able to initiate de novo crypt formation, suggesting that the PTEN-PI3K-Akt pathway probably governs stem cell activation by regulating nuclear localization of β-catenin [22].

The study of JPS has disclosed another crucial link between molecular genetics and developmental biology, revealing the importance of the bone morphogenetic protein (BMP) pathway. BMPs bind to receptor types I or II (BMPR1 or BMPR2), thus leading to the phosphorylation of the intracellular signal transducing factors SMAD1, 5 or 8, which then form a heterodimer with SMAD4, translocate to the nucleus, and act as transcriptional activators [23]. JPS is an autosomal-dominant gastrointestinal condition that predisposes to hamartomatous gastrointestinal polyp formation, which can turn into malignant lesions in approximately 20% of cases [24]. Germline mutations in the SMAD4 gene have been found in 15–20% of cases, and mutations in the BMPR1A gene in 25–40% of cases. Moreover, it has been shown in mice that conditional inactivation of BMPR1A as well as ectopic expression of Noggin or Gremlin (negative modulators of BMP signaling) results in an expansion of the stem and progenitor cell populations and in the formation of numerous ectopic crypt units, eventually leading to intestinal polyposis resembling human JPS [24, 25]. Considering that BMP stabilizes PTEN and leads to reduced Akt activity and subsequent reduction of β-catenin nuclear accumulation, the regulation of BMP signaling in the intestinal epithelium may contribute to the central role of the Wnt pathway in intestinal homeostasis [26]. In the intestine, BMP4 is secreted by intravillus stromal cells and BMPR1 is expressed in all intestinal epithelial cells [24, 25], suggesting that alterations in intestinal stem cell microenvironment might influence normal development and tumorigenicity.

4.1.3 Intestinal Epithelial Stem Cells

Stem cells (SCs) are defined as undifferentiated, primitive cells that persist throughout the lifetime of an organism due to their ability to maintain themselves (self-renewal) and to generate all the differentiated types of the pertinent tissue (multipotency). Despite the significant progress made in recent years in the field of stem cell biology, the identification, isolation, and characterization of SCs of the intestinal crypt remains elusive. Many obstacles have hindered the identification of intestinal SCs, including the lack of clonogenic and reconstitution assays, the complexity of the crypt structure that limits the retrieval of putative SCs from their niche (where they are interspersed among more differentiated daughter cells), and the absence of reliable markers. In 1974, Cheng and Leblond formulated the "Unitarian hypothesis," according to which all of the terminally differentiated cell repertoire in the intestinal crypts are derived from a single multipotent SC located at the bottom of the crypt where cellular migration originates [27–29]. Clonality studies in humans, mainly relying on natural mutations and polymorphisms, confirm the clonal origin of the crypt cell population. One such mutation was described in the gene coding for the enzyme *O*-acetyl transferase (OAT), responsible for O-acetylation of the sialic acid in goblet cell mucus. Mild periodic acid-Schiff reagent (mPAS) staining, which marks non–O-acetylated mucus, has shown that crypts from heterozygous (OAT+/−) individuals do not stain with mPAS unless further

mutation causes the loss of the remaining allele. When loss of heterozygosity occurs, the whole crypt is progressively colonized by the progeny of mutant cells [30]. The frequency of positive crypts is increased after irradiation and the time required for "chimeric" crypts to become uniform ("clonal stabilization time") is about 1 year [31]. Additional more convincing evidence for the clonality of human colonic crypts came from the work of Novelli et al., in which in situ hybridization analysis of the Y chromosome was performed on rare patients with XO/XY mosaicism who have undergone colectomy for FAP. None of the 12,000 crypts analyzed showed coexistence of Y chromosome positive and Y chromosome negative cells [32]. Further studies have demonstrated that the age-dependent accumulation of mitochondrial DNA (mtDNA) mutations in human colonic crypt SCs results in a significant biochemical defect in cytochrome C oxidase (COX) activity in their progeny. A number of crypts were uniformly negative for COX activity; however, a few crypts were found to have ribbons of COX-deficient cells moving from the bottom to top of the crypt, suggesting that one of the multipotent SCs within the niche has acquired enough mtDNA mutations to result in a functional deficit. More recently, the ability of a single mutated SC to repopulate a crypt has been confirmed by two-color enzyme histochemistry that simultaneously detects mitochondrial COX and the nuclear DNA-encoded succinate dehydrogenase [33].

Despite the fact that the "Unitarian concept" has been well documented in the mouse small intestine, the exact identity of the intestinal SCs has proven to be controversial over the last 30 years. During this time, many studies have been performed in order to indirectly localize intestinal SCs by using techniques such as long-term retention of labeled DNA [34] or transgenic mice expressing histone H2B-green fluorescent protein (GFP) [35]. Both approaches were aimed at identifying infrequently cycling cells and were based on the "immortal strand hypothesis" formulated by Cairns in 1975. According to this theory, SCs selectively retain their original DNA strands, while donating the newly synthesized DNA strand to their daughter cells. However, this hypothesis is currently the subject of controversy due to the demonstrated absence of asymmetric genetic material segregation in hematopoietic SCs that represent the milestones of SC identification [36].

The studies performed in the last 3 decades to identify intestinal SCs have led to the formulation of two different models, known, respectively, as the "+4 position" and the "stem cell zone" models. Both models are based on the assumption that every crypt contains approximately 4–6 independent SCs. According to the "+4 position" model, the crypt is essentially a tube of proliferating cells bounded from below by non-cycling Paneth cells, and SCs are located just above the Paneth cells at the so-called +4 position relative to the crypt bottom [37, 38]. A more recent model, "the stem cell zone," originated from the identification of a unique cell type population of small undifferentiated cycling cells, interspersed within the Paneth cells, termed crypt base columnar (CBC) cells, that are believed to be the true intestinal SCs [27, 39, 40]. Definitive proof for either model has proven elusive due to the lack of specific markers for these cells. This is different than other organ systems such as the hematopoietic system [41] or mammary gland [42], in which the recognition of specific cell surface markers has allowed the identification of SCs.

Several molecules have been proposed as markers of SCs in the intestine including the mammalian neural stem cell marker musashi-1 (Msi-1). Msi-1 is a RNA-binding protein whose function has been characterized in asymmetric division during neuronal development in *Drosophila melanogaster* [43]. Msi-1 was expressed in putative SCs in the neonatal and adult murine intestinal crypts [44]. Moreover, immunohistochemical analysis performed in normal human colon crypts revealed that the majority of cells expressing Msi-1 reside in the lower region of the crypt, which corresponds to the expected position of the colonic SCs [45]. However, immunoreactivity was also observed above the bottom of the crypt, suggesting that Msi-1 is still expressed by early transient amplifying progenitor cells. The ability of Msi-1 to up-regulate the expression of the transcriptional repressor Hes-1 led to the evaluation of Hes1 and Msi-1 co-expression in the mouse small intestine epithelium [46]. These two potential SC markers were co-expressed by the putative SCs at the crypt base, although Hes1 was expressed by a broader population of cells. However, an "ideal" SC marker would be a surface molecule suitable for selecting viable cells to test in functional repopulating assays and both these markers cannot be identified in intact cells. Other biomarkers have been evaluated to distinguish the SC population within the intestine, such as members of the integrin superfamily of heterodimeric transmembrane glycoproteins. The members of this superfamily (as well as their receptors) define basement membrane function and activate the cellular signaling pathways controlling epithelial cell survival, proliferation, and differentiation [47]. Integrin subunits have been identified as SC markers in the epidermis [48], and testes [49] and have been recently been suggested as markers on intestinal clonogenic cells on the bases of the expression of the $\alpha_2\beta_1$ integrin in the epithelial cells at the base of the crypts in the human small intestine [50].

More recently, Eph-B receptors have been described as important regulators of migration and proliferation in the intestinal epithelium. The expression of both Eph-B2 and Eph-B3 tyrosine kinase receptors has been reported at the bottom of the crypt in mouse colon [13]. Inhibition of Eph-B2/Eph-B3 signaling has shown to reduce the number of proliferating cells without altering the SC number, suggesting that Eph-B receptors are unlikely to be independent biomarkers of colonic SCs. Conversely, a more promising intestinal SC marker might be polycomb protein Bmi-1, known to be involved in the self-renewal of hematopoietic and neural stem cells [51]. Bmi-1 has recently been reported to be expressed within the bottom of the crypts in the small intestine mainly by the cells at the +4 position [52]. The long-term label retaining cells located in a four-cell *annulus* at the crypt base were long considered as intestinal SCs. However, this hypothesis was recently challenged when, during a study aimed at determining the genetic program deregulated in APC-mutant human colon cancer cells, Barker et al. selected several Wnt target genes with a restricted crypt expression [53]. Among these, *Lgr5* has been proposed as a biomarker of intestinal SCs. The *Lgr5* gene encodes a seven-transmembrane, leucine-rich repeat containing G-protein–coupled receptor, also known as Gpr49. Despite being predicted to be a receptor for a peptide ligand, its function is currently unknown. In situ hybridization on mouse small intestine revealed that Lgr5 expression

is restricted to cycling CBC cells and it has been demonstrated that Lgr5-expressing cells differentiate into the expected functional lineages of the colonic epithelium. Importantly, Lgr5 positive cells appear to fulfill the major criteria which define SCs in that they are both self-maintaining and multipotent. Indeed, more recently, it has been shown that single sorted $Lgr5^+$ cells are able to establish long-term cultures and to generate crypt–*villus* organoid, without requiring a mesenchymal niche [54]. These cultures can be established and maintained in a serum-free medium containing a defined set of growth factors including R-spondin 1, Noggin, and epidermal growth factor (EGF). Gene expression profiling of $Lgr5^+$ epithelial cells isolated from the bottom of murine small intestinal crypts led to the identification of a gene signature for these cells [55]. Not surprisingly, many genes on the list were previously identified as Wnt-dependent genes such as the transcription factor Achaete Scute-Like 2 (Ascl2). The *achaete-scute* genes are essential for the differentiation of the central as well as the peripheral nervous system and are the best known targets of the Notch pathway [56]. In the adult intestinal epithelium, Ascl2 controls Lgr5 SC fate and misexpression of *Ascl2* gene in non-stem cells results in crypt hyperplasia and in the formation of crypt-like invaginations on villi.

By in situ hybridization experiments, olfactomedin-4 (OLFM4) was also identified as a highly specific and robust marker for $Lgr5^+$ cells, even though its expression was not under the control of Wnt. The *OLFM-4* gene encodes a secreted molecule with unknown function, originally cloned from human myeloblasts [57], which is enriched in human colon crypts [58]. Due to the very low expression levels of Lgr5, OLFM-4 has been recently proposed as a more faithful SC marker highly expressed in CBC cells in human small intestine and colon [59].

4.1.4 Mutational Events in Colon Tumorigenesis

The gastrointestinal tract is one of the most rapidly proliferating tissues in the body with differentiated cells undergoing continuous replacement. Intestinal cells are also exposed to a hostile environment as they come into close contact with numerous toxins and carcinogens contained in digested foods. Thus, it is not surprising that there is a high cancer prevalence in the gastrointestinal epithelium which has become important for the understanding of cancer biology. Moreover, clinical and histopathological data have suggested that most, if not all, malignant CRC arise from benign tumors [60]. In 1990, Fearon and Vogelstein proposed a model of successive genetic changes leading to CRC, the so-called "adenoma–carcinoma sequence." In the original proposal, they stressed that mutational activation of a number of genes was essential for the development of CRC, but more than 10 years of research were needed to elucidate the function of the key genes involved in the model. Studies on the familial colonic cancer syndromes including FAP and hereditary non-polyposis colon cancer (HNPCC, also known as the Lynch syndrome) massively contributed to the understanding

of intestinal tumor initiation including the confirmation that many colonic adenocarcinomas arise from adenomas [8]. The hereditary nature of FAP was recognized at the end of nineteenth century; however, it was not until 1986 that a deletion of chromosome 5q was observed in a FAP patient [61]. Several years later, the tumor suppressor gene *APC* was mapped in the deleted region and identified as the initial mutation involved in the adenoma–carcinoma progression [62, 63]. Further studies revealed that mutations in APC are also found in 63% of sporadic adenomas [64] and up to 80% of sporadic colorectal cancers [65]. This observation led to the definition of a "gatekeeper" function for APC in the control of normal epithelial cell proliferation required for intestine homeostasis. A mutation of the gatekeeper leads to a permanent imbalance of cell division over cell death. Conversely, mutations of other genes in the presence of a normal gatekeeper function would not able to induce a sustainable growth perturbation. APC mutations typically affect the central domain of the protein containing the binding site for β-catenin, and thus determine the increase of nuclear β-catenin and the transcriptional activation of specific target genes, such as the oncogene c-myc [66, 67]. Furthermore, approximately 10% of CRCs carry activating mutations in the highly conserved serine/threonine residues of β-catenin, which are required for recognition and degradation of the protein [68]. Other hereditary bowel cancer syndromes have been used for the identification of an alternative pathogenic mechanism for colon tumorigenesis. HNPCC is a condition that predisposes patients to cancers of the colon, endometrium, and several other extracolonic sites without prior formation of polyps [69]. The use of microsatellite markers linked to HNPCC susceptibility to demonstrate allelic losses in this syndrome led to the identification of new microsatellite alleles in HNPCC tumor cells never observed in patient's normal cells. These new alleles were present in every di- and tri-nucleotide repeat examined, suggesting a genome-wide instability due to defects in DNA mismatch repair genes which normally recognize and repair single base pair and larger mismatches during DNA replication. The observation that 90% of HNPCC patients carry mutations of the mismatch repair genes *hMSH2* and *MLH1* led to the definition of the "caretaker" function [70, 71]. Taken together, the studies performed on FAP and HNPCC patients demonstrate the importance of both "gatekeeper" and "caretaker" gene functions. FAP results from an increased rate of tumor initiation due to the altered gatekeeper function of APC that leads to the development of numerous benign tumors. Each of these benign tumors slowly progresses to a malignancy, requiring the sequential accumulation of mutations in other genes including kRAS and p53. Thus, FAP can be considered as a disease of tumor initiation. In contrast, the mismatch repair defect in HNPCC results in an enhanced rate of mutation that greatly accelerates tumor progression. Interestingly, FAP and HNPCC patients both develop CRC at a median age of 42 years, suggesting that initiation and progression are the cardinal features leading to malignancy and that once one of these is hereditarily acquired, a similar time is needed to accumulate the other involved in either initiation or progression.

4.2 Colorectal Cancer Stem Cells: The Driving Force Behind Tumor Formation

The parallel evolution of knowledge concerning normal and tumor development in mouse models as well as in human CRC highlights the idea that cancer may be regarded as a disease of dysregulated intestinal SC homeostasis. Indeed, several lines of evidence support the SC origin of CRC, above all, the observation that differentiated epithelial cells have a short lifespan, whereas normal intestinal SCs are long lived and have more opportunity to accumulate mutations that give rise to a malignant phenotype. Moreover, studies on CRC pathogenesis have widely demonstrated that the most common mutations observed in patients involve pathways that also play a crucial role in intestinal ontogenesis.

For many years, the observation that tumors are composed of a heterogeneous population of cells differing in morphology, marker expression, proliferation ability, and tumorigenic potential has been explained on the basis of the "stochastic model" of tumor development. According to this traditional model, every tumor cell is equally capable of initiating neoplastic growth such that stochastic genetic events and microenvironmental influence lead to clonal selection. However, this theory has been recently challenged by the new hierarchical "cancer stem cell" (CSC) model, which suggests that within a tumor, only a small fraction of cells with stem cell-like properties (including the ability to self-renew and differentiate) possesses cancer-initiating potential and are therefore able to initiate and sustain tumors with heterogeneous histology.

Although normal colonic SCs have long been believed to be the logical origin of CRC, only recently has the development of new experimental methods facilitated the identification and isolation of this tumorigenic population of CSCs. The gold standard for ascertaining the existence of a subpopulation of CSCs within a tumor is the demonstration that these cells are able to initiate a tumor in mouse xenograft models and morphologically and histologically reproduce the parental tumor. However, similar to SCs, the identification and isolation of putative CSC subpopulations requires the definition of specific cell surface biomarkers which can be used to enrich a subfractionated population for cancer-initiating activity (Table 4.1). Several recent studies have evaluated the functionality of specific CRC-SC biomarkers. In 2007, the first two studies have suggested that the tumorigenic cell population of CRC can be isolated by means of the positive expression of the surface molecule CD133 [72, 73], which was originally classified as a marker of primitive hematopoietic and neural stem cells [74, 75]. CD133, also known as Prominin-1, is a cholesterol-interacting pentaspan, glycosylated membrane protein located in the apical plasma membrane protrusions of epithelial structures such as epithelial microvilli and epididymal ductal epithelial sterocilia [76]. Due to its location, a functional role was ascribed to CD133 as an "organizer" of the plasma membrane topology [77]. Interactions between CD133 and cholesterol within membrane microdomains suggested that CD133 might also be important in maintaining an appropriate

Table 4.1 Proposed stem cell markers for normal and cancer intestinal epithelium

	Marker	Function
Normal intestinal stem cells	Musashi-1	RNA-binding protein
	Hes-1	Transcriptional repressor
	EphB2/B3 receptors	Cell surface receptors
	Bmi-1	Polycomb-repressor protein
	Lgr5	Unknown
	ALDH1	Enzyme
	Olmf-4	Unknown
	α2β1 integrin	Cell surface receptor
Colon cancer stem cells	CD133	Unknown
	CD44	Hyaluronic acid receptor
	CD166	Adhesion molecule
	ALDH1	Enzyme
	Bmi-1	Polycomb-repressor protein

lipid composition within the plasma membrane [78]. However, the specific functions and ligands of this molecule are still relatively unclear. The tumorigenic potential of $CD133^+$ CRC-SCs, which account for approximately 2.5% of the bulk tumor cells, was evaluated in both studies by sorting freshly dissociated tumor cells and injecting them into immunocompromised mice [72, 73]. $CD133^+$ cells display an undifferentiated phenotype, characterized by the expression of the surface epithelial antigen BerEp4 (also known as ESA or EpCAM), and by the lack of intestinal epithelial differentiation markers such as cytokeratin 20 (CK20). $CD133^+$ cells have been also identified in normal colon tissue, although at lower frequency than tumor tissues, reinforcing the hypothesis that the increased number of $CD133^+$ cells in cancer samples might result from oncogenic transformation of normal colonic SCs. O'Brien et al. isolated $CD133^+$ cells from seven primary colon cancers and ten extracolonic (metastatic) sites [72]. When transplanted under the renal capsule of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, $CD133^+$ cells readily developed tumors that displayed equivalent morphologic features to the parental cancer. Using limiting dilution assays, the authors calculated that the frequency of CRC-SCs was approximately 1 out of 5.7×10^4 in an unfractionated population of cancer cells, and 1 out of 262 cells in a $CD133^+$-enriched fraction. Similarly, in the second study by Ricci-Vitiani et al., a population of $CD133^+$ cells was isolated from colon cancer specimens and subcutaneously injected into severe combined immunodeficiency (SCID) mice where they were able to give rise to tumors, whereas the $CD133^-$ fraction did not. The tumorigenic potential of freshly isolated $CD133^+$ cells was maintained upon serial transplantation, as was the ability of tumor cells to replicate the parental tumor phenotype. Importantly, colonic cells obtained from dissociation of cancer samples can be propagated in culture in a serum-free medium containing EGF and basic fibroblast growth factor [73]. In these conditions, CSCs and progenitor cells grow exponentially and give rise to floating $CD133^+$ cell aggregates named tumor spheres which express BerEp4 but not differentiation markers such as CK20 (Fig. 4.2). Such cultures could be maintained

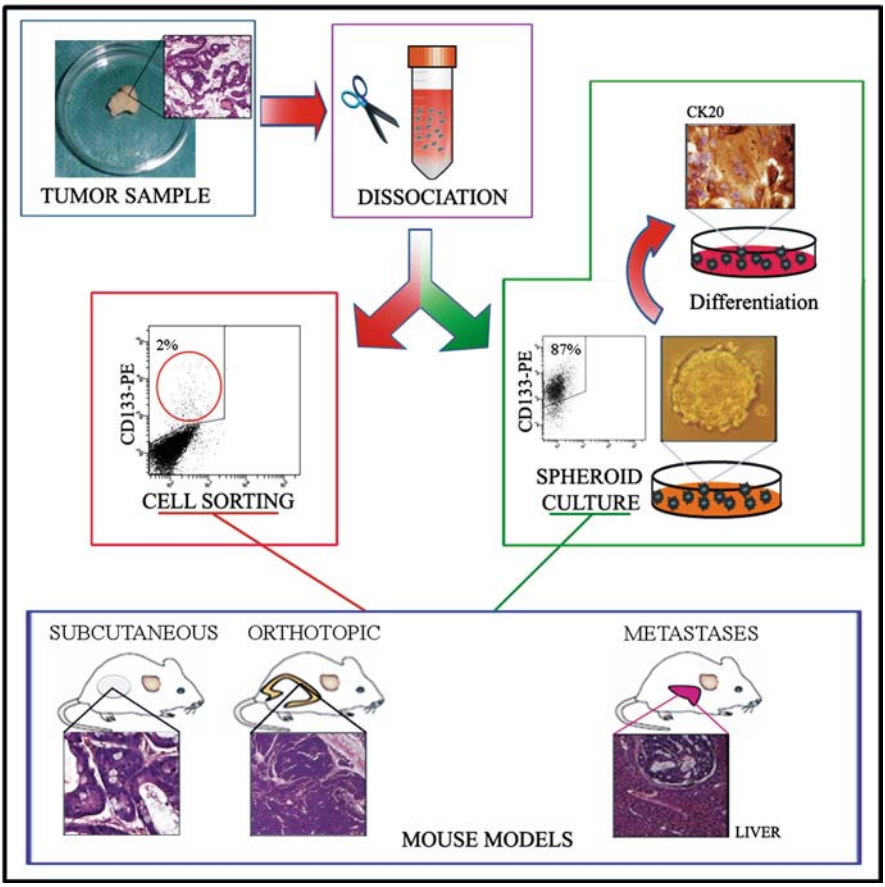

Fig. 4.2 In vitro selection of colon cancer stem cells from excised tumor samples. After dissociation of the surgical sample, cells are selected either on the basis of the expression of the CD133 surface marker (*left*) or by appropriate culture conditions (*right*). Cells obtained in either of the two ways can produce tumor xenografts in immunodeficient mice which recapitulate the complex histology of the parental tumor (*lower panel*). These cells can also be used to produce metastatic lesions in different organs

for serial in vitro passages without losing their ability to generate tumors in SCID mice. Upon growth factor deprivation and in presence of serum, $CD133^+$ tumor spheres gradually acquire an adherent phenotype characterized by the expression of CK20 and high levels of the colon carcinoma-specific marker CDX2, whereas CD133 expression is progressively down-regulated. As expected, differentiated cells obtained from $CD133^+$ tumor spheres lost the ability to initiate a tumor in SCID mice [73].

From the observed frequency of one cell with cancer-initiating potential in 262 $CD133^+$ cells, it can be deduced that not all $CD133^+$ are CRC-SCs. Consequently, searching for additional biomarkers appears mandatory in order to enrich

the CRC-SC population. To this end, Dalerba et al. proposed CD44 and EpCAM as CRC-SC–specific markers, with further enrichment by CD166 [79]. Subcutaneous injection of purified $CD44^{+}/EpCAM^{high}$ cells into NOD/SCID mice resulted in high-frequency generation of tumor xenograft. In contrast, $CD44^{-}/EpCAM^{low}$ cells lack tumor-initiating activity [79]. Further subfractionation of the $CD44^{+}/EpCAM^{high}$ cell population using the mesenchymal stem cell marker CD166 increased the success of tumor xenograft. However, immunohistochemical analysis of normal colonic epithelium shows that CD44 expression occurs not only in the stem cell compartment at the crypt bottom but also in cells within the proliferative compartment; thus the specificity of CD44 for colonic SC remains to be determined. More recently, aldehyde dehydrogenase 1 (ALDH1) has been proposed as a promising new marker for normal and malignant human colonic SCs [80]. ALDH is a detoxifying enzyme that oxidizes intracellular aldehydes and thereby confers resistance to alkylating agents [81]. ALDH also converts retinol to retinoic acid, a modulator of cell proliferation. Moreover, ALDH1 has been described as highly expressed in embryonal tissues as well as in adult SCs isolated from bone marrow, brain, and breast. As few as 25 $ALDH1^{+}$ cancer cells, isolated from CRC specimens by flow cytometry, were able to generate tumor xenografts. Notably, a subsequent isolation of cancer cells using a second marker (CD44 and CD133 serially) produced a modest further enrichment of tumor-initiating ability [80].

Significant controversy exists, however, over the functional role of these CRC-SC markers. Major questions have been raised regarding CD133. Studies using a transgenic mouse model in which the CD133 promoter drove *LacZ* reporter expression demonstrated that CD133 was expressed by both mature and undifferentiated colonic epithelial cells, suggesting that CD133 is not restricted to SC compartment. Moreover, in primary human colon cancer specimens, CD133 was expressed in most of the tumor cell population and sorting of $CD133^{+}$ cells from liver metastasis of colon cancer demonstrated that CD133 high- and low-expressing cells could generate tumors in NOD/SCID mice [82]. Regardless of the ongoing debate regarding CD133 as a CRC-SC marker and the lack of evidence for a functional role in tumorigenesis, growing evidence supports the clinical significance of this molecule in CRC. Indeed, Horst et al. have recently shown that CD133 expression correlates with poor prognosis and it is an independent prognostic marker for low survival in CRC [83]. The combined evaluation of CD133 and nuclear β-catenin can identify high-risk cases of low-stage CRC [84], and longer relapse-free interval with an increased overall survival has been observed in patients with lower levels of CD133 [85]. Moreover, a recent study comparing the expression of CD133, CD44, and CD166 (markers that have been associated with CRC-SCs) revealed that the expression of CD133 correlates with that of CD166, whereas neither correlates with CD44. The authors also showed that CD133 is the best single marker to predict poor patient survival [86] whereas the combination of the three markers allows stratification of patients into high-, intermediate-, and low-risk classes. To verify the clinical relevance of CD133 in CRC metastasis, CD133 expression has been evaluated in a matched case–control collection of 54 pairs of CRC patients with and without synchronous liver metastasis showing a strong correlation between high CD133 expression and

synchronous liver metastasis. However, no effect was observed in colon cancer cell lines after CD133 knocking down on proliferation, migration, invasion, and colony formation, suggesting that CD133 may be a marker with high prognostic impact for CRC, without relevant functional role as a determinant of tumor progression [87].

Taken together, these data confirm that the identification of biomarkers for CRC-SCs will enable greater understanding of the mechanisms underlying tumor growth and progression. Studies performed on mouse models have provided a great contribution to the identification of the CRC-SC population. Indeed, Barker et al. recently provided strong support for the hypothesis that the origin of intestinal cancers is from $Lgr5^+$ CBC cells. The deletion of APC in Lgr5 expressing cells leads to their transformation within days. Transformed SCs remain located at the crypt bottom while feeding a growing microadenoma that develops into a macroscopic adenoma within 3–5 weeks. These data suggest that Lgr5 may mark not only normal intestinal SCs but also the small population of CSCs [88]. Using knock-in *LacZ* reporter mice within the Prominin-1 (*Prom1*) locus, Zhu et al. have shown that $Prom1^+$ cells, located at the base of the crypts in the small intestine, co-express Lgr5, generate the entire intestinal epithelium, and are susceptible to neoplastic transformation [89]. Lgr5 was markedly over-expressed in the majority of advanced human CRCs compared with normal mucosal tissue [90]. As expected, in situ hybridization analysis confirmed the expression of Lgr5 in CBC cells in both small intestine and colon. This Lgr5 expression, which was variable among CRC cases, correlated significantly with lymphatic and vascular invasion, lymph node metastasis and tumor stage, suggesting the involvement of this marker in tumor progression. A similar correlation has been described for the "stemness" gene *Bmi1*, confirming that cells responsible for colon tumorigenesis and colon ontogenesis share common markers [91].

4.3 Therapeutic Implications of the CRC-SC Model

The CSC model has major implications for the development of new and more effective therapeutic strategies aimed at targeting and eradicating the tumor SC population. At present, anticancer therapies for CRC include surgery, radiation, chemotherapy, and anti-VEGF or EGFR monoclonal antibodies. Regardless of the therapeutic approach, none of these treatment modalities is curative in most of advanced cancer cases. One of the major concerns surrounding the use of cytotoxic agents is that they are designed to target the most rapidly dividing cells, which represent the majority of the tumor cell population, thus resulting in a remarkable but frequently transient clinical remission. Failure of conventional treatment options to eliminate the CSC compartment might result in tumor relapse and, more importantly, in the proliferation of therapy-resistant and more aggressive tumor cells, which ultimately reduce patient survival. Indeed, it has been shown that CRC-SCs are enriched in residual tumors following conventional chemotherapy regimens, and remain capable of rapidly regenerating the tumor from which they were derived [92]. According to the CSC hypothesis, it was expected that tumor-initiating cells may

display resistance to cytotoxic therapy, permitting the repopulation of treated tumors. Many mechanisms may contribute to the development of therapeutic resistance, including the stochastic selection of resistant genetic subclones, microenvironmental factors, and cell extrinsic factors. CSCs are relatively quiescent and this allows them to escape from chemotherapeutic regimens that typically target actively cycling cells. Moreover, CSCs share signaling pathways (i.e., Wnt, Hedgehog, and Notch) with their normal counterparts that regulate self-renewal of the normal colonic SC population and whose deregulation can lead to tumor development. Similarly to normal SCs, CSCs have been proposed to exhibit high-level expression of multidrug transporter family genes, likely resulting in more efficient efflux of chemotherapeutic drugs and innate multidrug resistance [93]. Thus, an efficient therapeutic approach would require the identification of distinctive molecular pathways active in CSCs and should identify agents that can block CSC proliferation without or minimally affecting normal tissues. Together with intrinsic factors, the microenvironment or niche may influence the ability of CSCs to proliferate, migrate, and/or invade. The niche is an anchoring site for CSCs and adhesion molecules or microenvironmental soluble molecules (including growth factors and cytokines), and these can significantly contribute to therapy resistance. In line with this hypothesis, it has been recently demonstrated that the production of interleukin-4 (IL-4) by $CD133^+$ CRC-SCs promote tumor resistance to the chemotherapeutic agents 5-fluorouracil and oxaliplatin. On the basis of this finding, a new therapeutic strategy can be devised in order to sensitize CRC-SCs to chemotherapy through the targeting of IL-4 [94]. Thus, in addition to its impact on our understanding of the efficacy of available therapies, the CSC model has an impact on the identification of future therapeutic targets.

To study new approaches to develop drugs that target CSCs, Boman et al. used computer modeling [95]. They demonstrated that an exponential increase in both SC and non-SC populations in CRC development involves an enhanced symmetric SC division. This finding suggests that any systemic therapy designed to effectively treat CRC and other cancers must control or eliminate symmetrical CSC division in tumors, while minimally affecting normal SCs. Thus, a systematic approach to identify and challenge the CSC survival machinery would be mandatory in order to develop novel and more efficient SC-targeted therapies. Genome-wide analyses of cancer have revealed the existence of a vast genetic variation among individual tumors, which makes the use of an exclusively genomic approach to cancer biology extremely complex. On the other hand, it is increasingly clear that tumors share common features at the protein pathway level, suggesting that a pathway-oriented perspective may represent the most effective approach to drug discovery and therapy. In a recent study, Fang et al. generated $CD133^+$ tumor sphere cultures from several colon cancer specimens and performed mass-spectrometry–based quantitative proteomics in order to identify cell surface proteins enriched on cultured tumor cells [96]. These cells retain the expression of cell surface markers such as CD133, CD166, CD44, and EpCAM as well as other stem cell–associated proteins including nestin, Bmi1, and Msi-1, thus confirming the value of this in vitro model for biological analysis of CSC populations and for drug screening experiments.

Finally, most of the currently available mouse models of CRC are based on chemically induced tumors, genetically engineered animals, and tumor implants in immunocompromised mice, but none of them faithfully replicates all aspects of human tumor development. CRC-SCs represent an excellent tool for the preclinical evaluation of new anticancer therapies both in vitro and in vivo, where they generate xenografts that phenocopy the human tumor of origin [97] (Fig. 4.2). Reliable mouse models of human CRC are essential to understand the mechanisms underlying tumor development or pathogenesis and for the preclinical evaluation of new therapies. CSCs both as a theoretical model and as innovative "reagents" could contribute to a significant advancement in cancer research.

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Chapter 5
Cancer Stem Cells in Pancreatic Cancer

Jorge Dorado, Alicia G. Serrano, and Christopher Heeschen

Abstract Over the past decade, increasing evidence has suggested that stem cells play a crucial role not only in the generation of complex multicellular organisms but also in the development and progression of malignant diseases. It has now been shown that many tumors harbor a subset of distinct cancer cells that bear stem cell characteristics and, therefore, these cells are termed cancer stem cells (CSCs) or tumor-initiating cells. CSCs are hypothesized to be exclusively responsible for tumor initiation, propagation, and metastasis. Indeed, it has been shown that human pancreatic CSCs contain a subpopulation of so-called migrating CSCs characterized by CXCR4 co-expression. Only these cells are capable of escaping the primary tumor and metastasizing to distant sites. Clinically even more important, however, is the observation that CSCs are highly resistant to chemo- and radiotherapy. Laboratories around the world are now aiming to further characterize these cells in hopes of identifying novel treatment modalities to conquer pancreatic cancer.

Abbreviations

ABC	ATP binding cassette
ALDH1	Aldehyde dehydrogenase 1a1
Arx	Aristalless-related homeobox
CAC	Centroacinar cells
CD	Cluster of differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A

C. Heeschen (✉)
Clinical Research Programme, Stem Cells & Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain
e-mail: christopher.heeschen@cnio.es

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CSC	Cancer stem cell
CXCR4	CXC chemokine receptor 4
EMT	Epithelial-to-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
Hes1	Hairy enhancer of split 1
IL-4	Interleukin 4
IPMN	Intraductal mucinous neoplasm
Klf4	Krueppel-like factor 4
MDR1	Multi-drug resistance 1
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
Ngn3	Neurogenin 3
PanIN	Pancreatic intraepithelial neoplasia
Pax4	Paired box gene 4
PDAC	Pancreatic ductal adenocarcinoma
Pdx1	Pancreatic and duodenal homeobox 1
PTEN	Phosphatase and tensin homolog
Ptf1	Pancreas-specific transcription factor 1
RBP-Jκ	Recombination signal-binding protein 1 for J-kappa
SDF-1	Stromal-derived factor 1
Shh	Sonic hedgehog
Sox2	Sex determining region Y-box 2
SP	Side population
TP53	Tumor protein 53 gene
ZEB1	Zinc finger E-box-binding homeobox 1

5.1 Introduction

Pancreatic ductal adenocarcinoma is the deadliest solid cancer and currently the fourth most frequent cause for cancer-related deaths. Pancreatic cancer is characterized by late diagnosis due to lack of early symptoms, extensive metastasis, and high resistance to chemotherapy and radiation. Despite increasing research activities in the field of pancreatic tumor and vascular biology, there has been very little substantial therapeutic progress regarding clinical endpoints over the past decades (Fig. 5.1). One of the more recent therapeutic advancements involving introduction of the antimetabolite gemcitabine in 1997 has improved clinical response in terms of pain reduction and weight loss [1]. However, with a 5-year survival rate of 1–4% and a median survival period of 4–6 months, the prognosis of patients with pancreatic cancer remains extremely poor [2–7]. The addition of the only other approved agent, erlotinib, to gemcitabine has not resulted in major improvement in survival [8]. Therefore, elucidation of the mechanisms governing pancreas biology and their deregulation during tumorigenesis is of crucial importance for the development of more effective therapies.

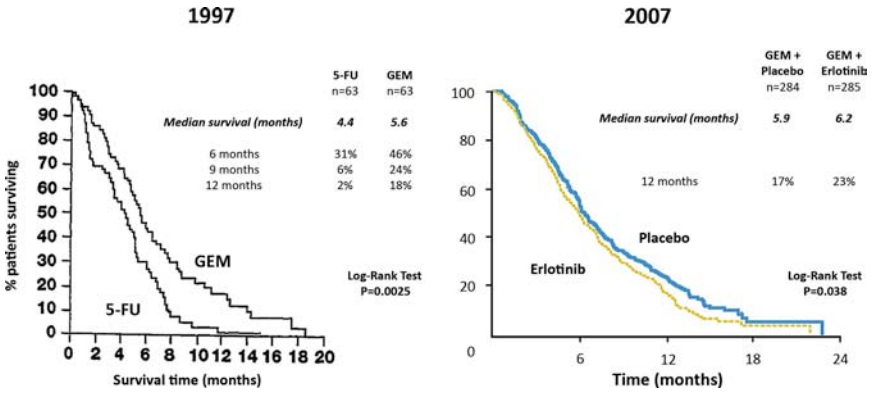

Fig. 5.1 In 1997, Gemcitabine was shown to significantly improve median survival in patients with advanced pancreatic cancer from 4.4 to 5.6 months (*left panel*) [1]. Ten years later, so far the only other approved targeted treatment modality using the EGF receptor inhibitor Erlotinib enhanced survival by no more than 10 days (*right panel*) [8]

5.2 Pancreatic Stem Cells

The pancreas is a glandule of both exocrine and endocrine nature, and is formed by a complex branching network of ducts that end in globular structures (acini), where the production and secretion of digestive fluids occurs. The exocrine component of the pancreas is responsible for the secretion of precursors of multiple digestive enzymes into the duodenum. The endocrine component, responsible for the secretion of hormones implicated in the regulation of carbohydrate metabolism, is made up of islets comprised of different cell types. These include α-cells which secrete glucagon and β-cells responsible for insulin production in response to varying blood glucose levels.

Extensive efforts have been undertaken to identify pancreatic stem cells, which could be involved in the maintenance and (more importantly) in regenerative processes in the pancreas in response to chronic pancreatitis as well as loss of β-cell mass. The characterization of such an elusive stem cell population could lead to the development of therapeutic strategies for the replacement of β-cells lost as a consequence of type I diabetes. Despite lacking a clear definition of postnatal pancreas stem cells for the different cellular types within the pancreas, we have gained deep knowledge regarding the characteristics of pancreatic stem cells during development. Thus, all pancreatic cells, from both exocrine and endocrine lineages, are believed to originate from an initial cell progenitor expressing the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) (Fig. 5.2). The expression of this factor together with silencing of signaling mediated by Sonic hedgehog (Shh) in the surrounding mesenchymal tissue initiates embryonic pancreas development. The implication of Shh in this process is supported by several observations, including a lack of Pdx1 expression in embryos with constitutively active hedgehog signaling [9].

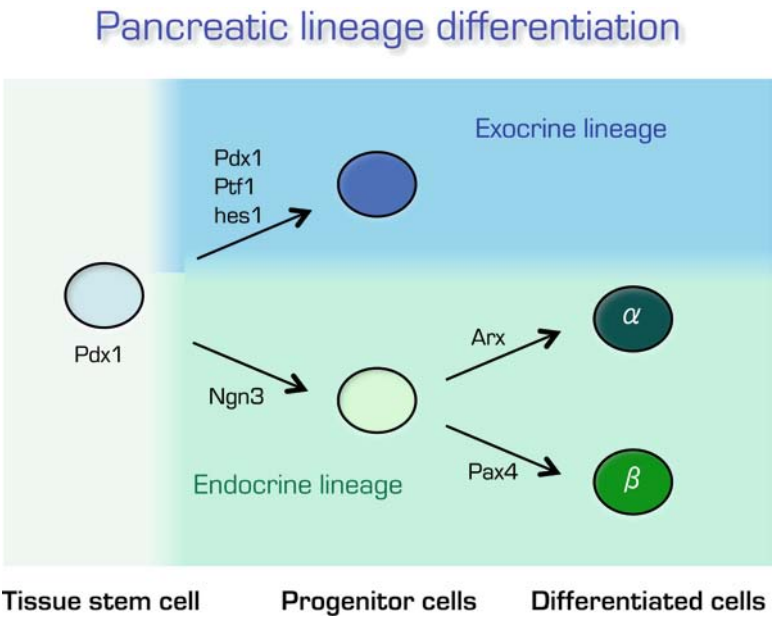

Fig. 5.2 Different transcription factors are responsible for the determination of cell fate during pancreas development. Cells retaining Pdx1 expression and initiating the expression of Ptf1 and Notch signaling progress towards an exocrine lineage. In contrast, the expression of Ngn3 determines an endocrine fate associated with differential expression of Arx and Pax4, which will then further differentiate these committed cells into α-cells and β-cell, respectively

However, Pdx1 null mutant mice show aberrant pancreas formation during embryonic development, although they are capable of forming an aberrant pancreas including insulin and glucagon expressing cells, which are unable to expand. Thus, Pdx1 can be considered as a critical transcription factor in pancreatic commitment, although there might be more actors implicated, since absence of this factor does not result in complete impairment of pancreas formation.

Subsequently, a second transcription factor seems to be critically involved in the differentiation of Pdx1-positive pancreas stem cells toward an exocrine phenotype. Shortly after the expression of Pdx1, a subset of these stem cells activate the expression of a second transcription factor known as pancreas-specific transcription factor 1 (Ptf1). Regardless of this apparent temporal sequence, the expression of both transcription factors seems to occur in an independent manner [10]. Similar to Pdx1, Ptf1 expression is an essential requirement for pancreas development in humans. Thus, malfunctioning mutations of the Ptf1 gene in humans result in impaired development of this organ [11] and, conversely, forced overexpression of Ptf1 induces pancreas formation in ectopic locations [12]. However, Ptf1 expression has been implicated in the commitment of precursor cells toward an exocrine phenotype, since Ptf1 null mutant mice show impaired pancreas development but are still capable of developing endocrine cells [10].

In addition, commitment toward an exocrine fate seems to be potentiated through signaling of the surrounding mesenchyma on Pdx1-positive cells, enhancing Notch signaling mediated by its downstream target Hes1 (hairy enhancer of split 1) and inhibiting expression of the pro-endocrine differentiation factor Neurogenin 3 (Ngn3) [13]. Thus, null mutant mice for both the Notch ligand delta-like or for the Notch target RBP-Jκ transcription factor are enriched in cells of the endocrine lineage and Hes1 null mice display severe hypoplasia of the pancreas as a result of lack of exocrine progenitor cells [13]. Determination of endocrine fate is induced by expression of the transcription factor Ngn3. In fact, Ngn3-positive cells represent the origin of all the heterogeneity of pancreatic endocrine cells [14]. Thus, α- and β-cells will arise from Ngn3-positive cells, although they will be generated in a different ratio. In early pancreatic development during mouse embryogenesis, the vast majority of cells derived from Ngn3-positive cells are glucagon-secreting α-cells, providing a rationale for the observation that Pdx1-Ngn3 forced expression primarily leads to the development of glucagon cells [15]. Subsequently, Pdx1 is then downregulated in α-cells as they progress toward a non-epithelial phenotype in a process that strongly resembles the epithelial-to-mesenchymal transition (EMT). Conversely, β-cells retain Pdx1 expression while remaining in rather low numbers as compared to glucagon-secreting cells until later in development, when an amplification of the pool of β-cells occurs, together with branching morphogenesis and acinar cell differentiation [14, 16].

Commitment toward α- or β-cell fate seems to depend on the mutually exclusive action of the transcription factors Aristalless-related homeobox (Arx) and paired box gene 4 (Pax4). Expression of Arx might induce formation of α-cells, since deletion of this gene results in impaired generation of this cell type [17], whereas Pax4 appears to be responsible for β-cell formation [18]. The existence of different sequential progenitor cells raises the question of whether these cells can also be reverted to a less differentiated phenotype in order to give rise to a broader number of cell types (plasticity). However, accumulating evidence suggests that β-cells are differentiated cells with very limited expansion capability. In fact, most β-cells seem to originate from a pool of already existing β-cells precursors rather than from expansion of ancient β-cells [19]. Notch is not capable of reverting mature endocrine cells toward a progenitor-like state [20]. In contrast, Ngn3-positive cells demonstrate greater plasticity, since they can be reverted to a ductal progenitor phenotype [21].

5.3 Cell of Origin for Pancreatic Cancer

The cell from which human pancreatic ductal adenocarcinoma originates still remains elusive. One of the hypotheses that have been proposed for tumor initiation in solid organs is the malignant transformation of stem cells resident in the normal tissue. These cells are intrinsically endowed with the capacity of self-renewal, and would therefore only need to accumulate sequential mutations to undergo malignant transformation and originate a tumor. Indeed, this hypothesis has just recently been validated for intestinal cancer [22]. The fact that adult pancreatic stem cells, while

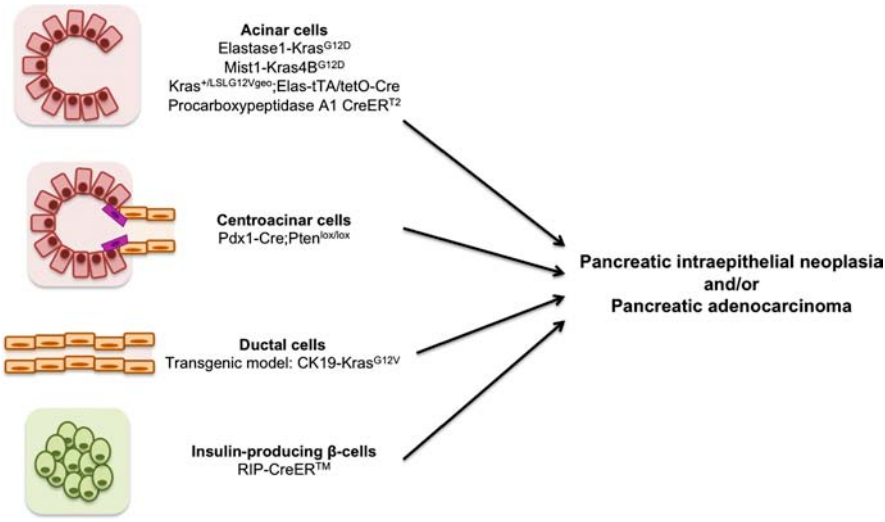

Fig. 5.3 Experimental models targeting different pancreatic cell types used to study the development of pancreatic intraepithelial neoplasias (PanIN) or pancreatic ductal adenocarcinoma (PDA). Elastase1-Kras^{G12D} described in [28]; Mist1-Kras4B^{G12D} in [88]; Kras$^{+/LSLG12Vgeo}$;Elas-tTA/tetO-Cre system detailed in Guerra et al. [37]; proCPA1CreER^{T2} in Zhou et al. [89]; Pdx1-Cre;Pten$^{lox/lox}$ in Stanger et al. [32]; CK19-Kras^{G12V} in Brembeck et al. [90]; and RIP-CreER^{TM} in Dor et al. [91]

having been proposed for mice several years ago [23], still cannot be tracked due to their rather vague description, has hindered the field in providing definitive proof for this postulate. Until further knowledge has been gained to verify this stem cell model of tumorigenesis in pancreatic cancer, other models still need to be considered as the main mechanism. Indeed, to clarify these aspects of tumor initiation and progression, different mouse models have served as important tools, especially genetically engineered mouse models expressing mutated Kras (Fig. 5.3).

Activating mutations in this oncogene are detected in almost every pancreatic premalignant lesion or adenocarcinoma in humans, pointing to Kras activation as one of the crucial and most likely initiating genetic hits leading to tumorigenic transformation. Independently of the cell of origin, expression of an activated mutant Kras allele (KrasG12V or KrasG12D) in the mouse pancreas recapitulates formation of premalignant pancreatic intraepithelial neoplasias (PanIN) and their progression toward pancreatic ductal adenocarcinoma. However, it is remarkable that even if all the pancreatic cells in this model express activated Kras, only a minor subset of them eventually progresses to neoplastic lesions. Furthermore, when this allele is conditionally activated in Pdx1-positive cells by use of a Cre system, mice develop pancreatic ductal adenocarcinoma in a process that highly resembles tumor progression in humans. Additional mutations such as loss of TP53 or CDKN2A are also found in a smaller percentage of pancreatic ductal adenocarcinomas, and therefore have been included in the current pancreatic ductal adenocarcinoma mouse models [24].

Considering the fact that pancreatic ductal adenocarcinoma has a ductal morphology and that its gene expression pattern is similar to that of ductal cells, it is tempting to affirm that a ductal cell would be the target for the tumorigenic transformation. Unfortunately, the poor performance of currently available ductal promoters for in vivo mouse models renders this hypothesis difficult to prove, and the evidence obtained so far is not yet conclusive. Specifically, expression of $Kras^{G12V}$ under the control of the ductal promoter cytokeratin-19 in a transgenic mouse model produces no apparent malignant phenotype. However, despite the ductal histology of pancreatic ductal adenocarcinoma, the lesions, which can be appreciated at the earliest stages of tumorigenesis, are actually embedded in islets mainly formed by clusters of α- and β-cells. This observation raises the possibility that transdifferentiation of β-cells may be the root of pancreatic cancer, a hypothesis which is further supported by the observation that chemical depletion of β-cells impairs tumor initiation. Indeed prior evidence suggests that transdifferentiation may occur in the pancreas, since markers of foregut differentiation are expressed in some premalignant pancreatic lesions [25]. While tracing experiments indicate that β-cells do not contribute to the generation of cells with acinar or ductal phenotype during tumorigenesis [26], a new mouse model of Kras activation in β-cells has provided new insights. Exclusive Kras activation in β-cells was not sufficient for transformation of these cells in unchallenged mice, although previous induction of pancreatitis did lead to the development of exocrine neoplasia [27].

During the early stages of pancreatic tumorigenesis, progression of acinar cells toward a ductal phenotype is also frequently detected, expanding the possibility toward acinar cells as the tumor-initiating cells. Mouse studies using the acinar-specific promoter of the Elastase gene have revealed that conditional activation of Kras exclusively in acinar cells results in tumors of mixed acinar and ductal morphology [28]. Moreover, Notch signaling cooperates with Kras activation during tumor initiation and progression [29]. Another observation pointing to acinar cells as the pancreatic cell type where tumorigenesis may initiate has been the recent discovery of a Bmi1-positive population within the acinar subset of cells, which is capable of maintaining pancreatic cell homeostasis [30]. Finally, centroacinar cells (CAC) have emerged as another candidate cell type for driving tumorigenesis. These cells are located at the junction of ductal and acinar compartments. The fact that Notch signaling and its target gene Hes1 remain active in these cells during adulthood [30, 31], together with the observation that Notch signaling maintains an undifferentiated state during pancreas embryogenesis [21], has lead to the hypothesis that these CACs are possible targets for tumor-initiating events. Consistent with this hypothesis, it has been observed that different Notch signaling mediators, including Hes1, are overexpressed in pancreatic ductal adenocarcinoma. Furthermore, specific deletion of PTEN gene in pancreatic tissue leads to expansion of CACs and eventual progression to carcinoma, pointing to these cells as the origin of tumorigenic processes within this organ in mice [32]. However, in this model, mice develop tumors morphologically different from pancreatic ductal adenocarcinoma and more related to human intraductal mucinous neoplasm (IPMN), an infrequent premalignant lesion in ductal cells than can progress to PDAC pancreatic ductal adenocarcinoma.

5.4 The Role of Chronic Pancreatitis

Pancreatitis is an inflammatory response of the pancreas toward autoimmune antibodies and liberation of pro-enzymatic content of the exocrine pancreas following external injury or damage caused by xenobiotics such as caerulein [33]. There is growing evidence that this inflammatory state facilitates pancreatic tumorigenesis, indicating that the physiological context can exert a strong influence on the susceptibility of a cell toward transforming events. Consistently, chronic pancreatitis has been identified as a prominent risk factor for pancreatic cancer in humans. This link between pancreatitis and human pancreatic cancer has been well documented in several epidemiological studies [34–36]. Accumulating experimental evidence from mouse models now also supports this notion. An inflammatory stimulus was necessary for the induction of tumorigenesis through activation of Kras in acinar cells of adult mice, which otherwise are refractory to this oncogenic input [37]. Interestingly, this process was not mediated by the pro-inflammatory transcription factor NF-κB, although such a mechanism has been demonstrated to be operative for other similar processes such as colitis-associated colon carcinogenesis [38].

Pancreatitis has not only been shown to have synergistic effects for promoting malignant transformation, but it has also been implicated in mobilization of tissue progenitor cells and induction of their proliferative capacity. Specifically, partial duct ligation of the pancreas results in activation of β-cell progenitors together with their expansion [39]. In a similar manner, pancreatic injury leading to pancreatitis has been demonstrated to affect the endocrine status of insulin-secreting cells, enabling them to behave as starting points for exocrine neoplasias [27]. Since, under normal conditions, pancreatic stem cells are likely to constitute only a minor population that can hardly be detected, experimental induction of chronic pancreatitis may increase the number of these cells facilitating their detection, characterization, and further investigation. Therefore, future studies should address the possibility of pancreatic stem cells being expanded in response to pancreatitis, rendering them more susceptible to transforming events and their subsequent conversion into cancer stem cells (CSCs) as the proposed root of pancreatic cancer as discussed in the following section.

5.5 Pancreatic Cancer Stem Cells

The CSC hypothesis is receiving increasing interest within the field of pancreatic cancer as well as other malignancies, since it also provides a rationale for the high resistance to chemotherapy leading to relapse of disease after treatment. In this context, a thorough understanding of the biological characteristics of CSCs will be crucial for their better identification, for their tracking during treatment and for the development of new therapies directed against these cells as the putative root of the tumor. To date, pancreatic CSC markers remain poorly defined. The first evidence for a distinct CSC population in pancreatic cancer was provided by Li and colleagues [40].

The authors identified a highly tumorigenic $CD44^+CD24^+EpCAM^+$ subpopulation using a xenograft model of immunocompromised mice for primary human pancreatic ductal adenocarcinoma. In contrast to their $CD44^-CD24^-EpCAM^-$ counterparts, these $CD44^+CD24^+EpCAM^+$ cells were able to form tumors at low numbers and displayed typical stem cell features such as self-renewal, activation of developmental signaling pathways (Shh), generation of differentiated progeny, and the ability to recapitulate the phenotype of the parental tumor from which they were derived [40]. Interestingly, the finding that tumorigenicity in pancreatic cancer is confined to $CD44^+CD24^+$ cells is in stark contrast to the original findings in breast cancer, where only $CD44^+CD24^{-/low}$ cells were tumorigenic [41]. However, these different findings have now been extended to other tumor entities such as ovarian cancer [42].

In a second study, Hermann et al. showed that CD133 expression in freshly isolated primary human pancreatic cancers discriminated for cells with capacity for self-renewal, sphere formation, and, most importantly, in vivo tumorigenicity upon serial transplantation [43]. Although $CD133^+$ cells show some overlap with the $CD44^+CD24^+EpCAM^+$ subpopulation, these data indicate that the putative CSCs isolated by different research groups are not identical. Further studies will be required in order to determine whether these markers ($CD44^+CD24^+EpCAM^+$ and $CD133^+$) define two distinct pancreatic CSC populations, or whether the use of a combination of these markers confers a higher enrichment in pancreatic tumor-initiating cells (Fig. 5.4).

More recently, additional markers have also been associated with pancreatic CSCs. Specifically, aldehyde dehydrogenase 1a1 (ALDH1) has been shown by several groups to label tumorigenic cells in pancreatic cancer [44–46]. However, although ALDH1 may indeed enrich for a tumorigenic population within the tumor tissue, ALDH1 has also been found to be abundantly expressed in normal pancreas tissue [47]. Therefore, ALDH1 can be best used for tumors whose corresponding normal tissues express ALDH1 in relatively restricted or limited levels such as breast, lung, ovarian, or colon cancer. Since the currently available cell surface markers are merely enriching for CSC populations, and therefore their use remains controversial, functional assays such as sphere-formation capacity in vitro and tumorigenicity in vivo are becoming even more important for the identification and subsequent characterization of CSCs and may also serve as a platform to find better CSC markers.

The main stem cell properties of CSCs are self-renewal and differentiation to generate the heterogeneous cancer cell population within a tumor, a process which is also recapitulated in metastatic spread. Metastasis is the major cause of death in pancreatic cancer patients and currently there is no effective treatment available for this deadly disease. Importantly, not all cells within a tumor (or even within the CSC population) possess the same metastatic potential, and only a small subset of cells is directed through lymphatic or blood vessels toward specific secondary sites to form metastases. In order to be able to establish secondary lesions, the migrating cells would require similar features to the cells initiating the primary tumor. For this reason, CSCs were proposed to represent the only cell population capable of spreading and giving rise to metastases. Indeed, Hermann et al. for the first time identified two

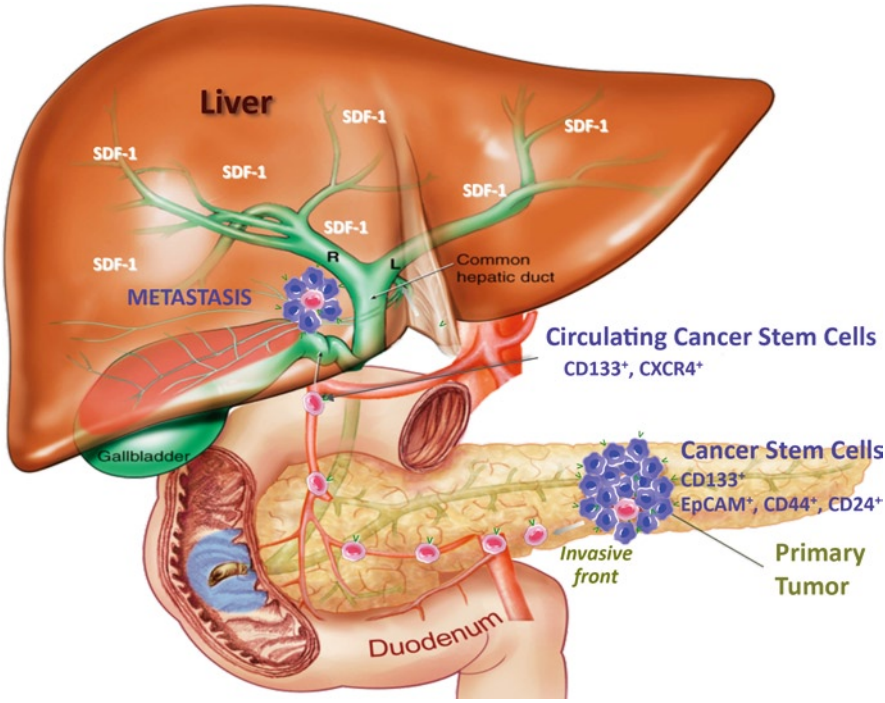

Fig. 5.4 Distinct populations of cancer stem cells (CSCs) in pancreatic cancer. In addition to the tumor resident EpCAM$^+$, CD44$^+$, CD24$^+$ [40] and/or CD133$^+$ [43] CSCs, a subpopulation of migrating CSCs, identified by CD133$^+$ and CXCR4$^+$ expression [43], can be detected in the invasive front in the pancreas as well as in the circulating blood. Typically, metastatic lesions in pancreatic cancer are found in organs with strong expression of stromal-derived factor-1 (SDF-1), the specific ligand for CXCR4. Detection of these circulating CSC could serve as prognostic and therapeutic biomarkers

distinct subsets of CSCs based on the expression of the chemokine receptor CXCR4 in pancreatic cancer [43]. CXCR4 is a chemokine receptor responding to chemotactic gradients of its specific ligand stromal cell derived factor 1 (SDF-1) that was originally found to be responsible for leukocyte and hematopoietic progenitor cell homing. Both are also obligatory players in the maintenance of pancreatic duct survival, proliferation, and migration during pancreatic organogenesis and regeneration [48]. Emerging evidence suggests that CXCR4 plays a pivotal role in the metastatic process of different tumor entities toward a gradient of SDF-1, which is highly expressed in secondary sites usually associated with metastasis such as lymph nodes, lung, liver, and bone marrow [49, 50].

Hermann et al. identified a "stationary" population expressing CD133, but not CXCR4, which is responsible for the initiation and maintenance of the primary tumor, and a "migrating" and highly metastatic population characterized by co-expression of CD133 and CXCR4. Only CD133$^+$CXCR4$^+$ cells had metastatic potential, while depletion of the CSC population for CD133$^+$CXCR4$^+$ cells completely abrogated

the usually strong metastatic phenotype of the implanted tumors. Consequently, pharmacological inhibition of the CXCR4 receptor by AMD3100 also prevented the metastatic activity of transplanted CSCs. These data provide convincing evidence for a crucial role of the SDF-1/CXCR4 axis in metastasis. Since most cancers initially spread to local lymph nodes long before solid organ colonization, the lymphatic system and lymph node metastases also need to be investigated for the presence and contribution of migrating CSCs. Indeed, Hermann et al. also found significantly higher numbers of $CD133^{+}CXCR4^{+}$ migrating CSCs in patients with lymph node metastasis (pN1+), demonstrating a close clinical correlation between migrating CSCs and advanced disease [43]. A different study by Nakata et al. suggested that CCR7, another chemokine receptor (also known as BLR2 or CD197), is also associated with lymph node metastasis in pancreatic cancer and, based on multivariate survival analysis, could serve as an independent prognostic factor [51].

CSCs may acquire a migrating phenotype through EMT in primary tumors, since the mesenchymal phenotype is usually associated with strong migration capacity while maintaining stemness, thus allowing the production of progenies during metastasis. Recently, Wellner et al. showed in pancreatic and colon cancer that the EMT-activator ZEB1 represents an important promoter of metastasis by suppressing E-cadherin. Furthermore, the stem cell phenotype was maintained by suppression of miR-200 family members that usually target stem cell factors such as Sox2 and Klf4 [52]. Together, these results suggest that the metastatic process is not random, but rather regulated by specific mechanisms related to the expression of adhesion molecules, chemokine receptors, and their respective ligands. Whether this is a reversible process in pancreatic cancer remains to be determined. Indeed, Hermann et al. did not find any evidence for the generation of $CD133^{+}CXCR4^{+}$ from $CD133^{+}CXCR4^{-}$ cells in the utilized model systems [43].

5.6 Therapeutic Implications of Pancreatic CSCs

Several studies have now shown that standard therapy has limited or no significant effect on CSCs, and in fact may enrich for these populations due to the elimination of more differentiated cells [43, 53, 54]. For this reason, it is important to identify new therapeutic approaches that can selectively eliminate this population and thus improve cancer treatment response. It has been consistently demonstrated that treatment of fresh and in vivo expanded patient-derived pancreatic cancer cells with the first-line chemotherapeutic agent gemcitabine preferentially targets more differentiated tumor cells, with a resulting enrichment of $CD133^{+}$ cells in which the tumorigenic population is contained. Similarly, gemcitabine treatment of orthotopically xenografted human tumors is merely effective in controlling tumor growth and prolonging survival, but does not affect CSCs as the putative root of the tumor [43, 55]. The basis of resistance to chemotherapy in this population is most likely linked to their quiescence, enhanced anti-apoptotic mechanisms [56], increased repair of DNA after damage, and by the presence of membrane transporters that

pump drugs out of these cells [57]; and this way the CSC population is protected from damage caused by external agents.

The functional identification of so-called side population (SP) cells has been linked to CSC in head and neck cancer [58–60] and many other types of cancer [61–65], including those of the gastrointestinal system [66]. This population shows the ability to efflux the fluorescent dye Hoechst 33342, producing a characteristic profile in flow cytometry analyses. This ability has been attributed to the expression of ABC transporters, in particular ABCG2 and MDR1, and has been related to tumor-initiating cells [62, 63, 67, 68]. Moreover, this efflux capacity may well be responsible for the resistance to some chemotherapeutic agents [69]. In the case of gemcitabine, a nucleoside analogue, it has also been suggested that the cause of resistance may be an increase in the expression of anti-apoptotic genes such as Bcl-X_L that can allow the incorporation of normal nucleosides but not toxic analogues [70]. Thus, withdrawal of gemcitabine treatment usually results in a rapid relapse of tumor growth and increased aggressiveness of the disease. Many investigators have already identified a "side population" in cultured pancreatic cancer cell lines [69] and fresh human pancreatic ductal adenocarcinoma samples [71], but to date, no data have been published demonstrating a direct relationship between side population cells and tumorigenicity in human pancreatic cancer specimens.

Although the identification of reliable pancreatic CSC markers and subsequent targeting of these cells will be a critical step toward improved treatment modalities for this devastating disease, more general approaches are also being developed. Telomeres play an essential role in the regulation of the lifespan of human cells and telomere elongation is usually mediated by telomerase. However, with increasing age, telomeres progressively shorten and also contribute to organismal ageing by limiting the proliferative capacity of stem cells. In contrast to normal somatic cells, telomerase appear also to be strongly activated in numerous cancer types, contributing to cell immortality and tumor growth [72, 73]. Growth-deregulated cells during tumorigenesis would rapidly deplete telomeres, leading to senescence and subsequent cell death unless telomerase or some other mechanism of telomere elongation is highly active in these cells. Indeed, despite increased expression of telomerase, tumors often still have significantly shorter telomeres as compared to normal somatic cells. Importantly, recent studies suggested that CSCs also express high levels of telomerase despite the quiescence of at least a subpopulation of these cells [74–77]. Therefore telomerase, which is essential for tumor progression, appears to be a critical marker in many cancers and most likely for all cancer cells despite the inherent cellular heterogeneity of solid tumors. Because of this, telomerase inhibition has emerged as an almost universal tumor target. It has been reported that the combination of standard chemotherapy with telomerase inhibitors is more effective for solid tumors such as prostate cancer [78]. Several therapeutic approaches for telomerase inhibition are now being developed and tested in solid tumors including pancreatic cancer, although most of the candidate molecules are still in preclinical development [76]. Most importantly, whether this new treatment modality will also be capable of eliminating CSCs still remains to be determined.

Extensive investigations concerning the development of the pancreas and global genomic analysis of human pancreatic ductal adenocarcinomas [79] have revealed the importance of several targetable stem cell regulatory pathways. The Sonic Hedgehog (Shh) pathway has been shown to be critical for the development of the pancreas [80] and also appears to play a role in the maintenance and progression of pancreatic cancer [24, 81]. More recently, this pathway has now also been considered as a crucial element for the maintenance of CSCs. Inhibition of Shh signaling increased survival in a mouse model of pancreatic cancer [82], and therapeutic blockade of this pathway in a xenograft model induced tumor regression and decreased the CSC content [45]. Moreover, Feldmann and colleagues [44] also showed that Hedgehog blockade abrogates pancreatic cancer metastases, a process which has been linked to the evasion of migrating CSCs from the primary tumor, as explained above. Surprisingly, however, Mueller et al. have recently shown that neither Shh inhibition alone nor as a supplement to chemotherapy were capable of effectively diminishing the CSC pool [55]. Only the combined inhibition of Shh and mTOR (mammalian target of rapamycin) together with chemotherapy reduced the number of CSCs to virtually undetectable levels in vitro and in vivo. Most importantly, in vivo administration of this triple combination in mice with established patient-derived pancreatic tumors was reasonably tolerated and translated into significantly prolonged long-term survival. Therefore, the combined blockade of Shh and mTOR signaling together with standard chemotherapy may provide the basis for the development of a novel therapeutic strategy to improve the devastating prognosis of patients with pancreatic cancer.

The Notch pathway is known to be critical in general developmental patterning and in cell fate determination. The main Notch pathway-mediated effect involves the ability to restrain differentiation and maintain cells in a precursor state. In the pancreas, Notch signaling modulates the differentiation of progenitors under physiological conditions [21]. Components of this pathway seem to be upregulated in invasive pancreatic cancer and precursor lesions [83] and there is a synergy between reactivation of Notch signaling and expression of Kras, leading to the formation and progression of early precursor lesions (PanIN) [29]. In addition, Notch1 downregulation has been shown to inhibit cell growth in pancreatic cancer cells [84] and more recently Plentz et al. showed that Notch blockade by gamma-secretase inhibition restrains tumor progression in a mouse model of pancreatic cancer [85]. Further studies are still needed to test whether inhibition of the Notch pathway is targeting all cancer cells including CSCs.

5.7 Summary and Perspectives

According to the cancer progression model postulated by Fearon and Vogelstein in 1990, at least 4–5 genetic events are required for the progression from normal epithelium to carcinoma [86]. Due to their very long lifespan, stem cells would represent a rather likely target for the accumulation of these genetic events. However,

irrespective of their actual cell of origin, CSCs seem to harbor mechanisms protecting them from standard therapy. While CSCs have been demonstrated to be responsible for therapy resistance in glioblastoma and pancreatic cancer [43, 53, 54], further evidence now points to similar mechanisms in colon CSCs. Todaro and colleagues have shown that $CD133^+$ colon CSC produce interleukin-4 (IL-4) in an autocrine manner, a feature that seems to protect them from chemotherapy, but which can be overcome by co-treatment with IL-4 inhibiting antibodies [87]. Therefore, it appears reasonable to conclude that there is sufficient evidence now for the existence of CSCs or tumor-initiating cells in several epithelial tumors, and that these CSCs pose a significant threat to the patient via their resistance to standard therapies. To further foster our understanding of CSC biology, synergy between development of novel probes such as nanoparticles and corresponding imaging modalities will be of paramount importance in building strategies for robust and efficient tracking and validation of CSCs and their niche, both under in vitro and in vivo conditions. These studies will pave the way to better elucidate the underlying regulatory mechanisms of CSCs and develop platforms for targeted theragnostics. Evidence is accumulating for putative therapeutic approaches to overcome these resistance mechanisms, thus promoting the search for new and better clinical therapies based on the CSC concept, which may eventually help improving the prognosis of patients suffering from this deadly disease.

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Chapter 6
Cancer Stem Cells in Prostate Cancer

Paula Kroon, Davide Pellacani, Fiona M. Frame, Norman J. Maitland, and Anne T. Collins

Abstract Prostate cancer is the most diagnosed cancer in men in the Western world. Currently, most treatments are directed toward an androgen receptor (AR)-expressing cell, which encompasses the vast majority of prostate tumors. Inevitably, the tumor recurs, thus the question remains: are cancer stem cells (CSCs) at the root of such recurrence, or is relapse the result of clonal evolution of an AR-expressing cell? There is also controversy regarding the phenotype of prostate CSCs: are they derived from an aberrant stem cell or AR responsive, progenitor cell? Here, we discuss the evidence for CSCs in prostate disease and why current therapies are not effective. How we specifically target these elusive cells is a question that is now being addressed for many solid tumors, including prostate cancer.

Abbreviations

AML	Acute myeloid leukemia
AR	Androgen receptor
ATM	Ataxia telangiectasia mutated
BER	Base excision repair
BPH	Benign prostatic hyperplasia
BRCA	Breast cancer susceptibility gene
CD	Cluster of differentiation
CK	Cytokeratin
CSC	Cancer stem cell
DSB	Double-strand break
ERG	v-ets erythroblastosis virus E26 oncogene homolog

P. Kroon (✉)
Cancer Research Unit, Department of Biology, University of York, York, UK
e-mail: Pk516@york.ac.uk

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ETS	Erythroblast transformation-specific
GSTP1	Glutathione *S*-transferase P1
HR	Homologous recombination
hTERT	Human telomerase reverse transciptase
IL-6	Interleukin-6
JAK	Janus kinase
MLH1	MutL homolog 1
MMR	Mismatch repair
NER	Nucleotide excision repair
PAP	Prostate acid phosphatase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PKB	Protein kinase B
PSA	Prostate-specific antigen
STAT	Signal transduction and activator of transcription
TMPRSS2	Transmembrane protease, serine 2
UGM	Urogenital sinus mesenchyme

6.1 Anatomy and Development of the Human Prostate

The prostate is located toward the base of the bladder surrounding the urethra. Its main function is to produce hormones and secrete proteins for semen production and is therefore essential for the reproductive system. It also functions as an endocrine gland, metabolizing the rapid conversion of testosterone to dihydrotestosterone, which is a more effective androgen [1].

The human adult prostate is a complex tubulo-alveolar gland composed of an epithelial parenchyma embedded within a connective tissue matrix. The epithelial cells are arranged in glands composed of ducts that branch out from the urethra and terminate into acini. It is a heterogeneous organ, and can be divided into central, transition, and peripheral zones [2]. The majority of prostate cancers arise in the peripheral zone (70%) compared to 20% in the transition zone and 10% within the central zone, whereas benign prostatic hyperplasia (BPH) mainly occurs within the transitional zone [3].

The development of the human prostate begins during the ninth week of embryogenesis [4], in response to testosterone stimulation, with the outgrowth of epithelial buds from the urogenital sinus epithelium into the surrounding urogenital sinus mesenchyme (UGM) [5]. These epithelial buds form ducts that elongate, branch out, and terminate into acini. From the 20th week of gestation up to puberty, the immature prostatic acini and ducts are lined by multiple layers of immature cells with round nuclei and very little cytoplasm. In the immature epithelium, cytokeratins (CK) of simple and stratified epithelium are expressed (primary cytokeratins; numbers 8, 18, and 19 and the large molecular weight forms; numbers 4, 5, 6, 7, 10, 11, 14, 15) [6]. Postnatal development includes a period of growth during the

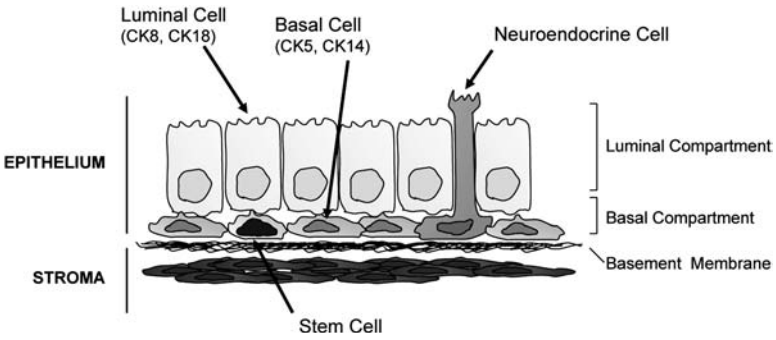

Fig. 6.1 Model of normal prostate tissue and the different cell types. Prostate tissue consists of a stromal layer, a basement membrane and an epithelial layer. The epithelial layer consists of a luminal compartment and a basal compartment, in which the stem cells are located

first year, quiescence during childhood and further growth with the testosterone surge at puberty. During puberty, the immature multilayered epithelium differentiates into a two-layered epithelium consisting of peripheral flattened to cuboidal basal cells and inner secretory cylindrical epithelium [7, 8]. In parallel with epithelial differentiation, the epithelial–mesenchymal interaction induces UGM to proliferate and differentiate into prostatic smooth muscle and interfascicular fibroblasts [9].

The main cell types within the mature prostate are basal, secretory luminal and neuroendocrine cells [7] (Fig. 6.1). The luminal epithelial cells represent the major cell type in normal prostate. They are terminally differentiated, express high levels of androgen receptor (AR) [10], and are dependent upon androgens for their survival [11]. Basal cells are relatively undifferentiated, express low/undetectable levels of AR [12] and are androgen independent for their survival [11]. Rare neuroendocrine cells are located within the basal layer and they are terminally differentiated and androgen insensitive [13].

6.2 Prostate Epithelial Stem Cells

The prostate is an androgen-dependent organ that undergoes involution following castration, but can completely regenerate if androgen levels are restored [14]. Isaacs showed that this cycle of involution, followed by regeneration, can be repeated numerous times and postulated the existence of a population of long-lived, androgen-independent stem cells responsible for regeneration of the gland [15]. This led to a model of prostate lineage in which androgen-independent stem cells give rise to androgen-responsive transit amplifying cells, which differentiate into secretory luminal cells that are both androgen dependent and terminally differentiated [14].

Basal and luminal cells can be discriminated on the basis of their localization, morphology, and expression of specific cytokeratins. For example, CK5 and CK14

are expressed by basal cells, whereas the luminal cells of the prostate predominantly express CK8 and CK18 [16]. Keratin expression patterns in the prostate have provided evidence of epithelial cells that are phenotypically intermediate between basal and luminal cells. Cells have been identified in the luminal layer that express both CK5 and CK18, while some basal cells lack CK14 expression but express low levels of CK18 and CK5 [17–19]. These results indicate that basal and luminal cells are linked in a hierarchical pathway.

Although the overall organization of the murine prostate differs from that of the human gland, studying the murine prostate provides a unique opportunity to study the biology of the prostate. It has been shown that the proliferating cells are located at the tips of ducts [20] and also that prostatic stem cells may be located in the distal region [21]. However, quiescent cells were subsequently shown to be located in the proximal region of the duct nearest the urethra. These cells also have a high proliferative potential and are capable of reconstituting large, branched glandular structures in collagen gels [22]. Tsujimura and co-workers proposed that the stem cells migrate distally toward the proliferating tips where they terminally differentiate [22].

The proposal that prostate stem cells are located within the basal layer of epithelial cells is supported by evidence provided by Signoretti et al., who showed that p63, which is expressed by basal cells [23], is essential for normal prostate development in the mouse [24]. By histological examination, they found that newborn p63(−/−) male mice do not develop a prostate, suggesting that p63 is necessary for the formation of ducts or epithelial budding structures [24].

Recently, using the murine hematopoietic stem cell marker Sca-1 [25], it was shown that Sca-1^+ prostate cells can self-renew (in a sphere-forming assay) for several generations. Moreover, Sca-1^+ cells can differentiate in vivo to produce prostatic tubule structures containing basal and luminal cells. Sca-1^+ cells are also localized to the basal cell layer within the proximal region of the murine prostate [26]. Leong and colleagues also showed enrichment of stem cells within the proximal region of the mouse prostate. They determined that lin^-/Sca-1^+/$CD133^+$/$CD44^+$/$CD177^+$ cells (localized to the basal compartment of mouse prostate) can generate a prostate after transplantation in vivo [27]. The regenerated prostate had a branching morphology with epithelial tubules composed of basal, luminal, and neuroendocrine cells. Nonetheless, there is still some controversy as to whether stem cells are located within the basal layer. The *Nkx3.1* gene regulates prostate epithelial differentiation, and is expressed within the luminal cells and rare basal cells in the mouse prostate. Expression is rapidly lost after castration and is restored following prostate regeneration when androgen levels are restored. Wang et al. [28] showed that in the castrate-resistant state, *Nkx3.1* expression is restricted to the luminal cells and only those genetically marked. They observed that *Nkx3.1*-marked luminal cells were able to give rise to both basal and luminal cells following androgen-induced regeneration.

In the human prostate, several studies have revealed that prostatic basal cells can differentiate into luminal cells in vitro [29, 30]. Basal epithelial cells, isolated on the basis of high surface expression of $\alpha_2\beta_1$-integrin, are clonogenic in vitro [31, 32] and have the potential to regenerate a fully differentiated human prostate epithelium

in vivo [31]. Use of the CD133 antigen, which was first identified as a marker for human hematopoietic stem cells [33], further enriched for the stem cell population [34]. The cells expressing CD133 are restricted to the $\alpha_2\beta_1^{hi}$ population and are located within the basal layer. Richardson and colleagues showed that these $\alpha_2\beta_1^{hi}$/CD133$^+$ cells had a greater colony-forming ability and proliferative potential in vitro than $\alpha_2\beta_1^{hi}$/CD133$^-$ cells. Moreover, when grafted together with prostate stromal cells into nude mice (which is necessary to produce a functional and morphological differentiated prostate [35]), $\alpha_2\beta_1^{hi}$/CD133$^+$ cells generated prostatic acini, unlike the $\alpha_2\beta_1^{hi}$/CD133$^-$ cells [34].

The identification and characterization of stem cells in the normal prostate is important, because they may represent a major target for carcinogenesis as well as a potential source of BPH [36]. It was hypothesized in the 1960s that cancers exist in a hierarchy consisting of cells with different proliferative potentials [37, 38]. The cancer stem cell (CSC) hypothesis presumes that the bulk population of cancerous cells arise from CSCs [39], defined as a rare population of cells that maintain the rest of the population. Normal stem cells and CSCs have shared properties, such as the capacity to self-renew and differentiate to give rise to multi-cellular lineages [40]. These properties are important for CSCs to maintain and spread the tumor.

6.3 Prostate Cancer

Prostate cancer is a major health problem as it is the most commonly diagnosed cancer in men in the Western world. It is mainly detected in men after the age of 50, at which time one in three men suffer from symptoms related to BPH [41]. Prostate cancer is thought to arise from high-grade prostate intraepithelial neoplastic lesions (PIN) [42, 43], although proliferative inflammatory atrophy (PIA) may also play a role [44]. Certain environmental factors, such as diet, are thought to have a role in the development of prostate cancer [45]. Risk increases with age, as the largest number of cases are diagnosed within the age range of 72–74, but ethnicity and family history are also thought to play a role in the development of the disease [46]. The most common diagnostic test to detect prostate cancer is blood prostate-specific antigen (PSA) levels, as PSA increases with prostate cancer, but can also rise with non-malignant growth of the prostate [47]. However, to complete the diagnosis of prostate cancer, a biopsy is necessary to assess histology using the Gleason grading system [48].

When the cancer is confined to the prostate gland, the disease can be treated with surgery, radiation therapy (brachytherapy), or cryotherapy [49]. For elderly men who have no symptoms at diagnosis, and have a relatively short life expectancy, symptoms are controlled as they occur; this is called “active surveillance” or “watchful waiting” [50]. For patients with metastatic prostate cancer, the widely used treatment remains androgen ablation therapy, as homeostasis of the prostate gland is dependent upon androgens [51, 52]. Androgen ablation reduces tumor growth but ultimately, in most cases, the therapy fails and the prostate cells become castrate resistant [53]. That the tumor initially responds well to androgen ablation

therapy is not surprising, as the main cell type found within prostate carcinomas is the AR-positive secretory luminal cell [54]. These cells express the AR and will be sensitive to the therapy. Treatment failure can be explained by the presence of tumor-initiating cells that are independent of androgens for their survival [55]. CSCs, isolated from patient samples, were found to be AR-negative [55]. It has also been suggested that cancer-initiating cells are more resistant to radiation [56] and chemotherapy [57]. These studies suggest that the cancer-initiating cells, also known as CSCs, are not affected by conventional therapies and therefore can be the cause of recurrence and/or spread of the tumor. Therefore, it is important to develop new, more effective therapies that will specifically target this population.

6.4 Prostate Cancer Stem Cells

The origin of prostate CSCs is still controversial. As prostate cancer mainly consists of luminal cells [54], it has been the prevailing view that the AR-expressing luminal cells are the tumor-initiating cells [reviewed in 58]. The observation that telomerase is expressed within the luminal compartment in high-grade PIN, thus extending the lifespan of these cells, has added weight to this proposal [59]. Others have suggested that an intermediate cell, which expresses both basal and luminal keratin markers, could give rise to prostate cancer [18]. However, it is more plausible that normal tissue stem cells are the targets for transformation given their longevity. This has been definitively demonstrated by Bonnet and Dick [60] who showed that the tumor-initiating cells in acute myeloid leukemia (AML) shared cell surface markers with normal hematopoietic stem cells. More recently, Barker and colleagues showed that crypt stem cells are the cells of origin of intestinal cancer [61]. There are several lines of evidence that support the proposal that prostate CSCs arise from normal stem cells. Metastases often include rare cells that are phenotypically undifferentiated, expressing basal cell markers, such as cytokeratins 5 and 14 [62, 63]. Advanced prostate cancers can respond to low levels of androgens, but the castrate-resistant state results from clonal expansion of androgen-independent cells that are present at a frequency of 1 per 10^5–10^6 androgen-responsive cells [64].

CSCs share numerous markers with normal stem cells. More recent work from our laboratory compared isolated populations, from primary prostate cancers, for clonogenic potential. We found that only the most primitive cells ($CD44^+/CD133^+/\alpha_2\beta_1^{hi}$), which were identical phenotypically to normal prostate stem cells, could self-renew in vitro [55]. Moreover, under differentiating conditions, $AR^+/PAP^+/CK18^+$ luminal cells could be identified in these cultures, suggesting that they were derived from the more primitive population. In support of this finding, the $CD44^+$ population from xenograft tumors and cell lines has enhanced proliferative potential and tumor-initiating ability in vivo compared to $CD44^-$ cells [65]. The $CD44^+$ cells are likewise AR^- and express higher mRNA levels of stemness genes, such as OCT3/4 and BMI 1. Using clonally derived human prostate cancer epithelial cells expressing human telomerase reverse transcriptase (hTERT), Gu and co-workers [66] demonstrated that these lines

could regenerate tumors in mice that resembled the original patient tumor with respect to Gleason score. The tumors contained luminal, basal, and neuroendocrine cells, implying that the clone of origin could differentiate into the epithelial cell lineages of the prostate. In this case, the tumor-initiating cell was AR^- and $p63^-$ and expressed the stem cell genes Oct-4, Nanog, Sox2, nestin, CD44, CD133, and c-kit. Moreover, Sca-1 sorted cells, enriched for cells with prostate-regenerating activity, showed evidence of basal and luminal lineage.

A recurrent genomic alteration in prostate cancer is the expression of *TMPRSS2-ETS* fusion genes [67], with *TMPRSS2-ERG* being the most frequently detected [68]. The presence of the fusion is associated with PSA biochemical failure [68] and occurs with a frequency of approximately 50% [67]. Identification of the *TMPRSS2-ETS* fusion gene in approximately 20% of PIN lesions suggests that it is an early event in prostate tumorigenesis [69] and our recent findings that *TMPRSS2-ERG* is expressed in $\alpha_2\beta_1^{hi}/CD133^+$ cells from prostate tumors [70] supports the hypothesis that the cell of origin of prostate cancer is a stem cell.

Gene expression studies on populations of prostate CSCs were carried out by Birnie et al. [70]. The resulting gene signature provided clear evidence of different gene sets expressed in the CSCs, their amplifying progeny, and their normal equivalents. Functional annotation of the CSC signature led to the identification of four main pathways: (1) JAK-STAT signaling; (2) cell adhesion and extracellular matrix interaction; (3) focal adhesion signaling, and (4) Wnt signaling. Verification that the cultures used in this study were tumorigenic came from the identification of the TMPRSS2-ERG translocation [67, 70].

6.5 Molecular Mechanisms Regulating Prostate Cancer Stem Cells

Similar pathways are involved in maintaining tumors and stem cells. For example, Wnt [71] and JAK-STAT signaling [72, 73] have been linked to stemness and malignancy, as have epigenetic mechanisms.

6.5.1 Wnt/β-Catenin Signaling

The Wnt/β-catenin signaling pathway plays an important role in multiple developmental events during embryogenesis, but it has also been implicated in adult tissue homeostasis [reviewed in 74] and cancer [75]. Wnt signaling can induce cell proliferation and self-renewal of adult hematopoietic stem cells [76], and in the intestinal epithelium it is important for the maintenance of stem cells [77]. Mutations that lead to constitutively active Wnt signaling are implicated in prostate cancer, where mutations of β-catenin are the most frequent [78, 79]. β-catenin increases AR transcription in a ligand-dependent manner [80], and it has been suggested that

there is a crosstalk between Wnt and androgen signaling in prostate cancer [81]. Yang et al. suggested that excessive free β-catenin, which occurs during prostate cancer progression, might maintain or even increase AR activity when androgen levels are low [81].

6.5.2 JAK-STAT Signaling

Normally, the human body only produces high levels of IL-6 as part of an inflammatory response. However, IL-6 is also elevated in the serum of patients with metastatic prostate cancer [82] and it acts as a positive regulator of prostate cancer cell growth [83]. It has also been shown that STAT3 is constitutively activated in prostate cancer tissue and high levels of STAT3 activation are associated with higher Gleason grade tumors [72]. Interestingly, activation of STAT3 is also important for maintenance of stem cell self-renewal and the undifferentiated state of embryonic stem cells [73], as well as glioblastoma stem cells [84]. Preliminary data from our group indicate that prostate CSCs secrete more IL-6 compared to the progenitor population and receptor levels are highest within the CSCs (unpublished data). Further investigation will determine whether the JAK-STAT signaling pathway is indeed important for maintaining (cancer) stemness.

6.5.3 Epigenetic Deregulation of Prostate Cancer Hierarchy

Epigenetic alteration of stem cells has been hypothesized to have an important role in prostate tumorigenesis; being involved both in the formation of prostate CSCs and their therapy resistant features [85, 86]. However, no direct study has confirmed epigenetic deregulation of prostate CSCs.

Epigenetic regulation of gene expression is defined as a heritable change in gene expression that does not involve changes in the DNA sequence [87] and includes DNA methylation, chromatin structure (mainly determined by histone posttranslational modifications) and small non-coding RNAs. Epigenetic mechanisms are both heritable and dynamic, allowing for fine regulation of gene expression throughout all the different cell types [88]. This regulation plays a crucial role in the maintenance of the hierarchical structure of tissues, being involved in both maintenance of stemness and fate determination of stem cells [89–93]. Moreover, compounds that inhibit DNA methylation or histone deacetylation can induce cell differentiation [92, 94]. In the prostate, the key epigenetic mechanisms responsible for the maintenance of hierarchy have not yet been identified. However, studies have been performed to identify genes that are differentially expressed in prostate stem and committed cells [70, 95], from which crucial epigenetic pathways can be identified for further study. Moreover, it is known that histone deacetylase inhibitors can induce differentiation to a neuroendocrine phenotype in prostate cells [96, 97], emphasizing the importance of epigenetic mechanisms in the maintenance of lineage determination.

Disruption of epigenetic mechanisms has been found in all cancer types and, together with genetic changes, plays a key role in cancer initiation and progression [98]. In the last few years, much effort has been put into defining the epigenetic alterations present in prostate cancer. This led to the identification of hundreds of hypermethylated genes, of which GSTP1 is the most studied [99], and alteration of chromatin structure through alteration of many histone-modifying enzymes and chromatin-associated proteins. In fact, global patterns of histone modification are linked to the risk of prostate cancer recurrence [100, 101]. However, these studies do not take into account the hierarchical structure present in cancer. In fact, the models typically used are cell lines adapted to culture conditions with crucial modification of epigenetic regulation [102] or tissues considered as a homogeneous population.

As previously discussed, epigenetic mechanisms are crucial for the maintenance of the correct hierarchical structure in normal tissues, suggesting that self-renewal and multipotency are, at least partially, under the control of epigenetic regulation. It has been proposed that disruption of this control may result in formation of self-renewing malignant stem cells [103], generating a deregulation of the hierarchical system, which ultimately leads to cancer [86]. Little is known about epigenetic deregulation of hierarchical structure in prostate cancer, but it undoubtedly plays a crucial role in prostate cancer development. Prostate cancer is characterized by an expansion of the luminal compartment [54], with a clear imbalance of the differentiation process that leads to the accumulation of aberrantly differentiated luminal cancer cells. Interestingly, this process is accompanied by epigenetic deregulation of genes that are usually expressed only in more undifferentiated cells. GSTP1 is only expressed in the basal compartment of normal prostate [104] and it is frequently downregulated by hypermethylation in prostate cancer [99]. Moreover, CD44, one of the molecules used as a marker to enrich for both benign and malignant prostate basal cells [58], is also downregulated by hypermethylation in the majority of prostate cancers [105]. These studies suggest that deregulation of epigenetic control is accompanying, or even driving, the expansion of the luminal compartment in prostate cancer, clearly indicating epigenetic deregulation of the hierarchy in prostate cancer. With the ability to now isolate different cell types, including prostate CSCs, it will be possible to elucidate the role of these epigenetic mechanisms in a cell-specific manner.

6.6 Prostate CSCs and Therapy Resistance

Existing therapies such as androgen ablation or radiation have been successful in reducing the bulk of cells within prostate tumors. These forms of therapies target the AR-expressing population and proliferating cells, respectively, but in most cases the tumors recur, which suggests that the tumor-initiating cells are a reservoir for recurrent disease following therapy, as they are more resistant to therapies currently used [58].

In the last decade, there has been an explosion in the number of papers published on CSCs. We are now reaching a consensus that CSCs must be taken into

consideration when designing therapies, particularly with tumors that are prone to relapse [57, 106–110]. However, before targeting prostate CSCs specifically, there needs to be proof that they are resistant to current therapies and preferably elucidation of the mechanism of resistance. Ultimately, we have to design diagnostic test(s) to determine whether, when the CSC component of a tumor is eliminated, there is a resulting tumor eradication or cure [111, 112].

Very little is known about therapy resistance of prostate CSCs, and most studies have been based on the use of cancer cell lines and tissue sections. However, increasing numbers of studies are now using primary epithelial cells from patient samples, a focus of which is the response of prostate cells to DNA damage caused by radiotherapy or chemotherapeutic agents. In terms of DNA repair, homologous recombination (HR), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) have all been examined. There are reduced levels of MMR proteins, including hMLH1 and hMSH2, in various prostate cancer cell lines [113]. More significantly, there are defects in MMR in prostate tumor foci as indicated by the absence of PMS1 and PMS2 proteins [114]. In contrast, another study found increased levels of PMS2 in recurrent prostate cancer patients and suggested this to be of use as a marker with prognostic potential [115]. Increased expression of this protein has been associated with increased mutation frequency and resistance to apoptosis. We can take from these studies that an increase or decrease in repair proteins has the potential to cause mutations that may be involved in tumor progression.

Combining radiotherapy with inhibitors of DNA repair has been explored by Bristow et al. [116]. Mutations in BRCA1 and BRCA2, key proteins in the double-strand break (DSB) response, are found in familial prostate cancers [117, 118]. Cells with these mutations are defective in DSB repair and are more sensitive to radiation [119]. Other DSB response proteins such as ATM (whose expression is increased in prostate tumors) and p53 are frequently mutated in prostate cancer [120, 121]. Mutations of Chk2 have also been observed [117]. These proteins are involved in cell cycle checkpoints, abrogation of which can lead to radioresistance and metastasis [116]. An altered BER pathway and response to oxidative stress have also been implicated in prostate cancer [114, 122–124]. With the study of CSCs, the prostate field can follow the lead of other fields. $CD133^{+}$ cells from hepatocellular carcinoma are resistant to doxorubicin and fluorouracil, which is due to expression of bcl-2, Akt, and PKB; components of an anti-apoptotic survival pathway [125]. Glioma stem cells are resistant to chemotherapeutic agents [126] and have increased activation of DNA damage checkpoints and more efficient DNA repair in response to irradiation, with inhibition of Chk1 and Chk2 kinase restoring radiosensitivity [127].

These studies on cell lines and whole populations of primary epithelial cells can be used as a basis for studies on prostate CSCs, as we now have the ability to isolate these cells and analyze their response, which is likely to be significantly different to the more differentiated cells. Ultimately, in cancer cells, there is an upset in control of DNA repair and cell cycle checkpoints, and depending on the mutation the cells may be either more sensitive to treatment or more resistant to treatment. Therefore, it is imperative to explore the specific response of prostate CSCs to

different treatments, in order to manipulate therapy. This would allow for prediction of success of certain therapies and also for manipulation of treatments to exploit defects in the prostate CSCs.

6.7 Conclusions and Future Perspectives

The identification and isolation of prostate CSCs was a major breakthrough in the understanding of prostate cancer progression and relapse following therapy [55]. To be able to study prostate CSCs is very important and increases hope of identifying a prostate CSC-specific target, but it is also very challenging. The main reason for this is that this population is very rare within the bulk of tumor cells (0.01%), and therefore only a few techniques are suitable for studying such a small cell number. However, we are able to expand these cells in culture, which can be used to study and have a better understanding of these prostate CSCs. Ultimately, it would be desirable to have a treatment specifically for the prostate CSCs that could be used in combination with androgen ablation to reduce tumor mass [62]. This will require further development of primary epithelial cell culture models and assays to detect stem cell-specific targeting. Novel treatments could include DNA repair inhibitors, inhibitors of anti-apoptotic proteins, and inhibitors of ABC transporters. With all these options, minimizing toxicity and maximizing patient benefit would be paramount. Ultimately, if the CSCs are responsible for the recurrence following therapy and metastasis, then their elimination is the best route to a longer lasting, or even permanent cure.

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Chapter 7
Cancer Stem Cells in Melanoma

Ping Jin, Qiuzhen Liu, Marianna Sabatino, David F. Stroncek, Francesco M. Marincola, and Ena Wang

Abstract Malignant melanoma is a significant health problem worldwide. Disease relapse due to the heterogeneity and instability of cancer cells may explain the persistence of disease in spite of primary response to therapy. Recent progress in cancer research suggests that melanomas, similar to other solid tumors, contain a subpopulation of cells which have unlimited self-renewal capability directly descending from the original founder cell and characterized by relatively stable genetic properties throughout disease evolution. This model also applies to the development of metastasis and may be responsible for drug resistance and cancer recurrence. These cells with tumor-initiating ability are termed cancer stem cells (CSCs). CSCs as well as tumor cells interact with their microenvironment (niche) to modulate the malignant phenotype. This chapter provides an overview of melanoma stem cell characterization and the interactions between melanoma stem cells and their niche.

Abbreviations

ABC	ATP-binding cassette
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
BRAF	B-Raf proto-oncogene serine/threonine-protein kinase
CD	Cluster of differentiation
CMC	Circulating melanoma cell

E. Wang (✉)
Infectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine, Clinical Center, National Institutes of Health,
10 Center Drive, Bethesda, MD 20892-1184, USA
e-mail: ewang@mail.cc.nih.gov

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CSC	Cancer stem cell
CXCR4	Chemokine receptor 4
DTC	Disseminated tumor cell
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
GAP-43	Growth-associated protein-43
GFP	Green fluorescent protein
HER2	Human epidermal growth factor receptor 2
HSC	Hematopoietic stem cell
hTERT	Human telomerase reverse transcriptase
IGF	Insulin-like growth factor
IL	Interleukin
MAGE	Melanoma antigen gene
MART1	Melanoma antigen recognized by T-cells
MCAM	Melanoma cell adhesion molecule
MCP-1	Monocyte chemoattractant protein-1
MEK	Map kinase kinase
MITF	Microphthalmia-associated transcription factor
MLL	Mixed lineage leukemia
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
NCAM	Neural cell adhesion molecule
NES	Nestin
NK	Natural killer
NOD/SCID	Non-obese diabetic/Severe combined immune deficiency
PDGF	Platelet-derived growth factor
PTEN	Phosphatase and tensin homolog
RANTES	Regulated on activation normal T cell expressed and secreted
SCA	Sphere cell formation assay
SCF	Stem cell factor
SDF	Stromal derived factor
SP	Side population
SSEA	Stage-specific embryonic antigen
TA	Transit amplifying
TGF-β	Transforming growth factor beta
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
VEGFR	Vascular endothelial growth factor receptor
VWF	von Willebrand factor

7.1 Introduction

Malignant melanoma is a disease with a very poor survival rate. Its incidence has increased 3–7% on average over several decades. In the US, the lifetime risk of melanoma in the year 2000 was estimated at 1 in 75 persons [1]. Patients with advanced disease have a poor prognosis with a reported median survival ranging between 3 and 11 months. Biological therapies including immune therapy with systemic administration of high-dose interleukin-2, interferon-alpha, antigen-specific immunization, and chemotherapy with dacarbazine or temozolomide can induce objective tumor regression in only 5–20% of patients [2, 3]. Adoptive transfer of autologous tumor-infiltrating lymphocytes following myeloid lympho-ablation has been reported to induce objective tumor regression in approximately 60% of patients [4–6]. However, these responses do not result, in most cases, in an overall survival benefit as the large majority of patients die with relapsing disease that is often resistant to further therapy.

It has been hypothesized that the stubborn recurrence of cancer following a primary response to treatment is due to the survival of a subset of cancer cells that display an intrinsic resistance to treatment-induced cell death [7, 8]. The existence of cancer stem cells (CSCs), characterized by a less differentiated status, lower immunogenicity and resistance to immune rejection [9] may be the source of cancer relapse and resistance to therapy [8, 10]. It is important to note that the term CSCs is more of a functional definition created to define a subgroup of cancer cells which can self-renew, initiate tumors, and differentiate into a heterogeneous progeny that partially maintains similarity to the original tissue from which they derived. Different from normal stem cells, CSCs share the accumulated genetic instability responsible for cancer development and acquire the genetic alterations required to promote the malignant process. As a result of the genetic and epigenetic changes, different subsets of cancer-initiating cells can be identified [11]. The concept of CSCs and the hierarchical model of tumorigenesis have implications that may help advance the understanding of tumor biology and the development of more effective anti-cancer treatments. Thus, the development of cancer therapy has expanded from targeting a population of cells derived from a stochastic model of chance variation to targeting cells transformed after a single or few random mutations followed by subsequent clonal selection and perpetuation. In this chapter, we review the characterization of melanoma CSCs and their biology as well as the interaction between melanoma and their microenvironment/niche.

7.2 Melanoma Genesis

Stem cells of the melanocytic lineage derive from the neural crest and migrate to the hair follicle or the basal layer of the epidermis during embryonic development. There, they remain in a quiescent state or asymmetrically divide when needed, with one

remaining a steady-state stem cell while the other one becomes a transit amplifying (TA) cell that proliferates and eventually results in a progeny of differentiated melanocytes. This asymmetrical proliferation property is a unique biological characteristic for stem cells and is the main mechanism of homeostasis and tissue repair. TA melanocytes further differentiate into pigmented melanocytes which are interspersed among keratinocytes at a constant ratio of approximately 1:35, forming an "epidermal melanin unit" [12]. TA melanocytes maintain a partial self-renewal capability and can return to a quiescent state in the hair bulge area if the original stem cells are missing. Although they share similar properties, TA cells are different from the originating stem cells [13]. In contrast to TA cells, melanocyte stem cells globally suppress transcription, including that of melanocytic genes, but express totipotent embryonic stem cell markers (i.e., nestin, slug, snail, twist, sow-9, bmp4, Nanog, and Oct4), which are less consistently expressed by TA cells [12, 14, 15]. Furthermore, melanocyte stem cells can differentiate in appropriate conditions not only into melanocytes but also into neuronal and smooth muscle cells, thus demonstrating their potential plasticity.

Cancer derives from the accumulation of genetic and epigenetic alterations. Mutations of critical growth regulatory genes contribute to its initiation and progression [16, 17]. Ras/Raf/MEK/ERK signaling is one of the most critical signaling pathways for melanoma proliferation, and hyper-activation of ERK is found in 90% of melanomas. *BRAF* mutations are found in 50–70% of melanomas and drive ERK signaling activation. Besides this common initiation mechanism, the transforming cell needs to accumulate other genetic and epigenetic changes to develop its full malignant potential, and this process may take years or even decades. There are two main models to explain how transformed cells retain their genetic code while at the same time sequentially accumulating further genetic mutations that could be relevant or irrelevant to their survival: one is a long-term survival of the founder(s) cell, and another is the continuous passage of genetic alterations through serial cell divisions that proceed vertically generation by generation. Because of their intrinsic long-term survival in the host and ability to generate a progeny, melanocyte adult stem cells and TA melanocytes are the critical target cells for melanoma development since adult melanocytes are less likely to survival long enough to accumulate the required repertoire of genetic alterations for a full-fledge transformation [18]. Mutated melanocyte stem cells transform, therefore, into melanoma stem cells and pass their self-renewal capacity to transformed stem cells [18].

CSCs derived from normal stem cells would be expected to bear markers similar to those borne by the latter, whereas CSCs derived from differentiated cells might have differentiation markers. In fact, CSCs identified in several kind of cancers share several phenotypic characteristics with their normal counterparts [19], and mouse leukemias induced by the fusion gene products MLL-AF9 and MOA-TIR2 contain leukemogenic cells with a phenotype closer to differentiated hematopoietic cells than HSCs [20]. A subtype of human acute myeloid leukemia cells that carry the hematopoietic stem cell phenotype CD34+CD38− can initiate the disease when engrafted in SCID mice [21]. However, this may not always be the case. In mouse models, mammary CSCs display lower expression of CD29 compared to normal mouse mammary stem cells [22]. As we will see later, in melanoma, this question remains open.

It has been suggested that reversal of epigenetic changes and genetic alterations could allow terminally differentiated cells to dedifferentiate back into stem cells. It has been documented that melanocytic quail cells can dedifferentiate into multipotent stem cells [23]. Furthermore, cultured differentiated normal melanocytes can be transformed into melanoma stem cells by introducing oncogenes [24, 25]. However, the question remains as to whether those populations of differentiated melanocytes contain a small percentage of normal melanocytic stem cells or TAs that could account for their plasticity. The clarification of this point is difficult, since the cancer genome may contain genetic patterns characterized by sporadic genetic alterations which do not necessarily contribute to malignant transformation and are due instead to stochastic accumulation of mutations related to the genetic instability of cancer cells. These "irrelevant" genetic patterns confuse the understanding of progression according to the CSC hypothesis, since it is difficult to sort variable phenotypes resulting from random occurrences from an orderly progression. Genomic and functional genomic analysis of metachronous melanoma metastases from a single patient, who underwent repeated treatments and experienced several recurrences over a decade, demonstrated that all metastases shared a unique genetic pattern derived from the original progenitor cell, while each metastasis displayed unique genetic alternations which appeared and disappeared in time without following a sequential pattern [26, 27]. Thus, only a small proportion of the genetic (and consequently) cellular make up of cancer is due to relevant alterations driving its malignant behavior but such specific mutational drivers may be difficult to identify unless the long-term progression of a disease can be followed.

It may be that the driving genetics of cancer are regulated by key transcription factors that control the pluripotent state [28, 29]. Mouse and human somatic cells can be reprogrammed to a pluripotent-like state by ectopic expression of various proteins such as OCT4, SOX2, KLF4 and c-MYC, NANOG, and LIN28, and sometimes only a combination of two such as Oct4 and Sox2 can be sufficient [30–35]. Yet, successful reprogramming may include sequential epigenetic alterations in culture similar to those that accumulate during normal stem cell development. Thus, both genetic and epigenetic changes are essential to the development of melanoma and the discrimination between a pure normal stem cell and a CSC compared to pluripotent-like phenotypes of differentiated cells may be difficult to completely define as they overlap in a continuum spectrum of genetic and epigenetic alterations of hierarchically decreasing relevance.

7.3 Melanoma Stem Cell Markers and Their Limitations

In many cases, CSC marker profiles are similar to those of their normal counterpart stem cells. For example, both human mammary stem cells and mammary CSCs lack CD24 expression [19, 36, 37]. Similarly, human acute myeloid leukemia stem cells and normal hematopoietic stem cells are enriched in the CD34+CD38− fraction of the bone marrow [21]. However, the markers of normal melanocyte stem cells have

not yet been identified, and therefore the markers for melanoma stem cells are deduced according to knowledge about common stem cell markers and common methods for identifying CSCs that are used in other cancer systems (i.e., sphere cell formation assays (SCA), cancer initiation properties, and side population (SP) identification using Hoechst 33342 stain) [38]. As we will see later, the adoption of neuronal crest markers may have led to the identification of CD271 as a useful melanoma stem cell marker [39].

Biomarker analysis using the SCA assay has demonstrated that melanoma spheres are negative for embryonic, endothelial, neural, and hematopoietic stem cells markers, such as SSEA-3, TRA-1-80, TRA-1-60, vWF, CD31, CD34, and VEGFR2, GAP-43, CD56/NCAM, and CD3, 4, 8, and 45; and positive for melanoma-associated markers such as MCAM, Sox10 and MITF [40]. This study also found that melanoma spheres are enriched for CD20+ positive cells. Since CD20 is present in 20% of human melanoma specimens, it is possible that this marker represents a subpopulation of melanoma-initiating cells. Na et al. [41] found that melanoma sphere cells from WM-266-4, a highly metastatic melanoma cell line, expressed stem cell markers such as ABCG2, Bmi1, WNT5A, CD133, Nestin, SCF, prox1, and VEGFR3. However, they could not demonstrate different tumorigenicity between WM-266-4 sphere-forming cells and the non-sphere counterparts, potentially because the WM-266-4 cell line is characterized by inherently high tumorigenicity.

7.4 Tumorigenic Potential of Melanoma Stem Cells

Because of the absence of credible markers that identify melanoma stem cells, testing their tumor-initiating capability has become the ultimate technique to demonstrate their most important characteristic: the ability to efficiently self-renew. Dou et al. identified a subpopulation (SP) of cells from B16F10 mouse melanoma cells with high expression of CD44+CD133+CD24+ that possess stronger tumorigenic potential in C57BL/6 mice compared to non-SP B16F10. Melanoma formed in 7 out of 8 mice injected with 3×10^4 SP- B16F10, while only 3 in 8 mice formed melanoma in the non-SP-B16F10 group [42].

Monzani et al. [43] demonstrated that a distinct subset of CD133+ cells existed in seven human melanoma specimens, which ranged in frequency from 0.2 to 0.8%. By injecting one NOD-SCID mouse with 1×10^5 CD133+ melanoma cells on one side and the same number of CD133− melanoma cells in other, they found that tumor occurred only in the CD133+ injected side. To further study CD133+ melanoma cell tumorigenicity, they investigated the WM115 melanoma cell line (which is 100% positive for CD133+ cells), and found that WM155 possess many properties of stem cells, such as expression of neurogenic markers and ability to differentiate into various mesenchymal lineages as adiposities. Moreover, WM155 cells could grow as spheres in serum-free media. More importantly, when injected in immunodeficient mice, they formed tumors which included a progeny of differentiated CD133− cells.

CD133 is a common marker for normal stem cells and some CSCs [44], and has also been used as a marker for melanoma stem cell identification. CD133+ melanoma cells not only have enhanced tumorigenic potential in mice but also express higher levels of angiogenic and lymphangiogenic genes which are related to melanoma initiation and metastasis [43]. Klein et al. [45] observed that CD133+ melanoma cells overexpress CD166 and nestin compared to melanocytic cells in nevi. On the other hand, the multi-drug resistance gene, a member of the ABC transporter family, has been reported to be enriched in melanoma sphere cells, which represent 1.3–9.7% of the entire melanoma population [46]. The multi-drug resistance-expressing cells also express stem cell markers such as ABCB5, Nanog, and hTERT, but are negative for CD133. Schatton et al. [47] suggested that ABCB5, an ABC transporter that mediates doxorubicin drug resistance in cancer, is a melanoma stem cell marker and showed that its expression correlates with clinical progression of melanoma. This marker was expressed by 1.6–20.4% cells in melanoma specimens, and cells bearing this marker were more effective in initiating tumors in immune deficient mice. ABCB5+ or ABCB5− melanoma cells isolated from patients displayed significantly different levels of tumorigenicity; 14/23 mice formed tumors when ABCB+ cells were injected compared to only 1/23 mice in ABCB5− group. ABCB5+ cell-derived xenografts re-established tumor heterogeneity and contained both ABCG5+ and ABCG5− progenies. The tumorigenic competence of ABCG5+ cells could be inhibited by anti-ABCB5 antibody. Histologically, ABCB5+ cells correlated with non-pigmented, undifferentiated regions of human samples, whereas pigmentation was more frequent in areas where ABCB5− cells were more abundant. ABCB5+ cells also expressed other melanoma progression-related markers, such as TIE1, CD144, CD133, and BMPR1. However, when purified, the ABCB5+ population did not lead invariably to tumor formation. This suggests that not every ABCB5+ cell represents a melanoma stem cell, and other factors may be necessary to achieve the complete stem cell phenotype, although ABCB5 may represent an essential component of the melanoma stem cell repertoire. This association between expression of multi-drug resistance-associated genes and melanoma stem cells may represent a useful marker for targeted therapy, and may have significant implications regarding their responsiveness to therapy [8].

Recently, Boiko et al. [39] reported that melanoma stem cells can be isolated prospectively according to the expression of the biomarker CD271, which has been used successfully to sort neural crest stem cells. It was observed that sorted CD271+ melanoma cells that were re-suspended in matrigel and implanted into T-, B-, and nature killer-deficient $Rag2^{-/-}$ $\gamma c^{-/-}$ mice resulted in xenograft tumor formation in 90% of injection sites, while CD271− subsets did not. CD271+ melanoma cells lacked expression of the melanoma differentiation antigens TYR, MART1, and MAGE, which may partially explain the ineffectiveness or brief responses of antigen-specific T-cell therapies. To date this is the most convincing characterization of melanoma stem cells and it may be used in the future to further analyze subcategories of melanoma and their responsiveness to treatment.

Quintana et al. [48] significantly reduced the number of cells needed for xenograft initiation by improving the conditions favoring engraftment. They used highly

immune compromised NOD/SCID IL-2Rγ−/− mice and infused cells within a matrigel that favored the growth of melanoma cells. With this model, they demonstrated that melanoma cells co-injected with matrigel grow faster than when injected alone. Moreover, they did not observe a substantial difference in tumorigenicity between cells bearing or lacking stem cell markers such as CD133, CD166, CD20, ABCG5. In fact, they were unable to identify any marker characteristic of melanoma-initiating ability. This study demonstrated that the number of cells needed to propagate melanoma is determined to a great extent by the environment in which cells are placed and not the frequency of CSCs. Moreover, this study suggests that any single cell in a melanoma population can form xenografts and, therefore, tumorigenic cells might be more common in melanoma than previously believed. This finding also shows that unlimited proliferation is an intrinsic property of all cancer cells and each cell maintains similar growth kinetics in favorable environmental conditions. Overall, this study questions some of the methods used to study CSCs/melanoma stem cells, and suggests that a bias may be imposed in the characterization of self-renewal properties by providing an environment that may not be representative of the natural conditions in human subjects. It suggests that one of the most important components for promoting tumor initiation and perhaps facilitating tumor metastasis is a favorable microenvironment or niche.

7.5 Plasticity of Melanoma Stem Cells

One reason that may account for the large variation of melanoma stem cell frequency in different studies might be due to their plasticity in switching phenotypes under different conditions. It has been observed that pathological confirmed melanoma can redirect its differentiation into chondrosarcoma [49] and melanoma derived from neuroglioma (unpublished clinical data). Highly aggressive melanoma cells have molecular signatures that are reminiscent of pluripotent stem cells [50, 51]. It has been reported that melanoma cells can switch reversibly between more and less pigmented states [52], and inter-conversion between proliferative and invasive states has been reported in primary tumors [53]. Inter-conversion has recently been reported between tumorigenic and non-tumorigenic cells in vivo using intra-vital imaging methods combined with a reporter construct [54]. A subpopulation of cells containing little or no pigment and high levels of Brn2:GFP expression have been shown to metastasize to secondary sites and lose the characteristics of the primary tumor, implying switching between states as melanoma cells metastasize. Pinner et al. also demonstrated that melanoma cells can switch in both directions between high- and low-pigment states. Therefore, a cell which is non-tumorigenic in one context could be tumorigenic in another context. The majority of melanoma cells might be in a state of TA and share some degree of self-renewal potential. They can, however, be easily dedifferentiated back to a melanoma stem cell state in favorable environmental conditions [13]. Held et al. [55] identified three subsets of melanoma cells in three different

melanoma mouse models that could be divided by surface markers as well as function: a CD34−p75− subset representing stem cells, a CD34+p75− subset representing TA cells (called “intermediate cells” by these investigators), and a CD34−p75+ subset representing differentiated cells. Tumor formation occurred at a high rate when CD34+p75− melanoma cells were injected, while intermediate and low rates of growth were observed when CD34−p75− or CD34−p75+ cells were, respectively, injected. Interestingly, individual xenografts derived from CD34−p75− cells (TA cells) recaptured cellular heterogeneity, whereas CD34+p75− melanoma stem cells underwent self-renewal only and remained homogeneous. This study suggests that TA cells can reverse to the melanoma stem cell state, and that tumor formation is not initiated by a single subset of CSCs.

The plasticity and TA dedifferentiation of melanoma cells may also contribute to the variation in melanoma stem cell biomarkers. Melanoma cells cultured in vitro are heterogeneous even when derived from a single cell expansion [56].Therefore, a great degree of heterogeneity exists in long-term dense cultures that may confuse the detection of the conversion between melanoma stem cells into TA cells and vice versa. It will be important to evaluate the stability of the immune phenotype in CSCs (and melanoma stem cells) over time to develop more confidence in the significance of their marker expression as stable predictors of self-renewal capacity among a continuously evolving and chaotic cancer cell population. If some markers prove to be transiently expressed, prospective isolation of CSCs will be an approach of limited validity.

7.6 Metastasis and Cancer Stem Cells

Metastasis is a fundamental characteristic of cancer. However, the targeted organ of cancer in some degree depends on the cancer tissue of origin. Not every cell in a tumor has the ability to metastasize to other organs. Similarly, the majority of circulating tumor cells are incapable of forming metastases, and it is possible that only CSCs can give rise to metastases. Using a combination of in vivo video-microscopy and immunohistochemical staining, it was observed that 80% of intraportally injected B16F1 melanoma cells can survive and extravasate by day 3, but only one out of 40 survived cells formed micrometastases and 1 in 100 micrometastases continued to grow into macrometastases [57]. Expression analysis of the stem cell markers nestin (NES) and CD133 on circulating melanoma cells (CMC) revealed that there are less than 1% of CMCs double positive for CD133 and NES. However, NES-positive cells represent 18% of the CMC (median percentage) independent of the absolute number of CMC, and are significantly correlated with tumor burden and number of metastases [58]. This suggests that cancer metastatic capability depends not only on multiple factors involving tumor cell growth, survival, angiogenesis, and invasion but also (and most importantly) on the microenvironment at an ectopic site, which is crucial for efficient tumor cell proliferation.

It has been hypothesized that primary tumors may influence the development of a niche even before they metastasize [59, 60]. Gene expression analysis of ovarian cancer revealed that histological normal sub-peritonea stromal tissues in ovarian cancer patients share the same gene expression signature as the cancer itself, suggesting that the stroma may facilitate regional spread of ovarian cancer [60]. Notwithstanding ectopic site contribution to metastasis, it has been reported that cancer cells may improve the efficiency of metastasis formation by recruiting mesenchymal and endothelial cells from the bone marrow, the niche for hematopoietic stem cells [61–63]. Moreover, fibroblasts cooperate actively in cancer development and progression within the niche [64]. It has also been observed that in BALB-neuT mice transgenic for transforming rat Her-2/neu and in the MMTV-polyomavirus middle T transgenic mice model, disseminated tumor cells (DTC) (CK+ and HER-2+) become detectable in bone marrow as early as 4–9 weeks of age when the most meticulous analysis of the mammary gland could detect areas of only atypical ductal hyperplasia, suggesting an early spread of cancer through the migration of CSC-like progenitors in the bone marrow [65]. Moreover, those disseminated tumor cells in bone marrow do not significantly increase in number during tumor growth and progression, suggesting a quiescent phenotype and asymmetrical self-renewal in the niche. Those DTCs in bone marrow have also been identified in breast cancer patient at different stages [65]. These observations suggest that migrating cancer cells leading to cancer metastasis act like stem cells and have tropism to their niche. Whether this concept applies to melanoma remains to be determined.

7.7 Melanoma Cancer Stem Cells and Microenvironment/Niche

7.7.1 Cancer Stem Cells and Microenvironment/Niche

To effectively exert their self-renewal and generation of differentiated progeny properties, both CSCs and normal stem cells require a favorable surrounding environment commonly referred to as the “niche.” In 1978, Schofield proposed the “niche” hypothesis to describe the physiological microenvironment within which stem cells reside to maintain their stemness [66]. Increasing evidence supports the theory that the tumor microenvironment plays a major role in all phases of tumorigenesis, including initiation, progression, maintenance, and metastasis. It may also influence the outcome of therapy in several cancers including melanoma [67].

Niches are specific anatomical locations that provide a nurturing microenvironment for stem cells to grow. By nourishing stem cells, the niche protects them from apoptosis and regulates the differentiation of their progeny. Components of a niche include fibroblasts, endothelial cells, and extracellular matrix (ECM); and each different stage of stem cells possesses a distinct relationship with its own niche cell population [68]. Stem cells, their progeny cells, and other cells in the

niche work together as a functional unit and stem cells cannot function and/or function less effectively in the absence of a niche [69]. The preferential homing of stem cells into the bone marrow niche has been observed in mouse models after marrow ablation and the infusion of candidate hematopoietic stem cells (HSCs). Those infused HSCs not only homed to the bone marrow but also reconstituted the entire hematopoietic system for the lifetime of the animal. The success of this model depended upon the preferential homing of candidate HSCs to the ablated marrow, which is their natural niche. It would be logical that in solid tissues, cancer cells with stem cell properties could reconstitute the structure of the tissue and niche of their normal residence and exert their multi-potent functions. However, little is known about the requirements for a conductive microenvironment for the development of CSC niches, although niches have been well characterized in different model systems of normal stem cells [70].

Attempts to create a self-organizing niche in mice that could favor the establishment of CSC-initiated tumors have been made by co-infusing potential "helper" cells [61]. Infusing breast cancer cells together with human mesenchymal cells can greatly reduce the number of cells needed to initiate xenografts in mice, suggesting that the co-injected cells may provide a necessary component to develop a "niche-like" environment in the mouse recipient [61]. In fact, as is the case for a niche, the cancer microenvironment is characterized by an intricate network of distinct supporting cells such as fibroblasts, endothelial cells, macrophages, mesenchymal stem cells, and immune-infiltrating cells as well as their products such as cytokines and receptors. However, the putative CSC niche remains different from normal stem cell niches that support a steady-state number of stem cells and their progeny with a characteristically large degree of heterogeneity. This balance in the niche of normal tissues maintains an organized structure where the self-renewal capabilities of stem cells are highly regulated. On the contrary, the cancer microenvironment has no capacity to control the growth and differentiation of CSCs into their progeny or to regulate self-renewal of CSC progeny. Thus, it may be unrealistic to attempt to reconstruct a CSC niche in animals since such a niche may not truly exist in the cancer-bearing status in humans. It could be hypothesized that in primary tumors, the tissue niches that are responsible for normal stem cell growth and behavior may nurture in part the early CSCs. However in metastases, migrating CSCs may be able to prime the targeted tissue and re-establish a surrogate niche that allows growth and differentiation, although this niche may be highly likely not to contain the complete repertoire of factors that regulate the function of a normal stem cell niche.

7.7.2 Melanoma and Microenvironment/Niche

The melanoma microenvironment/niche includes ECM, fibroblasts, microvasculature, infiltrating immune cells, growth factors, and cytokines. Melanoma cells actively interact with their microenvironment through the direct cell–cell and cell–matrix contact and secreted growth factors and cytokines. The development of melanoma

involves the interaction of environmental, genetic, and host factors. Under normal tissue homeostasis, melanocytes in the skin dwell on the basement membrane and in the hair follicles in close contact with keratinocytes, which play a regulatory role through an intricate system of growth factors and cell adhesion molecules such as E-cadherin, P-cadherin, desmoglein, and connexins [71]. To succeed in development and progression, melanoma cells need to override these regulatory mechanisms. Loss of dendrite formation is common in these autonomous melanoma cells. Normal melanocytes cultured in vitro in the absence of keratinocytes display altered genetic profiles similar to those observed in melanoma, suggesting an important homeostatic role of keratinocytes in normal conditions [72]. Cadherins are a family of transmembrane proteins. Melanoma cells escape keratinocyte control by changing cadherin expression via down-regulating E-cadherin and up-regulating N-cadherin, which allows melanoma cells to interact with other N-cadherin–expressing cells such as fibroblasts and endothelial cells [71].

Fibroblasts are the main cellular component of the tumor stroma, comprising an integral component of the tumor. In melanoma, tumor-associated fibroblasts generate ECM components and secrete growth factors such as bFGF, IGF-1, and TGF-β into the tumor microenvironment. The resident fibroblasts or circulating mesenchymal stem cells derived from bone marrow are recruited to the tumor stroma and are then stimulated by melanoma cells to proliferate and transform into myofibroblasts or fibrocytes. Melanoma and stromal cells carry on a continuous cross-talk. Melanoma cells secrete PDGF which stimulates fibroblast to secrete IGF-1. IGF-1 in turn stimulates melanoma proliferation and activates fibroblasts to release bFGF and endothelin to promote melanoma growth [73]. Melanoma microvasculature is derived from the sprouting of local vessels. Angiogenesis in melanoma is stimulated through autocrine and paracrine growth factors such as VEGF, bFGF, PDGF, and TGF-α and β. Significantly increased expression of VEGF and bFGF in melanoma is associated with reorganization of the ECM, enhanced secretion of matrix metalloproteinase (MMPs) which digest ECM, and stimulation of tumor-associated fibroblast and endothelial cell proliferation [74].

7.7.3 Bone Marrow-Derived Mesenchymal Stem Cells (MSC) and Tumor Microenvironment

Mesenchymal stem cells are typically characterized by their ability to differentiate into variety of cell types, including osteoblasts, chondrocytes, adipocytes, etc. In the bone marrow, they provide the microenvironmental regulation that control HSC quiescence and proliferation. MSCs have been attracting lots of attention recently in the tumor biology and tumor therapy field because of their ability to give rise to bone, cartilage, fat, and muscle; their role in inflammation and tissue repair; and their potential role in cancer progression.

MSCs can be recruited by tumors [75, 76]. The relationship between MSCs and tumor cells is twofold. Primary and metastatic tumors actively attract MSC from the bone marrow where they become tumor-associated fibroblasts and contribute to the tumor microenvironment, affecting tumor cell survival, angiogenesis, immune function, and establishment of distant metastasis [77]. Two potential roles for MSCs in metastasis have been recognized; including their ability to colonize metastatic tumors and their ability to promote the metastatic behavior of malignant cells in the primary tumor. MSCs also attract tumor cells to the bone marrow, support their growth, and support their survival during chemotherapy which underline possible mechanisms for the high frequency of bone metastasis.

The mechanisms involved in the recruitment of MSCs into tumors exhibit significant overlap with the mechanisms involved with migration and activation of inflammatory cells in the tissue repair process. The angiogenic molecule VEGF can induce the homing of MSCs to tumor sites in murine glioma models [78]. In addition, tumor-derived cytokines such as TGF-β, IL8, EGF. HGF bFGF, and PDGF also function as chemoattractants to recruit MSCs into tumor sites. Additionally, a number of chemokines and their receptors have been implicated in MSC homing, although their contribution is not clear. Along with soluble growth factors and chemokines, ECM proteases that are activated at injury sites can contribute to attracting MSCs [78].

Together with other cells such as myofibroblasts, endothelial cells, and immune cells, MSCs incorporate into the tumor and contribute to the tumor microenvironment. MSCs also can secrete some important inflammatory cytokines that affect tumor cells and immune cells, such as IL-6, IL-10, CCL5/RANTES, and VEGF [79]. The immune modulatory function of MSCs influence tumor development by inhibiting T-cell proliferation, dendritic cell maturation, and NK and B-cell activation, as well as simultaneously increasing regulatory T-cell (Treg) numbers [80]. In a preclinical study, co-injection of MSC allowed B16 melanoma cells to grow in mice with an allogeneic background, avoiding a vigorous immune rejection response [81].

In addition migration to tumor sites, MSCs contribute to a pro-tumorigenic environment in the bone marrow. Here, MSCs produce chemoattractants such as SDF-1 and MCP-1 that not only attract and retain HSCs but also are potent chemoattractants for circulating tumor cells in the bloodstream. In the bone marrow, tumor cells can interact with MSCs and their progeny through adhesion-dependent and adhesion-independent mechanisms [77]. Through the SDF-1/CXCR4 axis, MSCs have been suggested to mediate chemotaxis of CD34+ acute myelogenous leukemia cells, and to play an important role in the homing of these cells to the bone marrow microenvironment [82]. Therefore, the preferential homing of potential CSCs to the bone marrow via the production of SDF-1 has been proposed as a mechanism of chemoresistance in different hematological malignancies [82]. Adhesion-independent mechanisms of interaction between tumor cells and MSCs also play an important role in bone marrow and bone metastasis. Both myeloma and neuroblastoma models have shown that IL-6 appears at the center of the interaction between tumor cells and MSC in the bone marrow microenvironment, acting as a potent pro-tumorigenic factor [77].

Taking together, MSCs can migrate to the primary tumor and sites of metastasis, and can be recruited by tumors to become part of tumor microenvironment and modulate the immune reaction in tumor biology. MSCs can also attract tumor cells into the bone marrow in order to retain and protect them from chemotherapy.

7.8 Cancer Stem Cells and Drug Design for Melanoma Treatment

Melanoma is characterized by a peculiar resistance to chemotherapy. One reason could be a particular resistance of melanoma stem cells to standard treatment and/or immunotherapy. As previously discussed, CSCs and melanoma stem cells might maintain properties of normal stem cells that include their multiple self-protective mechanisms including drug resistance.

7.8.1 Limitations of CSC-Based Drug Discovery

It is possible that the successful treatment of cancer rests on the use of multiple therapeutic approaches targeting different cell types within the same cancer population. Cancer as a functional unit includes CSCs, TAs, and differentiated cancer cells. Each one of these cell types may have a different sensitivity to drugs. If an agent is effective against CSCs, because of the previously discussed plasticity of the system, TAs if resistant to the same treatment may restore the CSC populations and the same could happen in the other direction. Therefore, testing drug effectiveness cannot be limited to the elimination of CSCs.

Drug discovery relies heavily on the sensitivity of cancer cell lines in vitro. These cell lines are almost all monoclonal and they may not recapitulate the complexity of the tumor in vivo, where the various subpopulations of cancer cells ranging from CSCs to differentiated cancer cells may be more representative. This could partially explain the drug resistance heterogeneity observed using different cultured cell lines which represent only a subpopulation of CSCs. Similarly, in vivo xenograft models may not predict drug efficacy because models may not fully represent the niche-like environment that fosters cancer growth in humans which involves complex interactions between CSCs and other cells and its protective effects against therapy. Thus, an ex vivo primary tumor cell model may better test drug effects. Drug evaluation studies performed on primary human glioblastomas seem to have greater accuracy in predicting treatment results in a preclinical setting [83].

Thus, there is no good experimental model to study therapeutics for individual components of the different tumor populations. Mechanisms of asymmetric division, dedifferentiation into TAs, and their self-renewal capability need to be better understood before a rational approach can be applied for the identification of effective drugs. This applies to melanoma as well as many other cancers.

7.8.2 *Targeting Pathways Regulating CSCs Growth*

Many pathways of self-renewal involved in the propagation of CSCs appear to be shared by their normal counterparts, raising the possibility that therapies which target CSCs may also damage normal stem cells. Therefore, it is important to identify unique targets not shared with normal stem cells; some of which have been identified. For example, leukemic stem cells have lost PTEN tumor suppressor activity which promotes their self renewal, while hematopoietic stem cells employ different mechanisms for their survival. Rapamycin, which targets mTOR, eradicates leukemia-initiating cells in mice and restores normal HSC function [84, 85]. Moreover, Parthenolide selectively targets human leukemia stem cells, but not normal stem or progenitor cells [86]. Unfortunately, to date no pathways which are specific to melanoma stem cells compared to normal stem cells have been identified with the exception of Notch signaling which seems to be required for maintenance of the melanoma stem cell niche.

7.8.3 *Drug Delivery via Mesenchymal Stem Cells*

Because MSCs can home to tumors and metastatic sites, they can be considered as novel cell-based delivery agents to cancer [87, 88]. Recently, some preclinical models tested the efficacy of engineered MSCs to systemically deliver pro-drug activating enzymes or cytokines with anti-cancer activities to the primary tumor and metastatic sites. For example, the systemic administration of MSCs engineered to express recombinant TRAIL in brain glioma-bearing mice has an anti-tumor effect [89], and co-injection of human prostate cancer cells with adipose tissue-derived MSCs engineered to express the suicide gene cytosine deaminase induce complete tumor regression upon treatment of mice with the pro-drug 5 fluoro-cytosine [90]. However, to translate these observations to human clinical trials will require convincing evidence that MSCs can effectively colonize primary tumor and metastatic sites in cancer patients.

7.9 Conclusions

Since our last review of melanoma stem cells [91], progress has been made in the characterization of these cells particularly at the basic experimental level. From the inception of the CSCs hypothesis more than 100 years ago [92], evidence has grown that supports the existence of a subpopulation of cells within the tumor that is responsible for tumorigenesis, tumor maintenance, growth, and metastasis. As previously discussed, if CSCs bear dramatically different biological properties compared with the rest of the cancer cells, it is possible to explain the poor effectiveness of current therapies by the fact that most were developed by testing their activity against the bulk of cancer cells independent of functional subsets.

However, many questions remain. Most of the reported characterizations of CSCs, including melanoma stem cells, rest on the expression of surface markers; the ability to form spheres; and the capacity for self-renewal by initiating tumors in immunodeficient mice. These arbitrary criteria may suffer some limitations. The markers used to isolate CSCs are not unique to these cells and are often expressed by somatic cells in normal tissues [93]. Their expression can be modulated by different experimental and environmental conditions; for example hypoxia can induce increased expression of stem cell-like surface markers and interfere with the gene expression machinery of cancer cells [94]. Thus, surface markers may not be considered accurate indexes capable of identifying a pure population of CSCs, but they could be more realistically applied to enrich for a specific population bearing stem cell-like properties that could be then tested for their ability to initiate tumors in animals. These in vivo assays and their results can be difficult to interpret because of extremely variability due to the experimental conditions and the host microenvironment [95]. The ability of tumor cells to survive and regenerate in xenografts may be unrelated to stem cell-like features but instead may be due to random alterations in the regulation of apoptotic pathways, cell cycle regulation, or altered methylation patterns.

Research on melanoma stem cells suffers the same limitations experienced in other cancer models and may be additionally hampered by the high plasticity of this cancer, its unpredictable behavior, and its unique resistance to most therapies. As other aspects of cancer biology are being better understood including the understanding of the leading driver pathways that stimulate its growth and of the immune biology responsible for its survival/rejection, it is becoming clear that combination therapies may represent the most rational approach to treatment [96, 97]. Most therapies look to simultaneously target different pathways related to a stable phenotype of melanoma that are studied in its globality. It is possible that another level of complexity should be added to the algorithm used to design anti-melanoma therapy by considering a plastic interaction of different cell populations within each tumor that may differently respond to the treatments. For instance, immune therapy should consider alternate target antigens unrelated to tissue differentiation such as cancer testis antigens [98], whose expression is increasingly stabilized in the later stages of cancer progression or mutated neo antigens associated with the oncogenic process and most likely expressed by CSCs [99, 100]. However, it should be kept in mind that even these antigens may or may not represent good targets for melanoma stem cell due to a different sensitivity to cytotoxicity [101] and to an intrinsic down-regulation of some of them [39]. Similarly, chemotherapy should target pathways that are less strictly associated with the division rapidly dividing differentiated melanoma cells but more closely related to the metabolism of resting melanoma stem cells [102].

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Chapter 8
Cancer Stem Cells in Lung Cancer

Jun Shen and Feng Jiang

Abstract Lung cancer is the most common cancer worldwide, accounting for 1.2 million new cases annually. Furthermore, it is the most lethal of all cancers. A major challenge in treating this deadliest form of malignancy is the intrinsic resistance to conventional therapies. It is believed that cancer stem/progenitor cells are responsible for the sustained growth, survival, and invasion of tumors. Therefore, identifying lung cancer stem cells (CSCs) and studying the biologic functions necessary for their existence within lung tumors will provide new clinical approaches with the goal of improving clinical outcomes of the disease. This chapter will summarize our understanding of the identification of cancer stem cells in lung tumors, molecular mechanisms, and associated pathways that operate within cancer stem cells of lung tumors, and potential applications in clinic settings. We will also discuss future perspectives in lung cancer stem cell research.

Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
AC	Adenocarcinoma
ALDH1	Aldehyde dehydrogenase 1
ASCL	Achaete scute-like
BAC	Bronchioalveolar carcinomas
bHLH	Basic helix-loop-helix
CCSP	Clara cell secretory protein

F. Jiang (✉)
Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA
e-mail: fjiang@som.umaryland.edu

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CD	Cluster of differentiation
CSC	Cancer stem cell
EPC	Endothelial progenitor cell
Hh	Hedgehog
HSC	Hematopoietic stem cell
MDR1	Multi-drug resistance protein 1
mTOR	Mammalian target of rapamycin
NSCLC	Non small-cell lung cancer
PNEC	Pulmonary neuroendocrine cells
PTCH	Patched
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficiency
SCLC	Small-cell lung cancer
SDF-1	Stromal-derived factor 1
SMO	Smoothened
SP	Side population
SSEA	Stage-specific embryonic antigen
TAC	Transit amplifying cell
TRA	Tumor rejection antigen

8.1 Introduction

Lung cancer, the leading cause of cancer death worldwide, is comprised of four major histological types: small-cell lung cancer (SCLC) and three types of non–small-cell lung cancer (NSCLC) including squamous cell carcinoma (SCC), adenocarcinoma (AC), and large-cell carcinoma [1, 2]. Despite recent treatment advances, including modernization of drug cocktails and radiotherapeutic regimens over the past half century, the 5-year survival rate of patients with NSCLC is only 15%. Therefore, there is an urgent need to better understand the key molecular events driving lung tumorigenesis, such that we can find more effective ways for its prevention, diagnosis, prognosis, and treatment.

Accumulating evidence suggests that stem cells and cancer are inextricably linked, and perceived wisdom is that the process of carcinogenesis initially affects normal stem cells or their closely related progenitors. For instance, in animal models of intestinal cancer, a direct involvement of stem cells in adenoma formation has well been demonstrated [3]. Furthermore, during the progression of the tumor, neoplastic stem cells may evolve to maintain tumor growth. Many other types of solid tumors also have a population of self-renewing and/or expanding stem cells: cancer stem cells (CSCs). The CSC hypothesis provides an explanation for the origins of tumor self-renewal and heterogeneity [4, 5]. There are two components of the CSC hypothesis [6]. The first is that cancers directly arise from stem cells that have acquired sufficient oncogenic mutations for transformation [7]. Therefore, the

tumor cell of origin, referred to as a tumor-initiating cell, is likely a stem or progenitor cell that is already capable of self-renewal and differentiation. The second component of the hypothesis is that tumor progression could be driven by a subpopulation of self-renewing tumor cells. This vision is supported by the observation that most tumors are comprised of functionally heterogeneous cell subpopulations, including a population that differs in their ability for limitless proliferative potential and repopulation ability [8].

Lung cancer is a complex network consisting of cells at various stages of differentiation, neovascular structures, reactive inflammatory cells, recruited cells, and infiltrated parenchyma that interact within the tumor mass. Based on the CSC hypothesis, a lung tumor might be driven and maintained by antigenically distinct subpopulations of perpetually self-renewing CSCs that give rise to transit-amplifying cells (TACs) and terminally differentiated cells. Therefore, like normal cell populations, lung tumors may have a hierarchical structure. Adherents of the hypothesis imply that the bulk of the solid tumor is thus not the clinical problem, and that identifying CSCs and the associated factors that regulate CSCs' behavior may have an enormous bearing on the way we treat the deadliest form of malignancy in the clinical setting. The clinical implications of a tumorigenic hierarchy thus seem to be obvious, considering that therapies targeting the rapid reduction of tumor size in the lungs are not selected for their discriminatory ability to treat tumor-initiating cell subpopulation. When a therapy fails to kill all self-renewing lung CSCs, residual surviving CSCs will be able to repopulate the disease, leading to relapse of the tumors of the lungs.

Evidence for the existence of clonogenic cells in the lungs was first described in 1982 [9]. In this study, a small population of cells (<1.5%) isolated from surgically resected tumors from patients diagnosed with AC and SCLC of the lungs were able to form colonies in a soft agar cloning assay [9]. However, comparatively less is known about the biology of lung CSCs compared with other solid tumor stem cells. Considering lung cancer is the most common lethal type of cancer in the world [10], there is a pressing need for the development of new therapeutic agents that better manage the progression of highly aggressive lung cancer cells. Research in the area of lung CSCs might provide a new paradigm leading to improved therapies for the disease. This chapter will summarize our understanding of the cellular and molecular mechanisms that operate within CSCs or initiating cells of lung tumors and their potential applications in clinical practice.

8.2 Identification and Isolation of Lung Cancer CSCs

Because many of the molecules expressed by normal stem cells may also be found in their malignant counterparts, considerable effort has been made in the search for "markers" of CSCs based on the currently used markers for stem cells (Table 8.1).

Table 8.1 Commonly used markers for identification and isolation of lung CSCs

Marker	References
MDR1	[11]
CD24	[12]
CD34	[13]
CD44	[13, 14]
CD87	[11]
CD133	[15, 16]
ALDH1	[17, 18]
Side population (SP)	[19, 20]
Bmi-1	[21, 22]

Examples of well-known stem cell markers that have been applied to identify lung cancer CSCs include CD24, CD34, and CD133, among others. The aldehyde dehydrogenase (ALDH) gene superfamily encodes detoxifying enzymes for many pharmaceuticals and environmental pollutants [23]. In addition, murine and human hematopoietic and neural stem cells have high ALDH activity [24, 25]. Class 1 enzymes of the ALDH family (ALDH1) are the isoforms of ALDH that predominates in mammals [26, 27]. Increased ALDH1 activity has been found in stem cell populations in human multiple myeloma, acute myeloid leukemia, brain cancer, and breast cancer [28–31]. Therefore, ALDH1 activity might be usable as a common marker for both normal and malignant stem cell populations [31]. Our recent study further suggests that ALDH1 is a marker that can be used for isolation and identification of CSC population of lung cancer [17]. Furthermore, one of the common and important characteristics of stem cells is the ability to withstand cytotoxic insults either through efficient enzyme-based detoxification systems or by the ability to rapidly export potentially harmful xenobiotics [32]. Based on the characteristics of stem cells, Goodell et al. [33] were the first to develop a method by using "side population" (SP) for the isolation of hematopoietic stem cells (HSCs), because HSCs have the ability to efflux a fluorescent dye. Ho et al. [20] found that 16 clinical lung cancer samples displayed a smaller but persistent SP population in six human lung cancer cell lines, indicating that the SP could be an enriched source of lung tumor-initiating cells with stem cell properties. Isolation of SP cells might be a useful tool to study lung CSCs in the lung tumorigenic process. In addition, B lymphoma Moloney-murine leukemia virus insertion region 1 (Bmi-1) is required for the self-renewal of HSCs [21]. Therefore, Bmi-1 was applied as a marker for human cancers [22]. Koch et al. [22] found that in human small cell lung cancers, 98.4% (63/64) of cases ubiquitously expressed Bmi-1, a key player in self-renewal of stem cells. One of the commonly used markers for lung is Prominin-1 (CD133), the first identified member of the rapidly growing prominin family of pentaspan membrane proteins [15]. Moreover, in combination with other markers, CD133 has been used to isolate HSCs and endothelial progenitor cells (EPCs). Its utility to enrich CSCs of solid tumors including lung cancer has also been well documented [16]. For example, Eramo et al. [15] first used CD133 to isolate lung CSCs and subsequently demonstrated that CD133 positive cells had characteristics of CSCs.

8.3 CSCs Play Important Roles in Lung Tumorigenesis

Using the Aldefluor® assay followed by fluorescence-activated cell sorting analysis, we isolated lung cancer cells that had high ALDH1 activity [17]. ALDH1 positive lung cancer cells displayed in vitro features of CSCs, including enhanced capacity for proliferation, self-renewal, and differentiation; resistance to chemotherapy; and expression of the CSC surface marker CD133 [17]. In vivo experiments showed that the ALDH1 positive cells could generate tumors that recapitulate the heterogeneity of the parental cancer cells. Furthermore, immunohistochemical analysis of 303 clinical specimens from three independent cohorts of lung cancer patients and controls showed that expression of ALDH1 was positively correlated with the stage and grade of lung tumors, and related to a poor prognosis for the patients with early-stage lung cancer. ALDH1 is therefore a lung tumor stem cell–associated marker. These findings offer an important new tool for the study of lung CSCs, and provide a potential prognostic factor and therapeutic target for treatment of patients with lung cancer [17].

CD133 positive lung cancer cells have also been considered as CSCs in the lungs. However, as experienced for other tumors, somewhat disparate results have been reported. For example, from both the NSCLC A549 cell line and the SCLC H466 cell line, CD133 negative cells were as proficient as CD133 positive cells at colony forming ability in vitro and tumorigenic capacity in nude mice in vivo [34]. On the other hand, it has been shown that in NSCLC, 1×10^3 CD133 positive cells could form tumors in SCID mice; however, 1×10^4 CD133 negative cells could not [35]. CD133 positive cells also show enhanced expression of Oct-4 and ABCG2, and small interfering RNA (siRNA) knockdown of Oct-4 blocked clonogenicity and enhanced chemosensitivity. Expression of Oct-4 was observed in bronchioalveolar carcinomas (BACs), but the immunohistochemical expression data were unconvincing in comparison with the seminoma positive control [36].

Because CSCs are resistant to chemotherapeutic drugs, the perceived chemoresistance of CSCs has been investigated. For example, CSCs have been enriched in NSCLC cell lines treated with cisplatin and doxorubicin [37]. 5×10^3 drug selected cells regularly formed tumors in SCID mice. These cells expressed CD133, CD117, and the embryonic stem cell markers stage-specific embryonic antigen 3 (SSEA-3), tumor rejection antigen TRA1-81, Oct-4, and nuclear β-catenin. In the A549 cell line, the SP fraction was 5.2%; however, after drug treatment, this was increased to 35%. These studies clearly indicated that drug resistance and lung CSCs are heavily entwined. Furthermore, in the A549 NSCLC cell line, a large (24%) SP has been found. The SP cells displayed enhanced resistance to doxorubicin and methotrexate related to ABCG2 activity [38]. In the SCLC H-146 cell line, a “migratory” SP has been observed which is able to migrate toward conditioned media from hypoxic bone marrow–derived stromal cells, probably due to release of stromal cell derived factor 1 (SDF-1). This fraction was also enriched for CSCs in nude mice in vivo [39]. The β-helix-loop-helix (bHLH) transcription factor achaete scute complex homologue 1 (ASCL1/mammalian achaete scute homolog 1 [Mash1]) is essential

for neuroendocrine development, and it appears that both CD133 and ALDH1 are directly regulated by ASCL1 [18]. In SCLC, there might be a relatively abundant $CD133^{high}ASCL1^{high}ALDH^{high}$ subpopulation enriched for CSCs, with as few as 1×10^3 of these cells being capable of forming rapidly growing aggressive tumors in nude mice. In a number of SCLC cell lines, a subpopulation (1–4%) of urokinase plasminogen activator-positive cells was found. The positive cells were more resistant to traditional chemotherapies such as 5-fluorouracil, cisplatin, and etoposide. The resistance might be associated with enhanced MDR1 (ABCB1) activity and CD44 expression [11]. Ling et al. [40] showed that a serum-free system for primary neonatal pulmonary cells can support the growth of Oct-4+ epithelial colonies. Furthermore, besides Oct-4, these cells also expressed other stem cell markers such as SSEA-1, stem cell antigen 1 (Sca-1), and CCSP. These cells could be maintained for several weeks in primary cultures and undergo terminal differentiation to AT1 and AT2-like pneumocytes. They further demonstrated these Oct-4+ cells were located at the bronchoalveolar junction of neonatal lung. Taken together, all these findings provide strong evidence that CSCs play key role in the development and progression of lung cancer.

8.4 Cancer Stem Cell-Related Pathways of Self-Renewal in Lung Cancer

Stem cell self-renewal is a tightly controlled process governed by both signals from the stem cell niche as well as deliberate and regulated control of key developmental pathways. Examples of such pathways are Wnt, Hedgehog, and Notch signaling pathways [41]. Because CSCs comprise the self-renewing component of tumors, it is hypothesized that the same pathways that govern normal stem cell self-renewal could also govern CSC self-renewal. However, self-renewal in tumorigenesis is achieved by the deregulation of these key pathways. [4]. It is imperative to identify and understand the deregulated pathways involved in lung CSCs, because future development of potential therapeutic approaches to target these altered pathways in tumors might provide an important strategy for treating tumors that are often intractable to conventional therapy alone [42]. We will review three major pathways whose deregulations are involved in lung CSCs.

8.4.1 Wnt/β-Catenin Signaling

The Wnt/β-catenin pathway was originally found to play an important role in the regulation of HSC self-renewal [43]. Reynolds et al. [43] showed that activated Wnt/β-catenin signaling in the developing lung coincided with an expansion of BASCs and attenuated bronchiolar differentiation. On the other hand, conditional Cre-mediated deletion of Catnb (which encodes β-catenin) had no appreciable effect

on the repair and maintenance of the bronchiolar epithelium. These observations suggest that the role of Wnt/β-catenin signaling in lung stem cell self-renewal might be niche specific [44]. In lung cancer, evidence of activated Wnt signaling suggests aberrant Wnt signaling may be important for lung tumorigenesis [45, 46]. Furthermore, inhibition of Wnt signaling by a Wnt-2 monoclonal antibody resulted in the induction of apoptosis in NSCLC cells [47]. Taken together, deregulation of the Wnt signaling pathway clearly promotes lung carcinogenesis and stem cell self-renewal, making the Wnt signaling pathway an appealing target for the development of novel therapies for lung cancer.

8.4.2 Hedgehog Signaling

The Hedgehog (Hh) signaling pathway is activated when one of three extracellular Hh ligands (sonic hedgehog, desert hedgehog, and Indian hedgehog) binds to and inactivates its receptor patched (PTCH). The Hh signaling pathway is a key developmental pathway required for proper embryogenesis [35]. In the developing lungs, activated Hh signaling is involved in pulmonary cell fate determination and branching morphogenesis [36]. Aberrations in expression and activation of this pathway could lead to deformations in development, and hence contribute to tumorigenesis [37]. For example, during lung epithelial regeneration after injury, activated Hh signaling is observed in regions of repair and in pulmonary neuroendocrine stem cell niches [38] and cyclopamine-mediated suppression of aberrantly active Hh signaling in some SCLCs resulted in a dramatic drop in cell viability and tumorigenicity [39]. These findings suggest that SCLC is a malignancy that arises from a population of self-renewing pulmonary neuroendocrine cells (PNECs) that retain active Hh signaling as well as primitive neuroendocrine features. Furthermore, the observation implies that therapeutic targeting of the Hh signaling pathway may suppress stem-like tumor cell self-renewal [40]. The increasing evidence for the use of Hh signaling in tumor cell maintenance and cancer stem cell self-renewal has provoked the development of better and more specific inhibitors of the Hh pathway, some of which are currently in clinical trials for SCLC [41, 48, 49].

8.4.3 Notch Signaling

The Notch signaling pathway is involved in cell fate determination, organogenesis, and tissue homeostasis. Notch-mediated cell–cell interactions dictate the preservation or differentiation of stem cells [50]. Activation of Notch signaling begins when membrane-bound Notch ligands bind to receptors on adjacent cells. Upon binding, the intracellular domain of the receptor is cleaved by gamma-secretase, allowing for the activation of downstream targets, such as the inhibitory basic helix-loop-helix transcription factor Hes1 [51]. In lung development, Notch signaling appears

to be required for determining proximal and distal lung epithelial cell fates [52]. Forcing activation of Notch signaling in the developing lung tissue either through the ectopic expression of intracellular Notch domains or through gamma-secretase activation can result in the accumulation of distal airway stem cell differentiation [53]. Although elevated Notch signaling transcripts have been described in NSCLC, the role of Notch in tumor maintenance remains largely unknown. Suppression of Notch signaling in some NSCLC cells by treatment with a gamma-secretase inhibitor induced cell death and decreased tumor growth in mice [54]. In contrast, another study showed that activation of Notch signaling in A549 cells through overexpression of Notch1 resulted in a decrease in proliferation and tumorigenic growth in mice [55]. The apparent discrepancy between these results may be due to the perturbations of Notch signaling via different Notch receptors. However, although it is not yet clear how Notch signaling functions for self-renewal of lung CSCs, several reports suggest that Notch signaling components are expressed in putative lung CSC populations and are required for tumor initiation capacity [56].

Finally, it should be emphasized that for treatment of CSCs to have any significant future impact on the overall survival of patients with lung cancer, the underlying molecular signaling that drives tumorigenicity in these cells must be elucidated in much greater detail than is currently known.

8.5 Potential Applications of CSCs for Lung Cancer Treatment

As described above, Wnt signaling, Hedgehog signaling, Notch/Delta signaling, mTOR, ABC transporters, and the stem cell niche may provide a variety of druggable targets related to CSCs. The below examples could demonstrate the potential in developing innovative treatments of lung cancer in future.

In lung tissues, key regulators of stem cell renewal appear to be members of the Polycomb group protein family of transcriptional repressors, including Bmi-1, polyhomeotic-like 1, and melanoma surface molecular 18. Bmi-1 is a downstream target of the morphogen Sonic hedgehog (Shh) through the latter's activation of the glioma-associated oncogene homolog (Gli) family of transcription factors. Shh acts on the receptor complex of patched (PTCH) and smoothened (SMO), blocking the restraining influence of PTCH on SMO, resulting in SMO signaling that activates the Gli family of transcription factors, thus activating target genes like Bmi-1. Inhibiting the action of SMO with the antagonist cyclopamine is a highly effective strategy against some cancers. It has been shown that almost all SCLCs ubiquitously express Bmi-1 [22], and antisense Bmi-1 RNA therapy reduces proliferation of A549 cells [57].

Another example is that of the Notch family of receptors, which are critical for stem cell self-renewal. Engagement of ligands of the delta and jagged families could cause cleavage of the intracellular portion of Notch and its translocation to the nucleus where it binds to the transcription factor chisel, changing it from a transcriptional repressor to an activator. Interestingly, cleavage of the intracellular

portion of Notch is mediated by the γ-secretase protease complex. Therefore, the use of γ-secretase inhibitors may have promising utility in cancers where Notch signaling is inappropriately inactivated [58]. Furthermore, various pathways activated in human lung cancer converge on mTOR, with obvious therapeutic possibilities for the disease. Apart from these renewal and proliferation pathways, there are many other potential molecular targets relating to lung CSCs. For instance, because lung tumors have SP fractions, targeting ABC transporter activity might be a useful strategy for overcoming chemoresistance and directly eradicating stem cells [37, 59].

Techniques that could be applied for the potential targets include but are not limited to those described below. Antibody-based targeting of CSCs exploiting the overexpression of molecules such as CD133 is possible approach. Small molecule therapeutics that target growth factors, growth factor receptors, and their kinases and more specific tyrosine kinase inhibitors are gaining widespread usage in lung cancer treatment. RNA interference will become an increasingly useful strategy. In fact, targeting Oct-4 by using the technique in lung cancer has been shown to increase apoptosis of the cells [60]. Additionally, some new therapies for lung cancer are designed to make cells more sensitive to induced cell death, while others target self-renewal pathways. Although it is not clear whether these therapies specifically target lung cancer stem cells, in other cancers such as colon cancer [61], siRNA targeted to putative stem cell molecules like CD44 and Musashi-1 have been highly effective in blocking growth of xenografted tumors [61, 62].

8.6 Future Perspectives

The development of effective, safe CSC-based therapies for the treatment of lung cancer remains a tantalizing prospect [63]. However, before considering and initiating any possibility of CSC-based treatments, emphasis must be placed on understanding events occurring frequently in CSCs and their local microenvironment during cancer progression, and the molecular mechanisms involved in their resistance to current chemotherapeutics. The fact that CSCs and normal adult stem cells utilize common molecular machinery and have similar protein expression profiles is potentially the most challenging hurdle to overcome in the development of therapies to target CSCs while sparing normal stem cells. Therefore, more precise methods and specific markers need to be developed to discriminate CSCs from normal stem cells. Furthermore, it is unknown whether CSCs originate from pluripotent adult stem cells or more differentiated committed stem/progenitor cells. If CSCs originate from pluripotent adult stem cells, CSCs may be able to differentiate into all pathological types of lung cancer with more primitive characteristics. However, if CSCs come from more differentiated committed stem/progenitor cells, their fate will already be determined as it can only differentiate along a particular lineage. To answer this question, the lineage tracing technique should be used to identify the cell of origin. Niches along with trachea for normal

adult stem cells may be harnessed by CSCs, since aberrant signals within the niches are utilized for abnormal proliferation of CSCs. Therefore, there may be a possibility that lineage differentiation is already defined by the remaining extinctive niches with potential reactivation of a particular signal pathway. Successful answering of these and other questions will undoubtedly have profound implications for the treatment of lung cancer and ultimately decrease lethality from the disease.

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Chapter 9
Cancer Stem Cells in Ovarian Cancer

Fang Fang, Curt Balch, Meng Li, Jay M. Pilrose, and Kenneth P. Nephew

Abstract Ovarian cancer causes more deaths than any other gynecologic malignancy. Five-year survival rates have only marginally improved over the past 3 decades, with progression to drug resistance remaining the major therapeutic barrier. Similar to a number of other carcinomas, recent reports suggest that ovarian tumors may exhibit a hierarchical organization of cell types, with tumor development and progression driven by "cancer stem cells" that are inefficiently targeted by conventional therapies. This chapter will focus on the cancer stem cell (CSC) hypothesis as it may relate to ovarian cancer, examine reports of ovarian cancer stem cells (OCSCs), and discuss potentially improved therapeutic strategies based on the specific targeting of these tumor progenitors.

Abbreviations

ABC	Adenosine triphosphate binding cassette
ALDH	Aldehyde dehydrogenase
ATRA	All-trans retinoic acid
BCRP	Breast cancer resistance protein
BRCA	Breast cancer susceptibility gene
CD	Cluster of differentiation
CSC	Cancer stem cell
DNMT	DNA methyltransferases
DTEP	Drug-tolerant expanded persisters

K.P. Nephew (✉)
Medical Sciences Program, Indiana University, Bloomington, IN, USA

Indiana University Simon Cancer Center, Indianapolis, IN, USA
e-mail: knephew@indiana.edu

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DTP	Drug-tolerant persisters
EMT	Epithelial-to-mesenchymal transition
EOC	Epithelial ovarian cancer
ERK	Extracellular receptor kinase
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FTE	Fallopian tube epithelia
HDAC	Histone deacetylase
HOX	Homeobox
IFN-α	Interferon-alpha
IL	Interleukin
MDR	Multidrug resistance
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NICD	Notch intracellular domain
OCSC	Ovarian cancer stem cell
OS	Overall survival
OSE	Ovarian surface epithelium
PI3K	Phosphatidylinositol 3-kinase
SCF	Stem cell factor
SHH	Sonic hedgehog
SP	Side population
STIC	Serous tubal intraepithelial carcinomas
TGF-β	Transforming growth factor beta
uPA	Urokinase plasminogen activator
Wnt	Wingless

9.1 Ovarian Cancer Biology and Pathology

Globally, ovarian cancer is the seventh leading cause of total cancer-related death, claiming 125,000 lives per year [1]. In the United States, an estimated 21,550 women will be diagnosed with, and more than 14,600 women die from, ovarian cancer in 2009 [2]. It is estimated that one woman in 70 will develop ovarian cancer during her lifetime, and the lifetime risk of death for all woman is 1 in 98 [1].

Epithelial ovarian cancer (EOC) comprises the majority (over 80%) of malignant ovarian tumors in adult women and is further subclassified into serous, mucinous, endometrioid, clear cell, transitional cell, squamous, mixed, and undifferentiated subtypes [3]. Of these, the serous subtype is the most common (over 60%) and consequently, in most contexts, the generic term “ovarian cancer” refers to serous EOC. Unfortunately, serous EOC is also the most lethal subtype, generally revealing no symptoms until late in its course [4, 5], resulting in an overall 5-year survival rate of 46%. This ranges from 70 to 90% for early stage I–II disease, compared with 30.6% at the advanced stages (III–IV), which constitute the majority (over 75%) of

initial presentations [6]. The current standard treatment, comprised of cytoreductive surgery followed by a platinum/taxane-based regimen, results in clinical complete remissions in approximately 70% of patients [7, 8]. However, for the majority of those initially responsive patients, chemoresistant tumor recurrence is common, at which point the disease is essentially terminal, as current second-line therapies are largely ineffective [9]. Thus, similar to most metastatic malignancies, drug resistance represents the major therapeutic barrier to the effective treatment of EOC [10].

Although no direct cause(s) of EOC have been discovered, various hypotheses for transformation have been put forth, including ovulation-related wound healing, ovulation-associated inflammation, and prolonged exposure to gonadotropins (pituitary hormones that regulate the estrous cycle) [11–13]. These hypotheses are not mutually exclusive, and all are consistent with established EOC risk reduction factors, including multiple pregnancies, contraceptive use, and younger age group (thus having less cumulative ovulations and exposure to pituitary gonadotropins) [14–16].

While no precursor lesion for serous EOC has been identified, it has traditionally been hypothesized that tumors arise from the ovarian surface epithelium (OSE) and/or cortical inclusion cysts (stroma-entrapped OSE cells), possibly formed during ovulation, that are highly exposed to estrogen [3]. However, more recent hypotheses speculate that EOC can arise from secondary Müllerian tissues (remnants of the embryonic female Müllerian duct) or from the secretory epithelium of the fallopian tube fimbria, based on similarities in gene expression (specifically, overexpression of mutant p53) and the frequent detection of fibrial carcinomas in *BRCA1/BRCA2*-carrying women undergoing prophylactic salpingo-oophorectomies [17, 18]. A growing consensus, however, is that serous EOC can likely arise from any of these tissues (OSE, fimbrial epithelia, or secondary Müllerian structures) [19–21], and ongoing studies of ovarian cancer stem cells (OCSCs) may provide more definitive insight into origin(s) of this lethal gynecologic malignancy.

Similar to most epithelial malignancies, ovarian cancer metastasis can occur by direct extension into nearby organs, including other reproductive structures, and less frequently, the rectum or bladder [22]. However, hematogenous metastasis to distant organs is exceedingly rare, and unlike other carcinomas, tumor extension is followed by dissemination ("seeding") into the peritoneal cavity, with frequent involvement of pelvic lymph nodes [23, 24]. Individual tumor cells frequently form multiaggregate "spheroids," likely for the purpose of immunoevasion [22]. Peritoneal ascites act as a fluidic carrier for detached metastatic cells or spheroids, which upon attachment to the mesothelial cell monolayer typically disaggregate into individual cells with an invasive and motile phenotype, followed by implantation into the peritoneal lining [25, 26]. Peritoneal implantation is mediated by protein-mediated cellular attachment to extracellular matrix components, such as integrin/fibronectin interactions or binding to the glycosaminoglycan hyaluronate by its receptor, CD44. Additionally, reports of CD44 as an OCSC marker (see below) raise a number of questions regarding possible signal transduction propagated by this specific protein/polysaccharide (i.e., CD44/hyaluronan) interaction.

9.2 Isolation and Characterization of Ovarian Cancer Stem Cells

The existence of CSCs was first demonstrated in hematologic malignancies [27] and more recently in solid tumors [28, 29], including EOC [30–44]. Putative OCSCs have now been isolated from a number of sources, including established EOC cell lines, ascites, and primary tumors. Similar to the isolation of other tumor stem cells, enrichment of OCSCs relies on various phenotypes likely shared with normal stem cells, including the ability to form anchorage-independent spherical aggregates, express stem cell markers, undergo membrane efflux, display distinct surface proteins, form clones in culture, and exhibit greatly enhanced tumor-forming ability [45, 46].

9.2.1 Isolation of Putative OCSCs from Established Ovarian Cancer Cell Cultures

A number of studies have now been performed demonstrating the presence of a subpopulation of tumorigenic stem-like cells in cultures of established cancer cell lines. While all of the above-mentioned methods have been used to identify CSCs from cell cultures, the most widely used technique exploits the membrane efflux phenotype, by fluorescence-activated cell sorting (FACS) of cells capable of expelling a specific fluorophore ("side population" or SP cells), based on early studies of hematopoietic stem cells [47]. One of the most commonly used fluorescent reagents for SP isolation is the DNA-binding molecule Hoechst 33342 [48]. In ovarian cancer, SP cells have been identified in a number of cell culture lines, including the human lines OVCAR3, IGROV-1, SKOV3, and the mouse lines MOVCAR-7 and 4306 [38, 42]. In the latter study, mouse SP cells demonstrated stem cell properties not present in non-SP cells; including self-renewal, generation of non-SP cells (i.e., differentiation), and shorter tumor latency periods [42]. SP cells are characterized by their expression of ATP-binding-cassette (ABC) transporters including ABCG2 [49] and the multidrug resistance-associated transporter (MDR1) [50], membrane pumps that also mediate efflux of chemotherapeutics and other anticancer agents, thus contributing significantly to drug resistance [51]. However, in another study, Patrawala et al. demonstrated that while SP cells purified from various human EOC cell lines (including the SKOV-3 line) were more tumorigenic than their non-SP counterparts, $ABCG2^{+}$ and $ABCG2^{-}$ cells were similarly tumorigenic, and both overexpressed various "stemness" genes [52]. Moesele et al. further demonstrated that SP cells had a higher proliferation rate, reduced apoptosis, increased tumor-forming ability (based on tumor growth time and the reduced necessary number of engrafted cells), and interestingly, were also highly sensitive to treatment with interferon-α (IFN-α) [40]. Moreover, IFN-α treatment of purified SP cells was associated with a distinct change in their transcriptional profiles [40],

while in an orthotopic mouse EOC model, intraperitoneal delivery of a lentiviral human IFN-α gene construct caused more regression of isogenic tumors having a large SP fraction than tumors with low SP levels [40]. Additionally, the ABCG2/BCRP1 gene, first isolated from drug-resistant human tumor cell lines [53–55], has been reported to be more highly expressed in SP than in non-SP cells, supporting the concept that CSCs (including OCSCs) are more drug-resistant than their more differentiated progeny cells [52, 56, 57].

9.2.2 Isolation of Ovarian Cancer Stem Cells from Ascites

The first report by Bapat et al. of the isolation and identification of OCSCs from EOC patients described two ascites-derived clones able to form multiaggregate, anchorage-independent spheres in culture, and serially propagate xenograft tumors (i.e., re-isolation of stem cells capable of tumorigenesis in a newly engrafted animal) in nude mice that were histopathologically similar to their parental tumors [33]. A follow-up study by the same group demonstrated that in one of these ascites-derived clones, overexpression of mediators of the epithelial-to-mesenchymal transition (EMT, a facilitator of metastasis) was associated with chemotherapy and radiotherapy resistance [58]. In another study, SP cells isolated from ovarian cancer patient ascites were demonstrated by immunohistochemistry to express the stem cell markers Oct4, Nanog, Stellar and ABCG2/BCRP1, as compared to non-SP cells, while also exhibiting greater proliferation rates and tumor multiplicity in xenografted animals [39].

9.2.3 Isolation of Ovarian Cancer Stem Cells from Primary Ovarian Tumors

While sphere-forming and clonogenicity assays have been used to isolate CSCs from solid tumors, the use of cell surface markers or stem cell gene reporter assays has been employed most extensively. A number of surface markers have now been used to isolate OCSCs from primary patient ovarian carcinomas (Table 9.1); most of these are “cluster of differentiation” (CD) markers originally used for the identification of hematopoietic cells of distinct lineages and levels of differentiation [59].

9.2.3.1 CD117

The *c-kit* proto-oncogene CD117, encoding a tyrosine kinase receptor, is expressed in many normal and cancerous tissues, and has also been used to isolate

Table 9.1 Candidate ovarian cancer stem cell markers

Marker	Normal function(s)	Proposed ovarian cancer function(s)	References
CD44	Cell adhesion, hyaluronate degradation, lymphocyte homing	Cancer stemness, mesothelium binding, Nanog activation, cytoskeletal activation of MDR-1	[30, 44, 61, 186, 187]
CD117 (c-KIT)	Hematopoietic cell survival, proliferation, differentiation	Cancer stemness, tumor proliferation, metastasis, angiogenesis	[30, 188–190]
CD133 (PROM1)	Hematopoiesis, tissue development, differentiation	Cancer stemness, metastasis, angiogenesis	[31, 34, 36, 82]
LIN28	Stem cell self-renewal, maintenance of pluripotency	Cancer stemness, dedifferentiation	[41, 87, 89, 191]
MyD88	Immune response, inflammation	Chemoresistance, proliferation	[36, 44, 192]
Oct4	Maintenance of pluripotency, stem cell self-renewal	Cancer stemness, dedifferentiation	[39, 41, 193]
ALDH1	Metabolism of aldehydes, alcohol oxidation, differentiation (generation of retinoic acid)	Cancer stemness, tumor development	[35, 96, 194]

OCSCs from primary tumors. C-Kit kinase activity is induced by the binding of its ligand, stem cell factor (SCF), resulting in autophosphorylation of the receptor. Co-expression of *c-kit* and *SCF* has previously been examined in human EOC tumors, in normal ovaries, and in cultured ovarian surface epithelial (OSE) cells [60]. Normal OSE cells expressed *SCF* but not *c-kit*; *c-kit* expression was, however, found in epithelial invaginations and inclusion cysts. While c-Kit kinase activity is well documented to be oncogenic, it was also reported that c-Kit expression is decreased in advanced stage disease, with c-Kit-negative patients having a significantly shorter disease-free survival time than c-Kit-positive patients [60]. These results suggest that c-Kit may play an early role in ovarian carcinogenesis, while loss of c-Kit expression associates with poor prognosis in later stage disease. Correspondingly, our group demonstrated that ovarian tumor cells coexpressing CD117 and the hyaluronate-binding protein CD44 (see below) isolated from serous ovarian adenocarcinomas exhibited numerous CSC properties, including formation of anchorage-independent self-renewing spheres, self-renewal, and high tumorigenic potential (100 cells/mouse could form tumors, while 105 unsorted cells could not) [30]. $CD44^+$/$CD117^+$ cells could also serially propagate tumors identical to their original histology and expressed a number of genes associated with “stemness” (*BMI-1*,

SCF, *OCT4*, *NES*, *NOTCH1*, and *NANOG*) [30]. Those highly tumorigenic cells were also found to express the membrane efflux pump ABCG2, and demonstrated enhanced resistance to the conventional EOC chemotherapies cisplatin or paclitaxel [30].

9.2.3.2 CD44

The hyaluron receptor CD44, a single chain transmembrane glycoprotein widely expressed in both epithelial and nonepithelial tissues [61], plays a role in numerous physiological processes including cell–cell and cell–matrix interactions, cell adhesion, and cell migration [62]. CD44, however, has also been implicated in tumor progression, and its interaction with hyaluronan has been shown to play a role in the onset of drug resistance [63–66], and in EOC progression CD44 expression has been correlated with the multidrug resistance proteins MDR1, MRP2, and the invasion mediator uPA [67]. Moreover, EGF family member activation of oncogenic ErbB2-ERK signaling was found to result in hyaluronan synthase phosphorylation/activation, with the subsequent upregulation of hyaluronan leading to CD44-mediated ovarian cancer progression [68]. The hyaluronan-CD44 interaction has also been demonstrated to facilitate cytoskeletal protein binding to the multidrug resistance protein MDR1 to augment drug efflux [69], and hyaluronan-based prodrugs against CD44 have demonstrated antitumor and antimetastasis activity in vivo [70]. Paradoxically, however, CD44 expression has been found to correlate with well-differentiated, early-stage ovarian tumors and greater survival [71]; however, the specific CD44 isoform that was analyzed may contribute to these differences.

In coexpression studies with CD133 (see below), it was found that while $CD133^{high}$ and $CD133^{-/low}$ cells expressed similar CD44 levels, in the $CD133^{high}$ cells, CD44 demonstrated physical interactions with transporters, receptor tyrosine kinases, and the metastasis/invasion-associated plasma membrane protein emmprin (CD147) [72].

9.2.3.3 CD133

In addition to CD44 and CD117, CD133 (Prominin-1, formerly known as AC133), a plasma membrane glycoprotein and marker of neural stem cells [73], has now been described as a common CSC marker for numerous malignancies, including brain [74, 75], pancreas [56], liver [76], melanoma [77], prostate [78, 79], and colon [80, 81]. In ovarian cancer, it was also reported that CD133-positive EOC cell lines, primary tumor cells, and patient ascites-derived cells were more platinum-resistant than CD133-negative cells, in addition to forming more aggressive tumor grafts at lower inoculums [34]. In the latter study, $CD133^{+}$ cells sorted from primary ovarian carcinomas were more clonogenic in culture and more proliferative than

$CD133^-$ cells. Moreover, $CD133^+$ cells were found significantly more frequently in ovarian carcinomas than in normal ovaries, benign ovarian tumors, or omental lesions [34], while also showing increased tumorigenic capacity and recapitulation of their original tumor phenotype [34]. $CD133^+$ cells were also shown to differentiate into $CD133^-$ cells, with CD133 silenced in progeny cells by DNA methylation [31]. In another study, however, while $CD133^+$ cells were found to interact with OCSCs, these cells were not tumorigenic, although they could facilitate tumor development by augmenting vasculogenesis (thus suggesting the $CD133^+$ cells to be OCSC-associated endothelial stem cells) [82]. Additionally, CD133 expression was found nonpredictive of patient response to treatment, time to progression (TTP), clinical prognosis, or overall survival (OS) [83]. Consequently, the precise role of this glycoprotein in ovarian tumors remains somewhat uncertain.

9.2.3.4 MyD88

Myeloid differentiation factor 88 (MyD88) is a critical component of the toll-like receptor pathway (associated with immune response) and an activator of the proto-oncogenic NF-κB signaling pathway [44]. In ovarian cancer, Alvero et al. isolated $CD44^+$/$MyD88^+$ cells from solid ovarian tumors and ascites that demonstrated constitutive NF-κB activity, cytokine and chemokine production, a high capacity for DNA repair, and resistance to conventional chemotherapies [44]. Gene array analysis demonstrated that MyD88 was exclusively expressed in $CD44^+$ EOC cells, while 10% of all genes examined were differentially expressed between $CD44^+$ and $CD44^-$ cells, including genes related to apoptosis, signal transduction, and cell differentiation [44].

9.2.3.5 CD24

CD24 is a cell surface molecule upregulated in a large number of human malignancies, and in EOC, its expression has been correlated with poor prognosis [84]. CD24 has also been used as a marker to identify breast and pancreatic CSCs [85, 86], and $CD24^+$ ovarian tumor cells were reported to possess various stem-like characteristics, including quiescence, chemoresistance, self-renewal, and differentiation. In addition, low (5×10^3) numbers of $CD24^+$ cells were capable of xenograft formation in nude mice, while equal numbers of $CD24^-$ cells remained nontumorigenic. $CD24^+$ cells were also found to overexpress the stem cell markers *NES*, *CTNNBIP1*, *BMI-1*, *OCT4*, *NOTCH1*, and *NOTCH4*, while underexpressing *CDH1*, as compared to $CD24^-$ cells [37].

9.2.3.6 LIN28 and OCT4

The microRNA-binding protein Lin28 and the pluripotency-associated transcription factor Oct4 have also been linked to the initiation and/or progression of EOC.

In previous studies of "induced pluripotency," *LIN28* and *OCT4* coexpression with two other transcription factors was capable of eliciting "dedifferentiating" epidermal cells to an embryonic-like, pluripotent phenotype [87]. Lin28 is also known to inhibit processing of the tumor-suppressing microRNA let-7, whose downregulation is associated with poor prognosis and advanced stage of EOC [88]. Analogously, upregulation of (the let-7 antagonist) *LIN28* associated with higher-grade EOC and was also found capable of transforming fibroblasts and other normal cells [89]. Peng and colleagues also reported that a subpopulation of EOC cells and patient tumors coexpresses *LIN28* and *OCT4*, and that in tumors their coexpression correlated with advanced tumor grade, thus suggesting that *LIN28* and *OCT4* coexpression may be a genotype of OCSCs [41].

9.2.3.7 ALDH1

Aldehyde dehydrogenase-1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, has been reported to play a role in the early differentiation of stem cells through its oxidation of retinol to retinoic acid [90–95]. Additionally, several groups have now shown *ALDH1* expression to be a prognostic marker for a number of epithelial cancers [96–102]. Deng et al. [35] analyzed *ALDH1* expression in 24 types of normal tissues and a large collection of epithelial tumor specimens, in addition to a transgenic EOC mouse model and murine EOC cell lines. *ALDH1* expression, while minimal in ovarian cancer cells and tumors, was significantly associated with poor clinical outcomes in serous EOC patients [35]. This finding, however, was contradicted by another study of 266 serous and 176 nonserous EOC patients, in which *ALDH1* expression correlated with favorable prognosis [99]. These discrepancies may indicate that the prognostic value of *ALDH1* is tumor subtype-specific [99].

9.3 Origin of Ovarian Cancer Stem Cells

As mentioned in Sect. 9.1, a precursor lesion for serous EOC has yet to be identified, and it is unclear whether this disease originates from the ovarian surface epithelium (OSE) or from the epithelium of neighboring reproductive structures [103]. Epithelial cells from the three most common types of EOC tumors (endometrioid, mucinous, and serous) are morphologically identical to epithelia of the endometrium, endocervix, and fallopian tubes, respectively [104]. Because no ovarian structures have an epithelial lining similar to any of the aforementioned tissues, it has been hypothesized that EOC tumors may originate from those nonovarian tissues which (unlike the ovary) are embryologically developed from the Müllerian ducts [105]. During embryonic development, the cervix and uterus are formed from the Müllerian ducts fusing, while the fallopian tubes form from an unfused portion of the ducts [106]. Support for this possible origin of EOC is

found in two types of benign cysts found on the ovarian surface: inclusion cysts, which are lined by epithelial cells resembling the OSE, and metaplastic cysts, which are lined by cells identical to the epithelia of the nonovarian structures discussed above [107].

Gene expression analyses may further resolve this point-of-origin question. In particular, expression of the development-associated *HOX* gene family has shown that endometrioid, mucinous, and serous ovarian tumors express the same *HOX* genes as normal endometrioid, endocervix, and fallopian tube epithelia, respectively [108]. Within the fallopian tube, the structure implicated in the "shedding" of tumor cells onto the ovarian surface is the fimbria (Latin for "fringe"), an entity located at the distal end of the fallopian tube that during ovulation, is hormonally induced to rub the surface of the ovary in a sweeping motion, allowing extraction of the ovum into the tube. One compelling argument for a fimbrial origin for EOC is that overexpression of mutant p53 (vaguely designated as a "p53 signature"), a defining characteristic of fimbrial serous tubal intraepithelial carcinomas (STICs), is typically also found in high-grade (but not low-grade) serous EOCs [18]. Microarray gene expression studies comparing distal STICs, fallopian tube epithelia (FTE), and serous EOCs demonstrated indistinguishable expression profiles in *BRCA*-mutation-carrying women, implicating the FTE as a precursor to serous EOC [109]. Pax8, another developmentally associated transcription factor, is also expressed in mucinous, clear cell, serous EOCs, and nonciliated cells present in ovarian inclusion cysts; however, *PAX8* is not expressed in normal OSE cells [110].

In contrast to the hypothesis of the FTE as a precursor for serous EOC, the current argument for the OSE as an origin of serous EOC is that the Müllerian-like features of those malignant cells develop within inclusion cysts (stroma-entrapped OSE) following exposure to high levels of female hormones in that microenvironment [111]. Consequently, however, that hypothesis suggests that serous EOC cells are more differentiated than their cell(s) of origin, and this type of tumor progression (i.e., increased differentiation) runs counter to the known characteristics of all nonovarian carcinomas [112].

It has also been suggested that various CSC attributes can be conferred to normal/precancerous cells during drug treatment. A recent study by Sharma et. al. demonstrated that treating lung cancer cells with normal first-line anticancer drugs resulted in the transient expression of stem cell markers; however, these stem-like "drug-tolerant persisters" (DTPs) did not proliferate. However, while DTP cells were relatively quiescent, a transient subpopulation of these, designated "drug-tolerant expanded persister" (DTEP) cells (which did not express the stem cell markers) proliferated normally and possessed significantly greater cisplatin resistance than the original tumor cells [113]. Moreover, DTEP cells could be ablated by inhibition of histone deacetylases or a histone demethylase [113], demonstrating that chromatin-targeting agents might preferentially target cancer stem-like cells. In a related study, our group recently showed that in an EOC model of platinum resistance, drug resistance positively correlates with a linear increase in the total genomic number of hypermethylated gene promoters, while drug sensitivity was

subsequently restored by inhibitors of DNA methylation [114]. These results provide additional support for the association of epigenome alterations with chemotherapy resistance and/or cancer stemness.

9.4 Therapeutic Approaches for Eradicating Ovarian Cancer Stem Cells

As a consequence of the CSC theory, it is hypothesized that tumors that initially undergo complete remission, but subsequently relapse to a completely refractory state (e.g., ovarian cancer), are more likely to possess CSCs than tumors that do not respond well to primary therapy [115]. In this model, chemotherapeutics preferentially target the rapidly proliferating cells (presumably, CSC progeny cells) that comprise the bulk of the tumor, causing tumor regression, but fail to eradicate drug-resistant CSCs. Consequently, therapies are needed that target both the small percentage of tumorigenic progenitors as well as the more rapidly proliferating nontumorigenic progeny that comprise that bulk of the tumor [116]. Such potential CSC-targeted therapies can be categorized into four general classes: (1) elimination, (2) differentiation, (3) stem cell niche modification, and (4) epigenetic.

9.4.1 Elimination Therapies Targeting Cancer Stemness-Related Pathways

9.4.1.1 PI3K/Akt Signaling

One cascade upregulated in numerous solid cancers, possibly contributing to tumor initiation (and thus an attractive target for cancer therapeutics), is the phosphatidylinositol kinase-3 (PI3K)/Akt mitogenic signaling pathway [117]. Two potential Akt signaling inhibitors, daidzein-daunomycin and N-t-Boc-daidzein, were derived to from the promising cancer chemopreventative phytoestrogen daidzein, which is relatively unstable [118]. Daidzein-daunomycin was reported to improve therapeutic response in an animal EOC model [119], while N-t-Boc-daidzein could decrease the number of OCSCs isolated from patient ascites. N-t-Boc-daidzein was also found to elicit apoptosis of ascites-derived mature EOC primary cell lines, in a dose- and time-dependent manner, due in part to the degradation of Akt [120]. In other cancers, Akt inhibitors were found effective in targeting $CD133^+$ hepatocellular, $CD133^+$ glioblastoma, and $CD133^+/CD44^+$ prostate tumor-initiating cells [121–123]. In an impressive recent study of breast cancer, an antagonist of the IL8 receptor CXCR1 was demonstrated to reduce the number of $ALDH^+$ breast CSCs, followed by massive apoptosis of the remaining bulk of the tumor; and that antagonist was demonstrated to inhibit focal adhesion

kinase (FAK) signaling through Akt [124]. Together, these reports strongly implicate the PI3K/Akt cascade in CSC maintenance and self-renewal.

9.4.1.2 Sonic Hedgehog Pathway

Another signal cascade implicated in playing a role in cancer stemness is the Sonic hedgehog (SHH) embryogenesis-associated pathway. SHH signaling, initiated by SHH binding to its and its receptor, Patched-1, is a crucial mediator of cell fate during early mammalian development [125]. However, SHH deregulation has been hypothesized to contribute to CSC self-renewal, and therefore represents an attractive target for cancer therapy [126, 127]. In support of such an approach, it was demonstrated that cyclopamine, a naturally occurring alkaloid also found to be specific SHH pathway inhibitor, strongly inhibited the proliferation and clonogenic growth of ovarian tumor cells in vitro, while also arresting ovarian tumor growth, in vivo [128]. However, another study demonstrated minimal SHH signaling in ovarian cancer [129], leaving the specific role of this pathway in EOC largely unresolved.

9.4.1.3 Notch

The Notch pathway is a cell–cell contact signaling cascade intimately involved in normal development and tissue renewal [130]. Signal transduction occurs when a surface Notch ligand on one cell activates its receptor on a contiguous cell, resulting in cleavage of the Notch intracellular domain (NICD) [130]. The NICD then relocates from the cytoplasm to the nucleus, resulting in gene transactivation via its interaction with the transcription factor CBF (C element-binding factor) [130]. However, Notch dysregulation has also been implicated in maintenance of the CSC phenotype, and a number of specific Notch inhibitors are currently in various phases of development [131]. In ovarian cancer, various Notch pathway members are overexpressed in tumors, but not in adenomas [132]. Correspondingly, significant Notch signaling has also been observed in EOC cell lines and 76% of EOC patient tumors [133]. Moreover, *Notch1* was found overexpressed in candidate OCSCs, as compared to the bulk population of tumor cells or OCSCs placed under differentiating conditions [30], while *Notch3* amplification in EOC tumors was found to mediate their proliferation and survival [134]. Together, these and other reports strongly implicate Notch in ovarian tumorigenesis and OCSC maintenance, making this an attractive therapeutic target.

9.4.1.4 Wingless (Wnt) Signaling

The Wnt pathway is essential for embryonic morphogenesis and body axis specification and tissue homeostasis, due to its regulation of self-renewal of normal

stem cells [135]. Signal propagation occurs upon the binding of Wnt ligand to its receptor, Frizzled, resulting in a cascade that leads to nuclear translocation of beta-catenin, which upon binding to its transactivational cofactor, TCF, induces a number of protooncogenes (including *MYC*) [135]. Consequently, similar to other embryonic signaling pathways, Wnt dysregulation is also associated with carcinogenesis and tumor progression [135, 136]. In one EOC study, Rask et al. demonstrated increased expression of components of the Wnt pathway in malignant EOC tumors, as compared to normal ovarian tissues [137]. Towards the targeting of Wnt signaling as an effective cancer therapy, two small molecules (ZTM000990 and PKF118-310), were identified in a high-throughput screen (based on the structure of the beta-catenin/TCF complex) to target the canonical Wnt signaling cascade [138]. Additionally, anti-Wnt1 and anti-Wnt2 monoclonal antibodies were found to be potent inducers of apoptosis in melanoma, mesothelioma, and melanoma cells [139]. With further pharmacologic optimization, these small molecules or antibodies targeting the Wnt signaling pathway could represent effective ovarian cancer therapeutics.

9.4.2 Differentiation Therapies Targeting Cancer Stem Cells

Another potential approach to CSC targeting is the use of differentiating agents, which presumably would alter the embryo-like CSC phenotype toward that of its normal, mature tissue; it is hypothesized that disruption of the abovementioned self-renewal pathways might serve this purpose. Various differentiating agents have now been examined with varying degrees of success, including dietary polyphenols and phytoestrogens, and vitamin D3. However, the agent best studied (and to date, the most successful) differentiating agent is all-trans retinoic acid (ATRA), which has demonstrated impressive effectiveness against acute promyelocytic leukemia, head/neck squamous carcinomas, thyroid cancer, and in combination with interferon-γ, neuroblastoma cells [140–143]. In two serous EOC cell lines, ATRA was also demonstrated to alter cell morphology relative to that of differentiated epithelial cells, in addition to strongly inhibiting cell proliferation [144]. With regard to CSC differentiation, our group showed that OCSCs are more resistant to cisplatin and paclitaxel, but could be resensitized to both agents under differentiating conditions [30]. In a separate study, we also demonstrated that histone deacetylase inhibitors (a type of epigenetic therapy) induced morphological changes and epithelial differentiation markers in a platinum-resistant EOC cell line [145]. In a series of striking studies, differentiation of highly aggressive melanoma cells to normal melanocytes has also been demonstrated. Following plating the melanoma cells atop an embryonic stem cell-derived extracellular matrix, the reciprocal placement of melanocytes onto a melanoma-derived microenvironment resulted in restoration of the aggressive malignant phenotype [146, 147]. As normal differentiation is governed by epigenomic changes (see below), it is strongly believed that CSCs also possess a type of “epigenetic plasticity” capable of altering their degree of

differentiation (and thus their malignant phenotype) [148, 149]. Together, these results support the idea that differentiation therapy, possibly facilitated by epigenetic therapies of OCSCs, has strong potential as an effective therapeutic approach.

9.4.3 Destruction or Alteration of the Cancer Stem Cell Niche

In vivo, stem cell self-renewal and differentiation are tightly controlled by a complex niche that physically harbors those cells in an anatomically well-defined location within a tissue (reviewed in [150]), and there is increasing evidence that the microenvironment regulates tissue specificity and contributes significantly to tumorigenesis (reviewed in [151]). The extracellular environment provides the structural platform necessary for cell growth and intercellular communication; analogously, various growth factors and chemokines may enhance tumor cell proliferation and invasion [151]. Conversely, the tumor microenviroment may also stimulate production of antiangiogenic proteins and inhibitors of matrix metalloproteases that can obstruct tumorigenesis [152]. In brain cancer, it was found that $CD133^+/Nestin^+$ CSCs reside in a paravascular niche, and that inhibition of angiogenesis, via EGF signaling disruption or inhibition of vascular endothelial growth factor (VEGF), eradicated those self-renewing cells [153, 154]. Moreover, as noted previously, a number of studies have now demonstrated differentiation-associated "reprogramming" of aggressive melanoma and breast cancer cells into normal epithelial cells by culturing them in an embryonic stem cell–derived microenvironment [146]. Those studies demonstrate that even advanced stage cancers (likely having increased numbers of CSCs) exhibit a phenotypic "plasticity" for differentiation that is governed by epigenomic changes. In particular, the embryonic microenvironmental signaling molecule responsible for melanoma cell reprogramming was found to be an inhibitor of the embryonic morphogen Nodal, a member of the TGF-beta family [146]. Nodal was later found to effect "vascular mimicry," formation of tube-like structures capable of perfusing the tumor [155, 156] (thus possibly similar to brain CSC perivascular niche). In ovarian cancer specifically, components of the secondary Müllerian system (paraovarian/paratubal cysts, rete ovarii, endosalpingiosis, endometriosis, and endomucinosis) may similarly provide a source of cells and/or signaling molecules that contribute to the different histologic types of ovarian malignancies [103]. Analogously, a recent study suggested Müllerian inhibiting substance (MIS) as a possible adjuvant to conventional ovarian cancer chemotherapeutics that targets putative OCSCs, as MIS treatment inhibited proliferation of both SP and non-SP cells, while conventional chemotherapies primarily arrested non-SP cells [42].

9.4.4 Epigenetic Therapies

Epigenetic alterations have been demonstrated to govern gene expression both in embryonic and tissue stem cells, and thus likely play an important role in the

tumorigenic potential (and differentiation prevention) in OCSCs. These alterations include methylation of deoxycytosine, as well as numerous modifications of histones that regulate distinct gene expression patterns that allow for specific cell or organotypic phenotypes [157]. It is also well established that epigenetics contributes significantly to ovarian tumorigenesis [158], likely in association with its intricate role in differentiation.

It is hypothesized that ovarian tumor chemotherapy resistance results largely from the repression of tumor suppressor genes (specifically, chemotherapy-response genes) by DNA methylation [159–161]. In contrast to DNA mutations and deletions, however, aberrant gene-repressive epigenetic modifications are potentially reversible, by epigenetic therapies, including inhibitors of DNA methyltransferases (DNMTs) or various histone-modifying enzymes (reviewed in [161]). Although epigenetic monotherapies have shown little activity against solid tumors [162–164] including ovarian cancer [161, 165], preclinical studies of DNMT inhibitors by our group [114, 166] and others [167–170] have demonstrated potent resensitization of drug-resistant EOC cells and xenograft tumors to conventional chemotherapies, possibly by differentiation or apoptosis of CSCs [159, 164]. Interestingly, it was also demonstrated that DNMT inhibitor treatment induced silenced *SFRP5* (encoding an endogenous Wnt pathway inhibitor), similarly chemosensitizing drug-resistant EOC cells and xenografts [171].

Based on the abovementioned studies demonstrating chemotherapy sensitization of resistant EOC cells and tumors, several such combinatorial regimens are now being examined in cancer clinical trials [160, 161]. Our group recently completed a Phase I trial (NCT00477386, Study ID 0704-07, www.clinicaltrials.gov) using a DNMT inhibitor, decitabine (Dacogen; Eisai, Inc., Tokyo, Japan) combined with the standard chemotherapeutic, carboplatin, hypothesizing that low-dose decitabine may derepress silenced tumor suppressors to chemosensitize platinum-resistant EOCs [172]. In that study, a low dose (10 mg/m^2) of decitabine was administered for 5 consecutive days, followed by carboplatin (AUC5) administered 3 days later, with each treatment cycle lasting 28 days. That regimen demonstrated bioactivity in vivo, as assessed by decreased methylation of DNA repetitive elements (in patients' peripheral blood cells) and of specific genes (in patients' sera). Out of 10 patients, we observed 1 complete response, while 4 patients experienced stable disease for at least 6 months; although interestingly the complete response did not occur prior to eight treatment cycles [172]. While we cannot draw conclusions based on this small patient sample size, it is interesting that breast CSCs induce tumorigenesis only after a latency of 6 months, and it is conceivable that drug-associated hypomethylation of CSCs would similarly require an extended period based on the slow division of those cells and their delayed response in other carcinomas [29, 173].

Since histone deacetylation is another transcriptional silencing mechanism in ovarian cancer, HDAC inhibitors (HDACIs) can also relieve epigenetic gene repression, and these agents also exert anticancer effects by inhibiting the deacetylation of nonhistone proteins [174]. Similar to DNA methylation inhibitors, HDACIs are most promising in combination with conventional agents, and studies by our group [145] and others [175–180] have demonstrated chemosensitizing effects on

drug-resistant ovarian cancer cells and tumors. HDACIs have also been effectively combined with a differentiating [181] or a "death receptor" ligand [182] in ovarian cancer cell studies, similar demonstrating additive or synergistic effects. It is also possible that HDACIs might also have direct effects on CSCs. In one study, a subpopulation of rapidly proliferating, drug-resistant lung cancer cells could be eliminated by HDACI treatment [113], while an HDACI/imatinib combination was demonstrated to target chronic myelogenous leukemia stem cells [183]. In other studies, an HDACI was found to block self-renewal and aggregation of breast cancer spheroids [184], while another HDACI suppressed expression of the stemness gene *NANOG* in embryonic carcinoma cells, resulting in loss of a stem cell "gene signature" [185]. Thus this class of epigenetic agents holds promise for the treatment of drug-resistant ovarian cancer, possibly via their effects on ovarian CSCs.

9.5 Conclusions

Based on several studies to date, it is highly likely that ovarian tumors, similar to numerous other solid cancers, possess a hierarchy of cell types, with tumor initiation, progression, and chemoresistance driven by a distinct subpopulation of malignant progenitor cells. A further understanding of these "cancer stem cells" holds promise for the design of strategies toward their eradication, possibly by epigenetically inducing their differentiation or elimination via blockade of specific pathways essential for maintenance of their tumor propagating and undifferentiated phenotype.

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Chapter 10
Cancer Stem Cells in Hepatocellular Cancer

Russell C. Langan and Itzhak Avital

Abstract Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and typically portends a poor prognosis with a median survival ranging from 6 to 16 months. In the United States, a total of 24,120 new cases of primary liver cancers and 18,910 deaths are projected to occur in 2010. Associated factors potentially contributing to this abysmal prognosis include delayed diagnosis, underlying cirrhosis, and resistance to chemotherapy. Recently, compelling evidence has emerged in support of the cancer stem cell (CSC) hypothesis for many solid organ cancers including hepatocellular cancer (HCC). CSCs are postulated to account for tumor initiation, therapeutic resistance, and relapse following surgery or therapy. Identification, proper characterization, and understanding the biology of the HCC-derived CSCs (HCSCs) are imperative for improving early detection and treatment outcomes. If proven correct, the CSC hypothesis may herald a paradigm shift in the treatment of this deadly disease. This chapter summarizes the differences between HCSCs and normal liver stem cells through state-of-the-art identification and characterization, and then assesses the clinical correlation and potential novel therapeutic strategies based on HCSCs.

Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
AFP	Alpha-fetoprotein
ALDH	Aldehyde dehydrogenase
CD	Cluster of differentiation

I. Avital (✉)
National Cancer Institute (NIH), Surgery Branch,
Bethesda, MD, USA
e-mail: avitali@mail.nih.gov

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CK	Cytokeratin
CSC	Cancer stem cell
CYP	Cytochrome P450
EpCAM	Epithelial cell adhesion molecule
ESA	Epithelial specific antigen
FAH	Fumarylacetoacetate hydrolase
HCA	Hepatocellular adenoma
HCC	Hepatocellular cancer
HCSC	Hepatocellular cancer stem cell
HTAC	Hepatocellular transiently amplifying cell
ICAM	Intercellular adhesion molecule
IL	Interleukin
LRCC	Label retaining cancer cell
MDR	Multi drug resistance pump
NCAM	Neural cell adhesion molecule
NOD/SCID	Non-obese diabetic/severe combined immune deficiency
SC	Stem cell
SCF	Stem cell factor
SMO	Smoothened
SP	Side population
STAT3	Signal transducer and activator of transcription 3
TACSTD1	Tumor-associated calcium signal transducer 1
TBRII	TGF-beta type II receptor
TGF-β	Transforming growth factor beta

10.1 Introduction to Hepatic Stem Cells

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and typically portends a poor prognosis with a median survival ranging from 6 to 16 months [1]. In the United States, a total of 24,120 new cases of liver and intrahepatic bile duct cancer and 18,910 deaths are projected to occur in 2010 [2]. Systemic therapy for HCC is of limited efficacy [3]. The precise cell of origin of HCC is unknown. Currently, the cancer stem cell (CSC) hypothesis posits that HCC might be derived from liver stem cells or be driven by stem-like cancer cells. In order to elucidate the mechanisms of hepatocarcinogenesis and design more effective therapies, identification of the cell of origin of HCC or the hepatocellular cancer stem cell (HCSC) is of paramount importance.

Potential properties that define CSCs are: (1) self-renewal; (2) the capacity for differentiation, which allows for the recapitulation of all cell types of the original tumor; and (3) tumor-initiating capacity, which is the ability to propagate tumors when transplanted into a separate environment; and possibly, asymmetric cell division via non-random chromosomal co-segregation [4]. Investigators have been using

these properties and various cell-membrane markers for the isolation and testing of the HCSC. Below, we will begin with a discussion of normal hepatic stem cells followed by the potential role of stem cells in hepatocarcinogenesis and finally discuss potential therapeutic targets.

10.1.1 Hepatic Stem and Progenitor cells

Hepatoblasts (bipotential liver progenitors) originate during embryogenesis and differentiate into hepatocytes and cholangiocytes [5, 6]. Human fetal liver contains epithelial cell adhesion molecule (EpCAM)/CD326^{+} cells divided into two groups: hepatoblasts expressing intercellular adhesion molecule-1 (ICAM-1), alpha-fetoprotein (AFP), albumin CK19, and CD133; and another population negative for ICAM-1, AFP, and albumin but positive for CD133, CK19, and NCAM [7, 8]. Transplantation of this cell population into livers of NOD/SCID mice results in differentiation into human liver tissue [8]. Recent data have shown that liver stem cells exist both in fetal and in adult livers [7]. They are able to compensate for a daily loss of up to 3% of the parenchymal cell mass after injury [9–12]. However, they might represent transiently amplifying cells or mature hepatocytes rather than true liver stem cells.

10.1.2 Oval Cells and Human Liver Progenitors

Oval cells (rodent liver progenitors) can differentiate into hepatocytes and cholangiocytes [13–15]. Oval cells have appeared in the periportal spaces after treatment with carcinogens, and subsequently repopulated the injured liver. Oval cells may have been the first evidence for the HCSC. In humans, cells with similar characteristics have been identified in chronic inflammatory conditions and HCC. These cells were named hepatic progenitors or small hepatocytes, and they reside within terminal branches of the biliary system in the Canals of Hering [6]. When mature hepatocytes and cholangiocytes are damaged, these hepatic progenitors are activated (termed ductular reaction) [6]. They express markers of hepatocytic (albumin) and cholangiocytic (CK19) differentiation, and can differentiate into hepatocytes or cholangiocytes.

10.1.3 Liver Progenitors in Benign Liver Diseases Associated with Malignancy

Important risk factors for the development of HCC are chronic inflammatory conditions such as hepatitis and cirrhosis characterized by progenitor cell activation [16]. When the ability of mature hepatocytes or transiently amplifying cells to proliferate

is impaired, liver regeneration is initiated by liver progenitors [17–22]. This activation is correlated with the severity of the hepatic injury [12, 23–27]. However, evidence for a continuum represented by the presence of liver stem cells in the transition from ductular reaction metaplasia dysplasia adenoma to hepatocellular carcinoma is not robust at this time.

10.1.4 Liver Progenitors in Hepatocellular Adenoma

Hepatocellular adenoma (HCA) is a benign tumor. Approximately 10–20% of all reported surgical specimens of HCA have been observed to contain foci of HCC [28, 29]. Libbrecht et al. found liver progenitors in 5/10 patients with HCA. They identified a population of cells consisting of intermediate cells which were phenotypically balanced between liver progenitors and hepatocytes (expressing CK7, CK19, Chromogranin-A, and OV-6) [27]. These studies suggest that liver stem cells contribute not only to chronic liver disease but also to benign liver tumors and, thus, may be involved in the adenoma–carcinoma transition, and potentially may assist in early detection of HCC.

10.1.5 Liver Progenitors in Hepatocellular Carcinoma

Some HCC tumors have characteristics consistent with both HCC and cholangiocarcinoma. CK7 and CK19 expression is correlated with biliary differentiation, while CD34 and CD117 are associated with hepatocytic differentiation. Yamamoto et al. found among 217 HCCs that CK7, CK19, and CD117 were expressed by 40, 10, and 1% of cells, respectively, and none were positive for CD34, suggesting that some HCCs may have been derived from bipotential liver progenitors [30]. Several studies have demonstrated tropism of liver stem cells to HCC [11, 12]. Yao et al. found that 28–50% of HCCs expressed markers associated with liver progenitor cells [1]. Tumors with these characteristics had an inferior prognosis [1, 6]. Further, Lee et al. demonstrated that a subset of HCC, which carried a poor prognosis, had a genetic signature consistent with liver progenitor cell origins [31]. However, the question as to whether these cells are dedifferentiated hepatocytes or transformed progenitor cells remains open.

10.2 The Liver Stem Cell Niche

Normal stem cells (SC) reside in a restricted microenvironment, the stem cell niche [32, 33]. The SC niche is a location where SCs are kept in an undifferentiated state [34–36]. However, in response to specific signals (such as tissue injury), stem

cells exit the niche and differentiate. In humans, the liver stem cell niche is thought to be localized to the Canals of Hering at the terminal branches of the biliary tree [18, 37]. It is hypothesized that SCs in the niche and hepatocellular transiently amplifying cells (HTAC) are in balance. Disturbances in this balance (i.e., in chronic inflammatory diseases) can disrupt the physical niche resulting in the SC exiting the niche prematurely with subsequent aberrant differentiation and eventual cancer formation [38].

10.3 The Side Population (SP)

In 1996, Goodell et al. used the capacity of cells to efflux Hoechst 33342 dye via the ABCG2 pump to isolate hematopoietic SCs [39]. This population of cells was named the side population (SP). Subsequently, a SP was identified in several solid cancers and was hypothesized to contain stem-like cancer cells. Further testing showed that SP cells were able to generate both SP and non-SP cells recapitulating the parent tumor, while non-SP cells can only generate non-SP cells. Importantly, SP cells are more tumorigenic in vivo [40]. The SP is a helpful tool for SC studies when there is a lack of other specific markers. It should be noted that some oval cells and HCC cells highly express ABC transporters such as the ABCG2 [41].

10.3.1 The Side Population in Normal Livers

Asakura et al. reported on SP cells (45% CD45-positive) in normal livers with stem cell capacity [42]. Hussain et al. isolated CD45-negative SP cells (0.01% of the non-parenchymal cells) from human livers. They showed that SP cells could differentiate into mature hepatocytes expressing lipofuscin pigment, HepPar, CK8, human albumin, CK18, P450 enzyme CYP2B6, and α1-anti-trypsin [43]. In rodents, hepatic-SP cells were able to regenerate livers (hepatocytes and cholangiocytes) undergoing chemical injury. Overall, the SP cells comprise approximately 1% of the parenchymal liver cell mass. They express CD34 (4%), c-Kit (12%), Sca-1 (50%), and thy-1 (50%), markers which have all previously been described as liver and hematopoietic SC markers. Interestingly, the SP cells were negative for liver maturation markers fumarylacetoacetate (FAH), the biliary marker CK19, and A6.

10.3.2 The Side Population in Human HCC

In human HCC cell lines, approximately 0.25–3.2% of cells have been observed to exhibit the SP phenotype [7, 44]. These SP cells resemble stem cells: small, quiescent, immature, highly tumorigenic, and expressing low levels of hepatocyte

differentiation markers. However, there is a paucity of data on SP cells from fresh tumors. The SP phenotype is dependent on the expression of the ABCG2 pump. Zen et al. reported on the expression of ABCG2 in human normal livers ($n = 5$), low grade ($n = 10$), and high-grade dysplastic nodules, and two HCC cell lines ($n = 15$). ABCG2-positive cells were found to be concentrated around the periportal area of dysplastic nodules, and scattered or in clusters within foci of HCC [45]. ABCG2-positive cells could generate both ABCG2-positive and negative cells, while ABCG2-negative cells generated only ABCG2-negative cells [45]. ABCG2-positive cells expressed progenitor markers (AFP and CK19) while ABCG2-negative cells highly expressed albumin. Note, these results are consistent with the SP phenotype but are not equivalent to SP cells.

10.3.3 The Side Population in Human HCC: Self-Renewal and Tumor Initiation Capacity

Our group and others have tested the tumor-initiating capacity of human SP cells derived from HCC. Chiba et al. compared the tumor initiation capacity of SP and non-SP cells (HuH7 and PLC/PRF/5) [7]. They reported that 1×10^3 SP cells and 1×10^6 non-SP cells were required to initiate tumors, respectively. After serial xenotransplantation, SP cells generated both SP and non-SP cells while the non-SP cells generated only non-SP cells. Other investigators demonstrated that SP cells have further stem cell-like characteristics, including self-renewal, high clonogenicity and chemoresistance [46, 47].

10.3.4 The Side Population in Human HCC: Therapeutic Resistance

Chemoresistance of CSCs possibly contained within the SP fraction of HCC may be related to the expression of the ABCG2 transporter. Until recently, doxorubicin was the first-line chemotherapeutic agent for HCC. SP cells were found to be less sensitive to doxorubicin (a known ABCG2 substrate) than non-SP cells [44, 48]. However, SP cells demonstrated more variable resistance to other chemotherapeutic agents (Gemcitabine and 5-flurouracil) which are not substrates of the ABCG2 transporter. Therefore, although the ABCG2 transporter may be associated with treatment failure, it is not the definitive answer to the potential chemoresistance exhibited by the SP. Fan et al. studied the anti-apoptotic mechanisms of SP cells in two cell lines (MHCC97 and hHCC), which contained 0.25 and 0.5% SP cells, respectively [49]. Following apoptotic conditions, SP cells demonstrated greater proliferative capacity

that was correlated with inhibition of Bax and upregulation of Bcl-2, potentially indicating that SP cells have an efficient anti-apoptotic mechanism that may account for the relative therapy resistance [49].

10.4 Experimental Considerations for Stem-Like Cells in HCC

The concept of the CSC in hepatocellular carcinoma is not a new idea. In the past, prior to the recent interest in the CSC hypothesis, mouse models of hepatocarcinogenesis suggested the role of stem cells in HCC [4]. There are three cell types in the adult liver that can potentially undergo malignant transformation resulting in HCC: hepatocytes, cholangiocytes, and hepatic progenitors. Mature hepatocytes have a lifespan of over a year and near infinite capacity to proliferate (approximately 69 doubling times); as such, they potentially live long enough to propagate transformation events [9, 37]. In 1997, Overturf et al. demonstrated in serial transplantation experiments that hepatocytes have the characteristics of longevity, including the extensive capacity to proliferate and self-renew (clonogenic) both of which are fundamental stem cell properties. Hence, mature hepatocytes, with their innate stem-like traits, could be one type of stem-like cancer cells, i.e., a dedifferentiated mature hepatocyte. As stated above, extensive SC/progenitor cell proliferation has been observed in preneoplastic, inflammatory conditions of the liver, and the extent of progenitor cell proliferation correlated with the severity of the underlying liver insult. Also, following malignant conversion, a substantial number of HCCs demonstrate bipotential characteristics, i.e., tumor cells co-express biliary and hepatic markers such as CK7, CK19, AFP, and albumin. The presence of these traits was associated with a more aggressive phenotype and a worse overall outcome [7]. Therefore, the question remains as to what the relationship is between liver stem cells and HCC. Is it a liver stem cell that gives rise to HCC or do some HCC cells behave like stem cells but themselves are not derived from stem cells?

Currently, there are two general approaches for the isolation of CSC: the antigenic approach that use mainly cell surface markers to label putative CSCs, and the functional approach. The antigenic approach uses known stem cell markers and it is the most commonly reported approach. The problem with this approach is that studies report on different alleged CSC phenotypes from the same cancer. The function of these cells seems to differ from study to study, in particular their ability to reconstitute tumors after xenotransplantation. The clinical problem with this approach is such that targeting these cells will also target normal stem cells. In contradistinction, the functional approach uses basic stem cell functions to define the putative CSC populations; the theory being that it is truly their function that makes these stem cells unique. One such approach was reported by Hari et al., who isolated live putative HCC-derived CSCs by their ability to retain DNA labels and divide asymmetrically with non-random chromosomal cosegregation (LRCC, label retaining cancer cells) [50].

10.4.1 Markers for Hepatocellular Cancer Stem Cells

10.4.1.1 HCC-Derived CD133-Positive Cells

CD133 is a potential cell surface marker for HCSC. CD133-positive cells derived from HCC have been shown to exhibit greater tumorigenicity when compared to their negative counterparts (CD133-negative cells). CD133-1 (the glycosylated epitope of AC133) was first identified in 1977 within primitive hematopoietic stem cells as a subset of CD34-positive cells derived from human fetal bone marrow and liver [51, 52]. Subsequently, CD133 was found to be expressed by various normal primitive cells of hematopoietic, neural, and endothelial lineages [52]. It is a 120 kD cell surface glycoprotein with five transmembrane domains. It is hypothesized that CD133 participates in organization of plasma membrane topology, and its expression sustains the stem cell phenotype. CD133-positive cells derived from hematopoietic and somatic tissue have shown multipotential differentiation capabilities. These cells could differentiate into myogenic, endothelial, keratinocytic, cardiac, renal, prostatic, neural, islet, pancreatic, and liver lineages [53–56]. However, its true scope of function is yet to be elucidated [51, 57, 58].

CD133-positive cells have now been detected in several poorly differentiated human cancer cell lines derived from lung, prostate, brain, pancreatic, colon, and breast carcinomas. It was not detected in similar more differentiated cell lines, indicating that its expression might depend on the degree of cellular differentiation [59]. Subsequently, CD133 was reported to be a marker of putative solid organ CSCs in several organ systems (brain, prostate, colon, melanoma, pancreas, and liver) [60–66]. These CD133-positive cells were designated as putative solid organ CSCs because they were capable of self-renewal, tumor initiation, multilineage differentiation, and recapitulation of the original tumor phenotype in vivo, unlike their CD133-negative counterparts.

When specifically analyzing normal livers, CD133 is not detected by immunostaining; however, its messenger RNA can be detected by northern blot analysis. Honor et al. identified putative liver stem cells as blast-like cells in human livers after massive hepatic necrosis. These cells were CD133- and CD117- (SCF/stem cell factor/c-KIT) positive, and CD34−, CD45− and tryptase-negative [67]. Ma et al. reported that Prominin-1, the mouse orthologue of human CD133, is highly upregulated during liver regeneration [62]. These findings suggested that CD133 expression was associated with putative liver stem cells following liver injury.

Several reports have confirmed the high tumor-initiating capabilities of CD133-positive cells isolated from liver cancers. Xenograft transplantation experiments showed that CD133-positive cells are capable of initiating tumors in NOD/SCID mice, while CD133-negative cells failed to generate tumors to the same degree. Yin et al. transplanted cells both intraperitonealy and intrahepatically, and observed that 2×10^6 unsorted cells were required to grow a tumor intraperitoneally at 1 month [68]. In comparison, as few as 100 CD133-positive cells generated tumors in the abdominal cavity in 3/5 mice, and none were seen in the CD133-negative population.

Intrahepatically, 2,000 CD133-positive cells in 4/6 animals were required to grow tumors; while none grew after transplantation of CD133-negative cells [68]. However, reported results in the literature vary widely; 1×10^6 CD133-positive cells from the HuH7 cell line, and as few as 1×10^3 CD133-positive cells from the SMMC7721 cell line were able to initiate tumors in NOD/SCID mice [68, 69]. Suetsugu et al. reported that both CD133-positive and -negative cells initiated tumors in NOD/SCID mice, although CD133-negative cells induced only very small tumors [69].

Although CD133-positive cells have shown large variability in their tumor-initiating characteristics, Suetsugu et al. reported that HuH7 CD133-positive cells were able to regenerate and recapitulate the parent tumor. CD133-positive cells were able to regenerate both the CD133-negative and CD133-positive cells both in vitro (after only 7 days in culture) and in vivo. In addition, HuH7 CD133-positive cells had a significantly higher proliferative capacity [69].

Ma et al. examined stem-like properties other then tumor initiation capacity of CD133-positive cells. They reported that CD133-positive cells demonstrated significantly upregulated expression of Wnt/β-catenin, Notch, Hedgehog/SMO, Bmi, and Oct3/4 when compared to CD133-negative cells. These genes participate in pathways that govern stem cell pluripotency, proliferation, self-renewal, and differentiation and will be discussed in further detail below [62, 70]. Inhibition of the Notch pathway resulted in an approximately fivefold decrease in the CD133-positive population and an almost complete elimination of the side population [71].

As previously stated, it has been postulated that solid organ CSCs are resistant to chemotherapy. CD133-positive cells have been reported to be resistant to both chemotherapy and radiotherapy in several different models of cancer [72, 73]. Ma et al. tested HuH7 and PLC8024 CD133-positive cells with agents that are used clinically to treat HCC (doxorubicin and 5-fluorouracil). Treatment of unsorted cell populations resulted in significant enrichment of the CD133-positive subpopulations. The resistance to doxorubicin and 5-fluorouracil was based on the preferential activation of the Akt/PKB and Bcl-2 survival pathways [74]. Both pathways are thought to be pivotal in cytotoxic drug-mediated apoptosis in HCC [75]. Furthermore, inactivation of the Akt/PKB pathway by an AKT1 inhibitor abolished the preferential survival of CD133-positive HCC cells.

The variable results (and in certain reports, contradicting results) relating particularly to the tumor-initating capacity of CD133-positive cells suggests that the population of HCSCs is widely heterogeneous. Thus, CD133 as a marker of HCSC may not be specific or sensitive enough for clinical studies but might be a useful tool for preclinical investigations. It also highlights the need for better HCSC markers.

10.4.1.2 HCC-Derived EpCAM-Positive Cells

Epithelial Cell Adhesion Molecule (EpCAM; also known as ESA or TACSTD1) is a cell surface molecule expressed by several epithelial stem cells and by most

epithelial cells. EpCAM is now believed to be a CSC marker for a number of malignancies including liver cancer [4]. EpCAM-positive cells from two separate HCC cell lines have displayed CSC properties. When compared to their EpCAM-negative counterparts, these cells were more tumorigenic in vivo and they formed more spheres in anchorage-independent growth trials [76]. Isolated EpCAM-positive cells in HCC exhibited stem cell gene expression profiles via cluster analysis tested on the human stem cell pluripotency array, as compared to EpCAM-negative cells, which much closer resembled the mature hepatocyte subtype of HCC [4]. Furthermore, using primary liver tumor samples, it was found that increased EpCAM-positivity (likely caused by Wnt signaling) increased the tumorigenicity of these cells, whereas blocking EpCAM had the opposite effect on tumorigenicity [76]. This suggests that classification of HCC patients via the EpCAM marker may have prognostic significance [4].

10.4.1.3 HCC-Derived CD90-Positive Cells

CD90 is a mesenchymal stem cell marker and is expressed by hepatic stem/progenitor cells during liver development, but infrequently expressed in adult liver [6]. Although it is expressed on HCC less than CD133, 0–2.5% of HCC cells have been reported to be positive for CD90 [75]. In these samples, it was found that the CD90+/CD45− population was highly tumorigenic. Note, this expression was absent in normal and cirrhotic livers [76]. HCC CD90-positive cells exhibit stem cell-like properties including propagation of tumorigenicity after secondary transplantation into SCID mice [6]. Prognostically, CD90 may serve as an early tumor detection marker for potential early diagnosis of HCC [6]. Interestingly, the results of CD90 and EpCAM studies on standard cancer cell lines have been reproduced in fresh human HCC tissue. These data provide supporting evidence for the presence of putative hepatic CSCs in human patients with HCC [76].

10.4.2 Genomics and Signaling Pathways in Hepatocarcinogenesis

Over the last decade, there have been rapid advances in microarray technologies. Through these advances, microarray analysis of the liver has uncovered a number of molecular signatures, signaling pathways, and gene sets associated with hepatocarcinogenesis. How these exactly relate to liver stem cells waits to be elucidated. That being said, we now have data showing how many of the dysregulated pathways in HCC are involved in stem cell maintenance and self-renewal, including Wnt, Notch, Hedgehog, TGF-β, and IL-6. The relationship of HCC to these pathways may suggest the stem cell origin of HCC. Further evidence supporting the stem cell origin of HCC has recently been provided by comparative genomic investigations. Gene expression data from rat fetal hepatoblasts and adult hepatocytes were integrated with gene expression data from human HCC. The HCCs, which shared expression

data with fetal hepatoblasts (including hepatic oval cell markers), were profoundly different from other prognostic subtypes of HCC [4]. In addition, these HCCs were associated with a worse prognosis, and classification of HCC based on gene expression of EpCam and AFP revealed distinct HCC subtypes. The specific phenotype of EpCam+/AFP+ was associated with poor survival and was characterized by activation of WNT/β-catenin and TGF-β [4]. From this we can derive further support for the hypothesis that HCC is a disease characterized by a hepatic progenitor cell origin/CSC interplay. The pathway analysis findings (presented below) in HCC support the idea that molecular heterogeneity of HCC originates in the CSC compartment [76]. Therefore, these pathways could potentially serve as novel prognostic biomarkers and represent potential targets for novel therapeutic strategies.

10.4.2.1 Wnt Signaling

Extrapolating data from embryonic development, it has been found that Wnt signaling is involved in cell survival, proliferation, and cell fate, and plays a crucial role in stem and progenitor cell expansion. Disruption of Wnt signaling can result from both genetic and epigenetic changes and is frequently found in cancer, specifically colon and HCC [76]. In the colon, Wnt signaling is activated in the colonic crypts and keeps cells in a proliferative state [6]. Increased Wnt activity leads to enlarged crypts and intestinal tumors, whereas inhibition of Wnt leads to loss of the stem cell compartment all together [6]. Therefore, we know that Wnt signaling is essential for maintenance of the stem cell compartment in the colonic crypts. Up to 40% of HCCs analyzed have mutations and deletions in this pathway leading to overexpression of β-catenin [6]. However, not all studies demonstrate a correlation between elevated β-catenin and expression of its transcriptional targets in HCC, indicating that these target genes are also regulated by alternative pathways [1]. The effects of Wnt signaling on stem cells are modulated through the association with other signaling pathways such as Notch, Hedgehog, and TGF-β. Disruption by mutational and non-mutational events such as “cross-talk” with TGF-β is seen in 30% of all HCCs and emphasizes the magnitude of this pathway in hepatocarcinogenesis [76]. Elevated expression of Wnt has been found in CD133+ and EpCAM+ liver CSCs along with SP cells [76]. Exactly how the Wnt pathway promotes stem cell self-renewal and its involvement in HCC is still unclear. In select situations, Notch acts jointly with Wnt to sustain stem cell proliferation and is essential for the differentiation of specific cell types [6]. It may be the interplay between Wnt and its associated pathways that determines whether stem cells self-renew or differentiate. That being said, the Wnt pathway may have therapeutic potential in the future being that it is a central pathway for putative HCC-derived stem cells.

10.4.2.2 TGF-β Signaling

Recent evidence suggests a critical role for TGF-β signaling in both foregut cancer suppression and normal gut endoderm development. The data suggest a dual role in

liver tumor suppression as well as the transition of stem cells to a progenitor and fully differentiated phenotype [1]. Theory suggests that TGF-β is important for the transition of stem cells into progenitor cells with an ultimate conversion to a fully differentiated liver or biliary phenotype [1]. TGF-β appears to have its most prominent role at the interface between development and cancer in liver and foregut epithelial cells [6]. Specifically, Smad signaling is crucial for embryonic hepatocyte proliferation as well as the formation of gastrointestinal cancers [6]. Also, the formation of bile ducts can be upregulated by treatment with TGF-β in liver explant cultures [1]. The addition of TGF-β in haploinsufficiency studies causes an increase in Smad levels which leads to the formation of a limiting plate and bile ducts [1].

In many gastrointestinal tumors analyzed, it has been found that there is a disruption of at least one of the TGF-β signaling components [1]. Yao et al. believe that tumors arise in organs lacking crucial differentiating factors such as Smad2 and Smad3. This occurs at the progenitor cell to transitional cell stage or at the stage when stem cells divide into progenitor cells which then further develop into immature epithelial cells. Thus carcinogenesis is potentially favored by a lack of TGF-β–driven epithelial differentiation [1]. Essentially, TGF-β signaling may help to discriminate between normal stem cells and CSCs and help identify a human progenitor cell pool and other pathways that become activated in cancer stem/progenitor cells [1].

Disrupted TGF-β signaling has been observed in potential HCC-derived CSCs. Some investigators suggested that lack of responsiveness to the TGF-β signaling pathway in liver stem cells leads to the generation of liver CSCs [76]. This was also observed in the EpCAM+ putative liver CSC [76]. Loss of expression of certain components of the TGF-β pathway such as TBRII, ELF, and Smad4 in cells that express stem cell markers such as Nanog, STAT3, and Oct3/4 could represent a prognostic event in HCC [6]. Furthermore, genetic studies in mice suggest that loss of ELF/TGF-β signaling and an increase in STAT3 contribute to the transformation of a normal hepatic stem cell to putative CSC [6]. However, the low numbers of stem cells and difficulties in isolation have precluded clear delineation of stages of differentiation [6]. There is therefore a need to define clear experimental conditions to show the role of all of these markers in the stage of stem cell to a differentiated hepatocyte or HCC [6].

10.4.2.3 Notch Signaling

The Notch pathway is involved in numerous cell processes including differentiation, cell fate, proliferation, apoptosis, and cellular adhesion [76]. In the liver, Notch is involved in the coordination of biliary cell differentiation and morphogenesis. Disruption of Notch signaling has been recognized in several HCC samples studied [76]. Moreover, activation of the Notch pathway has been demonstrated in putative HCSC and in HCC-derived CD133-positive cancer cells as compared to CD133-negative cells [62, 76]. The exact role of Notch in hepatocarcinogenesis demands further delineation.

10.4.2.4 Hedgehog Signaling

Hedgehog signaling is crucial in cellular processes associated with stem cell physiology. Upregulation of this pathway has been observed in a number of cancers including hepatobiliary cancers, specifically in the CD44/24/EpCAM-positive pancreatic CSCs at the invasive front of tumors [76]. Hedgehog signaling was investigated in HCC, and it was observed that upregulation of genes involved in this pathway was found in the highly tumorigenic CD133+ HCSCs [76]. Potentially, in the future, this may provide targets for treatment of HCC via targeting pathways more specific for HCSC. However, currently, how this pathway relates to HCC, HCSC, and its clinical implications have yet to be elucidated.

10.4.2.5 MYC

It is known that the proto-oncogene MYC is involved in regulation of approximately 15% of all genes [75]. MYC can be activated by numerous pathways, including Wnt and Hedgehog. Over-expression or modifications in MYC are seen in a plethora of cancers [76]. Although MYC has an eclectic role in general cellular processes, it is also involved in stem cell pluripotency. Over-expression of MYC has been seen in side population cells of colon cancer, and knockdown of MYC caused cell cycle arrest and apoptosis in the SP cells [76]. Studies have now found that MYC is involved in the malignant transformation of hepatocarcinogenesis in murine and human models [76]. Using transcriptomic analysis to compare hepatic dysplasia, cirrhosis, and early HCC, the MYC-associated genes were found to be activated only in early HCC [4]. This therefore alludes to the involvement of MYC in driving conversion of preneoplastic lesions to malignancy and discriminating between preneoplastic lesions and early HCC [4]. That being said, the exact role in liver CSCs is not fully understood. An important confounding issue is the dual oncogenic and pro-apoptotic characteristics of MYC; therefore, targeting of MYC should be approached with caution [76].

10.5 Potential Therapeutic Implications

Understanding the potential mechanisms of liver progenitor cells and liver CSCs as the cells of origin of HCC is essential to design novel therapeutic approaches. If proven true, the CSC hypothesis and HCSCs will alter the way we treat HCC and may herald a paradigm shift in this deadly disease. Currently, we believe that the majority of cancer therapies address the highly proliferative cells within a tumor mass but not the tumor-initiating cells (solid organ CSCs) which are thought to be quiescent. When targeting the bulk of the tumor, one may often achieve tumor shrinkage but without specific therapies against the tumor-initiating cells (HCSC), rates of recurrence are high.

Clinical complete response to conventional chemotherapy in HCC is extremely rare. The relative resistance and recurrence rate in HCC after chemotherapy

suggests that a subpopulation of cells exists that are highly resistant and potentially relatively dormant. However, the relative quiescence of HCSCs is a much debated subject, suggesting that different mechanisms impart chemotherapy resistance on HCSC. These need to be further studied to potentially target HCSCs. Using blood born malignancies as a comparative example, it was found that approximately 70% of liver CSCs survive cell cycle–dependent cytotoxic treatment, whereas the leukemic stem cells can be eradicated [7]. Furthermore, since large populations of HCC SP cells are in the G_0 phase, it is conceivable that these cells are also resistant to cell cycle–specific agents [7]. The ABC transporters represent an important protective mechanism. It has been demonstrated in several cancers including HCC that there exists a subpopulation of cells that highly express the MDR/ABC family of genes (SP cells) [77]. High drug efflux capacity through ABC transporters is one of the most striking characteristics of SP cells [7]. Therefore, strategies for tumor eradication must look outside of chemotherapeutics.

Inhibitors of pathways that cause therapy resistance would be an optimal target for tumor eradication. One particular strategy would be to target the stem cell niche since it is known to be the specific microenvironment in which stem cells reside and assists in self-renewal and reproduction [1]. The goal of targeting the niche would be to change the fate of stem cells. It is known that human embryonic stem cell–derived fibroblast-like cells provide a supportive environment for stem cells through insulin-like growth factor 2 [1]. Targeting insulin-like growth factor 2 may therefore manipulate the microenvironment. How this applies to clinical practice remains to be elucidated [1, 6].

The inhibition of specific HCSC pathways also shows promise. It has previously been shown that activation of the Akt/PKB and Bcl-2 pathways contributes to chemoresistance in CD133-positive HCC cells [7]. Following treatment with an Akt1 inhibitor, the previously chemoresistant CD133 cells became sensitized to 5-flurouricil. CD133-derived HCC cells also harbor strong aldehyde dehydrogenase (ALDH) enzymatic activity. ALDH is a detoxifying enzyme which eliminates toxic byproducts of reactive oxygen species and is a marker of both normal cells and CSCs [7]. It was subsequently found that ALDH-positive cells are resistant to alkylating agents. Therefore, an inhibitor of ALDH may show clinical significance in the future. In pancreatic cancer, cyclopamine (a small molecule hedgehog inhibitor) reduces ALDH and is currently in phase II trials for pancreatic cancer [6]. Targeting of the Notch pathway through gamma secretase inhibitors may decrease tumor growth by inhibiting CSC self-renewal [6]. Similarly, blocking IL-6 signaling may show promise in HCC therapy. IL-6 may be linked to the self-renewal of hepatocellular stem cells; therefore, blocking IL-6 may inhibit this pathway [6].

A separate approach to tumor eradication would be differentiation therapy. Theory states that the tumorigenicity of CSCs is determined partly by self-renewal [6]. Therefore, it is presumed that the differentiation of a CSC results in suppression of carcinogenesis. In transgenic mice where c-Myc was conditionally regulatable, c-Myc expression induced HCC. However, following inactivation of c-Myc, the HCC cells lost their neoplastic properties and differentiated into hepatocytes and cholangiocytes [78]. These mice then showed decreased tumor burden which was

associated with improved survival [78]. Interferon-alpha has also been shown to expedite differentiation into hepatocytes and cholangiocytes in oval cell lines and may have a role in the treatment of HCC CSCs [7].

With the recent attention to monoclonal antibody therapy as a treatment modality for cancer, attention has been placed on CD44 with regard to hepatocellular carcinoma. CD44 is a receptor for hyaluronic acid and osteopontin and is expressed by hepatocellular stem cells, leukemia stem cells, and other CSCs [7]. Anti-CD44 treatment in a xenograft mouse model of human acute myelogenous leukemia (AML) was found to eradicate leukemia stem cells without disturbing normal stem cells. The antibody diminished the capacity of leukemia stem cells to hone to their supportive microenvironment and also promoted a terminal differentiation of leukemia stem cells in vivo [7]. Extrapolating these data to hepatocellular cancer, one may postulate that the administration of a CD44 antibody might be a promising CSC therapy in HCC while not effecting normal hepatic stem cells.

In summary, clear identification and definition of markers specific to stages of CSC formation such as cell surface markers (CD90, CD133, CD45), stem cell markers (Nanog, Oct3/4, STAT3), and pathways (TGF-β/Smad/ELF) are crucial for the development of CSC focused treatments.

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Chapter 11
Cancer Stem Cells in Head and Neck Cancer

Mark E.P. Prince and Samantha J. Davis

Abstract Each year, malignancies of the head and neck account for approximately 500,000 new cases cancer diagnoses worldwide. The prognosis for patients affected by head and neck cancer has remained largely unchanged in recent years, despite significant advances in the understanding of tumor biology and etiologic factors. This is largely due to the fact that two-thirds of patients present with disease that has already spread regionally or metastasized. Unfortunately, for patients presenting with advanced-stage disease, complete cure is often not possible with our current treatment modalities. The consistently poor prognosis of head and neck cancer patients underscores the need for a better understanding of tumor biology and how to target malignant cells. The re-emergence of the cancer stem cell (CSC) hypothesis offers hope in this area. This chapter reviews the current knowledge about CSCs in head and neck cancer, including markers used for CSC identification and isolation, as well as their potential clinical implications.

Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ALDH	Aldehyde dehydrogenase
ATP	Adenosine triphosphate
BCRP1	Breast cancer resistance protein 1
CD	Cluster of differentiation
CSC	Cancer stem cell
CXCR1	Chemokine receptor 1

M.E.P. Prince (✉)
Department of Otolaryngology-Head and Neck Surgery,
University of Michigan, Ann Arbor, MI, USA
e-mail: mepp@med.umich.edu

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DEAB	Diethylaminobenzaldehyde
ESA	Epithelial specific antigen
HNSCC	Head and neck squamous cell carcinoma
IL	Interleukin
MDR1	Multidrug resistance pump 1
NOD/SCID	Nonobese diabetic/severe combined immune deficiency
SP	Side population

11.1 Introduction

Each year, malignancies of the head and neck account for approximately 500,000 new cases cancer diagnoses worldwide [1]. The prognosis for patients affected by head and neck cancer has remained largely unchanged in recent years, despite significant advances in the understanding of tumor biology and etiologic factors. In the U.S., the overall 5-year survival for patients with cancer of the oral cavity or oropharynx is around 59%. This statistic reflects the fact that two-thirds of patients present with disease that has already spread regionally or metastasized [2]. Unfortunately, for patients presenting with advanced stage disease, complete cure is often not possible with our current treatment modalities. The consistently poor prognosis of head and neck cancer patients underscores the need for a better understanding of tumor biology and how to target malignant cells.

Although introduced over a century ago, the cancer stem cell (CSC) hypothesis has only recently gained a strong foothold in the research community. Advances in stem cell biology have revealed that most adult tissues contain a stem cell population, and many markers have been identified as being characteristic of a stem cell phenotype. In the field of oncology, it is now widely accepted that most, if not all, cancers arise from a small population of cells within a given tissue that have a unique set of characteristics. These characteristics include (a) the ability to self-renew in order to preserve a "stem cell" population; and (b) the ability to produce differentiated progeny, thereby forming a heterogeneous tumor. Both of these requirements are fulfilled by the process of asymmetric division, when a progenitor cell divides not to form two identical daughter cells, but rather to form another progenitor cell and a differentiated daughter cell. While normal stem cells are usually quiescent until signaled to divide by some insult or growth factor, CSCs have undergone some transformation, the result of which is deregulated self-renewal [3].

Initially, research into CSC biology focused almost exclusively on hematopoietic malignancies. This was largely due to the ease of obtaining samples and existing knowledge regarding cell markers that define the lineage of normal blood cells [4]. The first CSCs isolated from a solid tumor were derived from breast cancer [5]. Since then, CSCs have been identified in brain, colon, pancreatic, prostate, and head and neck cancers, among others [6]. Several markers have been shown to designate a stem-like phenotype in more than one type of solid tumor, and CSC research continues at an ever-increasing pace as oncologists in one field discover new avenues to explore from researchers in another area.

11.2 HNSCC Markers

11.2.1 CD44

CD44 is a transmembrane glycoprotein that binds to hyaluronan and, with less affinity, to other extracellular matrix components. There are several variant isoforms of CD44 (CD44v) that are generated via alternative splicing and glycosylation, all of which can have slightly different functions and altered ligand affinities [7]. Specifically, CD44 is encoded by 20 exons, 12 of which are variant, meaning they are differentially included in the final transcript. Although theoretically, there could be hundreds of CD44 variants, only a couple dozen appear to be expressed [8]. The most commonly expressed human isoform is standard CD44 (CD44s), which does not contain any of the variant exons and, thus, is quite small (85–95 kDa) [9]. CD44s, whose function was the first described, helps circulating lymphocytes home to lymph nodes in the periphery [10]. Like some other malignancies, squamous cell carcinomas of the head and neck (HNSCC) have been shown to express high levels of CD44s, CD44v5, and CD44v6 as compared to nonmalignant squamous epithelia controls. Expression of these isoforms has also been correlated with poor prognosis in HNSCC patients [11].

A study by Prince et al. established CD44 as a CSC marker in HNSCC using primary tumor specimens [12]. Primary HNSCC sorted for CD44 expression reveals a small population of cancer cells that exhibit the properties that define the CSC phenotype (Fig. 11.1). Additional analysis of tumor sections with areas of identifiable squamous cell differentiation revealed that the cells at the basal level showed marked CD44 expression, whereas no CD44 staining was seen at the most differentiated levels of epithelium. In addition, CD44+ cells costained with cytokeratin 5/14, a marker of normal epithelial progenitor cells, but did not costain with involucrin, which is a marker of differentiated keratinocytes. This provided strong evidence that CD44 was expressed by cells with CSC-like properties [12].

To further support this hypothesis, a possible relationship between CD44 and BMI1 was investigated. BMI1 is a proto-oncogene that plays a key role in stem cell self-renewal by inhibiting expression of the Ink4a/Arf locus, which encodes a tumor suppressor and is often deleted in malignant cells. BMI1 is expressed at high levels in normal adult stem cells in many tissues, including the epithelium. Quantitative RT-PCR was used to assess the expression of BMI1 in CD44+ and CD44− cells from four primary tumors. This analysis showed that CD44+ cells had high levels of BMI1 expression, while the CD44− cells had little or no detectable expression of the proto-oncogene. In addition, BMI1 costained with CD44 in tumor sections [12].

To determine whether this stem-like phenotype conferred any tumorigenicity in vivo, an immunodeficient mouse model was used. HNSCC cells were separated for CD44 expression, and the purified cell population was injected in the flank of a mouse. In total, 20/31 injections of CD44+ cells resulted in tumors, whereas just 1/40 injections of CD44− cells formed a growth. These xenograft tumors resembled the original tumor histologically, and only a small fraction of the tumor cells were now CD44+, proving that the implanted cells had retained a CD44+ population while also producing differentiated progeny [12].

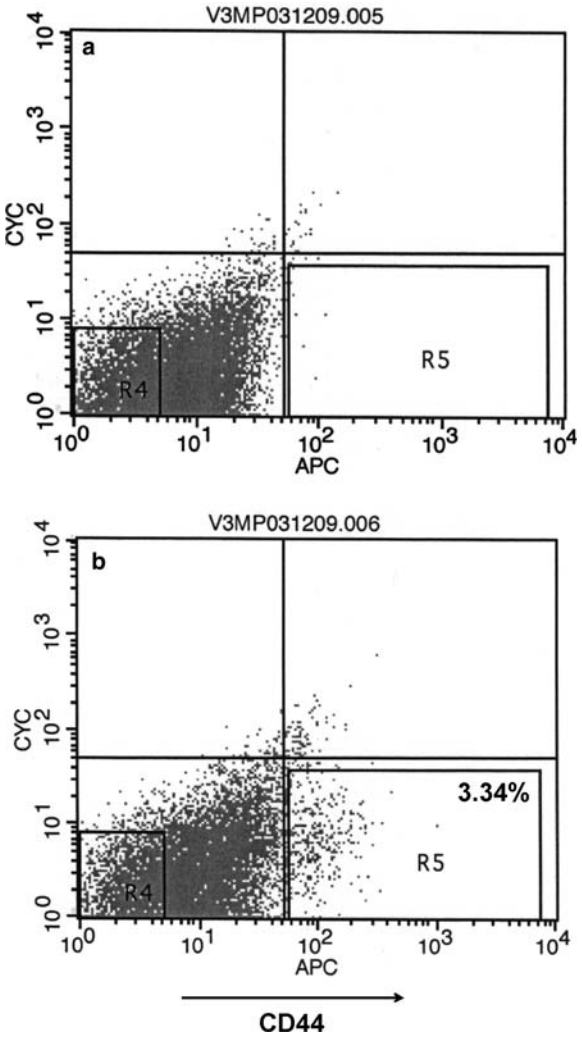

Fig. 11.1 Primary head and neck squamous cell cancer sorted for CD44 expression (**a**) Isotype control. (**b**) Sample sorted for CD44

Recently, Okamoto et al. further elucidated the stem cell–like properties of CD44+ cells from a HNSCC cell line established from a tumor of the hypopharynx [13]. Co-staining with two other solid tumor CSC markers, CD133 and ABCG-2, was performed to assess whether cells with high CD44 expression coincided with other CSC phenotypes. Indeed, CD44+ cells were more likely than CD44− cells to express CD133 and ABCG-2, supporting the hypothesis that CSCs in HNSCC are contained within the CD44+ population.

Both normal stem cells and CSCs are known to form free-floating spheres in vitro in serum-free media containing some combination of growth factors and/or supplements [14]. This culture method has been used to study tumor biology for decades, since it provides a model that is thought to more closely resemble an in vivo system [15].

Recently, spheroid formation has been established as one of the defining features of CSCs, and it is often used for identifying cells with stem-like properties and enriching for CSCs from an unsorted cell population [4]. Using a HNSCC cell line, Okamoto et al. cultured unsorted cells in serum-free conditions and successfully grew spheroid colonies of cells. These spheroids stained positively for CD44, CD133, and ABCG-2, suggesting that they are composed of CSCs [13].

CSCs are thought to be more resistant to radiation and chemotherapeutics, thus explaining why a cancer can recur even after it has been treated aggressively with multiple modalities. Using a PCR gene array, Okamoto et al. compared the expression of 84 genes known to play key roles in metabolism and drug resistance in CD44+ vs. CD44− cells. Eleven of these genes were found to be upregulated in CD44+ cells. Moreover, CD44+ cells were significantly less sensitive to five chemotherapeutic agents that are commonly used in treatment of HNSCC: 5-fluorouracil, paclitaxel, cisplatin, carboplatin, and docetaxel [13]. The chemoresistance of CSCs underscores the importance of continuing to study these tumorigenic cells so that they can eventually be targeted with more specific treatment modalities.

11.2.2 Aldehyde Dehydrogenase

In addition to exploiting cell surface markers, functional assays can also be used to isolate CSCs. Aldehyde dehydrogenase (ALDH) is an intracellular enzyme that detoxifies aldehydes through oxidation. In humans, there are at least 13 different genes that encode various ALDH isotypes [16]. Research in the field of embryology has shown that ALDH plays a well-defined role in early organogenesis via signaling through the retinoic acid pathway [17]. In addition, it helps cells metabolize both endogenous and exogenous toxins, protecting them and allowing them to thrive. ALDH has been identified as a CSC marker in breast cancer, lung cancer, pancreatic cancer, prostate cancer, multiple myeloma, leukemia, and head and neck cancer [18]. It has been proposed that cells with a high level of ALDH enzymatic activity have the ability to differentiate into a tumor, similar to how they have the ability to differentiate in embryonic development. In addition, ALDH's detoxifying properties may protect CSCs from chemotherapeutic insults, perhaps allowing them to survive even the most aggressive treatments.

Using six primary tumors collected from patients with HNSCC, Prince et al. isolated cells with high ALDH activity using the Aldefluor enzymatic assay (StemCo Biomedical, Durham, NC) followed by flow cytometry [19]. The ALDH+ cells made up a small percentage of the total population (<10%), and the majority of these cells (50–75%) also expressed high levels of CD44 (Fig. 11.2). To test the ability of these cells to form tumors in vivo, NOD/SCID mice were injected with either ALDH+ or ALDH− cells. In total, 24/45 ALDH+ injections and 3/37 ALDH− injections resulted in tumor growth ($p < 0.00001$, χ^2 test). When the xenografts were harvested from the mice and analyzed, the original tumor heterogeneity had been reestablished, and the percentage of cells with high ALDH activity was similar to that seen with the original tumor cells [19]. This study provided strong evidence that high ALDH activity is a characteristic of CSCs in HNSCC.

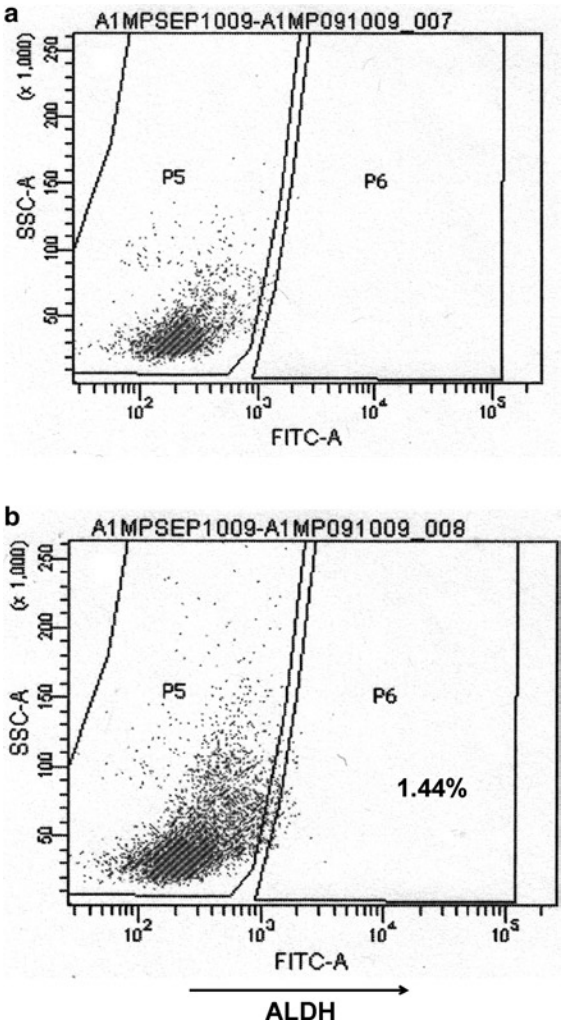

Fig. 11.2 Primary head and neck squamous cell cancer sorted for ALDH expression. (**a**) Aldefluor substrate inhibited with the specific inhibitor DEAB. (**b**) Aldefluor substrate uninhibited. The population of cells exhibiting ALDH expression is 1.44%

11.2.3 Side Population

The ATP-binding cassette (ABC) transporters make up the largest family of transmembrane proteins. These transporters use the energy from ATP to move molecules into and/or out the cell, and they can be located on the cytoplasmic or nuclear membrane. Of the 46 known human ABC transports, ABCG2 (a.k.a. MXR, ABCP, BRCP) and MDR (a.k.a. ABCB1, PGY1) are the most well described. Both of these transmembrane proteins cause efflux of toxins and, thus, can confer multidrug resistance [20]. They can be found in both the blood–brain and blood–testis barriers, where protection from toxic substances is most important. In addition, there is evidence that ABC transporters can inhibit cellular differentiation, thereby helping to

maintain a stem cell phenotype. Side populations (SPs) of cells with highly active ABC transporters have been identified using a Hoechst 33342 dye exclusion assay. Cells able to pump out the dye will appear as an unstained fraction in flow cytometric analysis. These SPs are thought to be enriched in stem cells, as they have been shown to express markers of a stem-like phenotype [21]. SPs have been reported in several tumor types, both solid and hematopoietic. Although these cell populations do not appear to be enriched in CSCs, SP cells have still proven to be highly tumorigenic [22].

11.3 Future Directions

While the markers studied in HNSCC thus far have allowed for separation of a cell fraction that contains a large number of highly tumorigenic cells, it is likely that no single marker will be specific enough to select for a pure CSC subpopulation. Therefore, it is imperative to identify new properties of CSCs so that, by combining these characteristics, a profile of sorts can be used to isolate and target these high-risk cells. In breast cancer, the stem cell subpopulation has been isolated by identifying cells with high CD44 expression in combination with low CD24 expression. The CD44+/CD24− population formed tumors very efficiently, while the CD44+/CD24+ cells did not [5]. Similarly, CSCs from colon cancers were characterized by expression of multiple markers: CD44+/CD166+/ESA+ [23].

Costaining has also been used with the Aldefluor assay to narrow in on the CSC population. In breast cancer cells, it was recently reported that CXCR1, one of the receptors for IL-8, is necessary for reproducing the original tumor heterogeneity in a mouse xenograft model. Aldefluor+/CXCR1+ cells isolated from human breast cancer cell lines were able to reconstitute the original tumor cell population, while Aldefluor+/CXCR1− cells formed a tumor but remained Aldefluor+/CXCR1−. Moreover, the CSCs expressing CXCR1 had a higher rate of metastasis after intraventricular injection into NOD/SCID mice [24].

It is important to keep in mind that each tumor type is distinct from any other, and the corresponding CSC population will likely have a unique gene expression profile. Nevertheless, it is reasonable to explore avenues that have proven fruitful in other areas of CSC research, at least as a starting point. Improved understanding of HNSCC tumor biology, therefore, will likely rely on the use of multiple CSC markers in the future.

11.4 Clinical Implications

According to the CSC theory of tumorigenesis, a cancer can only be completely eradicated by killing cells with a stem cell–like phenotype. This may explain why many malignancies recur after being treated with multiple modalities that debulk the tumor, even to the point of appearing eradicated on imaging, but do not specifically

target the CSC subpopulation. Several studies have shown that CSCs are less sensitive to chemotherapy agents and radiation in several tumor types, though some argue that the data are still inconclusive [25, 26].

Regardless of their sensitivity or resistance, CSCs are believed to be responsible overall for the persistence or distant spread of disease in patients who have previously undergone treatment for their cancer. Upregulated detoxifying mechanisms, such as overexpression of ALDH and ABC transporters, likely confer a great deal of resistance to chemotherapeutics. In addition, CSCs are relatively quiescent compared to the rest of the tumor population and have enhanced DNA repair mechanisms, which may make them less likely to succumb to the cytotoxic hits induced by radiation or drugs [27, 28].

CSCs are thought to be preferentially localized in well-vascularized areas of tumor, and the use of antiangiogenic drugs in combination with classical chemotherapeutics have been shown to kill more CSCs than does chemotherapy alone [27]. Other potential mechanisms of targeting stem-like cells include the use of small molecule inhibitors, monoclonal antibodies, and micro-RNAs aimed at pathways known to be exploited in CSCs. This has shown promise in breast CSCs, in which CXCR1 signaling has been blocked using both a small molecule inhibitor, repertaxin, and a monoclonal, resulting in a drastic reduction in the CSC population [24]. Since many of the known CSC cell surface markers, including CD44, are also present on the surface of normal human cells, targets specific to the stem-like phenotype must be identified to refine our treatments and reduce toxicity to healthy tissue. While many obstacles and unanswered questions still stand in way of developing targeted anti-CSC therapies for HNSCC, recent advances in the field of tumor biology have given us hope that such a goal is within reach.

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Part III
Cancer Stem Cell Gene Expression and Mechanisms: Convergence of Embryonic and Tumorigenic Signaling Pathways

Chapter 12
Relationship Between Regulatory Pathways in Pluripotent Stem Cells and Human Tumors

Olga Gaidarenko and Yang Xu

Abstract Embryonic stem cells have the ability to undergo unlimited self-renewal and retain the pluripotent capacity to differentiate into all of the cell types of the body. Their pluripotency is maintained by the core transcription factors Nanog, Oct4, and Sox2, as well as signaling pathways, which include Wnt, FGF, and TGFβ. Recent studies have shown that somatic cells can be reprogrammed into the pluripotent state with defined reprogramming factors, including Oct4, Sox2, Nanog, and c-Myc. Considering the oncogenic potential of the reprogramming factors, the induced pluripotency mimics the cellular transformation process and is inhibited by the tumor suppressor p53. Since the Wnt/FGF/TGFβ pathways are implicated in human cancers and pluripotency/reprogramming factors Oct4/Sox2/Nanog/c-Myc/Lin28 are overexpressed in some human cancers, there is a possibility that dedifferentiation is a mechanism for the generation of tumor-initiating cells in human cancer. Therefore, there appears to be a strong link between the regulatory pathways in pluripotent stem cells and human tumors.

Abbreviations

ALL	Acute lymphoblastic leukemia
AML1-ETO	Acute myelogenous leukemia (AML) with t(8;21)(q22;q22)
APC	Adenomatous polyposis coli
BMP	Bone morphogenic protein
CKI	Cyclin-dependent kinase inhibitors
ERK	Extracellular signal-regulated kinase

Y. Xu (✉)
Section of Molecular Biology, Division of Biological Sciences,
University of California, San Diego, La Jolla, CA, USA
e-mail: yangxu@ucsd.edu

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ES	Embryonic stem
FGF	Basic fibroblast growth factor
FGFR	Basic fibroblast growth factor receptor
GSK	Glycogen synthase kinase
hESC	Human embryonic stem cell
HSC	Hematopoietic stem cell
ICM	Inner cell mass
iPSC	Induced pluripotent stem cells
Klf4	Kruppel-like factor 4
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
PcG	Polycomb group
PI3K	Phosphoinositide 3-kinase
PIP	Phosphotidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLZF	Promyelocytic leukemia zinc finger
RAR	Retinoic acid receptor
SSEA	Stage-specific embryonic antigen
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGF-β	Transforming growth factor

12.1 Regulatory Pathways in Embryonic Stem Cells

Mammalian embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the pre-implantation or peri-implantation blastocyst. The typical stage of isolation for human ES cells (hESCs) is from day 5 to 6 blastocysts. These cells are karyotypically normal, have high levels of telomerase activity, are capable of prolonged self-renewal in culture, and are pluripotent. As such, they are able to give rise to all cell types found in the human body, derived from all three embryonic germ layers: endoderm, mesoderm, and ectoderm. This capacity is maintained even after repeated passaging in long-term, and potentially indefinite, culture [1–5].

Pluripotency, self-renewal, and differentiation of ES cells are regulated by a complex network that includes transcription factors, signaling pathways, micro-RNAs (miRNAs), and epigenetics. Pluripotency-associated transcription factors such as Oct4, Nanog, and Sox2 function in concert to maintain ES cells in their pluripotent, self-renewing state. They repress the transcription of genes involved in differentiation and activate those that are important for stem cell characteristics.

Oct4 is a POU transcription factor that is expressed in blastomeres, germ cells, and cells of early embryos. It is encoded by the Pou5f1 gene and is also referred to as Oct3. It is essential for the establishment of a pluripotent cell population in

the blastocyst ICM. Precise regulation of Oct4 levels is crucial to ES cells, as its downregulation results in dedifferentiation into trophectoderm, whereas less than a twofold increase in its expression leads to differentiation into primitive endoderm and mesoderm [6, 7]. Nanog is a homeodomain transcription factor whose expression in the developing mammalian embryo is restricted to the transient ICM population from which ES cells can be established. Its expression is downregulated rapidly during further differentiation from ICM and is tightly associated with and required for pluripotency [8–10]. Sox2 belongs to the SRY-related HMG box (Sox) family of transcription factors. Its pattern of expression during early embryogenesis is similar to that of Oct4. It is a major transcriptional regulator involved in the perpetuation of the self-renewing, pluripotent state of ES cells. At present, it remains less extensively studied than Oct4 and Nanog [11, 12].

Oct4, Nanog, and Sox2 co-occupy the promoters of at least 353 genes in hESCs, some of which are active in ES cells, while others are not. The transcriptionally inactive population is enriched for transcription factor genes that are involved in developmental processes such as differentiation. The transcriptionally active genes include Oct4, Nanog, and Sox2 themselves, as well as other transcription factors and components of signaling pathways that have been implicated in the self-renewal and pluripotency. The promoter co-occupancy is regulatory in nature, promoting stem cell programming while suppressing developmental processes [13]. In addition to regulating the expression of protein-coding genes, Oct4, Sox2, and Nanog occupy the promoter regions of some miRNAs that are preferentially expressed in ES cells. The miRNA expression profiles differ for undifferentiated ES cells, ES cells undergoing differentiation, and fully differentiated cells. Thus, miRNAs participate in the network that regulates “stemness” and differentiation [14]. Of note is the miR-302-367 cluster in hESCs, whose transcription is regulated by Oct4, Sox2, and Nanog. Its expression is restricted to pluripotent ES cells and is repressed upon differentiation. Much remains to be understood about the mechanisms of its action in ES cells; however, evidence exists that implicates it in apoptosis, differentiation, cell cycle regulation, and maintenance of the stem cell state [15]. As differentiation ensues, pluripotency factors themselves become targets of miRNA-mediated silencing. As hESCs differentiate, miR-145 serves to downregulate Oct4, Sox2, and Klf4, another transcription factor of importance to pluripotency. Deficiency in miR-145 results in impaired differentiation and elevated levels of the three aforementioned transcription factors [16].

Epigenetics also play a prominent role in maintaining the stem cell fate, as well as during differentiation. In hESCs, a variety of genes that function in differentiation, development, and transcription are repressed by polycomb group (PcG) proteins that posttranslationally modify histones. A significant portion of these genes are also occupied by the three key transcription factors, Oct4, Sox2, and Nanog. This repression is alleviated for the majority of the targets during differentiation of ES cells. Conversely, Oct4, Sox2, and Nanog themselves become repressed by PcG proteins as they become downregulated during differentiation. CpG methylation is also involved in the epigenetic regulation of “stemness,” and a significant subpopulation of PcG bound regions are within 1 kb of CpG islands [17, 18].

12.2 Similarities Between ES cells and Cancer Cells

Cancer cells have striking similarities to ES cells. For instance, most cancers can overcome their proliferative limit by expression of telomerase, which is also active in ES cells, but not in normal somatic cells [19, 20]. Other cancer cells rely on an alternative lengthening of telomeres (ALT) mechanism for telomere maintenance [21]. However it is managed, maintained telomeres are necessary for the immortalization of cancer cells as well as the self-renewal of ES cells. Additionally, numerous genes that are reversibly repressed by the PcG proteins in ES cells are permanently silenced by promoter methylation in cancer cells [22]. Cancer cells and hESCs have common miRNA expression patterns [23]. Some cancer types such as germinomas, seminomas, dysgerminomas, and embryonal carcinomas are able to give rise to various somatic and extraembryonic tissues, thus exhibiting pluripotency [24]. Various signaling pathways of importance to the maintenance of ES cells also play a role in driving tumorigenesis, and these are described below.

12.2.1 Wnt Signaling

Wnt signaling occurs via canonical and non-canonical pathways. Canonical Wnt signaling is involved in the regulatory circuitry of ES cells, maintenance of adult stem cells, and, if gone awry, tumorigenesis. It proceeds via stabilization of β-catenin, which then translocates to the nucleus and activates the expression of genes that are constitutively bound by Tcf/Lef proteins. In the absence of β-catenin/Wnt signaling, the expression of those genes is repressed. The Wnt signaling cascade begins when Wnt binds two receptors on the cell surface: a serpentine receptor belonging to the Frizzled family and Lrp5/6, an LDL receptor family member. This binding inhibits the activity of the destruction complex which otherwise acts upon β-catenin. The destruction complex has two scaffolding proteins, axin and adenomatous polyposis coli (APC) that bind to β-catenin, whose N-terminus is then phosphorylated by two kinases of the destruction complex, CKI and GSK3. This phosphorylation recruits an E3 ubiquitin ligase, which targets β-catenin to the proteasome for degradation [25–29].

It has been demonstrated that stimulation of the Wnt canonical pathway is sufficient for the maintenance of self-renewal in human, as well as mouse, ES cells. When Wnt signaling is activated in ES cells experimentally in a variety of ways, these cells are able to resist differentiation, remain pluripotent, and maintain the expression of Oct4, Nanog, and Rex-1 transcription factors associated with pluripotency [30, 31]. The mechanism of Wnt action in hESCs remains to be elucidated. However, studies in mouse ES cells suggest potential roles that Wnt signaling may play in hESCs, if conserved across species. In mouse ES cells, Tcf3 has been found to have genome-wide promoter co-occupancy with Oct4 and Nanog. It occupies its own promoter, along with the promoters of the Oct4, Nanog, and Sox2 genes, thus

participating in an autoregulatory loop with them. In the absence of Wnt signaling, Tcf3 plays a repressive role; when the pathway is active, Tcf3-bound genes are activated, supporting pluripotency and self-renewal in ES cells [29].

Wnt signaling is involved in the maintenance of adult stem/progenitor cells in a variety of tissues, such as the colorectal crypts and the hematopoietic system. Aberrations in the pathway result in neoplastic malignancies. In the small intestine, Tcf-4 is responsible for crypt cell maintenance [32]. Tcf-4 target genes are constitutively activated in colon carcinomas that harbor mutations in β-catenin or APC genes. Thus, colorectal cancer cells share the gene expression program specified by Tcf-4 with the crypt stem and progenitor cells. Activating mutations of the Wnt pathway components are typically an early transforming event in colorectal cancers [28, 32–35].

There is also mounting evidence that Wnts, produced by hematopoietic stem cells (HSCs) as well as their microenvironment, are important for the maintenance of the HSCs and their niche [28, 36–41]. Various cancers of the blood have been found to have inappropriately active Wnt signaling. For instance, self-renewing granulocyte–macrophage progenitors from patients with chronic myelogenous leukemia have abnormally high levels of nuclear β-catenin [42]. In acute myelogenous leukemia (AML), the associated translocation products (AML1-ETO, RARα, and PLZF-RARα) encode transcription factors that activate Wnt signaling via induction of β-catenin expression as well as expression of its homologue plakoglobin (which can also interact with TCF/LEF proteins and activate targets of Wnt signaling) [43–45]. E2A-Pbx1, the product of the t(1;19) chromosomal translocation found in a significant portion of pre-B acute lymphoblastoid leukemias (ALL), transcriptionally activates a Wnt gene. It is likely that this Wnt promotes the development of such pre-B ALL cancers via an autocrine mechanism [46]. Additionally, Wnt signaling has been shown to be important for the growth of multiple myeloma cells [47].

12.2.2 FGF Signaling

At present, the human fibroblast growth factor (FGF) family boasts at least 22 known members that share 13–71% sequence identity. FGFs interact with four tyrosine kinase receptors; FGFR1-4. Alternative splicing of FGFR1-3 results in a diverse array of tissue-specific receptors with varying affinities for different FGF family members. FGFs also interact with heparin or heparin sulfate proteoglycans via a region distinct from the one that engages FGFRs. This interaction facilitates FGF binding of FGFRs and subsequent initiation of signal transduction. Once the FGF ligand is bound, FGFRs dimerize and autophosphorylate, primed to activate downstream signaling pathways such as Ras-Raf-MAPK, PLC(gamma)-PKC, and PI3K-Akt [48–52].

FGF2, also known as basic FGF or bFGF, is typically included in hESC media and has been demonstrated to maintain hESCs in an undifferentiated state even in the absence of feeder cells or feeder-conditioned media [53–55]. Inhibition of FGFRs in

hESCs results in suppressed activation of downstream kinases and leads to rapid differentiation [56]. Although the mechanisms by which FGF2 maintains hESC pluripotency remain to be fully elucidated, several groups have found evidence that the MAPK pathway plays a role in the maintenance of pluripotency. When this pathway is activated downstream of FGFR, ERK is phosphorylated. It then translocates to the nucleus and phosphorylates various transcription factors, including c-Jun, c-Fos, and c-Myc, the latter being one of the factors originally employed by Yamanaka et al. in generating iPS cells [52, 57]. Inhibition of the MAPK pathway in hESCs results in the downregulation of pluripotency markers such as Oct4, Nanog, and SSEA-4, and ultimately, differentiation [51, 52, 58, 59]. The PI3K/AKT pathway also appears to be involved in the maintenance of pluripotency in hESCs, as its inhibition leads to differentiation [52, 58]. Once PI3K is activated, it phophorylates PIP2, generating PIP3, a second messenger that then communicates with AKT and recruits it to the plasma membrane. At the membrane, AKT is phosphorylated, and thus activated, by PDKI, which enables it to activate its downstream targets. Of note is the ability of this pathway to stimulate the Wnt/β-catenin signaling. Additionally, the ERK/MAPK pathway appears to lie downstream of PI3K/AKT [52, 53].

Various cancers also utilize FGF signaling to carry out important malignant behaviors such as increased proliferation, angiogenesis, invasiveness, metastasis, and resistance to chemo- and radiotherapy. For example, prostate cancer has increased levels of FGF2, as well as FGF1, FGF6, and FGF8, and expresses all four FGFR types [48]. Levels of FGF2 are elevated in esophageal and gastroesophageal junction adenocarcinomas, and overexpression of FGF2 is correlated with tumor recurrence in patients with esophageal cancer [59, 60]. FGF2 confers apoptotic resistance to small cell lung cancer cells, and, if present at elevated serum levels, is a predictor of a poor clinical outcome [61]. The FGFs may function in an autocrine or paracrine manner. In addition to FGFs produced by cancer cells themselves, cancer tissues may have increased access to FGFs due to their enhanced release from the extracellular matrix [48]. Another way cancers make use of this pathway is through activating mutations in FGFR genes. Mutations resulting in constitutively active FGFR3 are frequently found in bladder and cervical carcinomas [62]. Likewise, 12% of endometrial carcinomas feature constitutively activated mutant FGFR2 [63]. Additionally, certain cancers such as gastric cancer and oral squamous cell carcinoma have amplification and overexpression of FGFRs, which also leads to aberrant FGF signaling [64, 65].

12.2.3 TGFβ/Activin/Nodal Signaling

Another ES cell pathway that is utilized by cancer cells proceeds via Activin/Nodal signaling. Activin and Nodal are both related to the transforming growth factor-β (TGF-β) family. The Activin signaling cascade is initiated when this secreted peptide binds its cognate heterodimeric receptor complex, bringing together two transmembrane serine/threonine kinase receptor types: type I and type II. The type II receptor

phosphorylates the type I receptor, which in turn phosphorylates Smad2 and Smad3. The activated Smads translocate to the nucleus where they are able to bind DNA directly. Smads associate with a variety of DNA-binding cofactors in order to fully activate the transcription of their target genes. This allows for flexibility and versatility of the pathway [66–69]. Nodal signaling proceeds analogously [70].

This signaling pathway has been shown to be important for sustaining self-renewal and pluripotency in hESCs [71], and its ability to maintain hESCs is augmented by cooperation with FGF signaling [72]. Smad 2/3 are able to bind the Nanog gene directly and maintain its expression [73, 74]. Nodal is secreted by the hESCs themselves, providing an autocrine mechanism for reinforcing their pluripotent state. Along with it, they secrete Lefty, a Nodal inhibitor, thus keeping the signaling pathway regulated. Nodal expression is rapidly downregulated upon differentiation [71, 72, 75, 76]. Activin A, secreted by mouse embryonic fibroblasts (MEFs) which are used as feeder layers for hESC culture, is a major regulator of pluripotency and self-renewal of hESCs. It is able to stimulate the expression of Nanog, Oct4, bFGF, FGF8, Wnt3, and Nodal. Additionally, it suppresses BMP signaling, which promotes hESC differentiation [77, 78].

The TGFβ/Activin/Nodal Signaling pathway also plays a role in various cancers, albeit a complex, context-dependent one. At the onset of cancer development, it has an inhibitory role. However, as the disease becomes more mature, the pathway may become oncogenic, promoting invasion and metastasis. In epithelial cells, this usually proceeds by increased ligand secretion coupled with decreased or altered cancer cell responsiveness to the tumor-suppressive effects of the pathway. The majority of human tumors possess functional TGFβ signaling. Ligand secretion by tumor cells also affects the microenvironmental interacting cells, such as the stroma [68, 79–82]. High levels of TGFβ1 correlate with poor clinical outcome in patients with a variety of cancers, such as colorectal, lung, and prostate [82–86]. Elevated expression of Activin A is observed in a variety of carcinomas, including esophageal, prostate, and pancreatic [87–90]. Aggressive melanoma and breast carcinoma cells express Nodal, but not its inhibitor Lefty, whose expression is restricted to hESCs, thus allowing for uncontrolled signaling to maintain their multipotent, dedifferentiated phenotype [75, 91].

12.3 Induced Pluripotent Stem Cells (iPSCs) and Cancer Cells

Pluripotency is not necessarily a property that is restricted to cells of the early embryo and lost irreversibly upon differentiation. Terminally differentiated adult cells can be reprogrammed back to the pluripotent state by forced expression of transcription factors that maintain the “stemness” of ES cells. Such cells are referred to as induced pluripotent stem or iPS cells. The first iPSCs were reported in 2006, obtained by expressing Oct4, Sox2, c-Myc and Klf4 in mouse fibroblasts. The reprogrammed cells expressed a variety of ES cell markers. Like ES cells, they formed teratomas comprising all three germ layers (ectoderm, mesoderm, and endoderm) when injected

into nude mice, and were able to contribute to developing embryos after injection into blastocysts [92]. Just a year later, two groups obtained human iPS cells, one using the same four factors, the other substituting Nanog and Lin28 for c-Myc and Klf4. The gene expression profiles, surface antigens, and epigenetic status of genes involved in pluripotency were very similar, but not identical, between iPSCs and ES cells [56, 93]. Since then, various groups have reported new strategies for cell reprogramming, employing different methods, factors, and chemicals intended to promote or improve the process. Human somatic cells from all three germ layers, including hepatocytes (endoderm), hematopoetic progenitors (mesoderm), and neural stem cells (ectoderm), have been successfully reprogrammed into iPS cells [56, 92–96].

Induction of pluripotency with the defined reprogramming factors, most of which have oncogenic properties, provides an additional link between pluripotency and cancer [97]. Additionally, there is evidence that, during reprogramming, iPSCs aquire epigenetic changes that are associated with cancer [98, 99]. Findings that the tumor suppressor p53 also inhibits pluripotency induction further underscore the potential transforming nature of the reprogramming process [97, 100]. The presence of tumor-initiating cells in some human cancers has been suggested to be a primary cause for drug resistance and metastasis [91, 101–104]. These tumor-initiating cells are thought to possess some stem cell/progenitor characteristics, such as the ability to self-renew and the potential to differentiate into multiple lineages. The fact that many human cancers overexpress some of the reprogramming factors, including Oct4, Sox2, Nanog, Lin28, and c-Myc, raises the possibility that the dedifferentiation process within the tumor cells could lead to dynamic generation of tumor-initiating cells [24, 105–112]. Therefore, there appears to be a strong link between the regulatory pathways in pluripotent stem cells and human tumors.

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Chapter 13
Influence of the Embryonic Microenvironment on Tumor Progression

Daniela Quail, Meghan Taylor, Michael Jewer, and Lynne-Marie Postovit

Abstract Recent advancements in stem cell biology have revealed remarkable plasticity in cell fate specification. For example, fully differentiated somatic cells can be reprogrammed to pluripotent stem cells following the expression of specific stem cell associated transcription factors. This extraordinary process of cellular reprogramming or dedifferentiation shares many similarities with tumor progression, such that cancer cells often acquire stem cell-like plasticity concomitant with metastatic disease. Evidence suggests that cancer cells co-opt stem cell associated signaling factors to sustain plasticity. However, in contrast to normal stem cells, which have a complement of inhibitors and activators of pluripotency, cancer cells lack this critical balance. Here, we describe stem cell associated proteins and microenvironments that sustain and promote cellular plasticity in embryonic and neoplastic populations. We also review evidence that embryonic microenvironments may be capitalized upon to rebalance cancer cells toward a benign well-differentiated phenotype.

Abbreviations

ADAM	Disintegrin and metalloproteinase
ALK	Activin-like kinase receptor type I
ATP	Adenosine triphosphate
BCR-ABL	Breakpoint cluster region-abelson
CBF-1	C-promoter binding factor 1
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation

L.-M. Postovit (✉)
Department of Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada
e-mail: Lynne.postovit@schulich.uwo.ca

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DAPT	*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester
EGF-CFC	Epidermal growth factor-cripto FRL1 cryptic
EPO	Erythropoietin
FOXH1	Forkhead box HI
Gdf-1	Growth differentiation factor-1
GLUT	Glucose transporters
GPI	Glycosyl-phosphatidylinositol
Gsc	Goosecoid
HIF	Hypoxia-inducible factor
HPV	Human papilloma virus
hESCs	Human embryonic stem cells
INK4	Inhibitor of cyclin-dependent kinase 4
iPSC	Induced pluripotent stem cells
MAML	Mastermind/Lag
NICD	Notch intracellular domain
NOD/SCID	Non-obese diabetic/Severe combined immune deficiency
PI3K	Phosphoinositol-3-kinase
RB	Retinoblastoma
siRNA	Small interfering RNA
T-ALL	T-acute lymphoblastic leukemia
VEGF	Vascular endothelial growth factor

13.1 Introduction: Cancer as a Disease of Development Undone

With few exceptions, all cells in an organism share the same DNA code; yet, different cell types have vastly different gene expression profiles. Such phenotypic diversity is dictated during development by microenvironmental mediators such as morphogen gradients and oxygen availability. The instructive cues of the embryonic program culminate in epigenetic alterations in cell signaling and DNA structure (i.e., DNA methylation). The result of embryogenesis is an organism made of tissues and cell types that maintain homeostatic balance concomitant with exquisite structure–function relationships. In cancer, the embryonic program is "undone," resulting in the manifestation of stem cell-like characteristics (Fig. 13.1) [1]. Alternatively, some cancers may arise from resident stem cell populations [2]. This stem cell-like nature of cancer is correlated with metastatic progression, resistance to therapy, and a poor clinical prognosis. Hence, understanding and targeting the molecular mediators of tumor cell plasticity could be of tremendous therapeutic value.

Recent advancements in the area of stem cell biology have revealed that somatic cells can be reprogrammed to pluripotent stem cells following the expression of the embryonic transcription factors Oct-4, Klf-4, c-Myc, and Sox-2 or Oct-4, Nanog, Sox2, and Lin28 [3, 4]. These groundbreaking studies have illuminated the epigenetic plasticity of the genome and have generated a powerful tool with which to

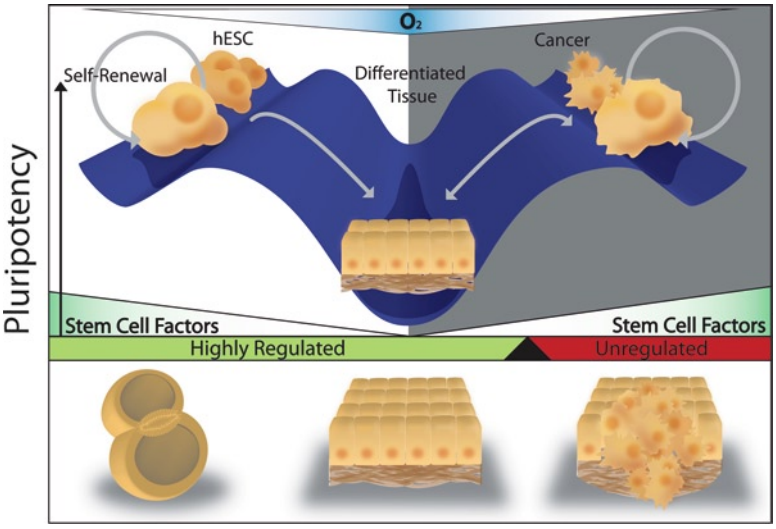

Fig. 13.1 Tumor progression as a disease of "development undone": Tumor progression is characterized by a loss of tissue structure and by the acquisition of a more pluripotent phenotype concomitant with the expression of stem cell associated factors such as Nodal and Notch. In many ways this process represents an "undoing" of the differentiation that occurs during development and mimics certain aspects of induced pluripotency. The mechanism by which cancer cells aberrantly acquire the expression of pluripotency-associated genes likely involves epigenetic alterations facilitated by reduced oxygen levels, a microenvironmental characteristic of both solid tumors and early embryonic development. Normal embryonic stem cells maintain a balance of activators and inhibitors of self-renewal, in order to facilitate differentiation in response to specific cues. In contrast, cancer cells hijack these elegant signaling pathways in a manner that favors uncontrolled growth in the absence of normal differentiation

understand dedifferentiation. In an attempt to improve the efficiency of pluripotent stem cell induction, researchers have found that inhibition of key tumor suppressor genes (such as p53 and INK4a) greatly enhances the ability of cells to undergo reprogramming [5]. Similarly, culture in microenvironments that promote tumor progression, such as hypoxia, have been shown to enhance the generation of induced pluripotent stem cells (IPSCs) [6]. An elegant body of work by Tlsty and colleagues has similarly shown that mutations in Ras or silencing of p16 can promote de novo methylation and cellular reprogramming in human mammary epithelial cells [7, 8]. These studies draw an interesting comparison between induced pluripotency and tumor cell plasticity such that phenomena classically associated with tumor progression (such as p53 inhibition) are similarly permissive to the acquisition of immortality in normal somatic cells. Moreover, oncogenic signaling events may precipitate heritable alterations in epigenetic signatures. The parallels between the acquisition of embryonic stem cell-like characteristics and tumor cell plasticity also suggest that these processes may capitalize upon similar microenvironmental mediators.

13.2 Cellular Mediators of Plasticity

A number of cell-derived proteins have been shown to promote cellular plasticity in both normal and neoplastic stem cell populations. Moreover, many of these factors work together in co-ordinated regulatory networks. Two such proteins are Nodal and Notch.

13.2.1 Nodal

Nodal is a member of the transforming growth factor beta (TGF-β) superfamily. During embryogenesis and in cancer, Nodal confers its signal as a homodimer by binding to activin-like kinase receptor type I (ALK4/7) and type II (ActRIIB). Upon activating this receptor complex (ALK receptor complex), SMAD2 (and possibly SMAD3) are phosphorylated intracellularly and interact with SMAD4 before translocating to the nucleus [9]. In the nucleus, transcription factors such as forkhead box HI (FOXH1) are activated to increase the expression of Nodal responsive genes such as Goosecoid (Gsc) and Lefty. Nodal also induces its own expression, thereby creating a positive feedback loop [10, 11]. The specificity of Nodal is further established by the epidermal growth factor-cripto FRL1 cryptic (EGF-CFC) family co-receptor, Cripto-1 [10, 12]. Cripto-1 has an N-terminal signal peptide, an EGF-like domain which directly interacts with Nodal, a conserved cysteine-rich (CFC) domain which interacts with ALK4, and a hydrophobic C-terminal glycosylphosphatidylinositol (GPI) anchor [13]. The adjacent positioning of the EGF-like domain and the CFC domain helps bring Nodal into proximity with ALK4 to facilitate enhanced binding [13]. Nodal signaling is inhibited spatially and temporally during development by proteins such as Lefty A, Lefty B, and Cerberus [11, 14]. These inhibitors are transcribed in response to Nodal signaling, and act as a negative feedback mechanism to control Nodal localization and action in the developing embryo [9]. In particular, Lefty is also regulated by alternate SMAD pathways, Wnt, and Oct3/4 signaling, and is upregulated during differentiation events [15]. Lefty inhibits Nodal signaling through interactions with Nodal and/or Cripto-1 that prevent activation of the ALK receptor complex [9].

The primary role of Nodal during embryonic development is to establish anterior–posterior axis patterning and left–right asymmetry [9]. Nodal is first expressed in the mouse epiblast shortly after implantation, and is maintained and enhanced by autoregulation [16]. Convertases expressed in the adjacent extraembryonic ectoderm process Nodal predominantly in the proximal epiblast [9, 17]. Activated Nodal signaling subsequently induces Lefty1 and cerberus-like (cerl) in the distal visceral endoderm, which later becomes the anterior visceral endoderm [9]. Eventually, Nodal signaling becomes restricted by Lefty1 and cerl to the proximal posterior region of the epiblast, where the embryonic ectoderm and primitive endoderm are developing, and where the primitive streak will form [12, 14, 18–20]. As development proceeds and cells undergo gastrulation, Nodal becomes restricted to the node at the anterior of the primitive streak, hence the name “Nodal” [9]. The node initiates left–right axis

formation [19, 21, 22]. Nodal and growth differentiation factor-1 (Gdf-1) from the ventral node pattern the left side of the embryo through interactions with Cryptic in lateral plate mesoderm [14]. On the right side of the embryo, Nodal inhibitors such as Cerl and Lefty, and physical leftward flow from cilia restrict Nodal signaling [14]. During somitogenesis, Nodal becomes more specifically restricted to mesoderm cells on the left side of the embryo, and is downregulated with differentiation until it is no longer present at approximately 8 days post fertilization [20].

Several studies with human embryonic stem cells have sought to elucidate the role of Nodal in human development. Vallier et al. [23] showed that Nodal signaling maintained pluripotency in human embryonic stem cells (hESCs) through SMAD2/3-induced activation of Nanog transcription. In turn, Nanog was shown to interact with SMAD2/3 to limit transcriptional activity of the Nodal signaling pathway, and inhibit endoderm differentiation [23]. Several studies have also shown that Nodal/Activin inhibition in human embryonic stem cells by receptor inhibition with SB431542 induces neuroectoderm specification [23–25]. Together, these studies exemplify the role of Nodal in maintaining pluripotency by inhibiting differentiation into neuroectoderm and mesendoderm lineages in hESCs.

Recent studies have demonstrated that Nodal is also expressed in several cancers, and that this expression is correlated with disease progression. Indeed, Nodal expression has been described in testicular cancer, glioma, melanoma, breast cancer, prostate cancer, and endometrial cancer lesions [10, 26–31]. Studies suggest that Nodal plays a pro-tumorigenic role in many of these cancers. For example, when Nodal signaling was inhibited with a small molecule inhibitory drug (SB431542) or morpholino oligonucleotides in melanoma cells, there was a marked reduction in cellular invasion and tumor formation [10]. A recent study demonstrated that Nodal similarly promotes tumor growth, invasion, and dedifferentiation in glioma cells [27]. In another study, Adkins et al. [26] determined that inhibition of Cripto–Nodal signaling via an anti-EGF antibody (A27.F6.1) was able to inhibit tumor growth of NCCIT testicular cancer cells in nude mice. Ongoing studies have recently correlated Nodal expression with tumor progression in prostate cancer [30]. Indeed, compared to poorly aggressive LNCaP prostate cancer cells that express low levels of Nodal, aggressive DU145 prostate cancer cells express high levels of Nodal, and undergo anchorage-independent growth and invasion in vitro [30]. Furthermore, transfection of LNCaP cells with a Nodal expression vector increases clonogenicity in vitro [30].

Nodal signaling has also been described in endometrial cancer [31]. Normally, the female endometrium undergoes constant remodeling and turnover throughout the menstrual cycle. In cancer, regulatory mechanisms of these remodeling events go amiss. One study by Papageorgiou et al. [31] showed that Nodal and its co-receptor, Cripto, were expressed during normal proliferative phases of the menstrual cycle in stromal and epithelial cells. Interestingly, patient biopsies of endometrial carcinoma that ranged from Grade 1 to Grade 3 in severity showed a positive correlation between Nodal/Cripto expression and cancer progression [31]. Lefty, a potent inhibitor of Nodal signaling in embryonic stem cells, was absent in all endometrial cancer biopsies [31]. These results are important for understanding normal mechanisms of proliferation in the endometrium, and aberrant mechanisms of endometrial carcinoma progression.

Recent studies have demonstrated a pro-metastatic role for Nodal and Cripto in breast cancer. Tissue microarray analyses of human breast tissue samples revealed a positive correlation between Nodal and breast cancer progression [29]. Furthermore, Nodal was completely absent in normal breast tissue samples from these experiments. When MDA-MB-231 cells were treated with Lefty from hESCs, invasion and clonogenicity was reduced concomitant with a downregulation of Nodal gene and protein expression [29]. Recently, Meyer et al. [32] demonstrated that CD44+/CD24+ breast cancer cell populations are able to convert to CD44+/CD24− breast cancer stem cells (and vice versa) both in vitro and in vivo. This suggests that differentiated cells outside the breast cancer stem cell subpopulation exhibit a dynamic plastic phenotype. It was found that the central regulator of this dynamic phenotypic switching was the Activin/Nodal pathway. When the ALK receptor was inhibited with SB431542 in either of these populations, phenotypic switching was significantly impaired, implying an important role for Nodal-associated signaling pathways in mediating plasticity in cancer.

Of note, although studies have described the presence of Nodal and Cripto in several cancers, none have reported the concomitant expression of Nodal inhibitors such as Lefty. In fact, studies have shown that breast cancers, melanomas, and endometrial cancers do not express the Lefty proteins [29, 31]. This is in sharp contrast with hESCs, where Lefty is among the mostly highly expressed genes [15]. Hence, it is possible that Nodal-associated increases in tumorigenic phenotypes are due to an imbalance in this signaling pathway, rather than its presence per se.

13.2.2 Notch

Signaling between Notch receptors and their ligands is known to play a role in a wide variety of cellular processes including stem cell maintenance, cell specification, differentiation, proliferation, and apoptosis [33]. There are four known mammalian Notch receptors (Notch 1–4) and five ligands (Jagged1, Jagged2, Delta1, Delta3, and Delta4) [34]. The Notch receptors are activated by binding to ligands that are expressed on adjacent cells, exposing a site in the extracellular portion of the transmembrane domain for disintegrin and metalloproteinase (ADAM) mediated cleavage [35]. The Notch intracellular domain (NICD) is subsequently released as a consequence of a second proteolytic cleavage mediated by γ-secretase [36]. The liberated NICD translocates to the nucleus where it interacts with C-promoter binding factor 1 (CBF-1), suppressor of hairless (Su(H)), lin-12, and glp-1 (Lag-1) (CSL), a DNA-binding transcription factor that normally inhibits transcription by interacting with co-repressor proteins [37]. The NICD competes with these repressor proteins to form a NICD-CSL complex which recruits the Mastermind/Lag (MAML) co-activator to form a transcriptional activation complex [38]. This complex initiates the transcription of target genes such as *Hes* and *Hey* [39] that have been shown to prevent the transcription of lineage-specific genes including *Myo-D* and *Mash-1* [34, 40–42].

The role of Notch in the maintenance of the undifferentiated state is generally thought to arise from its involvement in maintaining the balance between the

progenitor cell pool and differentiating cells [43, 44]. Studies using constitutively active NICD in both frog and chicken show that increased Notch signaling results in decreased neurogenesis, whereas blocking this pathway leads to a depletion of the pool of progenitor cells and unbalanced neurogenesis [45, 46]. In addition to contributing to stem cell maintenance, Notch has also been found to be involved in binary cell fate decisions through lateral and inductive signaling [47]. Lateral signaling was first elucidated in *Drosophila,* where it was determined that neuronal precursor cells, which have the capacity to differentiate into either neuronal cells or epidermal cells, initially express both Notch and its ligand, but over time exclusively express one or the other [47]. The Notch-expressing cell, which receives its signal upon binding neighboring receptors, adopts an epidermal cell fate, whereas the cells that exclusively express ligand pursue a neuronal cell fate [48]. Inductive signaling occurs between two developmentally distinct cells that express exclusively either receptor or ligand [49]. An example of inductive signaling is seen in mouse thymic epithelial cells: Populations that express Notch1 are able to induce early lymphocyte precursors to adopt a T-cell fate as soon as they enter the thymus, whereas populations that lack Notch1 adopt the B-cell fate as a default pathway [50]. Hence, Notch is a factor that has a notable influence over stem cell maintenance and cell fate decisions, which have the potential to become deregulated in the pathological state of cancer.

Just as Notch signaling has been demonstrated to play a role in maintaining cells in a proliferative and undifferentiated state, it has been postulated that its role in cancer is to prevent cells from responding to differentiation cues that they may receive from their immediate environment [51, 52]. How Notch contributes to the tumorigenic process is perhaps the least known aspect of Notch signaling. Depending on the tumor type, Notch can either promote or limit tumor growth through its effects on differentiation, cellular metabolism, cell cycle progression, and possibly self-renewal and immune function [53]. Much of this depends on cellular context and crosstalk with other signal transduction pathways. For example, NOTCH1 prevents cellular proliferation in normal epithelia by increased expression of the cell cycle regulator WAF1; however, it does not appear to play the same role in HPV-containing cervical cancer cells, primarily because RB and WAF1 are inactivated by HPV oncoprotein E7 [54–57]. Furthermore, it has recently been shown that Notch signaling may have a role in the maintenance of normal and malignant stem cells [58–61].

Oncogenic Notch signaling is most clearly understood in T-acute lymphoblastic leukemia (T-ALL), an aggressive neoplasm of immature T-cells [47]. *NOTCH1* was first identified by its involvement in a chromosomal translocation, and more recently, has been found to have two types of activating mutations, at least one of which is found in 55–60% cases of human T-ALL [62, 63]. Beyond T-ALL, there is increasing evidence that Notch signals are oncogenic in other cellular contexts, particularly breast cancer and melanoma [64]. The expression of Notch receptors is upregulated in human breast cancers, and the expression of ligands such as Jagged1 correlates with a more aggressive phenotype in both breast and prostate cancer [64–67]. Also, low levels of the Notch antagonist, Numb, correlate with both high levels of Notch signaling and sensitivity to γ-secretase inhibitors [68]. Another report on the role of Numb reveals that Notch1 signaling is increased in a variety of molecularly different breast cancers, and that with enforced expression of Numb, such cancers are reverted

to a more benign phenotype [69]. In primary human melanomas, expression of Notch1 promotes cancer progression, concomitant with an upregulation of Notch receptors and their downstream target genes [70–72]. Furthermore, in both breast cancer and melanoma cells, an interplay between increased Notch activity and activation of the P13K-Akt pathway involved in cellular metabolism has also been established; however, the specifics of the interplay are still not understood [72]. As is the case with normal Notch signaling, the role of Notch in cancer is known to be somewhat dependent on context. In many cases, Notch can alternatively serve as a tumor suppressor as opposed to an oncogene [73]. In breast cancer, Notch2 is known to function as a tumor suppressor, whereas the other Notch receptors function as oncogenes [73]. Furthermore, *Notch1* knockout mice develop basal cell carcinoma-like lesions, and mice with dominant negative MAML1 develop cutaneous squamous cell carcinomas [74].

A complexity between the Notch and Nodal signaling pathways has recently emerged, providing further evidence for co-opted stem cell pathways in cancer that may interact in a similar manner to their normal stem cell context [75]. Mouse embryos lacking functional components of the Notch pathway exhibited disorganized left–right asymmetry patterning, a phenomenon traditionally attributed to Nodal signaling [40, 41]. Analysis of the NDE region upstream from the *Nodal* gene locus lead to the identification of two binding sites for CSL, the primary transcriptional mediator of Notch signaling [40, 41]. This region has been shown to be responsive to Notch signaling in mouse embryos [40, 41]. Furthermore, Baf60c, a subunit of the Swi/Snf-like BAF chromatin remodeling complex, is essential for the integration of Notch signaling components for transcriptional activation of *Nodal* in the mouse node [76]. These studies revealed a role for the Notch pathway working upstream of *Nodal* expression in left–right asymmetry patterning in mice. Furthermore, it was determined that inhibiting Notch in metastatic melanoma cells with a γ-secretase inhibitor (DAPT) or by Notch4-specific small interfering RNA (siRNA) results in a decrease in Nodal expression [77]. Moreover, these studies indicate that human Nodal, like mouse Nodal, is upregulated by Notch signaling in cancer cells [77]. Recently, more complexity has emerged in the crosstalk between the Notch and Nodal signaling pathways, with the finding that the Nodal co-receptor Cripto-1 is able to facilitate the posttranslational maturation of Notch receptors [78, 79]. The similar appearance of Notch and Nodal signaling in breast cancer and melanoma, as well as their interaction in development, demonstrates a recapitulation of the interaction found in a normal stem cell context and indicates a way in which the interplay between two stem cell pathways may be mediating a stem cell phenotype in cancer.

13.3 Oxygen as a Regulator of Cellular Plasticity

Cell-derived factors potentiate the acquisition and maintenance of self-renewal and plasticity. However, such parameters do not operate autonomously; rather, they are dynamically regulated by alterations in the external milieu. As a solid tumor grows,

the rate of cell proliferation exceeds that of normal tissue growth and consequently interferes with the ability of the existing vasculature to supply growth factors, nutrients, and oxygen, and to remove waste products from the cells [80]. This results in areas within the tumor that have pathologically low levels of oxygen, low glucose levels, and low extracellular pH [80, 81]. These phenomena result in heterogeneous populations of cells within the tumor, and furthermore, differences between tumors [80]. Such aspects of the external tumor environment have been extensively linked to tumor progression and metastatic disease, and thus are important considerations when investigating the role of cancer stem cells within a complex and deregulated microenvironment.

Oxygen availability dramatically alters the expression profile and behavior of cells. Hypoxia, which is defined as a state of low oxygen tension that falls below critical levels, initiates complex and specialized responses at the molecular, cellular, tissue, and organismal level so as to re-establish oxygen homeostasis and minimize the detrimental effects of low oxygen [81]. The oxygen concentrations in mammalian tissues can range from 150 mmHg in the upper airways to 5 mmHg in tissues such as the retina [82, 83]. The first model of tumor hypoxia proposed by Thomlinson and Gray [84] suggested that as a result of diffusion-limited hypoxia (a consequence of tumor cells being sufficiently distant from the vasculature), cells within a tumor could remain viable in chronic levels of hypoxia for a few hours to a few days [85, 86]. More recently, studies of blood flow and oxygen levels in animal tumors have suggested a model of perfusion-limited hypoxia (acute or fluctuating hypoxia), in which perfusion of blood vessels is dynamic and can result in transient periods of severe hypoxia within the tumor [87–89]. As a result, it is now believed that the oxygenation levels within a tumor can vary, not just by the diffusion to cells that are distant from the vasculature but also by the fluctuating levels of blood flowing through the vessels to the tumor [90]. In response to hypoxia, there is reduced oxidative phosphorylation in the mitochondria, and those activities that require large amounts of ATP, such as ATP-dependent ion channel function and protein translation, are repressed [91]. Moreover, glycolysis becomes immediately upregulated to compensate for the lower-than-normal ATP production in the cell [91]. Generally, most transcription is repressed during times of hypoxia; however, transcription of subsets of genes, including vascular endothelial growth factor (VEGF), erythropoeitin (EPO), and glucose transporters (GLUT1, GLUT3) are increased dramatically [81, 92]. At the tissue level, angiogenesis and EPO concentrations are enhanced in hypoxia to increase the oxygen delivery to affected areas [81, 93]. Specifically, the development of tumor hypoxia is fundamentally linked to the formation of neovasculature by the process of angiogenesis.

Intratumoral oxygen tensions are strikingly similar to the low levels of oxygen experienced in early development [94]. This correlation suggests that specific pathways regulated by low oxygen in stem cells may also manifest in cancer. Early embryonic development occurs in a low oxygen environment (1–2%), and increased oxygen levels are detrimental to the proper execution of early developmental events and stem cell differentiation [95, 96]. Also, many human stem cells inhabit hypoxic niches throughout the body in adult life. For example, bone marrow–derived stem

cells reside in a microenvironment that has been estimated to have a relatively low oxygen tension (1–2%) [97]. When these cells are cultured ex vivo, they exhibit greater proliferation and ability to reconstitute the bone marrow of NOD/SCID mice if they are cultured in hypoxia vs. atmospheric oxygen [97, 98]. Other stem cells have also been reported to thrive in a hypoxic environment, such that their proliferation and maintenance of stem cell identity is promoted [99, 100]. Neuronal stem cells exhibit increased proliferation and preferential differentiation into certain cell types when cultured in hypoxic conditions (1–5%) over normoxic conditions [99, 100]. Furthermore, culturing hESCs in hypoxic conditions (3–5%) results in maintenance of an undifferentiated state, whereas culture at atmospheric oxygen causes differentiation of the cells [101]. Exposure to hypoxia also supports cancer stem cells [80]. For example, recent studies demonstrated that hypoxia promotes CD133-positive cancer stem cell populations in glioblastomas [102]. Furthermore, breast cancer cells have been shown to decrease estrogen receptor expression, and increase cytokertain 19 (an epithelial stem cell marker), following 3 days of exposure to 1% oxygen. Neuroblastoma cells exposed to hypoxia similarly assume a dedifferentiated phenotype, characterized by a reduction in neuronal/neuroendocrine markers such as dHAND, concomitant with an enhancement of markers including cKit and Notch-1, which characterize neural crest progenitors [103]. The parallel between the oxygen-mediated regulation of cell fate in stem cells and cancer cells is strong, supporting the notion that low oxygen has the ability to promote stem-like phenotypes in cancer, ultimately leading to metastatic spread.

Several studies have demonstrated that hypoxia promotes pluripotency and self-renewal in stem cells, and that it characterizes several stem cell niches [101, 104]. Hypoxia is similarly able to sustain cancer cell immortality and plasticity [94]. An important protein that has been found to be upregulated by hypoxia is the embryonic stem cell associated transcription factor Oct4 [105]. Oct4 is expressed in the embryonic stem cells of the inner cell mass of the blastocyst [106]. This pivotal protein is downregulated in somatic cells around the time of gastrulation, but is retained in primordial germ cells and in some adult stem cell populations [106]. A recent study revealed a significant link between hypoxia inducible transcription factors (HIFs) and Oct4 expression during development. In this study, a HIF-2α gene "knock-in" mouse was generated on a HIF-1α deficient background. Surprisingly, this knock-in did not rescue the effects of the HIF-1α knockout. Rather, the mice showed gross developmental abnormalities and embryonic lethality between E3.5 and E7.5 (earlier than HIF-1α deficient embryos alone which died at E9.5–E10.5). These HIF-2α knock-in mice had an enhanced expression of Oct4 [105]. ChIP analyses further revealed that HIF-2α binds to hypoxic regulatory regions in the mouse Oct4 promoter, and that this ability is not shared by HIF-1α [105]. Hence, by preventing terminal differentiation, the HIF-2 induced expression of Oct4 resulted in embryonic lethality. Of note, Oct4 expression is found in numerous tumor cell types, as well as human adult stem cells, and increased expression of Oct4 in embryonic stem cells causes them to form tumors in a dose-dependent manner [106, 107]. Thus, it is quite plausible that hypoxia promotes and/or sustains stem cell phenotypes and tumorigenicity via the HIF-2α mediated upregulation of Oct4.

An equally impressive finding is the discovery that HIF proteins also regulate the activity of c-Myc, an oncogene of central importance in many cancers and in embryological development [108]. HIF-1α was found to antagonize c-Myc activity by competing for binding to its transcription factor Sp1 in hypoxia, and thus inhibiting c-Myc dependent cell cycle progression [109]. However, HIF-2α had the opposite effect, and was able to promote cell cycle progression and also contribute to oncogenic transformation by enhancing the transcriptional effects of c-Myc [110]. This resulted in the activation and repression of c-Myc target genes in multiple cancer cell lines, mouse embryonic fibroblasts, and embryonic stem cell lines [110]. It is important to note that Oct4 and c-Myc are two of the four genes that were shown to be sufficient to generate iPSCs and therefore, it is highly probable that their hypoxia-induced expression in cancer plays a pivotal role in the development of a cancer stem cell phenotype [4, 111]. Furthermore, this provides a clear example of how hypoxia contributes to a poorly differentiated phenotype and the existence of stem-like cancer cells.

As mentioned previously, Notch is another stem cell factor known to play a role in cancer. Of note, hypoxia upregulates the activity of Notch signaling in a HIF-1 dependent manner [112]. Specifically, HIF-1α binds and stabilizes the cleaved NICD, thereby increasing the efficiency of NICD binding to CSL and initiating transcription of Notch target genes [112]. The interaction of HIF-1α with Notch was shown to inhibit neuronal and myogenic differentiation, and HIF-1α deficient mouse embryonic fibroblasts showed a decrease in activation of Notch target genes under hypoxia [112]. Furthermore, the interaction between Notch and HIF-1α shows that hypoxia is able to increase Notch activity and contribute to the maintenance of pluripotency in embryonic stem cells [113]. As previously described, Notch signaling is also involved in various forms of cancer. Hence, hypoxia may promote cancer stem cell phenotypes via the activation of Notch.

Notch is also known to regulate the potent stem cell factor Nodal in an embryonic environment, and is thought to have a similar activating effect in cancer. The relationship between Notch and Nodal is further supported by the finding that both converge on hypoxia and HIF-1α signaling. Preliminary studies done in our laboratory suggest that Nodal is regulated by low oxygen, and that hypoxia exerts its influence through HIF signaling [114]. When poorly metastatic breast cancer and melanoma cell lines (MCF-7, T47D, C81-61) that do not endogenously express Nodal were exposed to 0.5% oxygen for 48 hours, induction of Nodal mRNA expression and protein expression was observed. By examining the role of the HIF pathway using HIF-1α-specific siRNA in hypoxia and introducing at HIF-1α containing expression vector in normoxia, it was also determined that hypoxia-induced Nodal expression is dependent on the HIF-1α pathway. As discussed above, it was determined in embryonic studies that Notch binds to the NDE region of the Nodal gene. In this respect, it is possible that Notch, through its activation by hypoxia and HIF-1α, may be contributing to the undifferentiated state of cancer cells by inducing the expression of Nodal.

13.4 Targeting Tumor Plasticity: Restoring the Embryonic Program

The plasticity that allows cancer to progress also presents a unique and powerful target for treatment. There are two main anti-cancer strategies that capitalize on the embryonic microenvironment: (1) Normal embryonic niches, containing a balance of activators and inhibitors of stem cell fate, can be used to restore balance to aberrant signaling in cancer cells. As a consequence, the cancer cells would either stop growing due to an inhibition of self-renewal cues or reprogram toward a more differentiated phenotype. (2) Cancer cells can be fully reprogrammed back to an induced pluripotent stem cell, and then differentiated into a cell with a relatively normal epigenetic status and phenotype. Such a cell could then be targeted for treatment.

Numerous studies have revealed that embryonic microenvironments reduce tumorigenicity by inducing senescence and differentiation in cancer cells. For example, Pierce and Wells discovered that B16 murine melanoma cells are unable to form tumors and appear to differentiate toward a neuronal phenotype following exposure to microenvironmental factors derived from the embryonic skin of a developing mouse [115]. A number of zebrafish models have confirmed these findings. For example, extracts derived from zebrafish embryos have been shown to inhibit proliferation and induce apoptosis in several cancer cell types [116]. Furthermore, following transplantation into zebrafish embryos, metastatic melanoma cells lay dormant and are unable to form tumors [117]. Interestingly, this phenomenon is unique to the embryonic zebrafish, as human melanoma cells transplanted into zebrafish 2 days after fertilization (after morphogenesis and organogenesis are complete) form tumors and even induce angiogenesis [117]. Bissell and colleagues documented that Rous sarcoma virus, which causes a rapidly growing tumor when injected into hatched chicks, is non-tumorigenic when injected into 4-day-old chick embryos, despite viral replication and v-src oncogene activation [118]. In another set of experiments, transplantation of melanoma cells in ovo adjacent to host chick premigratory neural crest cells caused the transplanted melanoma cells to respond to neural crest cues by populating structures such as the brachial arches, sympathetic ganglia, and dorsal root, in a manner similar to neural crest cells [119]. Interestingly, a subpopulation of melanoma cells that invaded the chick periphery was reprogrammed to express the melanocyte-associated protein Mart-1/Melan-A, thus confirming that melanoma cells can respond to developmental cues by undergoing differentiation [119].

As an extension to these findings, an in vitro 3D model was developed to examine whether the microenvironment of hESCs could similarly reprogram metastatic phenotypes [29, 120]. In this model, hESCs were cultured on 3D extracellular matrices and were removed, thereby generating a cell autonomous hESC conditioned environment. Utilizing this approach, exposure of melanoma and breast cancer cells to extracellular matrices conditioned by hESCs (CMTX) resulted in reduced in vivo tumorigenicity concomitant with increased apoptosis [29]. More recently, a study demonstrated that conditioned medium from hESCs reduces the proliferation of ovarian, breast, and prostate cancer cells, but does not affect normal fibroblasts [121].

Collectively, these studies suggest that hESC-derived factors have anti-tumor activities. In an effort to isolate factor(s) that confer such properties, separation of conditioned medium has been conducted, and anti-tumorigenic entities appear to be enriched in low molecular weight (<10 kDa), heat stable fractions [121]. The identities of such factors have not, however, been described. Using the 3D CMTX model, Lefty was isolated as a protein with major anti-tumorigenic properties, largely due to its ability to inhibit Nodal signaling [29]. Of note, the hESC-derived CMTX was able to inhibit tumorigenic phenotypes even when Lefty was depleted, suggesting that other factors are also involved in this complex process [29]. Proteomic analyses of the hESC microenvironment, combined with functional assays, are vital to discovering which proteins in the hESC milieu may be harnessed for the treatment of cancers [122].

A number of studies have demonstrated that embryonic microenvironments can inhibit the tumorigenicity of cancer cells through the process of reprogramming followed by differentiation. One of the first examples was presented by Mintz and Illmensee [123]. In this seminal study, embryonal carcinoma cells were injected into blastocysts. Surprisingly, these cancer cells did not form tumors, nor did they mitigate embryological development. Rather, they partook in the development of all tissues, inclusive of the germ line. In corroboration with these findings, Jaenisch and colleagues demonstrated that nuclear transplantation of a RAS-inducible melanoma nucleus into an oocyte leads to the reprogramming of the melanoma genome, giving rise to ESCs with the capacity to differentiate into cell types such as melanocytes and fibroblasts [124]. While predisposing mice to RAS-induced tumors later in life, this reprogramming event illustrated the ability of the oocyte environment to epigenetically reprogram cancers to a well-differentiated phenotype. More recently, a number of studies have shown that cancer cells can be programmed to iPSCs, despite karyotypic abnormalities. For example, iPSCs have been generated from leukemic cells, colon cancer cells, and melanoma [125–127]. Notably, iPSCs were generated from human chronic myeloid leukemia cells containing the Philadelphia translocation (BCR-Abl). Prior to reprogramming, these cells had acquired resistance to Gleevec; however, hematopoietic cells differentiated from the iPSCs had regained sensitivity to this drug [125]. These findings point to the epigenetic nature of many phenomena associated with cancer progression, such as drug resistance. Moreover, they suggest that reprogramming followed by differentiation is a powerful strategy for the normalization and treatment of advanced cancers.

13.5 Conclusions

Tumor progression is characterized by a loss of differentiation and by the acquisition of an embryonic stem cell-like gene expression pattern (summarized in Fig. 13.1) [1, 128]. Several stem cell–derived proteins such as Nodal and the Notch receptors facilitate this transition. The mechanism by which cancer cells aberrantly acquire the expression of such pluripotency-associated genes is not clearly understood, but

likely involves epigenetic alterations induced by biophysical parameters such as reduced oxygenation. Normal embryonic stem cells maintain a balance of activators and inhibitors of self-renewal, in order to facilitate differentiation in response to specific cues. For example, hESC populations express high levels of both Nodal and the Nodal inhibitors Lefty and Cerl [29, 129]. In contrast, cancer cells hijack these elegant signaling pathways in a manner that favors uncontrolled growth in the absence of normal differentiation. This imbalance of stem cell associated cues in cancer presents a unique and powerful target for treatment. Indeed, mounting evidence suggests that normal embryonic niches, containing a balance of activators and inhibitors of stem cell fate, can be harnessed to reprogram cancer cells toward a more differentiated phenotype. This approach of re-establishing normal developmental signaling may be of great therapeutic value for the prevention and eradication of cancer stem cell-like populations.

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Chapter 14
The Epithelial-to-Mesenchymal Transition and Cancer Stem Cells

Jonas Fuxe

Abstract The epithelial-to-mesenchymal transition (EMT) is a developmental process which is reactivated during carcinoma progression, providing tumor cells with enhanced migratory properties, the capacity to invade the stroma, and the ability to metastasize. Tumor cells undergoing EMT also acquire stem cell characteristics, suggesting that there is crosstalk between pathways promoting EMT and self-renewal, and that the EMT process contributes to the generation of cancer stem cells. This chapter summarizes findings pointing to molecular links between EMT and cancer stem cells. The focus is crosstalk between signaling by the transforming growth factor-beta (TGF-β)/Smad pathway, a major inducer of EMT, and stem cell pathways including Wnt, Ras, Hedgehog, and Notch. Finally, the existence of EMT/stem cell niches in tumors where cooperative signaling between TGF-β and self-renewal pathways is activated is discussed.

Abbreviations

ALDH1	Aldehyde dehydrogenase 1
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
bHLH	Basic helix-loop-helix
CAR	Coxsackie- and adenovirus receptor
CD	Cluster of differentiation
EMT	Epithelial-to-mesenchymal transition
EpR	Epithelial repressors
EPSC	EMT promoting Smad complexes

J. Fuxe (✉)
Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden
e-mail: jonas.fuxe@ki.se

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ERK	Extracellular signal-regulated kinase
ERα	Estrogen receptor-α
GSK-3β	Glycogen synthase kinase-3beta
HIF-1α	Hypoxia-inducible factor 1 alpha
HMGA2	High mobility group A2
LDL	Low density lipoprotein
LEF	Lymphoid enhancer factor
MAPK	Mitogen-activated protein kinase
MeA	Mesenchymal activators
MMP	Matrix metalloproteinase
NF B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAI-1	Plasminogen activator inhibitor 1
PI3K	Phosphoinositol-3-kinase
RTK	Receptor tyrosine kinase
Sp1	Specificity protein 1
TCF	T cell factor
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
α-SMA	Alpha smooth muscle actin
β-Cat	Beta Catenin

14.1 Epithelial-to-Mesenchymal Transition in Tumor Metastasis

Epithelial-to-mesenchymal transition (EMT) is transdifferentiation process whereby epithelial cells acquire mesenchymal features, including the capacity to migrate to distant sites. EMT is important for various stages of development including gastrulation, neural crest formation, and heart development [1]. In addition, EMT is reactivated in pathological conditions such as organ fibrosis and cancer. Tumor epithelial cells undergoing EMT lose the expression of epithelial proteins involved in cell–cell interactions [2]. As a consequence, tumor cells can detach from the primary tumor. In parallel, EMT cells gain expression of mesenchymal cytoskeletal proteins such as vimentin and alpha-smooth muscle actin (α-SMA). This provides tumor cells with the capacity to invade the surrounding stroma and subsequently spread via blood and lymphatic vessels to distant sites [2]. Thus, activation of an EMT program in tumor cells constitutes a switch that converts benign tumors into invasive and metastatic counterparts [1, 3–5].

14.1.1 Consequences of EMT in Cancer

14.1.1.1 Loss of Cell–Cell Adhesion: Detachment from the Primary Tumor

Loss of cell–cell adhesion is a hallmark of EMT in cancer [1, 4]. Components of intercellular junctions including E-cadherin (adherens junctions) and the tight junction

proteins claudins, occludin, and the coxsackie and adenovirus receptor (CAR) are downregulated during EMT. These proteins are essential for the establishment of epithelial cell–cell interactions and for the barrier function and integrity of all epithelial cell layers. During EMT, transcription factors acting as repressors are activated and bind to specific DNA sequences in the gene promoters of these junction proteins. As a result, the genes are turned off.

Various EMT promoting transcriptional repressors of junction proteins have been identified, including members of the Snail, Zeb, and Twist families [6]. These transcriptional repressors recognize and bind a core 5′-CACCTG-3′ motif (E-box) within gene promoters. Snail1 was the first transcription factor identified to repress E-cadherin through direct binding to these E-boxes [7, 8]. Subsequently, additional transcription factors including Snail2 (Slug) [9], members of the bHLH family including Twist, E47 (TCF3), and TCF4 (E2-2) [6, 10, 11], and the two zinc-finger E-box binding homeobox factors Zeb1 (dEF1 or Tcf8) and Zeb2 (SIP1) [12, 13] have been identified as repressors of the E-cadherin promoter.

Overexpression of Snail1 results in EMT and the expression of other repressors, suggesting that Snail1 induces an EMT program [6, 14]. Snail1 expression in human cancer is confined to tumor cells at the invasive front [15–17]. However, Snail1 expression in normal adult tissues is limited and generally absent in mesenchymal cells [16]. The precise contribution of Snail, Zeb1/2, and bHLH factors to the repression of E-cadherin during EMT is not well understood. Twist promotes metastatic properties in breast tumor cells and stem-like properties in epithelial cells [11, 18, 19], and its expression is associated with high-grade ductal carcinomas and poor prognosis [20, 21]. Twist also promotes cellular migration, invasion, and resistance to paclitaxel treatment in breast cancer cells [22]. Twist is induced by hypoxia or overexpression of hypoxia inducible factor-1 alpha (HIF-1α), showing a link between the tumor microenvironment and the expression of EMT promoting transcription factors [23]. Twist can also be upregulated by Wnt signaling in mammary epithelial cells [24].

14.1.1.2 Activation of Mesenchymal Genes: Gain of Migratory Capacity

In parallel to inactivation of epithelial genes, EMT is characterized by activation of genes encoding mesenchymal proteins including N-cadherin, vimentin, fibronectin, alpha smooth muscle actin (α-SMA), the plasminogen activator inhibitor (PAI-1), and matrix metalloproteases (MMPs) [1, 4]. These proteins are involved in organization of the cytoskeleton during cell movement and in interactions with the extracellular matrix, therefore providing cells with migratory properties.

Various transcription factors acting as activators of mesenchymal genes during EMT have been identified. The activator protein-1 (AP-1) transcription factor, which is formed by Jun-Jun homodimers or Jun-Fos/Fra-2 heterodimers, induces the expression of Snail1, Snail2, and vimentin during EMT in colon cancer cells [25, 26]. AP-1 also promotes MMP expression, cancer cell invasion and metastasis [27]. Inhibition of AP-1 blocks EMT in human keratinocytes [28]. β-catenin is a transcription factor sequestered at adherens junctions through

interaction with E-cadherin in normal epithelial cells, but upon activation of Wnt signaling, β-catenin translocates to the nucleus where it interacts with TCF4/LEF transcription factors to induce the expression of EMT-related genes including Snail2, fibronectin, and vimentin [29–31]. β-catenin also interacts with Snail and other EMT-related factors, such as HIF-1α, Foxo3a, Foxo4, and estrogen receptor alpha (ERα) suggesting that β-catenin complexes play important roles in EMT [32–35]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which plays a key role in regulating immune responses, induces EMT in mammary epithelial cells by activating Snail1 and other mesenchymal genes [36]. Specificity protein 1 (Sp1) is a transcriptional activator required for TGF-β induced expression of vimentin and EMT in pancreatic cancer cells [37]. Sp1 seems also to be involved in the induction of MMP-9 in Snail expressing cells [37, 38].

14.2 EMT and Cancer Stem Cells

Due to the role of EMT in promoting tumor cell invasiveness and the capacity of cancer stem cells to metastasize to distant sites, it was proposed that EMT might provide a link between metastasis and cancer stem cells [39, 40]. Indeed, such a link was identified by results showing that cells undergoing EMT (by Twist/Snail/TGF-β) acquire a $CD44^{high}/CD24^{low}$ signature, a feature of breast cancer stem cells showing a unique ability to form tumors in xenograft models [18, 41]. Moreover, tumor cells undergoing EMT were found to form mammospheres, differentiate into cells of different lineages (i.e., myoepithelial or luminal epithelial cells), and reconstitute a heterogeneous tumor, thus displaying many properties of stem cells [18]. Subsequently, it was shown that tumor cells induced to undergo EMT by activation of the Ras-MAPK pathway display stem-like properties and a $CD44^{low}/CD24^{high}$ signature [19].

Further studies have reinforced a link between EMT and cancer stem cells. The capacity of mammary stem cells to form mammospheres is inhibited by overexpression of miR200c, a microRNA which turns off the EMT program [42]. Circulating tumor cells from breast cancer patients classified as non-responders to chemotherapy display both EMT and cancer stem cell signatures and express aldehyde dehydrogenase isoform 1 (ALDH1), a stem cell marker in breast, colon, lung, and head and neck carcinomas [43, 44]. Metaplastic breast cancer, a rare and aggressive form of human breast cancer, displays both EMT and cancer stem cell properties [45]. Similarly, human breast cancer cell lines with enhanced invasive properties classified as “Basal B” cell lines due to their basal-like/mesenchymal features show both an EMT and a cancer stem cell-like gene expression profile, including the $CD44^{low}/CD24^{high}$ signature [46]. Furthermore, the EMT promoting transcription factors Snail1 and Snail2 (Slug) induce expression of genes promoting cellular stemness including Nanog, KLF4, and TCF4 [47].

14.2.1 Signaling Pathways in EMT

TGF-β is a major inducer of EMT during development and is overexpressed in many types of human cancer, suggesting a role for TGF-β as an inducer of EMT in tumors [48, 49]. Paradoxically, TGF-β also has anti-proliferative tumor suppressive effects and inactivating mutations or epigenetic silencing of various components of the TGF-β signaling pathway predisposes tissues to cancer and inflammation, indicating that the capacity of TGF-β to induce EMT is contextual [50, 51]. TGF-β cooperates with pathways including Wnt, Hedgehog, Notch, and Ras to induce EMT. Interestingly, these are pathways involved in the induction and maintenance of stem cell niches. Thus, co-activation of TGF-β and stem cell pathways may shift the cellular response to TGF-β toward EMT. An explanation for this may lie in the subtle design of the TGF-β signaling pathway.

14.2.1.1 TGF-β/Smad Signaling in EMT

TGF-β binding to its receptors leads to phosphorylation and activation of downstream effectors of the Smad family [52, 53]. The receptor-activated Smad2 and Smad3 (R-Smads) become phosphorylated, associate with cytoplasmic Smad4 (co-Smad) and translocate to the nucleus where Smad complexes regulate transcription of target genes through interaction with specific binding motifs in gene regulatory regions [50]. However, Smad transcription factors have low affinity for DNA and need to interact with cofactors to achieve high affinity and selectivity for target genes. Through this delicate design of the Smad signaling pathway, cells read TGF-β signals differently and the outcome of the TGF-β response ultimately depends on the accessibility of Smad cofactors [50].

Smad signaling is essential for TGF-β–induced EMT [51]. Renal tubular epithelial cells deficient in Smad3 fail to undergo EMT and keratinocytes derived from Smad3−/− mice show reduced migration in response to TGF-β [54, 55]. Knockdown of Smad4 through RNA-interference or dominant negative approaches prevents E-cadherin repression upon TGF-β treatment [17, 56–59]. Smad4 deficiency also suppresses fibrotic type I collagen synthesis in vitro [57], and leads to decreased bone metastasis in vivo [56]. Furthermore, Smad4 promotes tumor cell invasion in advanced pancreatic tumors [60].

14.2.1.2 Wnt Signaling in EMT

Wnt signaling regulates stem cell renewal and is implicated in the induction of EMT in cancer. Overexpression of Wnt ligands or silencing of endogenous Wnt inhibitors has been reported in numerous types of human cancer including colon, breast, melanoma, and prostate carcinomas, and has been linked to EMT [61–66].

Activation of Wnt signaling is initiated by binding of Wnt ligands (typically Wnt1 or Wnt3a) to cell surface receptors composed by Frizzled (Fzd) and the LDL receptor-related proteins LRP5 or LRP6. Signaling from the receptor complex

via Dishevelled (Dvl) and Axin results in stabilization and nuclear translocation of β-catenin through inhibition of the destruction complex, consisting of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3beta (GSK-3β), which in the absence of Wnt signaling promotes phosphorylation and proteolytic degradation of β-catenin [67]. Wnt activation leads to inhibition of GSK-3β and stabilization and nuclear translocation of β-catenin, which forms a complex with T cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors and regulates the transcription of Wnt target genes [68].

Constitutively activated β-catenin signaling is a precursor to carcinogenesis and leads to excessive stem cell renewal/proliferation [69, 70]. Nuclear β-catenin is detected in tumor cells at the invasive front in colorectal cancer, suggesting that it can be used as a marker of EMT in vivo [71]. GSK-3β regulates stability and activity of β-catenin and other EMT-related transcription factors, such as Snail1 [72].

14.2.1.3 Ras Signaling in EMT

Activation of Ras signaling is a key event downstream of receptor tyrosine kinases (RTKs) activated by growth factors such as EGF and FGF [73]. This leads to the activation of PI3Kinase and Raf/ERK/MAPK pathways that regulate cell migration, proliferation, survival, and cell cycle processes. Ras cooperates with TGF-β to induce EMT but the mechanisms of cooperation between these pathways are not completely understood [74–77]. Raf and TGF-β cooperate to repress E-cadherin both at transcriptional and at posttranslational levels [78]. Activation of Ras cooperates with TGF-β to induce Snail1 expression during EMT [79, 80].

14.2.1.4 Hedgehog Signaling in EMT

Hedgehog signaling is activated in many forms of human cancer and has been linked to the expression of both stem cell and EMT markers (reviewed in [81]). Binding of Hedgehog family members to cell surface receptors results in stabilization and nuclear accumulation of GLI transcription factors that bind and regulate EMT-associated target genes like SNAI1, ZEB1, ZEB2, TWIST2 [82], and FOXC2. GLI1 promotes nuclear signaling by β-catenin through SNAIL1 and E-cadherin [83]. Hedgehog signaling has been proposed to promote the generation of CD44-positive invasive prostate cancer cells with cancer stem cell properties [84] and to regulate self-renewal of stem cells [85].

14.2.2 Transcriptional Crosstalk Between EMT and Stem Cell Pathways

Some of the transcription factors identified to play roles in EMT have the capacity to induce a whole EMT program, while others more specifically regulate a distinct subset of epithelial/mesenchymal target genes. Based on their mechanism of action,

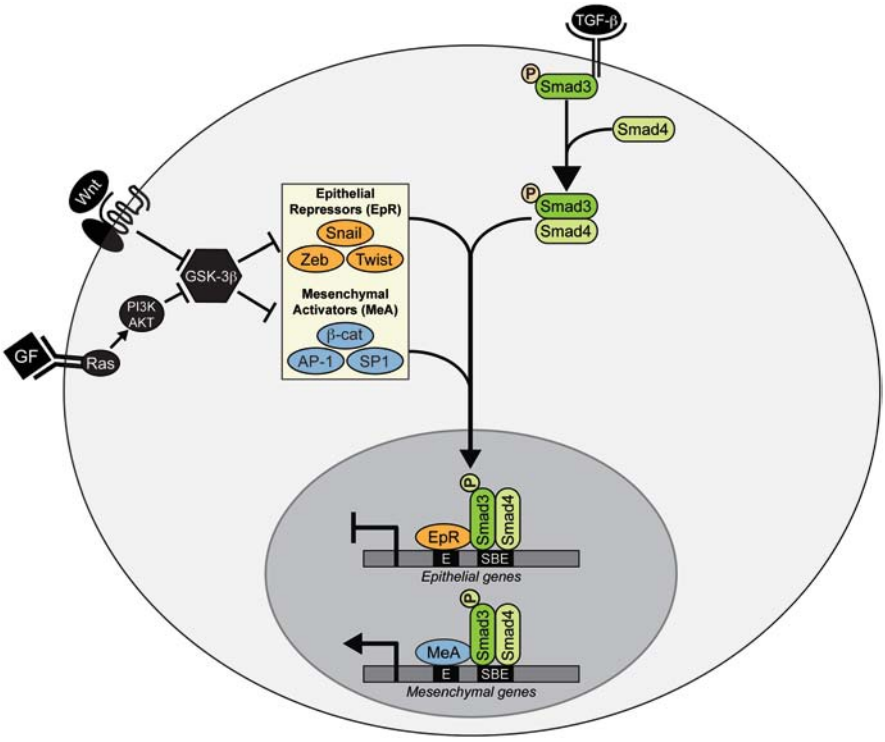

Fig. 14.1 Transcriptional crosstalk in EMT. TGF-β binding to its receptor results in phosphorylation and nuclear translocation of Smad transcription factors, which achieve target gene specificity through interaction with transcriptional cofactors. Transcription factors associated with EMT including epithelial repressors (EpR), such as Snail, Zeb and Twist, and mesenchymal activators (MeA), such as β-catenin (β-cat), AP-1, and Sp1 interact with Smads and form EMT promoting Smad complexes (EPSC). These complexes drive EMT by repressing epithelial genes, such as E-cadherin, or activating mesenchymal genes, such as vimentin. Thus, the formation of EPSC represents a point of convergence between TGF-β, Wnt and Ras pathways. Activation of Wnt and Ras/PI3K/AKT pathways leads to inhibition of GSK-3β and thus stabilization of Snail and β-catenin

EMT promoting transcription factors can be divided into two groups: (i) repressors of epithelial genes and (ii) activators of mesenchymal genes (Fig. 14.1). Recent data show that many EMT promoting transcription factors including Snail1, Zeb1/2, Twist, β-catenin, and AP-1 interact with Smads. This results in the formation of EMT promoting Smad complexes (EPSC), which engage in either the repression of epithelial genes or the activation of mesenchymal genes.

14.2.2.1 EMT Promoting Smad Repressor Complexes

Snail1 binds and form complexes together with Smad3 and Smad4 [17]. Snail1-Smad3/4 complexes repress CAR and E-cadherin during EMT in mammary epithelial cells through binding to adjacent E-boxes and Smad-binding elements in

the gene promoters (SBE) [17]. Zeb2 was initially characterized as a Smad-binding protein [86] and Zeb1 also interacts with R-Smads [87]. The specific role of Snail1-Smad3/4 vs. Zeb1/2-Smad3/4 complexes during EMT is not clear. Snail1 is more rapidly induced upon TGF-β stimulation compared to Zeb1/2 and it is therefore possible that Snail1-Smad3/4 complexes facilitate recruitment of Zeb1/2 to CAR and E-cadherin promoters.

14.2.2.2 EMT Promoting Smad Activator Complexes

β-catenin-Smad2 complexes are formed during TGF-β–induced EMT in alveolar epithelial cells and promote transcriptional activation of mesenchymal genes like α-SMA and PAI-1 [88]. Formation of β-catenin–Smad2 complexes is dependent on α3β1-integrin–mediated phosphorylation of β-catenin, which releases β-catenin from its interaction with E-cadherin. β-catenin-Smad3/4 complexes stabilize and promote nuclear translocation and transcriptional activity of β-catenin [89]. Silencing of Smad4 in pancreatic carcinoma cells leads to decreased β-catenin levels and signaling activity, suggesting a role for β-catenin-Smad3/4 complexes in controlling EMT in tumor cells [90]. Smad3-AP-1 and Smad3-Sp1/Sp3 complexes regulate the expression of vimentin in myogenic cells in response to TGF-β1 [91]. Smad3 and Jun proteins cooperate to activate AP-1–dependent promoters [92]. Sp1-Smad3 complexes activate the plasminogen activator inhibitor-1 (PAI-1) promoter in response to TGF-β [93]. Sp1-Smad3 complexes also induce the expression of the TGF-β receptor endoglin [94], the type alpha2 (I) collagen [95], and the type VII collagen [96] in response to TGF-β.

Finally, Smad proteins can interact with other cofactors to induce EMT activators. It has been found that high mobility group A2 (HMGA2), a non-histone chromatin modifier, regulates Snail1 expression through interaction with Smads [97]. HMGA2 is required for TGF-β–induced EMT in mammary epithelial cells [98] and maintains oncogenic Ras-induced EMT in human pancreatic cells [99].

14.3 EMT/Cancer Stem Cell Niches in Solid Tumors

As discussed, the proposed link between EMT and cancer stem cells has gained support by experimental data showing crosstalk between EMT and stem cell pathways. Some data hint that crosstalk between these pathways may specifically occur in certain niches within tumor tissues. For example, Wnt signaling is specifically active in stem cell/progenitor cell niches in adult tissues where it maintains self-renewal [100]. If such niches are hit by carcinogenic events, such as oncogenic activation of Ras, a foundation for the induction of EMT may be created during tumor development. This would imply that progenitor cells are more sensitive to EMT stimuli compared to more differentiated epithelial cells. Thus, the invasive drive of tumors may be affected by the state of differentiation of the tumor originating cells.

Inflammation represents another niche within the tumor microenvironment and is linked to EMT and tumor cell invasion [101–103]. Immune cells such as macrophages and T-lymphocytes infiltrating the tumor stroma contribute to EMT by secreting TGF-β, other cytokines, proteases, and growth factors. Macrophages can activate Wnt signaling in gastric tumor cells via secretion of TNF-α [104]. CD8 T cells can induce an EMT/stem cell phenotype in breast cancer cells upon co-culture, suggesting that immune responses may contribute to the generation of breast cancer stem cells [105]. In combination with intrinsic mutagenic events, the inflammatory milieu of the tumor microenvironment may provide a fundament for crosstalk between EMT and stem cell pathways.

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Part IV
Model Systems for Studying Cancer Stem Cell Biology and Therapeutic Development

Chapter 15
Application of Stem Cell Assays for the Characterization of Cancer Stem Cells

Pamela M. Willan and Gillian Farnie

Abstract Cancer stem cells (CSCs) have been defined as a population of cells capable of initiating a tumor and more recently for causing tumor recurrence, attributed so by their ability to preferentially survive current therapeutic strategies. The elimination of CSCs is therefore thought to be crucial for the improvement of long-term patient survival. Consequently, the ability to specifically investigate the CSC population and compare its biological functions to the non-CSC component of the tumor will be an essential step toward this goal. This chapter discusses the current methodologies that are widely used to identify and characterize CSCs, with an emphasis on recent developments in the breast cancer field.

Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
BRCA1	Breast cancer susceptibility gene
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CNS	Central nervous system
CSC	Cancer stem cell
CXCR4	Chemokine receptor 4
DNER	Delta/notch-like EGF repeat containing protein
DCIS	Ductal carcinoma in situ
DLL1	Delta-like-1

G. Farnie (✉)
Cancer Stem Cell Research, University of Manchester, School of Cancer and Enabling Sciences, Paterson Institute for Cancer Research, Manchester, UK
e-mail: gillian.farnie@manchester.ac.uk

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DNER	Delta/notch-like EGF repeat containing protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ESA	Epithelial specific antigen
FACS	Fluorescence activated cell sorting
IL	Interleukin
MaSC	Mammary epithelial stem cell
MDR1	Multi drug resistance pump 1
NOD/SCID	Non-obese diabetic/Severe combined immune deficiency
PR	Progesterone receptor
PROCR	Protein C receptor
SA-βgal	Senescence-associated β-galactosidase
SP	Side population
TGF-β	Transforming growth factor beta
UV	Ultraviolet
WT	Wildtype

15.1 Introduction

In normal tissue, a cellular hierarchy exists where a normal adult stem cell gives rise to progenitor and fully differentiated lineages of the normal tissue. It is now suggested that cancer also has a cellular hierarchy, where the cancer stem cells (CSCs) are the cells capable of producing heterogeneous tumors. CSCs are also termed tumor-initiating cells and can be defined as having a number of distinct properties: they have a selective capacity to initiate tumors and drive neoplastic growth; a capacity for endless self renewal; and the potential to give rise to more mature non-stem cell cancer progeny via differentiation. The CSC hypothesis suggests that every tumor contains a cellular component of CSCs, which retain the key stem cell properties to initiate and drive tumorigenesis [1].

It is widely accepted that like normal tissue, cancers are composed of morphologically and phenotypically heterogeneous cell populations [2, 3], and it has become increasingly more evident that to eliminate recurrence and improve survival in cancer patients, choosing the correct cells within the cancer to target will be crucial. Studies of CSC populations within a number of different cancers have proposed that CSCs are resistant to current anticancer therapies such as radiotherapy and chemotherapy [4–8]. One of the most prominent studies in breast cancer used in vitro and in vivo techniques to assess the number of CSCs and determined that biopsies taken from breast cancer patients after 6 weeks of chemotherapy treatment had an increased number of CSCs when compared with biopsies before treatment [6].

There has long been a movement toward a more targeted approach to cancer treatment to improve patient survival. A crucial step will be the ability to identify and characterize CSCs in the laboratory using both in vitro and in vivo assays, thus

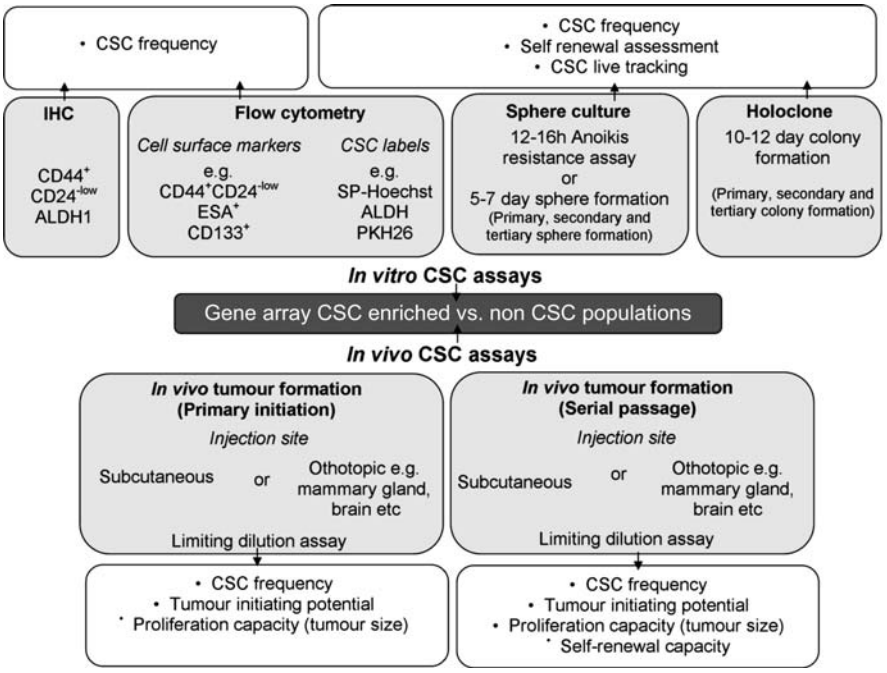

Fig. 15.1 Flow diagram representing current methodologies for cancer stem cell identification and characterization

allowing the identification of new targets for the development of new therapies. This chapter will address the current methodologies and assays used to identify and characterize CSCs (Fig. 15.1).

15.2 Lessons Learned from the Normal Breast

Numerous methods and tools now used in the breast cancer field have been learned from investigations into the normal breast. These methods include the in vivo transplantation method pioneered by DeOme in 1959, which still represents the "gold standard" assay for mammary gland reconstitution in mice [9]. This assay forged the way to numerous groups discovering the clonality of mammary outgrowths [10–13], leading to the prospective isolation of the mouse mammary stem cell (MaSC) and the description of their cell surface marker phenotype. In a double Nature publication in 2006, studies revealed that within the mouse a single cell with cell surface markers $CD24^{med}Sca\text{-}1^{low}CD29^{high}CD49f^{high}$ was capable of reconstituting a fully functional mammary gland in the cleared fat pad of a recipient mouse [14, 15].

However, to verify that any cell population has stem cell characteristics, they must undergo rigorous in vitro and in vivo assays. These include colony forming

assays determining the differentiation potential of the sorted cells, nonadherent mammosphere assays, and in vivo repopulating experiments with a particular emphasis on serial transplantation to assess repopulating capacity (reviewed in detail in [16, 17]). Many of these assays and ideas from normal breast stem cells now have been applied to breast cancer studies, and are reviewed later.

15.3 In Vitro Methods of Identification and Isolation of CSCs

15.3.1 Side Population

A side population (SP) is defined by its ability to efflux the dye Hoechst 33342 due to the high expression of ATP-binding cassette (ABC) transporter family members such as MDR1 and ABGC2 [18, 19]. First described in 1997, the SP population was found to contain hematopoietic stem cells from bone marrow [20]. Moreover, numerous studies have found a SP population within cancers including the skin [21], lung [22], brain [23], and breast [24]. SP cells from the mouse mammary gland were found to contain cells capable of regenerating a functional mammary gland system in a cleared fat pad [25], and this was also true in the human where the SP cells contained breast stem cells [26–29]. In MCF7 breast cancer cells, the SP population (0.2%) had a greater tumorigenic capacity than the non-SP fraction when determined by tumor formation subcutaneously in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. The MCF7 SP also expressed higher levels of Notch1 and β-catenin mRNA compared with the non-SP population, suggesting that the SP cancer cells have some intrinsic properties of stem cells [24]. A recent study has also reported that SP cells from human primary breast cancers and cell lines have tumor initiating properties [30]. Further analysis showed that the SP was predominantly found in luminal cancers but was not concomitant with other tumor initiating phenotypes such as $CD44^{+}CD24^{-/low}$ and aldehyde dehydrogenase $(ALDH)^{+}$, which are mainly found in the basal subtypes.

However, Hoechst 33342 is highly toxic, more so to non-SP cells, which do not efflux the dye, and thus presents difficulties in interpreting in vivo studies where non-SP cells appear less tumorigenic than SP cells in immunodeficient mice. Some studies have also indicated that non-SP cells can form tumors just as readily as SP cells, examples of which can be seen in mesothelioma and thyroid cancer cell lines [31, 32]. The toxicity issues surrounding Hoechst and the fact that studies have shown that the SP did not exclusively contain the tumorigenic cells have caused the SP technique to fall out of favor. However, with the emergence of new dyes that are not toxic such as calcein and DyeCycle Violet, which are also effluxed by cells expressing ABGC2, there may be more conclusive evidence emerging which is not influenced by Hoechst toxicity or UV damage during analysis [33, 34].

15.3.2 Cell Surface Markers

Fluorescence activated cell sorting (FACS) can allow the measurement and isolation of populations of cells with differential expression of single or multiple cell surface markers. The rationale for using cell surface markers to identify CSCs has been largely based on the known and shared markers of normal stem cells. In leukemia, the cell surface markers $CD34^{+}CD38^{-}$ have been successfully used [35, 36], and in gliomas CD133 also enriched for CSCs that were capable of tumor initiation [37, 38]. In the breast MaSC, surface markers are not as well defined and although the markers in the mouse have been described, the translation from mouse to human is not always consistent. To date there are a number of cell surface markers that have been used in the identification of CSCs, some of which are highlighted below and summarized in Table 15.1.

A seminal paper published in 2003 showed the ability of as few as 200 passaged or 1,000 unpassaged ESA (epithelial specific antigen)$^{+}CD44^{+}CD24^{-/low}$ $Lineage^{-}$ cells to give rise to tumors that could be serially transplanted in NOD/SCID mice [39]. These cells had extensive proliferative potential and had the ability to recapitulate the entire heterogeneity of the initial tumor. The cell surface makers ESA^{+} $CD44^{+}CD24^{-/low}$ have been used in a number of studies to enrich for CSCs, which have greater tumor initiating capacity, although the expression is highly heterogeneous within the different subtypes of breast cancer. Sorting for these cell surface markers selected for CSCs within breast cancer cell lines too, enriching tumor initiating cells in SUM159, SUM1315, SUM149 [5], and MCF7 cells [40] similar to that found in primary tumors [39]. These cells were capable of recapitulating the tumor heterogeneity in vivo and were enriched for mammosphere formation in vitro.

A study in 2008 utilized the $CD44^{+}CD24^{-/low}$ phenotype and showed that biopsies taken from invasive breast cancer patients after 12 weeks of chemotherapy treatment had a greater number of $CD44^{+}CD24^{-/low}$ cells compared with the pretreatment biopsy. These results and additional data showing post-biopsy samples had an increased potential for sphere formation in vitro and tumor initiation in vivo indicated that cells with the phenotype $CD44^{+}CD24^{-/low}$ were not only more tumorigenic but were also preferentially surviving chemotherapy treatment [6].

In contrast, a number of other studies have not favored this cell surface marker combination. For example, a study profiling eight breast cancer cell lines with known CSC markers CD44, CD24, CD133, PROCR, ABGC2, CXCR4, ESA, and ALDH concluded that $CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ were not universal markers of CSC isolation using a soft agar culture to measure tumorigenicity. They concluded that a combination of $ESA^{+}PROCR^{+}$ could define an uncharacterized type of breast cancer stem cell from both primary and breast cancer cell lines [41]. In particular, the study shows the MDA-MB-231 cell line, which had a 90% population of $CD44^{+}CD24^{-/low}$ cells, was further enriched for tumorigenic cells by selecting the $ESA^{+}PROCR^{+}$ vs. $ESA^{-}PROCR^{-}$. However, previous enrichment for tumor initiating cells within this cell line used a triple marker selection $ESA^{+}CD44^{+}CD24^{-/low}$ indicating that the selection for ESA^{+} cells may have been the enriching marker in this cell line [5].

Table 15.1 Common cell surface markers used to enrich for CSCs in solid tumors

Enrichment technique	Tumor type	References
$CD44^+$	Breast	[39, 40, 54, 55]
	Prostate	[48]
	Colon	[103, 104]
	HNSCC	[105]
	Pancreas	[106]
	Ovarian	[107]
CD24[a]	Breast	[39, 40, 55]
	Ovarian	[107]
	Pancreas	[106]
$CD133^+$	Breast	[54, 55]
	Brain	[37, 38]
	Prostate	[48]
	Colon	[49–51]
	Liver	[108, 109]
	Ovarian	[110, 111]
ESA^+	Breast	[39, 40]
	Pancreas	[106]
EpCAM	Colon	[104]
$CD49f^+$	Breast	[54, 56]
Side population (SP)	Breast	[24]
	Brain	[23]
	Lung	[22]
	Ovarian	[112]
	Skin	[21]
$ALDH^+$	Breast	[63, 66]
	AML	[65]
	MM	[64]
$CD34^+$	AML	[35, 36]
	Lung	[23]
$DLL1^{high}DNER^{high}$	Breast	[56]
CD271	Skin	[113]

HNSCC head and neck squamous cell carcinoma; *AML* acute myeloid leukemia; *MM* multiple myeloma; *ESA* epithelial specific antigen

[a]In breast and ovarian tumors $CD24^{low/-}$, whereas in the pancreas $CD24^+$ both in combination with $CD44^+$

Similarly, Meyer et al. showed that $CD44^+CD24^+$ cells from breast cancer cell lines can form tumors as readily as the $CD44^+CD24^{-/low}$ population in NCr-nu/nu mice [42]. Further investigation found that CD24 expression was dynamically regulated in cell lines; with $CD44^+CD24^{-/low}$ being capable of giving rise to $CD44^+CD24^+$ and vice versa, thus explaining the inconsistent effects of CD24 expression on tumorigenesis in a number of studies [42]. Furthermore, a subsequent study by the same research group showed that in primary triple negative breast cancers, the tumor initiating cells were not always restricted to the $CD44^+CD24^{-/low}$ population, although the $CD44^-$ population were nearly always non-tumorigenic in NOD/SCID mice.

They further investigated markers that would segregate $CD44^+$ cells into tumorigenic and non-tumorigenic populations using a panel of known stem cell markers. One of the markers investigated was CD133, which has been shown to be expressed by normal stem cells of the hematopoietic system [43, 44], brain [45], skin [46], and prostate [47]. CD133 has also been shown to be expressed in solid cancers from the brain [37, 38], prostate [48], and colon [49–52]. The expression of CD133 in the breast is less clear cut and information from the mouse mammary gland indicates that cells with CD133 expression, which also were $CD24^+$ are the luminal progenitor cells and not capable of in vivo stem/progenitor activity [53]. This is yet to be fully investigated in the human mammary gland, but recent studies have shown evidence for CD133 expression in CSCs from both mouse and human breast cancer [54, 55].

For example, Meyer et al. have demonstrated that triple staining for $CD44^+CD49f^{high}CD133/2^{hi}$ in four human primary triple negative tumors selected cells that were more tumorigenic in a xenograft NOD/SCID mouse model compared with cells expressing $CD44^+CD49f^{-/low}CD133/2^{-/low}$. Cells expressing $CD44^+CD49f^{high}CD133/2^{hi}$ also had other hallmarks of CSCs, shown by their ability to form spheres in vitro and give rise to functional and molecular heterogeneous tumors [54]. A study utilizing a BRCA mutated mouse model demonstrated that the $CD44^+CD24^{-/low}$ population did not overlap with the $CD133^+$ population, although both showed the ability to enrich for tumor initiating cells and shared the expression of common stem cell genes such as *Oct4*, *Notch1*, *Aldh1*, *Fgfr 1*, and *Sox-1* [55]. They detected 2–4% of $CD133^+$ cells in multiple cell lines derived from one BRCA1 tumor with characteristics similar to those found in $CD44^+CD24^{-/low}$ cells, including drug resistance, the ability to form spheres with further enrichment in $CD133^+$ cells, expression of stem cell genes, and in vivo reconstitution of tumors with as few as 100 cells [55].

A recent study investigated a number of cell surface markers alone and in combination, including CD24, CD49f, Delta/notch-like EGF repeat containing protein (DNER), and Delta-like-1 (DLL1). DNER and DLL1 were found to be overexpressed in PKH positive (human normal mammary stem cell shNMSC) cells and were shown to be the most efficient surface marker (used alone) to enrich for mammosphere formation compared with CD24 or CD49f, showing a 49-fold and 40-fold enrichment compared with 33-fold and 16-fold, respectively, relative to unsorted cells [56]. Using multiparametric cell sorting, the cell surface markers $CD49f^+DLL^{high}DNER^{high}$ were found to have the greatest enrichment for sphere forming cells (530-fold) from reduction mammoplasties and also enriched for tumor initiating cells from primary human tumors grown in NOD/SCID mice. Interestingly, this study found that high CD24 expressing cells from the normal breast formed spheres better than the CD24 low or medium expressing cells, which adds to the studies of others being shown that the expression of CD24 did not affect the tumorigenic potential of $CD44^+$ cells [42].

The inconsistency of CSC enrichment between studies using the same cell surface markers may be partly attributed to the implementation of different FACS "cut offs" particularly in low or high expression phenotypes, which are often not described within the Materials and Methods sections of manuscripts. Antibodies,

particularly for ESA, also vary between publications. Clones recognizing different epitopes on the ESA antibody do not always mark the same population of cells (*data not shown*) and should be taken into account if trying to compare the enrichment ability of different studies seemingly using the same cell surface markers.

A number of different cell surface marker combinations have been shown to enrich for breast CSCs; however, the limitation of our assays to "read out" CSCs must be taken into account particularly in the instance of human xenograft systems. The current methodologies used to create a "humanized" environment within the mouse may influence the cells ability to form a tumor, therefore generating data that may not translate into the human. This will be discussed later in the chapter.

15.3.3 ALDEFLUOR Assay

The aldehyde dehydrogenase (ALDH) family of enzymes have important functions in the development of epithelial homeostasis, and as a result deregulation of this class of enzymes has been implicated in multiple cancers [57]. The Aldefluor assay is based on the enzymatic function of ALDH1, a detoxifying enzyme responsible for the oxidation of intracellular aldehydes [58]. ALDH is suggested to have a role in early differentiation of stem cells via its role in oxidizing retinol to retinoic acid [59]. High activity of ALDH has been found in hematopoietic and neural stem/progenitor cells [60–62], and when used to select for human normal MaSCs only $ALDH^+$ cells had the capacity to reconstitute a mammary tree in a humanized cleared mammary fat pad [63]. $ALDH^+$ cells within malignancies also enrich for the CSC population of multiple myeloma and acute myeloid leukemia (AML) [64, 65]. The Aldefluor assay is therefore thought to be an almost universal marker of stem cell activity in both normal and cancer tissue.

Ginestier and colleagues utilized this assay to enrich for CSCs from xenotransplants generated from human invasive ductal carcinomas and found that three cancers had an $ALDH^+$ population representing 3–10% of the total cell population. These xenografts were serially passaged in vivo using limiting dilution of $ALDH^+$, $ALDH^-$, and unsorted cells, and at each passage only the $ALDH^+$ population formed tumors, even when implanted in low numbers (500 cells). The $ALDH^+$ population showed consistencies with stem cell characteristics, generating tumors that recapitulated the phenotypic heterogeneity of the initial tumor. Measurement of ALDH populations revealed that a similar ratio of $ALDH^+$ and $ADLH^-$ were found in tumors grown from an $ALDH^+$ population, suggesting that the $ALDH^+$ cells were capable of self renewing into both $ALDH^+$ and $ALDH^-$ cells [63]. Further investigation of the overlap of the $ALDH^+$ population and the previously described $CD44^+CD24^{-/low}$ $Lineage^-$ population [39] revealed that within the xenografted tumors both populations are represented (6.08 and 4.34%, respectively), and that the overlap of $ALDH^+$ and $CD44^+CD24^{-/low}$ $Lineage^-$ expressing cells from three xenografts ranged between 0.1 and 1.2%. Cells bearing both CSC phenotypes were

highly tumorigenic and generated a tumor with a few as 20 cells. However, cells with an $ALDH^-$ and $CD44^+CD24^{-/low}$ $Lineage^-$ were not tumorigenic, even when implanting up to 5,000 cells per fat pad [63].

$ALDH^+$ cells have also been discovered in 23 out of 33 breast cancer cell lines, representing the spectrum of molecular subtypes, in which populations ranged from 0.2 to ~100%. Further analysis showed that all of the 16 basal/mesenchymal breast cancer cell lines contained an $ALDH^+$ population, whereas 7 out of 12 luminal cell lines did not have detectable $ALDH^+$ cells. In vivo xeno-transplantation studies using three estrogen receptor α (ER)-negative breast cell lines (MDA-MB-435, SUM159 and BrCa-MZ-01) showed that in two of the cell lines (MDA-MB-435, SUM159) only the $ALDH^+$ cells had tumor generating capacity, which was maintained through serial passage. However, $ALDH^-$ cells from the BrCa-MZ-01 cell line were also shown to be capable of generating tumors but, in contrast to the $ALDH^+$ tumors, the $ALDH^-$ tumors had slower growth, only contained $ALDH^-$ cells and failed to reform tumors after 3 serial passages, suggesting that the $ALDH^-$ cell population may contain progenitor cells, which are able to undergo limited growth but not self renewal [66]. Another group investigating ER negative breast cancer cell lines have confirmed these findings, showing $ALDH^+CD44^+CD24^-$ (MDA-MB-231) and $ALDH^+CD44^+CD133^+$(MDA-MB-468) cells demonstrated increased colony formation, migration, and invasion in vitro compared to $ALDH^-CD44^{low/-}$ cells. Furthermore, in vivo experiments demonstrated that following tail vein or mammary fat pad injection of cells into NOD/SCID/IL2gamma receptor null mice, $ALDH^+CD44^+CD24^-$ and $ALDH^+CD44^+CD133^+$ cells had enhanced tumorigenicity and metastasis relative to $ALDH^-CD44^{low/-}$ expressing cells [67].

In contrast, a recent study in melanoma describes the capacity for $ALDH^+$ and $ALDH^-$ subpopulations isolated from different xenografts and two patient biopsies to form tumors in NOD/SCID/IL2gamma receptor null mice. Both $ALDH^+$ and $ALDH^-$ cells derived from the xenografts or patient biopsies were capable of efficient formation of first generation tumors following injection of as few as 100 or 2,000 cells, respectively. Tumor growth rate was also not dependent on ALDH expression and both $ALDH^+$ and $ALDH^-$ could be serially transplanted in vivo. The only difference seen between the two experimental groups was that the $ALDH^+$ cells could reestablish tumor heterogeneity, showing a mixed phenotype where 20–40% of the melanoma cells did not show ALDH activity, whereas the $ALDH^-$ tumors remained $ALDH^-$ [68]. Similarly, in a lung cancer cell line H225, both $ALDH^{bright}$ and $ALDH^{low}$ populations could generate tumors in recipient NOD/SCID mice, which were capable of serial passage. The in vivo data also demonstrated that the tumors formed from $ALDH^{bright}$ cell had a significantly slower growth rate [69].

These data demonstrate that the Aldefluor assay can recognize a subpopulation of cells showing ALDH activity, which are more tumorigenic in vivo. However, there are conflicting studies, which indicate ALDH alone may not be sufficient for CSC selection in other cancers, and within breast cancer studies further limitations are apparent as the studies demonstrating the tumorigenic capacity of $ALDH^+$ cells have predominantly utilized ER negative cell lines and primary samples. ER^+ cell lines have shown little or no $ALDH^+$ cells indicating that Aldefluor will not be a

universal marker for all molecular subtypes of breast cancer, although further studies are needed to confirm this. In spite of this, studies in breast cancer combining other CSC surface markers with Aldefluor have improved tumorigenic enrichment and this combination may prove to be a better strategy for enriching for CSCs.

15.3.4 Immunohistochemical Staining for CSC Markers

The ability to immunohistochemically stain in situ for CSCs would be an ideal methodology to enable the assessment of CSCs within large cohorts of patients with known survival, recurrence, and metastasis, and would allow the assessment of CSCs in samples from clinical trials without the need for in vitro or in vivo growth assays. Unfortunately, to date no specific marker or markers have been found to specifically pinpoint the CSCs, but a number of groups have investigated the expression of ALDH1 as a surrogate for the ALDH activity measured via the Aldefluor assay and have seen some success. Positive ALDH1 protein expression in two cohorts of breast cancer patient samples ($n = 136$ and $n = 341$) had a significant association with poor overall survival, and within the larger cohort ALDH1 expression was an independent prognostic factor when compared with tumor size, lymph node metastasis, grade, ER, PR, Ki67, and ErbB2 receptor status [63].

A retrospective study of 109 inflammatory breast cancers also reported that ALDH1 expression correlated with the development of distant metastasis and with decreased survival [70]. Characterization of ALDH1 expression in 203 breast cancer patients revealed that 10% were found to be ALDH1 positive and these cancers were significantly more likely to be ER^-, PR^-, $ErbB2^+$, and $Ki67^+$ [71], reiterating that ALDH1 expression or activity may be predominantly observed in ER^- breast cancers. However, ALDH1 expression to identify CSCs is also contentious as there are studies in colorectal cancer showing ALDH1 expression has no correlation with prognosis [72], and in ovarian cancer ALDH1 expression correlates with favorable prognosis [73].

Other markers used for in situ staining in the breast are the markers $CD44^+CD24^{-/low}$. How effective these markers are at identifying the CSCs is not clear, as expression of these markers in formalin fixed paraffin embedded sections may be very different in comparison to cell surface expression levels seen in FACS analysis studies. A retrospective study of 240 breast cancers stained for CD44 and CD24 demonstrated that $CD44^+/CD24^-$ cells were detected in 31% of the tumors. The $CD44^+/CD24^-$ phenotype was most common in the basal-like subgroup (ER^-, PR^-, $ErbB2^-$, $CK5/14^+$, and $EGFR^+$) and was found in 94% of BRCA1 hereditary tumors [74]. This suggests that immunohistochemical staining for $CD44^+CD24^{-/low}$ may not mark the CSCs in all breast cancer subtypes. Furthermore, another study in 122 breast cancer patients showed that the $CD44^+CD24^{-/low}$ phenotype was neither associated with overall survival nor clinicopathological characteristics [75].

Overall the expression markers being used have some ability to predict for overall survival, and in the case of ALDH1 in breast cancer can also predict for response

to chemotherapy [76]. However, to advance the detection of CSCs in situ, there needs to be further characterization of CSCs to find new markers or possibly combining multiple known markers to improve current strategies.

15.3.5 PKH26 Labeling of CSCs

Long-term label retention is used frequently for the identification of stem cells and has been successful in identifying MaSCs [25, 77, 78]. BrdU labeling has some disadvantages as it is known to be toxic [79–81] and does not allow the tracking of live cells. However, other labeling dyes such as CFSE and PKH26 can be measured in live cells, thus allowing more complex functional analysis of CSCs including their division and proliferation. PKH26 is a fluorescent dye that binds to cell membranes and segregates in daughter cells after each cell division, such that the intensity of staining correlates inversely, at the single cell level, with the number of previous cell divisions [82]. The hematopoietic field has used this dye to identify long-term and short term repopulating cells in blood [82, 83] and to follow the proliferation of leukemic cells from AML patients [84]. PKH^{Hi} cells isolated from mammosphere cultures from human normal breast and implanted into humanised cleared fat pads of NOD/SCID mice had the capacity to reconstitute normal mammary epithelium. Conversely, the PKH^{-} cells could not reconstitute the mammary gland even when high concentrations of cells were injected [56], indicating the PKH^{Hi} cells are enriched for MaSCs. Cells capable of forming a mammosphere from human or murine normal or breast cancer tissue have also been shown to retain PHK labeling [40, 56, 85]. Furthermore, in a panel of human tumor cell lines including ovarian, acute promyelocytic leukemia, glioma, lung, and breast, PKH26 labeling experiments showed that label retaining PKH^{Hi} cells have a greater colony forming capacity in vitro and a high tumorigenic potential in vivo compared with PKH^{-} cells, which lacked both colony and tumor forming capabilities [86]. PKH^{Hi} cells were also found to express the stem cell markers *Oct4*, *Nestin*, *Nanog*, and *Bmi*, whereas the PKH^{-} cell lacked all four markers, further indicating that label retaining PKH^{Hi} cells have CSC characteristics. To investigate the effect of chemotherapy on CSCs, xenograft tumors formed from PKH^{Hi} ovarian cells were treated with five doses of paclitaxel over a 6-week time period, and during this time tumors stopped growing and reduced marginally in size. Analysis of the tumors show that paclitaxel treatment increased the number of PKH^{Hi} cells, suggesting a treatment induced enrichment of CSCs and highlighting the need for targeting CSCs in the clinic [86].

PKH26 can also be used as a tool to analyse symmetric or asymmetric division. Cicalese and colleagues investigated modes of self-renewing divisions by time-lapse video microscopy by plating PKH^{Hi} cells from mammospheres grown from wild type (WT) mouse mammary gland or from tumor (ErbB2) mammary tissue into methylcellulose and monitoring these cells for 7 days, at 1 h intervals. The first division was defined as asymmetric if one of the first-generation daughter cells remained quiescent, whereas the other divided further, giving rise to a total of five

cells by day 3. It was defined as symmetric when both daughter cells continued to divide, giving rise to eight cells with dim fluorescence at 3 days. The PKH^{Hi} WT cells predominantly divided asymmetrically, generating daughter cells with different developmental fate, whereas the PKH^{Hi} ErbB2 tumor cells predominantly divided symmetrically and as a consequence increased their numbers [85]. Using this technique in combination with inhibitors of CSC pathways or conventional anti-cancer therapies will provide further insight into the effects of these inhibitors/agents on CSC self renewal/division.

15.3.6 Nonadherent Sphere Culture

Sphere forming ability as a measure of stem cells was first developed for the central nervous system (CNS) where a subset of cells isolated from human fetal brain and human CNS tumors formed spheres when cultured in serum free suspension [37, 38, 45, 87]. The methodology involves seeding single cell suspensions from primary samples or cell lines into nonadherent plates in a phenol-red free media usually containing the serum free supplement B27 and other growth factors (depending on tissue type). Over a 12–18 h time period, the more differentiated cells undergo cell death via anoikis, leaving the cells with self-renewal capacity to divide and proliferate into floating spheres of cells over 5–7 days. This culture system has since been adapted for the growth of normal breast stem and progenitor cells [26] and breast cancer [40, 88, 89], and the floating colonies that are produced have been termed mammospheres.

Mammosphere culture can be used as a tool to investigate stem cell activity, measuring the ability of the surviving stem and progenitor cells to undergo self renewal and proliferate to form spherical colonies of stem and progenitor cells. Using serial passage of mammospheres, the self renewal capacity can be measured. When comparing human normal breast to ductal carcinoma in situ (DCIS), the mammosphere regeneration capacity of normal breast produced a maximum of three new generations of mammospheres with a significantly lower average mammosphere regeneration ratio of 0.13 (a regeneration ratio of 1 = one mammosphere passaged and reseeded to make one mammosphere), whereas the DCIS cells were capable of regenerating mammospheres up to six times at an average mammosphere regeneration ratio of 0.85 at each passage [88]. PKH26 labeling can also be used in the sphere culture assay to analyse the self-renewal of CSCs within the mammospheres by measuring the number of PKH^{Hi} cells. Mammospheres generated from grade 3 breast cancers ($n = 15$) were found to have approximately fourfold more PKH^{Hi} cells when compared with mammospheres from grade 1 breast cancer ($n = 5$), indicating an increased number of CSCs [56].

The number of CSCs can be measured using mammosphere culture at primary, secondary, and tertiary generations. Studies have shown that compared with normal breast tissue, the number of sphere forming cells is significantly greater in preinvasive and invasive breast cancer samples. Similarly, when comparing the number of

spheres forming from grade 1 or grade 3 preinvasive or invasive breast cancer samples, the grade 3 samples produced greater numbers of mammospheres [56, 88]. Cells that survive the sphere culture conditions and are capable of initiating a mammosphere have been found to predominantly express CSC cell surface markers $ESA^{+}CD44^{+}CD24^{-/low}$ and have been shown to be $ALDH^{+}$. In both instances, if cells are FACS sorted for these CSC markers they can enrich for mammosphere initiating cells between 2 and 12-fold and 10-fold, respectively [40, 63].

Cells can also be enriched for mammosphere and tumor initiating cells by seeding cells into the mammosphere culture system but instead of leaving them to form spheres over 5–7 days the cells are harvested after ~16 h. At this timepoint, 85% of MCF7 cells have died by anoikis and the remaining viable cells have been shown to be 5.7-fold enriched for mammosphere formation and 12-fold enriched for tumor initiating cells after implantation into NOD/SCID mice when compared with monolayer cells [40]. The link between mammosphere initiating cells and tumor initiating cells was corroborated in primary human breast cancers when the growth rate and numbers of CSCs (measured by PKH) from human breast xenografts were found to be comparable regardless of whether the cells were directly implanted or were cells derived from mammospheres generated from the same patient samples [56].

Mammosphere culture is extremely useful for primary culture where only small numbers of cells are available and FACs staining for CSC markers is not feasible. Additionally, due to some controversy over which cell surface markers or labels enrich for CSCs and the suggestion that some markers may not identify CSCs from different molecular subtypes of breast cancer, studies have shown that mammospheres can be formed from cell lines and primary tissues from all molecular subtypes, allowing the measurement of CSC numbers regardless of origin of tissue.

15.3.7 Holoclone Formation

Another successful method of CSC identification is based on their ability to form dense colonies of a specific morphology, called holoclones [90, 91]. The three types of colony formed are as follows: holoclones containing the most undifferentiated cells including the CSCs; meroclones containing a mixture of progenitor cells and more differentiated cells; and paraclones which are fully differentiated. Holoclones consist of tightly packed small cells, paraclones contain larger cells with smaller cell numbers, and meroclones are an intermediate between the two.

Studies in a prostate cancer cell line PC3 have shown that the holoclones were negative for senescence-associated β-galactosidase (SA-βgal) and cells were homogenously small. In contrast, most cells in paraclones were positive for SA-βgal and cells were large and flat, suggesting that cells within paraclones were mostly senescent and nonproliferative [92]. Holoclones obtained from different cancer cell lines, including head and neck squamous cell carcinomas and breast cancer have also been shown to have cells with cancer stem cell characteristics [90–92]. When three independent holoclones from PC3 cells were harvested and injected into

NOD/SCID mice at 1,000 or 10,000 cells, all initiated a tumor 80–100% of the time. In contrast, meroclones ($n=2$) or paraclones were not capable of initiating any tumors even after 5–6 months with 10,000 or 10,00,000 cells injected. The holoclone derived tumors had the capacity for serial transplantation, and analysis of the cells generated from the tumors demonstrated that all three colony types could be formed, indicating that holoclones can regenerate or maintain the clonal heterogeneity in vivo [92].

Holoclone formation is a valuable in vitro technique for measuring CSCs, although, as with the mammosphere technique, combining holoclone formation with in vivo tumor growth aids the definition of the colonies measured. In addition, holoclone formation does not require specialized equipment or media and can be use with relatively low numbers of cells.

15.4 Assessing Biological Properties of CSCs Using Gene Array Analysis

Once CSCs have been enriched using the in vitro assays discussed earlier, gene expression arrays can be used to compare enriched CSC vs. non-CSC populations or normal tissue. These types of studies can identify differentially expressed genes within the CSC population, which may lead to the discovery of new biological processes (Table 15.2). Genes must then be subjected to revalidation using both the in vitro assays described above and ultimately in the in vivo models which are discussed next, to establish the functional effects of the genes identified.

Table 15.2 Differential gene expression in CSC vs. non-CSC populations

Tumor type	Enrichment technique	Examples of genes of interest found	References
Colon	$CD133^{high}$ vs. $CD133^{high}$-depleted cells (HCT116 cell lines)	988 differentially expressed genes including FOXO3, NFKB1A, IL-8, Shh and Oct4	[114]
Breast	$CD44^{+}CD24^{-/low}$ breast cancer cells vs. normal breast epithelium (human primary tissue)	Prognostic 186 gene signature, including CASP8, BCL2, LRP2, MAPK14, CXCL2, MMP7, RAD23B and ERBB4	[93]
	$ALDH^{+}$ vs. $ALDH^{-}$ cells from 8 breast cancer cell lines	413 differentially expressed genes including CXCR1/IL-8RA, Notch2, RAD51L1 and FBX021	[66]
	$CD44^{+}$ or $PROCR^{+}$ vs. $CD24^{+}$ cells in normal and breast cancer tissue	BMI1, Gli1, Gli2, and TGF-β	[95]
Prostate	$CD133^{+}/\alpha2\beta1^{hi}$ vs. $CD133^{-}/\alpha2\beta1^{low}$ human primary prostate cancer	518 differentially expressed genes including NFKB1, IL6, ITGAV and IFNGR1	[115]
Lung	SP and non-SP cells (A549 cell line)	Genes included AKR1C1/AKR1C2, TM4SF1, NR0B1, ABCG2 and IL6R	[116]

The comparison of CSCs enriched using cell surface markers $CD44^{+}CD24^{-/low}$ from 6 human breast cancer patients vs. normal breast epithelium from 3 mammoplasties revealed 186 genes that were differentially expressed in the CSC population [93]. When compared with published breast cancer gene signatures, a significant association between the CSC signature and worse overall survival and metastasis-free survival was found, suggesting more aggressive tumors may contain a higher percentage of CSCs [93]. When gene expression profiles of $ALDH^{+}$ vs. $ALDH^{-}$ cell populations (using the Aldefluor assay) from 8 breast cancer cells lines were compared, 413 genes were found to be differentially expressed [66]. Some of these genes were validated including CXCR1, which was found to be up-regulated in CSCs, and results show that inhibition of the IL-8 receptor CXCR1 in three breast cancer cell lines could selectively deplete the CSC population ($ALDH^{+}$ cells) and reduce tumor growth in vivo [66, 94]. CSCs enriched using CD44 from human normal breast and breast cancers samples ($CD44^{+}$) were compared with $CD24^{+}$ expressing non-CSC population. Profiles indicated that cells within the CSC enriched population expressed stem/progenitor-associated genes such as *BMI1*, *Gli1*, and *Gli2* whereas the non-CSC population expressed differentiation associated genes such as *GATA3* and *ERS1* [95]. This CSC gene expression signature also correlated with patient clinical outcome and specifically identified the TGF-β pathway to be activated within the CSC population, and inhibition of this pathway in vitro led to differentiation [95]. These data demonstrate that enrichment of CSCs can facilitate the discovery of biological properties of CSCs that are unique and may not have otherwise been revealed.

15.5 In Vivo Models for the Identification and Characterization of CSCs

The in vitro methodologies described are the foundation of identifying CSCs, but ultimately to validate these findings CSCs need to be tested in a human xenograft model. It is defined that a true CSC should be a cell that can reconstitute, in a recipient animal, a tumor identical to the original tumor in the patient, which can be serially xenotransplanted indefinitely. To date this xenograft model is the best experimental strategy to mimic tumors in human patients, allowing the primary tumor xenografts to grow in the presence of vasculature and stroma, which are not as easily mimicked in vitro. However, depending on the injection location, there may be differences in the mouse stromal environment compared to the human, for example the murine mammary fat pad was found to have an inferior stromal environment resulting in poor human mammary gland development. A major improvement to the cleared fat pad environment was published in 2004, where human mammary fibroblasts were implanted into cleared fat pad of NOD/SCID mice, creating a “humanized” fat pad resulting in the improved implantation of normal MaSCs [96]. These humanizing techniques have also been used for the improved implantation of human breast cancer cells [5, 6, 39, 56, 63, 70]; however, published

human xenograft models of human breast cancer used to investigate CSCs have used different variations of implantation techniques and mouse strains (Table 15.3).

Even though the xenotransplantation method is deemed as the "gold standard" to measure CSCs, there are still many limitations that may ultimately influence tumor transplantation and these have been highlighted in a number of different cancers. With regards to immunity, estimated CSC frequencies may vary with the immune status of tumor xenotransplantation recipients [35, 36, 97, 98]. In studies of human AML, 2×10^5 $CD34^+CD38^-$ cells were required to initiate leukemia in SCID mice [36] compared with 5×10^3 $CD34^+CD38^-$ cells (a 40-fold reduction) in more severely immunocompromised NOD/SCID mice [35]. These studies also demonstrated that CSC phenotype and function was dependent on the recipients' immune status in AML, as the $CD34^+CD38^-$ AML cells could be serially passaged in NOD/SCID mice [35] but failed to do so in SCID hosts [36]. Similar variability has been found in human melanomas, where the CSC frequency (measured by tumor initiation) was enriched from ~1 in 10^5 in NOD/SCID mice to 1 in 5.5×10^3 in IL2R$\gamma^{-/-}$ NOD/SCID recipients, which calculates as an 18-fold enrichment [97].

This also raises controversy about the CSC model, as the frequency of CSC numbers in the published melanoma model was 1 in 4 using the IL2R$\gamma^{-/-}$ NOD/SCID mice. However, by definition the CSC model does not dictate the number of CSCs (rather just the presence of a hierarchy), and this study did not directly address CSC functions such as self-renewal and differentiation capacity in serial xenotransplantation experiments. This study and others, however, do highlight the complications of the tumor microenvironment, which also include nonimmune host factors [99]. This has been highlighted in the breast with the humanization of the murine mammary fat pad improving implantation of human normal and breast cancer cells [39, 56, 63, 69, 70, 96] but also in other cancers such as glioblastoma [100]. This study demonstrated that intracranial orthotopic inoculation of stem-like glioblastoma cells enabled consistent tumor formation; however, this was significantly reduced when cells were injected subcutaneously [100]. $CD133^+Nestin^+$ brain CSCs were also found to reside within a perivascular niche and within proximity to endothelial cells [101], and increasing the number of cografted endothelial cells in orthotopic human brain xenografts caused expansion of the CSC population and increased tumor initiation and growth [101]. A recent publication also described that the engraftment of human hematopoietic stem cells is more efficient in female IL2R$\gamma^{-/-}$ NOD/SCID mice and that secondary transplantation from primary recipients also indicated that females more efficiently supported the self-renewal of human hematopoietic stem cells. This suggests that sex-associated factors may play a role in the survival, proliferation, and self renewal of human hematopoietic stem cells in xenograft models and as such the recipient sex should be carefully monitored in the future experimental design [102]. In addition, the coadministration of tumor growth promoting factors, such as extracellular matrix component laminin, can enable tumor cells, which would not in the absence of laminin initiate tumors, to contribute to experimental tumor formation [97]. These results emphasize the importance of CSC interactions with the tumor host microenvironment and therefore should be considered for the design of effective and clinically relevant assays to allow accurate CSC identification and targeting.

Table 15.3 Human xenograft models of breast cancer depicting the varied methodologies to assess CSCs number and tumorigenicity

CSC isolation method	# CSC or fold enrichment	ECM	Cell type	Estrogen pellet	Site of injection	Humanization of fat pad	Other additions	Mouse strain used	References
Limiting dilution	G1, 1:68,000 G3, 1:21,000	–	Human primary breast cancer	+	Mammary gland	2×10^5 non IR HMF	Non-IR & IR HMF with breast cancer cells	5 week old NOD/SCID	[56]
$CD44^+CD49f^{Hi}$ $CD133/2^{Hi}$	More tumorigenic	+	Human primary breast cancer	+	Mammary gland	None	i.p etoposide 5 days prior to cell injection	4–8 week old NOD/SCID	[54]
ESA^+CD44^+ $CD24^{low/-}$	100 fold	–	Human breast cancer cell lines	+	Subcutaneous	N/A	–	6 week old NOD/SCID	[40]
16 h Mammosphere culture	12 fold	–	Human breast cancer cell lines	+	Subcutaneous	N/A	–	6 week old NOD/SCID	[40]
ESA^+CD44^+ $CD24^{low/-}$	100 fold	–	Human primary breast cancer	+	Mammary gland	None	i.p etoposide 5 days prior to cell injection	8 week old NOD/SCID	[39]
$PROCR^+ESA^+$	More tumorigenic	+	Human breast cancer cell lines	–	Mammary gland	None	CAFs added 1:1 with breast cancer cells	NOD/SCID	[41]
$ALDH^+$	More tumorigenic	+	Human breast cancer cell lines	+	Mammary gland	1:1 IR and non-IR HMF in matrigel (5×10^4)	–	NOD/SCID	[66]
$ALDH^+$	More tumorigenic	+	Human primary breast cancer	+	Mammary gland	1:1 IR and non-IR HMF in matrigel (5×10^4)	–	NOD/SCID	[63]
$ALDH^{Hi}CD44^+$ $CD133^+$	More tumorigenic & metastatic	–	Human breast cancer cell lines	–	Mammary gland	None	–	7–10 week old NOD/SCID/ $IL2\gamma^{-/-}$	[67]

ECM extra cellular matrix (Matrigel); *IR* irradiated; *HMF* human mammary fibroblasts; *CAFs* cancer associated fibroblasts; *NOD/SCID* nonobese diabetic/severe combined immunodeficiency

15.6 Conclusions

With the growing evidence that cancers have a hierarchical population and that cells capable of initiating tumors (CSCs) also have the ability to avoid current therapeutic strategies, the ability to identify and characterize CSCs will be critical to the future development of therapeutic targets for many cancers. Therefore, the impact of both in vitro and in vivo CSC assays and their robustness in identifying CSCs will be very important. Identifying CSCs is very challenging and there is not, as yet, one definitive assay that will consistently identify CSCs in all cancers. However, the development of xenograft models that fully mimic the tumor's original microenvironment will aid the advancement of the field. With the combination of multiple in vitro assays and retransplantation xenograft models already available, studies should lead to a better understanding of the molecular mechanisms controlling self-renewal and differentiation. These studies may reveal whether CSCs from different types of cancers or from different subgroups within cancer types have similar CSC markers and biological function, knowledge of which will also be essential if the ultimate aim of eliminating CSCs is to be achieved.

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Chapter 16
Zebrafish as a Model to Study Stem Cells in Development, Disease, and Cancer

Viviana Anelli, Cristina Santoriello, and Marina C. Mione

Abstract Throughout the lifespan of an organism, stem cells help maintain normal homeostasis by choosing one of the several alternate fates: self-renewal, differentiation, senescence, or death. They are fundamental to development, but are also susceptible to generate disease and especially cancer. This chapter will review the information available to date on the study of stem cell biology and dysfunction using the zebrafish, an optically transparent vertebrate that is gaining increasing attention in experimental and preclinical disease studies. The first section will review studies that identified tissue-specific stem cells in developing zebrafish. The second section will deal with reports of stem cell involvement in regeneration and in diseases other than cancer. The last two sections will describe the increasing number of zebrafish cancer models and studies leading to the identification of cancer stem cells (CSCs) in these models, as well as the use of the zebrafish for heterologous CSC studies.

Abbreviations

AGM	Aorta-gonad-mesonephron
APC	Adenomatous polyposis coli
CHT	Caudal hematopoietic tissue
CNS	Central nervous system
CSC	Cancer stem cell
DA	Dorsal aorta
dpf	Days postfertilization
EGFR	Epidermal growth factor receptor
ERMS	Embryonal rhabdomyosarcoma

M.C. Mione (✉)
IFOM, The FIRC Institute of Molecular Oncology, Milan, Italy
e-mail: marina.mione@ifom-ieo-campus.it

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GFP	Green fluorescent protein
GSEA	Gene set enrichment analysis
hpf	Hours post fertilization
HSC	Hematopoietic stem cell
ICM	Inner cell mass
MPD	Myeloproliferative disorder
MPNST	Malignant peripheral nerve sheath tumors
MSC	Melanocyte stem cell
NSC	Neural stem cell
PGE2	Prostaglandin E2
RBI	Rostral blood island
RFP	Red fluorescent protein
RMS	Rhabdomyosarcoma
RUNX1	Runt-related transcription factor
T-ALL	T-cell acute lymphoblastic leukemia
VZ	Ventricular zone

16.1 Stem Cells in Development

There are three areas where tissue-specific stem cells have been thoroughly studied in developing zebrafish: the hematopoietic system, the nervous system, and the pigment cells (melanocytes).

16.1.1 Hematopoietic Stem Cells

The hematopoietic system has evolved to ensure nutrient supply and protection from external challenges in multicellular organisms. The blood is composed of a large variety of mature cell types with different life spans that need to be constantly replenished from a pool of hematopoietic stem cells (HSCs), and this process is known as hematopoiesis.

During embryonic development, the major site of hematopoiesis shifts from one organ to another in a dynamic temporal spatial manner. Indeed, in most vertebrates, hematopoiesis occurs in sequential waves, termed primitive and definitive. For example, in mammals there is first a transient primitive wave of hematopoiesis, during which the HSCs appear in the blood islands in the extraembryonic yolk sac, giving rise to erythrocytes and macrophages [1]. The successive definitive wave starts intraembryonically in the aorta-gonad-mesonephron (AGM) region, giving rise to all different blood lineages. Subsequently, HSCs born from the AGM migrate into the fetal liver where they will proliferate and ultimately seed the bone marrow, which is the adult hematopoietic organ in mammals [2].

In zebrafish, there are waves of hematopoiesis, which occur in a spatial and temporal sequence unique to this vertebrate model. The primitive or embryonic

hematopoietic wave occurs in two intraembryonic sites: (1) in the ventral mesoderm-derived tissue called the intermediate cell mass (ICM); and (2) in the rostral blood island (RBI) arising from the cephalic mesoderm [3]. During this wave the anterior part of embryo generates myeloid cells, while the posterior part generates mostly erythrocytes and some myeloid cells. From 24 h postfertilization, these primitive blood cells start to circulate through the embryo. Subsequently, the definitive HSCs emerge from the ventral wall of the dorsal aorta (DA) [4–6] and these HSCs migrate to the posterior region in the tail called the caudal hematopoietic tissue (CHT) [7]. From 3dpf lymphopoiesis initiates in the thymus. By 4dpf HSCs seed the kidney marrow, which is equivalent to the bone marrow in mammals. Although mammals and zebrafish show differences between the two hematopoietic waves, they share similar genetic programs. Definitive hematopoiesis requires the expression of transcription factors such as *runx1* and *c-myb* in mammals as well as in zebrafish. Excellent reviews on zebrafish hematopoiesis [3, 8] provide a detailed account of the similarities and differences between the two species.

16.1.1.1 Common Precursors for Hematopoietic and Endothelial Lineages

Several observations in the mouse related to the close time and space development of hematopoietic and endothelial cells raised the possibility of a common mesodermal progenitor, or of a hemogenic endothelium [9]. Now it is widely accepted that hemogenic endothelial cells of the DA are the source of HSCs within the AGM region. In fact, recent studies in zebrafish where they directly imaged the generation of HSCs using a combination of fluorescent transgene reporters and confocal time-lapse imaging have shown that HSCs arise from the hemogenic endothelium lining the ventral wall of the DA, indicating that the cellular processes underlying the generation of these multipotent progenitors are similar in fish and mammals [10, 11]. Moreover, it has been shown using the transcription factor *runx1* as early marker of very early HSC commitment that it is possible to visualize cells acquiring hemogenic properties and emerging as presumptive HSCs [12].

16.1.1.2 Zebrafish Shine a Light on HSCs

Large scale forward genetic screens in Boston and Tuebingen have generated numerous zebrafish blood mutants. More than 40 mutants with hematopoietic defects were identified, and many of these mutants (but not all) have been characterized and have been useful in elucidating specific signaling pathways important for HSCs [3].

These mutants also represent useful animal models for human diseases. Since HSC transplantation is an effective treatment for blood diseases, autoimmune diseases, leukemia, and lymphoma, improving the efficiency of homing and engraftment of HSCs is crucial. Using the zebrafish as a “platform” for chemical genetic screening aimed at identifying new pathways modulating definitive HSC formation, it was possible to discover that prostaglandin E2 (PGE2) is an important and conserved

regulator of HSC number [13]. These results have been confirmed *in vitro* and *in vivo* in a murine model, and prostaglandins are currently in clinical trials as a drug useful to enhance hematopoietic stem engraftment after bone marrow depletion (personal communication) [13].

16.1.2 Neural Stem Cells and Adult Neurogenesis

Vertebrate neurogenesis occurs during embryogenesis as well as in adults. The discovery of new neurons being generated in the adult brain opened up the way to new therapeutic treatments for brain injury and neurological diseases based on cell replacement therapy.

In mammals, neurogenesis occurs in two areas of the brain: (1) in the anterior part of the subventricular zone of the lateral ventricle; and (2) in the subgranular zone of the dentate gyrus [14]. In the first area, immature neurons migrate rostrally to the olfactory bulb; in the second, newly generated cells migrate to the granule cell layer of the hippocampus. In contrast, in teleosts and more specifically in zebrafish, there are dozens of such areas in the brain with high mitotic activity, including regions homologous to the olfactory bulb and hippocampus in mammals. In particular, 16 distinct proliferative zones have been described in the whole brain mostly located in the ventricular surface, but also deeper in the brain parenchyma [15]. The persistence of such proliferative areas indicates the presence of stem cells. In particular, the presence of different types of proliferating cells within the proliferative domain has been reported: these include cells that once generated, migrate from their place of birth, and cells that show an active cycling behavior and remain in place. This last population of cells represents the neuronal stem cell pool and shows ability to self renew and to retain fluorescent labeling [16].

There are a limited number of new neurons generated during adulthood, and this number must be tightly controlled by the balance between quiescent cells and neural stem cells (NSCs) recruited into the cell cycle. The signals that instruct NSCs to exit from a quiescent state are unknown, but there is evidence in mice of an involvement of Notch signaling [17], where it has been observed that cell cycle reentry of ependymal cells after injury is blocked upon Notch activation. In zebrafish, it has been shown that Notch activity controls the balance between quiescent and proliferating NSCs. In particular, radial glial cells lining the ventricular zone (VZ) have been seen to switch back and forth between a quiescent and proliferating state, in response to changes in Notch activity levels [18]. The authors propose that dividing progenitors use Notch to impose transitory quiescence to their neighboring glia. Moreover, it has been suggested that Notch (via the ligand Jagged) is important for the maintenance and differentiation of neural progenitors cells during late neurogenesis in the zebrafish embryo [19]. Thus, it appears that Notch signaling, in mouse as well as in zebrafish, is involved in the mechanisms controlling the frequency of adult stem cell recruitment.

Stem cells have also been identified in the adult retina of the zebrafish [20]. These cells support the multi lineage retinal progenitors in the developing, adult, and regenerating teleost retina and have features of neuroepithelial cells [21].

16.1.3 Melanocyte Stem Cell and Regeneration

An example of an adult stem cell that maintains specific cells is the melanocyte stem cell (MSC). A way to analyze the mechanisms underlying adult stem cell formation and regulation is to study regeneration of melanocytes. The zebrafish has been used extensively to study melanocyte regeneration during larval stages and in adults, following fin amputation [22]. During embryonic development, melanocytes first appear at 24hpf and by 3dpf the pigment pattern is fully established, and comprise a precise number of melanocytes (460). After 2 weeks, there is a new wave of melanocyte production, which will give rise to the typical striped pattern of the adult fish.

In recent years, some researchers have developed a protocol for ablating larval melanocytes [23] using the chemical MoTP, which was originally identified during a small molecule screen for drugs that affect zebrafish development [24]. The use of this protocol in combination with a zebrafish mutant called *picasso*, which has deficits in forming new melanocytes after metamorphosis, thus shedding light on the nature of adult MSCs.

Indeed it has been suggested that already during larval stages, there is a quiescent population of adult MSCs, dependent on epidermal growth factor receptor (EGFR)-3b (*erbb3b*), which give rise to the adult melanocyte [25]. The researchers therefore speculated that *erbb3b* is required for the development of adult melanocytes, and it is responsible for generating a niche where MSCs remain quiescent till metamorphosis.

A different approach to study regeneration is to look at melanocyte appearance following fin amputation. In particular, studies on fin regeneration propose the existence of two waves of melanocyte regeneration, the first or primary is dependent on the *kit* receptor tyrosine kinase, and the second one is *kit* independent [22]. These studies support a model of two distinct populations of regenerating melanocytes and probably of different populations of MSCs [25]. In a recent elegant study, the number of MSCs in the adult caudal fin has been estimated to be less than 10 cells [26].

Therefore, the use of zebrafish pigment mutants has been crucial for the understanding of basic melanocyte development and melanocytes stem cells. There are more zebrafish mutants that have been generated during forward genetic screens, and they have not yet been fully characterized or identified. The characterization/identification of these mutants in combination with the use of chemical biology applied to the zebrafish will help to clarify many aspects of adult MSC biology.

16.2 Stem Cells in Tissue Regeneration and Disease

Tissue-specific stem cells are very important for the homeostasis of an organism. Physiological aging, pathological aging, and regeneration are all conditions where tissue stem cells are called upon for extra work. Failure of stem cells to meet an increased demand will result in disease.

Pioneer studies on the involvement of tissue stem cells in disease in zebrafish have been concentrated on regeneration studies. The zebrafish shares with urodele amphibians an enhanced regenerative capacity, and cells ranging from CNS neurons to differentiated cardiomyocytes as well as entire organs (i.e., fins and scales) can regenerate to perfection in just a few days [27]. There is consensus on the involvement of stem cells in repairing missing tissue or damaged organs in both zebrafish and mammals. However, what is not clear (and is intensely investigated) is the source of the stem cells that repair the injury in the different tissues and the molecular, genetic, and epigenetic steps that are necessary for regeneration to occur. Thanks to its increased regenerative abilities, the zebrafish is starting to provide answers to these questions, in at least two areas: heart and fin regeneration.

16.2.1 Heart Regeneration and Stem Cells

Following the initial report on adult heart regeneration in zebrafish [28], the source of regenerating cardiomyocytes has been actively sought using a variety of lineage labeling approaches [29, 30]. The question of whether the regenerative response is sustained by quiescent cardiac progenitor cells or terminally differentiated cardiomyocytes has obtained different answers. Using the different stability of red fluorescent protein (RFP) and green fluorescent protein (GFP) driven by the cardiomyocyte specific promoter (*cml2*), Lepilina et al. [29] claimed that new cardiomyocytes are generated by progenitor cells, lending support to the hypothesis that stem cells may play an important role in heart regeneration. On the contrary, Jopling et al. [30] used a cre/*lox* system to demonstrate that upon ventricular resection, terminally differentiated cardiomyocytes undergo limited de-differentiation and re-enter the cell cycle. This latter report argues against a contribution of stem cells to heart regeneration in adult zebrafish.

16.2.2 Fin Regeneration and Stem Cells

The regeneration of a complex organ such as the adult fin composed of epithelial tissue, pigment cells, blood vessels, peripheral nerves and bony rays proceeds through the formation of multiple blastema, transient structures of undifferentiated

and proliferating cells. The existence of tissue specific stem cells to sustain blastema formation has been postulated; however, the origin of the lineages that are generated by blastemal cells is still unknown. The results obtained by several laboratories point to the origin of blastemal cells from de-differentiated fibroblasts sitting in the inter-ray region (as suggested by [28]). By contrast, for at least one of the populations of regenerating cells in amputated fins, the melanocytes, a discrete group of MSCs has been clearly demonstrated [30].

16.2.3 Stem Cells in Physiological and Pathological Aging

A reduction in the number or properties of tissue stem cells has been hypothesized as an important mechanism in physiological aging [31]. The zebrafish is a diurnal vertebrate showing progressive aging [32], and is a good model to study aging mechanisms and the behavior of tissue stem cells with aging. A number of mutants have been identified that show premature senescence phenotypes [33], and it would be interesting to investigate whether tissue stem cells are affected in these mutants. A recently described genetic screen for retinal degeneration phenotypes [34] is likely to uncover some of the genes that are important for retinal stem cell activity in aged zebrafish. Similarly, chemical screens for hair cell loss or protection in neuromasts of the lateral line in aging zebrafish [35] may identify drugs that help prevent sensory neuron progenitor/stem cell loss during aging.

In a zebrafish model of Costello syndrome, a rare disease resulting from *de novo* germline activating mutations of HRAS, we found that adult progenitor/stem cells in the brain and heart undergo oncogene induced senescence, a process that hampers their ability to proliferate or differentiate, leading to severe impairment of the functions of these two organs [36]. This is one example of the involvement of stem cells in pathological aging and in diseases with a degenerative component. It is highly probable that many more degenerative processes will be related to pathologies of the stem cell compartment in the coming years.

16.3 Zebrafish Cancer Models

The zebrafish has long been used as a model organism for the identification of genes required for early vertebrate development [37]. However, while keeping zebrafish in the laboratory environment, researchers have observed different diseases in adults, including cancer. Studies on the latter revealed that zebrafish spontaneously develop almost any type of tumor (reviewed in [38]). The most common target tissues for spontaneous tumors are the testis, gut, thyroid, liver, peripheral nerve, connective tissue, and ultimobranchial gland. Cancer progression in these animals recapitulates many aspects of human disease. After this discovery, several approaches have been developed to induce tumors in zebrafish, including chemical treatment, forward and

reverse genetic screens, transplantation of mammalian cancer cells, and ectopic expression of transgenes. Although many zebrafish cancer types have been generated using all these techniques, this section will focus on the more recent zebrafish cancer models, expressing mammalian oncogenes in specific cells.

A powerful demonstration of the oncogenic activity of a human protein modeled in zebrafish was the phenotype generated by expressing human RUNX1-CBF2T1 cDNA in zebrafish embryos in 2002 [6]. However, although this fusion protein is frequently found in human acute myeloid leukemia, the fish showed circulation defects, hemorrhages, abnormal vascular development, and defective hematopoiesis, but not leukemia. To overcome this problem, Langenau and colleagues expressed mouse c-*myc* under control of the zebrafish *Rag2* promoter, leading to a clonally derived T-cell acute lymphoblastic leukemia in transgenic zebrafish [39]. The *Rag2* promoter is expressed in immature T- and B-cell lineages, olfactory rosettes, sperm, and skeletal musculature. After injection of the transgene at the one-cell stage, 6% of injected fish develop tumors, showing distended abdominal cavities and splayed eyes due to retro-orbital infiltration by malignant cells. Interestingly, through the construction of a chimeric transgene expressing *c-myc* fused to GFP, it was possible to visualize and discover that leukemic cells arose in the thymus, spread locally into gill arches and retro-orbital soft tissue, and then disseminated into skeletal muscle and abdominal organs. However, as leukemia after germline transmission of the transgene was lethal before reproductive age, the line could only be propagated via *in vitro* fertilization. To overcome this problem, the same group generated a conditional transgenic zebrafish by using Cre/*lox* technology. After injection of Cre mRNA into one-cell-stage embryos, T-cell acute lymphoblastic leukemia (T-ALL) developed that recapitulates the human disease both molecularly and pathologically [39]. This transgenic strategy was subsequently improved by crossing the conditional line to a transgenic line expressing Cre under a heat-shock promoter, making it possible to express the oncogene at a specific time in the adult zebrafish.

After generation of the leukemia model, several transgenic models for solid tumors have also been developed. In 2005, a zebrafish model of RAS-induced embryonal rhabdomyosarcoma (ERMS) in which animals develop externally visible tumors by 10 days of life was generated by injecting zebrafish embryos with a human *KRASG12*-containing plasmid under the promoter *Rag2* [40]. These zebrafish tumors express clinical diagnostic markers of human rhabdomyosarcoma (RMS) and are morphologically similar to human ERMS. Microarray analysis and gene set enrichment analysis (GSEA) revealed that zebrafish RMS is similar to the human embryonal subtype of disease but not the alveolar subtype. Tumors were highly invasive, being found in the intestine, liver, kidney, and testis. Interestingly, dual fluorescently labeled RMS were created that allowed for the identification of discrete subpopulations of cells (cancer stem cells, CSCs) within the tumor mass based on muscle differentiation status that will be discussed in the next paragraph. Also in this type of tumor, a heat shock inducible Cre/*lox*-mediated transgenic approach was used in which activated human KRASG12 was conditionally induced within transgenic animals by heat shock treatment [41]. Using this system, four types of

tumors and hyperplasia were generated: RMS, myeloproliferative disorder (MPD), intestinal hyperplasia, and malignant peripheral nerve sheath tumors (MPNST); and all these RAS-induced zebrafish diseases are morphologically and molecularly similar to those described in humans.

Pancreatic neuroendocrine tumors in zebrafish were developed in the Look laboratory in 2005 by targeted expression of the human *MYCN* transgene under the control of the *myod* promoter. These zebrafish develop carcinomas between 3 and 6 months of age, and the tumors express insulin and histologically resemble pancreatic neuroendocrine carcinomas [42]. More recently, by using a BAC transgene under the regulation of *ptf1a* regulatory elements, GFP fused to oncogenic KRAS was expressed in the developing zebrafish pancreas [42, 43]. Pancreatic progenitor cells expressing the transgene underwent normal specification and migration, but failed to differentiate. The block in differentiation resulted in the abnormal persistence of an undifferentiated progenitor pool and was associated with the subsequent formation of invasive pancreatic cancer, showing several features common to the human disease.

Another tumor that has been extensively studied in zebrafish, thanks to availability of many transgenic models, is melanoma. The first transgenic melanoma model was generated in 2005 by Patton and colleagues through the injection of a transgene containing the most common BRAF mutant form (V600E) under the *mitfa* promoter [44]. In these fish, focal sites of melanocyte proliferation (designated "fish-nevi") were clearly evident by 8 weeks in 10% of fish. However, melanocytes in F-nevi appeared well differentiated, not dysplastic and without evidence of local tissue invasion. After injection of *mitfa*-BRAFV600E into zebrafish embryos harboring homozygous missense mutation of the *p53* gene, by 4 months of age half of these animals developed malignant melanoma that was highly invasive, with nuclear pleomorphism similar to human melanoma [44]. A more recent melanoma model was developed by Santoriello and colleagues [45] through the generation of transgenic lines specifically expressing oncogenic human HRAS in the melanocytic lineage using the combinatorial Gal4-UAS system, an expression system in which ectopically expressed Gal4 activates the transcription of a reporter gene that is downstream of an upstream activation sequence (UAS). In this model, when transgenic fish expressing Gal4 containing GAL4 under the control of *kita* promoter were crossed with reporter fish containing the constitutive active mutant form of HRAS (G12V), they develop melanoma by 1–3 months of age, without the need of coactivating mutations in tumor suppressors. Interestingly, analysis of the methylation status of histones showed that, like in human melanoma, important epigenetic changes occur that could be responsible for the global repression of gene expression observed in the zebrafish melanoma [46]. Moreover, the larvae show a hyper-pigmentation phenotype as the earliest evidence of abnormal melanocyte growth, offering the advantage of a larval phenotype suitable for large scale drug and genetic screens. The Gal4-UAS system has been adopted by several zebrafish laboratories and hundreds of specific enhancer-trap lines have been generated. These lines can be crossed with reporter lines expressing different oncogenes thus generating different combinations of oncogenes and cell types, with the possibility of targeting stem cells, progenitors, and differentiated cells.

In conclusion, the zebrafish cancer model show several features in common with the human disease and represent a useful tool for advancing and understanding the biology of the human disease.

16.4 Zebrafish and Cancer Stem Cells

A growing number of studies provide supporting evidence for the existence within tumors of cells with tumor-initiating abilities, the cancer initiating cells. Cancer initiating cells may evolve in CSCs, a population of hierarchically superior cancer cells that maintain the ability to generate the diversity of cancer cells (multipotentiality), self renew and possess properties that prevent their eradication by commonly used therapies. Not all cancer cells can sustain cancer development as demonstrated by the most commonly used *in vivo* assays for CSC identification, i.e., the transplantation and limiting dilution assays. CSCs have been identified both in hematopoietic malignancies and in solid tumors, as described in the other chapters of this book. In zebrafish, CSCs have been demonstrated through transplantation assays in hematopoietic malignancies [47], in RMS [40], in hepatocellular carcinoma [48], and in melanoma [49]. The use of transplantation and limiting dilution assays as gold standard tests for the presence and number of CSCs in zebrafish will take advantage of the development of clonal zebrafish that will permit cancer cell transplantation in syngeneic individuals without the need for immunosuppression [48].

Instead of being a stable population of cells, CSCs could represent a temporary state acquired in turns by different groups of cancer cells, and, as recently shown for melanoma, the properties of CSCs could derive from the transient expression of certain genes, or more likely by the transient epigenetic landscape of a group of cells [50]. The dynamic status of CSCs could justify the difficulties in identifying these elusive cells in solid tumors and the problems in eradicating them. The zebrafish may provide additional tools to identify the transient status of CSCs through different fluorescent reporters; careful design of probe-sets will be important and may be followed by genetic or molecular manipulation of the CSC determinant(s). Besides CSCs being responsible for tumor growth, metastasis formation and resistance to therapy, another important aspect is the identification of cancer initiating cells in different types of solid tumors. Many animal models of cancer are based on transgenic lines where the oncogene expression is driven by a specific promoter in a cell/tissue and time-specific manner. However, there are a limited number of cell types targeted by specific promoters and the process of generating the models is lengthy, especially for the mouse, further limiting the number of combinations of cancer relevant factors used in these models. The zebrafish has the potential to bridge this gap, as the generation of cancer model is somewhat quicker and more flexible than in the mouse. Moreover, studies where cancer initiating cells have been unequivocally identified have shown that a deep relationship

exists between the development of normal cell lineage and the CSC lineages that sustain tumor development in a particular organ, thus confirming that the study of normal developmental processes will also shine a light on cancer. Cancer initiating cells have been identified and targeted in transgenic zebrafish models of hematopoietic malignancies (reviewed in [51]) and shown to correspond to the same cells giving rise to different types of leukemia in humans. The origin of pancreatic adenocarcinoma from ductal or acinar cells has been investigated by the Leach laboratory [43, 52] using a *ptf1a* promoter, but the identification of CSCs in pancreatic adenocarcinoma is still unclear. In the gut, the pivotal role of the tumor suppressor APC in regulating cancer initiation by preventing global hypomethylation (which characterizes the initial stages of transformation) has been recently described by Rai et al. [53]. Cancer initiating cells seem to correspond to intestinal stem cells residing at the bases of the intestinal cryptae [54]. Melanoma initiating cells have been identified as cells that express the melanoblast/melanocyte-specific transcription factor *mitfa* [44, 55] but also (and more efficiently) as their precursors expressing *kita* [45].

Transgenic zebrafish expressing a fluorescent reporter or a fluorescent oncogene are being used to compare the same cell lineage in normal development or in transformation and cancer, and we will soon be able to follow the transformation events in real time *in vivo*. The ease of generating a large number of transgenic lines and the advantages of the zebrafish as an ideal model to study developmental processes will turn out to be a winning point in the study of cancer initiating cells and CSCs *in vivo*. Finally, the zebrafish has been used to study CSCs from human tumor samples and primary cancer cell lines, by serving as a recipient of transplanted cells [49]. Although providing a new platform for the analysis of CSCs, some aspects of the procedure still require optimization and tool development [56].

16.5 Conclusions

In complex organisms that have evolved to support a life-span of several years, stem cells are fundamental to maintain tissue homeostasis and ensure repair and regeneration. At the same time, stem cells have become susceptible to diseases that are more severe than those affecting replaceable progenitors or differentiated cells. Some of these diseases such as cancer may be able to generate their own stem cells, which will ensure perpetuation of the disease status. The parallels between development and disease for stem cell biology are clear. For this reason, the zebrafish, a transparent vertebrate which is widely used for genetic and *in vivo* imaging of tissue morphogenesis and regeneration, can provide a unique angle to study normal and pathological stem cell biology. An increasing number of scientific reports on stem cells in zebrafish organogenesis and disease modeling document the potential of this model for the study of stem cell and cancer biology.

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Chapter 17
Imaging Cancer Stem Cells

Paula Foster

Abstract With increasing evidence of a role for cancer stem cells (CSC) in tumor initiation, proliferation, and metastasis, and a multitude of advanced imaging technologies being developed for noninvasive in vivo cell tracking, the need for imaging studies with a focus on monitoring the fate of CSCs in vivo appears clear. Preclinical investigations of CSCs would benefit from techniques that could dynamically monitor cells from their earliest appearance in tissues and throughout the processes of tumor development and metastasis in entire organs or animals. Traditionally, the assays used to identify and examine CSC are labor-intensive, time-consuming, invasive, and provide little information on the dynamics of cancer cells in vivo. CSC studies should take advantage of advanced imaging technology to increase our understanding of the CSC model, dormancy, tumor growth, and metastasis. With the ability to reliably track the metastasis and proliferation of small numbers of cancer cells, and specific subsets of cancer cells, will come new knowledge of the behavior of these cells in a relatively undisturbed environment.

Abbreviations

18FDG	18-Fluoro-2-deoxyglucose
2D	Two-dimensional
3D	Three-dimensional
BLI	Bioluminescence imaging
CCD	Charge-coupled device
CD	Cluster of differentiation

P. Foster (✉)
Robarts Research Institute, London, ON, Canada

Department of Medical Biophysics, University of Western Ontario, London, ON, Canada
e-mail: pfoster@imaging.robarts.ca

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cODC	Carboxyl-terminal degron of ornithine decarboxylase
CSC	Cancer stem cell
CT	Computed tomography
Cu-64	Copper 64
FI	Fluorescence imaging
FITC	Fluorescein isothiocyanate
GFP	Green fluorescence protein
GRE	Gradient echo
HSV1-TK	Herpes simplex virus type 1 thymidine kinase
MRI	Magnetic resonance imaging
NIR	Near-infrared
PET	Positron emission tomography
PTSM	Pyruvalde-hyde-bis (*N*4-methylthiosemicarbazone)
RFP	Red fluorescence protein
SE	Spin echo
SPECT	Single photon emission computed tomography
SPIO	Superparamagnetic iron oxide

17.1 Introduction

With increasing evidence of a role for cancer stem cells (CSC) in tumor initiation, proliferation, and metastasis, and a multitude of advanced imaging technologies being developed for noninvasive in vivo cell tracking, the need for imaging studies with a focus on monitoring the fate of CSCs in vivo appears clear. Preclinical investigations of CSCs would benefit from techniques that could dynamically monitor cells from their earliest appearance in tissues and throughout the processes of tumor development and metastasis in entire organs or animals. The optimal techniques would be minimally or noninvasive, sensitive to small numbers of cells, and able to assess cell distribution throughout the organ at early stages, to differentiate between dormant and proliferating cells, and to follow the growth of tumors over time.

A number of imaging modalities can be used to detect cancer cells in vivo in preclinical animal models. These include optical techniques [1], computed tomography (CT) [2], ultrasound (US) [3], nuclear imaging techniques such as positron emission tomography (PET) [4] and single photon emission computed tomography (SPECT) [5], and magnetic resonance imaging (MRI) [6]. Each of these modalities has certain advantages and limitations for in vivo cell tracking of CSCs. To image CSCs, one of two approaches may be taken. First, CSCs could be isolated and prelabeled with a probe or reporter in vitro, prior to their injection or implantation into preclinical models. Second, CSCs could be imaged after in vivo labeling through the administration of a targeted or specific CSC probe (Fig. 17.1). The choice of which imaging modality to use depends mainly on the spatial resolution and sensitivity required, the depth of the tissue, and the prospective for clinical translation.

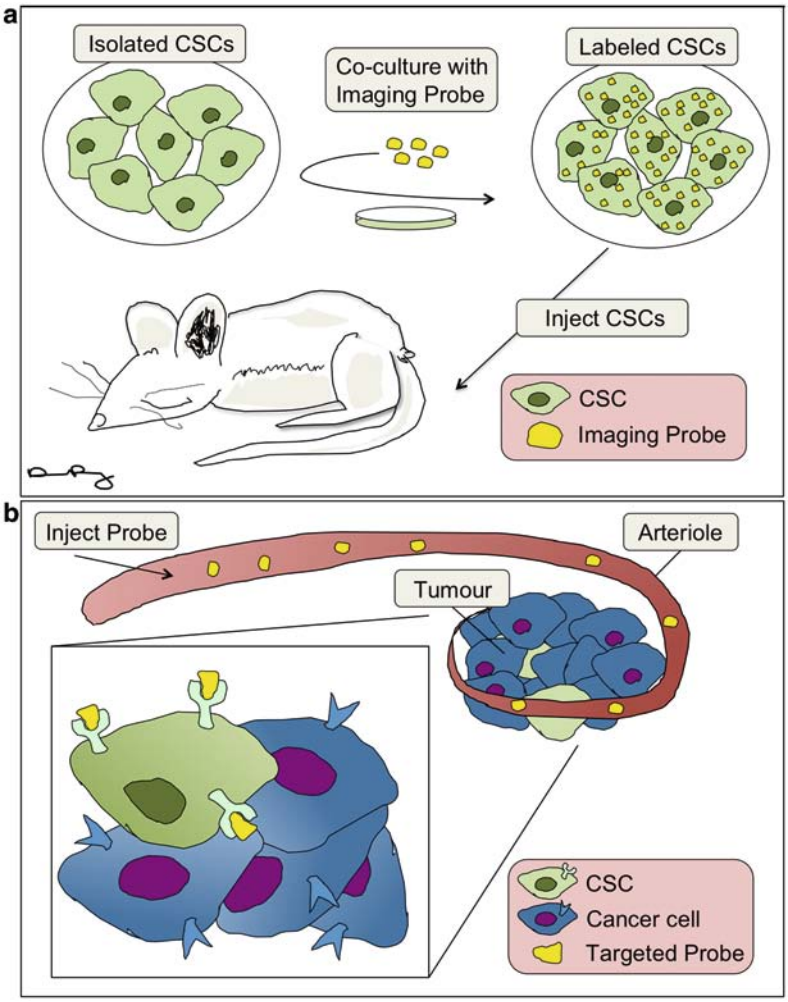

Fig. 17.1 There are two main strategies for labeling CSC for their detection using in vivo imaging techniques: (**a**) cells can be prelabeled in culture prior to their injection; or (**b**) a targeted imaging probe can be administered systemically to label cells in vivo

Over the past 10 years or so, the tracking of normal stem cell engraftment, migration, and homing clearly emerged as a key area for in vivo cell tracking and all of the modalities mentioned above have been used to track stem cells in preclinical models of disease and injury [7–14]. So far, nearly all studies have been of the "proof-of-principle" type, demonstrating that various probes or reporters and imaging modalities are useful for the long-term noninvasive tracking of transplanted cells. A variety of imaging modalities have also been used to track cancer cells. Optical and nuclear imaging methods have been widely used in cancer models to investigate factors involved in malignant transformation, invasion, and metastasis,

and to monitor responses to cancer therapy [15–20]. At present, there are very few reports on the use of noninvasive in vivo imaging of CSCs; however, the potential is great, especially for optical, nuclear, and MRI techniques.

17.2 Optical Imaging

Optical imaging techniques are based on fluorescence (i.e., enhanced green fluorescent protein; GFP) or bioluminescence (BLI) (i.e., luciferase) reporters [21]. Optical imaging techniques have several advantages: they are relatively noninvasive, inexpensive, convenient to use, allow for high throughput, and have high photon sensitivity, which allows for low levels of gene expression to be detected. However, they have limitations when compared with MRI and PET due to high scattering and absorption of light in tissue, resulting in limited depth penetration [22].

For BLI, cells are often transfected with the firefly luciferase reporter prior to their injection into animals. The substrate of luciferase, luciferin, is injected into the cell-bearing animals in vivo prior to imaging. The luciferase is called the reporter because it "reports" its location by emission of light following the chemical reaction of the luciferase enzyme with its substrate [23]. Images are acquired by placing the animal in a dark chamber and using a charge-coupled device (CCD), which is a light-sensitive camera. The time to acquire images ranges from 1 s to 10 min. Most imaging systems provide two-dimensional (2D) information in rodents, showing the locations and intensity of light emitted from the animal in pseudo-color scaling. A three-dimensional (3D) capability for BLI is now available, but is more expensive and less efficient. Whole mice BLI models have also been created [24].

Fluorescence imaging (FI) differs from BLI in that an excitation light source is required to detect the emission of light. Typically, a filtered excitation light source is used to excite a fluorophore, which emits light at a higher wavelength. There are two types of fluorophores: endogenous (i.e. GFP) and exogenous (i.e. FITC, rhodamine, Cy5.5) [25]. Similar to BLI, endogenous imaging requires the transfection of a cell line with a gene that produces a fluorescent protein such as GFP. A significant technical challenge is the autofluorescence present in normal tissues. To overcome the photon attenuation in living tissue, fluorophores with long emissions in the near-infrared (NIR) region are generally preferred, including widely used small indocarbocyanine dyes. The list of NIR probes continues to grow with the recent addition of fluorescent organic, inorganic, and biological nanoparticles [26].

The limitations of optical imaging make it unlikely that the method will be extended to human studies. However, in small animal models, BLI and FI are routinely applied to serially detect the location and burden of xenografted tumors, or identify and measure the number of immune or stem cells after an adoptive transfer. To date, the majority of preclinical studies that have imaged CSC in vivo have employed optical imaging methods.

Suetsugu et al. have shown that color-coded FI can be used to distinguish CSC-like and non-CSC cells in the same tumor in vivo in mice [27]. Human hepatocellular carcinoma cells were sorted based on expression of the surface protein CD133.

The sorted cell populations were then genetically labeled with GFP (CD133+ CSC-like) or red fluorescent protein (RFP, CD133− non-CSC). Cells were mixed and injected subcutaneously or into the spleen of nude mice. CSCs (GFP+) were observed to be highly tumorigenic and metastatic as well as highly resistant to chemotherapy compared with non-CSCs (RFP+). Vlashi et al. have used in vivo FI to demonstrate that reduced 26S proteasome activity is a general feature of CSCs that can be exploited to identify, track, and target them [28]. Human glioma and breast cancer cells were engineered to stably express ZsGreen fused to the carboxyl-terminal degron of ornithine decarboxylase (cODC), resulting in a fluorescent fusion protein that accumulates in cells in the absence of 26S proteasome activity; a ZsGreen-cODC reporter for FI. In vivo, ZsGreen-positive cells were approximately 100-fold more tumorigenic than ZsGreen-negative cells when injected into nude mice and the number of CSCs in tumors increased after 72 h postradiation treatment [28]. Lui et al. imaged breast CSCs in vivo using BLI by generating human-in-mouse breast cancer orthotopic models using patient tumor specimens labeled with optical reporter fusion genes [29]. As few as 10 cells could be detected in vivo, which allowed for the early visualization of tumor growth and metastasis. This study revealed that CD44+ cells from both primary breast tumors and lung metastases are highly enriched for tumor-initiating cells.

These optical imaging studies clearly show that the ability to distinguish stem-like cancer cells in vivo will be useful for preclinical investigations of the roles of CSC in tumor initiation, proliferation, and metastasis, and for investigating therapies targeting CSCs.

17.3 PET/SPECT

Nuclear imaging techniques such as SPECT and PET are highly sensitive systems that can detect trace amounts of γ- and β-emitting radionuclides, respectively. Thus, radionuclide imaging modalities are well suited for tracking and mapping the systemic biodistribution of cells. The sensitivity of PET and SPECT reaches nano- to pico-molar levels, and both methods have good penetration depth in tissues [30]. The images are also quantifiable, allowing for the number of cells to be determined in the whole body, and to follow the distribution of cells over time. PET and SPECT are limited by their low spatial resolution. Dedicated micro-PET and SPECT small animal scanners are able to achieve the spatial resolution (1–2 mm) necessary for imaging cells; however, this technology is not yet at the level of resolution necessary to detect single cells.

To date, most cell tracking PET studies have used radioactive metals such as ^{18}F-fluoro-2-deoxyglucose (^{18}FDG) and copper 64 (Cu-64), as imaging markers [31, 32]. A number of reporter genes have been developed for radionuclide imaging. These can be divided into three different classes: receptors, transporters, or enzymes. Enzyme-based systems are most common for cell tracking and use reporter gene production of a specific enzyme, such as herpes simplex virus type 1 thymidine kinase (HSVl-TK). This is a stable labeling method, which uses an F18-fluoropenciclovir probe which when phosphorylated by HSV is retained within cells [33].

The alternative to the reporter gene system is direct labeling of cells. Cells are incubated with the radioactive tracer, allowing the lipophilic molecules to diffuse across the cell membrane, and the isotope becomes trapped [34]. Following incubation, the cells are washed to remove any unbound activity, and the cells injected into the host. Cu-64 can be delivered into cells via a lipophilic redox-active carrier molecule pyruvalde-hyde-bis (*N*4-methylthiosemicarbazone) (PTSM). Cu-64 is one of the longer-lived PET radionuclides, with a half-life of 12.7 h, allowing labeled cells to be tracked for 2–3 days [35]. Although direct cell labeling has many attractive features, the drawbacks include radiotoxicity effects, loss of label from cells, dilution of signal from cell division, and lack of information on cell function or viability.

Yoshii et al. showed that in a mouse colon carcinoma model (colon-26), Cu-64-ATSM localized preferentially in tumor regions with a high density of CD133+ cells with characteristics of CSCs [36]. Most nuclear imaging studies are performed with the addition of either MRI or CT so that the low resolution PET or SPECT images can be superimposed onto high resolution anatomical images.

17.4 Magnetic Resonance Imaging

MRI can produce images with high spatial resolution and exquisite soft tissue contrast. Current micro-MRI technology can achieve three-dimensional spatial resolutions on the order of tens of microns [37]. MRI uses no ionizing radiation and is considered safe and noninvasive. Cellular MRI is a relatively young field of imaging research that combines the ability to obtain high resolution MR data with the use of magnetic contrast agents for labeling specific cells, thereby enhancing their detection [38, 39]. Superparamagnetic iron oxide (SPIO) nanoparticles represent a class of magnetic contrast agents used for cellular MRI that exhibit extremely high relaxivity [40]. A variety of iron oxide-based labels are now available [38]. Iron-labeled cells are usually imaged using either gradient echo (GRE) or spin echo (SE) sequences [41, 42]. The presence of the magnetic label causes a distortion in the magnetic field and leads to abnormal signal hypointensities in T2 or T2* sensitive images. Areas containing iron labeled cells appear as regions of low signal intensity, creating negative contrast. The large magnetic susceptibility of these particles affects an area much larger than the actual particle size. This effect is known as a “blooming artifact,” and leads to an exaggeration of the region occupied by iron oxide [43]. SPIO has been used to label and track a wide variety of cell types including T-lymphocytes [44, 45], macrophages [46, 47], pancreatic islets [48, 49], cancer cells [50, 51], and stem cells [42, 52] with minimal impact on cell function over a period of several weeks and over multiple cell divisions.

Pushing the limits of detection of SPIO-labeled cells has become an active area of research in the field of cellular MRI. This has been driven by interest in the following: (i) tracking the migration of small numbers of cells; (ii) detecting cells with

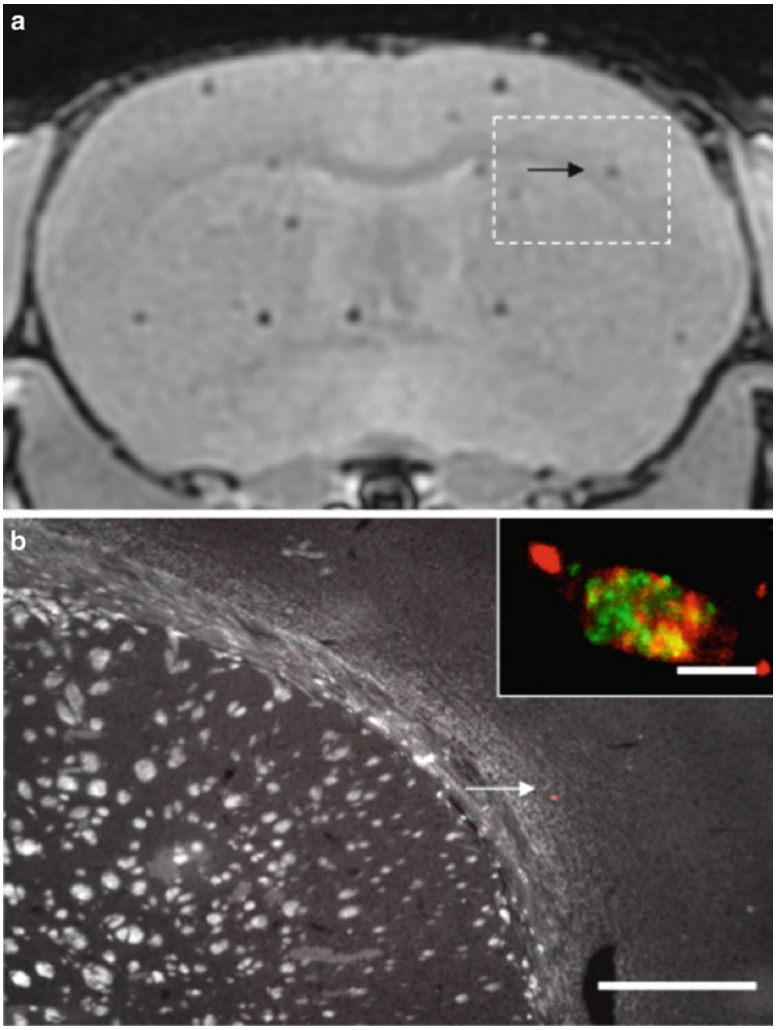

Fig. 17.2 Single iron-labeled cells can be detected in vivo in the mouse brain by MRI. (**a**) High resolution (100 μm^3) MRI of mouse brain demonstrates the presence of discrete signal voids (*black arrow*) throughout the mouse brain. (**b**) A discrete signal void detected in the MRI (*black arrow in* **a**) was correlated optically to a DiI positive region (*white arrow*). High resolution confocal microscopy revealed this region to be a solitary DiI (*red*) labeled cell with green fluorescence attributable to GFP expression (*inset of* **b**). From Heyn et al. [53], with permission

low levels of iron, which may occur in cells that are more difficult to label or in proliferating cells; and (iii) detecting small numbers of metastatic cancer cells. Heyn et al. were first to demonstrate the in vivo detection of single cells using MRI for SPIO-labeled cells in mouse brain (Fig. 17.2) [53].

The use of iron nanoparticles and MRI for cancer cell tracking has some limitations. These include relatively low sensitivity and difficulty in quantifying the signal loss caused by iron labeled cells. In addition, iron-labeled cells, which die in vivo, may be engulfed by bystander cells in tissue and this may confuse the image interpretation. The dilution of iron particles with cell division is also a drawback, since eventually in proliferating cells the iron label is diluted to a level below the detection threshold of MRI. MRI has yet to be used to track the fate of individual CSCs; however, the potential for this is clear. The fact that quiescent cancer cells retain the iron label for long periods compared with proliferating cells may represent a novel in vivo assay for differentiating between cancer cell types with various metastatic potentials.

17.5 Multimodality Imaging

It is becoming quite common to see multiple imaging modalities used in a complementary fashion to acquire multilayered information. The goal of multimodality imaging is to combine the best features of separate modalities. For instance, high-resolution anatomical images acquired with MRI or CT are often combined with functional or metabolic imaging such as PET or SPECT. Many studies have shown the benefits of using multiple imaging modalities to achieve different, complementary information about the fate of cells in vivo [54–59]. Along with this has come the development of multi-modality imaging probes [60–63].

Garzia et al. used PET/CT to investigate the relationship between miRNAs targeting the Notch pathway and medulloblastoma (MB) tumors. Notch regulates a subset of the MB cells that have stem-cell-like properties (CD44+) and can promote tumor growth. They showed that miR-199b-5p expression correlates with metastasis spread and that in a xenograft model, MB tumor burden can be reduced, indicating the use of miR199b-5p as an adjuvant therapy for the improvement of anti-cancer MB treatments [64].

Today hybrid multi-modality imaging systems are being developed that will allow the simultaneous acquisition of different types of images in the same animal during the same imaging session. These systems include integrated PET/CT, SPECT/CT, and PET/MRI. PET/CT studies are now quite common in cancer patients. A number of groups are pushing the frontier of PET/MRI, which will provide high resolution morphological, molecular, and functional information [65, 66].

An important recent development is the concept of multimodality fusion reporter systems. Ray et al. [67] and Ponomarv et al. [68] have described triple modality reporter genes for whole mouse body fluorescent, bioluminescent, and nuclear imaging. Fusion reporters have the potential to accelerate translational cancer research and will be important for defining the potential roles of each modality in specific applications.

17.6 Cell Detection Thresholds: A Comparison of MRI and PET

Radionuclide imaging techniques are generally considered to have superior sensitivity compared with MRI. This argument is based on the detectable concentration of the probe, which is ~10^{-12} M for PET vs. ~10^{-5} M for MRI [69]. Although the sensitivity of PET and SPECT tracers expressed in terms of concentration of the probe is an appropriate convention when the target is large (on the order of the resolution of a PET and SPECT scan), it may not be appropriate for cases in which the target is microscopic. Such a case arises when cells (especially small numbers of cells or single cells) are imaged. In this case, the probe is concentrated within a small volume within the voxel (the cell). For these microscopic targets, increasing image resolution would result in a significant increase in the "apparent concentration" of probe (number of moles of probe in a cell/voxel volume). For MRI, the high resolution of this technique results in a pessimistic estimate of sensitivity when expressed in terms of concentration, while the intrinsic low resolution of PET and SPECT techniques provide an apparent advantage. This advantage, however, would quickly deteriorate if the resolution of PET or SPECT could approach the resolution of MR. For the specific application of detecting a microscopic target, such as a cell, a proper calculation of sensitivity would involve a comparison of the number of moles of tracer that must be loaded into a cell to permit its detection.

An analysis by Heyn et al. indicates that on a mole basis, the detection threshold of Fe in SPIO is in fact not very different from that of PET tracers [70]. The detection of cells labeled with ^{64}Cu allows for the in vivo detection of a few hundred cells (~10^{-17} mol of ^{64}Cu per cell) or ~10^{-15} mol of ^{64}Cu (1 fmol) in a 10 microl voxel volume using a small animal PET scanner [71]. Heyn showed that for a typical MRI microimaging acquisition with an image SNR of 60 and 100 μm isotropic resolution, the minimum detectable amount of iron is approximately 1.3 pg Fe/cell, which corresponds to a detection threshold of 2.33×10^{-14} mol of Fe (23.3 fmol) [70]. Stated this way, the sensitivity levels of MRI and PET for SPIO and radionuclide tracer, respectively, are not very different after all.

17.7 Implications of Imaging for CSC Detection

Traditionally, the assays used to identify and examine CSC are labor-intensive, time-consuming, invasive, and provide little information on the dynamics of cancer cells in vivo. CSC studies should take advantage of advanced imaging technology to increase our understanding of the CSC model, dormancy, tumor growth, and metastasis. With the ability to reliably track the metastasis and proliferation of small numbers of cancer cells and specific subsets of cancer cells, will come new knowledge of the behavior of these cells in a relatively undisturbed environment.

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Chapter 18
Mouse Models for Studying Normal and Cancer Stem Cells

David A. Hess

Abstract Adult stem and progenitor cell functions have traditionally been studied by transplantation into immune-deficient mice. Over the years, the mouse strains capable of accepting human cells and models used to study regenerative or tumorigenic processes have grown in number and complexity. Because of these developments, it is now possible to study the establishment and metastasis of malignant human cancer cells, and this has generated an explosion of studies identifying cancer cells with robust tumor-initiating potential. In hematopoietic malignancies and some solid tumor types, the cells driving tumor growth, angiogenic vessel formation, and metastasis share characteristics with human stem cells; namely self-renewal and the ability to differentiate into multiple cell types. As our knowledge of the mechanisms of human tumor progression expands, we will hopefully be able to employ *humanized* mouse models to study cancer biology and to develop novel anti-cancer therapies.

Abbreviations

ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
BM	Bone marrow
CSC	Cancer stem cell
FACS	Fluorescent activated cell sorting
HSC	Hematopoietic stem cell

D.A. Hess (✉)
Department of Physiology & Pharmacology, The University of Western Ontario, London, ON, Canada

Vascular Biology Group, Krembil Centre for Stem Cell Biology, Robarts Research Institute, London, ON, Canada
e-mail: dhess@robarts.ca

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HSPCs	Hematopoietic stem and progenitor cells
IHC	Immunohistochemistry
IL	Interleukin
IL-2Rγ	Interleukin-2 receptor common gamma chain
NK	Natural killer cell
NOD	Nonobese diabetic
NOD/SCID	Nonobese diabetic plus severe combined immunodeficient
SCID	Severe combined immunodeficient
SL-IC	SCID leukemia-initiating cells
SP	Side population
SRC	NOD/SCID repopulating cell
UCB	Umbilical cord blood
β2M	Beta-2-microglobullin

18.1 Introduction

Immunodeficient mouse xenograft models have been used extensively in recent years to establish the fundamentals of human stem cell biology. Classically, intravenously transplanted human hematopoietic stem cells (HSC) have been shown to home to the murine bone marrow (BM) and differentiate into multilineage progeny following isolation, purification, and/or ex vivo manipulation [1–7]. Starting in the early 1990s, John Dick and collaborators also identified human cells that can establish human myeloid leukemia in transplanted murine recipients [8–12]. These groundbreaking findings were made possible by the development of the first and second generation immune deficient murine hosts, namely the severe combined immune deficient (SCID) and nonobese diabetic (NOD) plus severe combined immune deficient (NOD/SCID) recipients. Throughout the 1990s, the NOD/SCID recipient served as the "gold standard" for the study of normal human hematopoiesis and leukemogenesis in vivo.

The number and quality of immune-deficient murine models for xenotransplanation has grown immensely over the past 10 years. The explosive growth in this field is due in part to the limitations of the NOD/SCID model and the lack of in vitro assays to model complex biological processes such as human hematopoiesis, the regeneration or repair of damaged organs and tissues, and tumor development. Recently, improved immunodeficient murine strains, such as the NOD/SCID beta2-microglobulin (β2M) null [13–15] and NOD/SCID IL-2 receptor common gamma chain (IL-2Rγ) null mice [16–20], have increased the survival and engraftment of human cells after transplantation. These novel strains permit extensive human cell engraftment without rejection due to reduced innate immunity (NOD mutation), complete T- and B-lymphocyte deficiency (SCID mutation), and reduced NK-cell function (β2M or IL-2Rγ mutation) [20]. These strains are commercially available and provide transplanted human cells the highest rate of survival in xenograft models developed to date. Although these strains do not represent an exhaustive list of the immunodeficient mice described in the literature, this chapter will focus on the recent progress

made with commonly-used human-into-mouse xenotransplantation models to study the biology of normal human stem cells and tumor initiating cells from both hematopoietic and solid tumors.

18.2 Mouse Models to Study Normal Human Stem Cells

Although large animal models exist for the study of human xenografts in utero [21–24] or for studying nonhuman primate stem cells [25–28], the use of the immune-deficient mouse as a recipient of human stem cell grafts has emerged as the most common and cost effective strategy. The first immune-deficient mouse characterized was the athymic nude mouse described by Isaason and Cattanach in 1962. Early transplantation studies with the T-lymphocyte-deficient nude mice were disappointing, as these mice failed to support the growth of transferred human hematopoietic BM stem cells [29]. Although the nude mouse has been used to study murine tumor growth due to its hairless phenotype, the immunodeficiency elicited in this strain is insufficient for xenotransplantation studies using human HSC and poorly permits human tumor establishment and metastasis unless highly aggressive and/or large numbers of tumor cells are implanted. Thus, to effectively study complex biological processes such as human hematopoiesis or tumor biology in vivo, a systematic progression of genetic modifications was required to develop the modern immunodeficient host mouse, and these are described later. Table 18.1 also provides a summary of the immunodeficient mouse strains commonly used for human stem cell transplantation studies, and contrasts the advantages and disadvantages of each model common to experimental use.

18.2.1 The Severe Combined Immunodeficient Mouse

The ability to study human hematopoiesis in a murine model was greatly facilitated by the discovery of mice with the severe combined immunodeficiency (SCID) mutation [30]. SCID mice have defects in T- and B-lymphocyte development due to a mutation in the gene for DNA-dependent protein kinase on chromosome 16 (*Prkdc*^{SCID}). DNA-dependent protein kinase is required for successful rearrangement of the T-cell receptor and immunoglobulin gene segments, leaving the mice unable to produce functional T- and B-lymphocytes. Because DNA-dependent protein kinase is a DNA-proofreading enzyme, the SCID mutation also confers extreme sensitivity to radiotherapy, due to the inability of sublethally irradiated cells to mediate repair of double stranded breaks. Nonetheless, the SCID mouse was the first mouse to support the survival of human neoplasms [31] and human hematopoietic cells [32, 33]. One of the drawbacks to using the SCID mouse was low-level human cell engraftment due to elevated natural killer (NK) cell activity and innate immunity conferred by the background CB17 strain. Therefore, this strain is acceptable for the implantation of solid fetal liver or thymus (SCID/hu model) or sections of tumors, but human peripheral blood and BM cell suspensions (hu-PBL-model) were rapidly recognized

Table 18.1 Summary of immune deficient mouse models for xenotransplantation

Strain	Mutations	Phenotype	Advantages	Disadvantages
CB17-SCID	• Spontaneous mutation in DNA-dependant protein kinase ($Prkdc^{scid}$)	• Defects in TCR and IgG rearrangement • No T- or B-cells • Radiosensitive	• First model to engraft human cells • Acceptable for solid tissue implantation	• High innate immunity and NK-cell function • Low engraftment of human HSC
NOD/SCID	• Spontaneous mutations (NOD genome) • Spontaneous mutation in DNA-dependant protein kinase	• Decreased NK-cells and innate immunity • No T- or B-cells • Radiosensitive	• Higher engraftment of human HSC • Gold standard (SRC model) • Commonly used	• Residual NK-cell activity and innate immunity • Reduced life-span (thymic lymphoma) • No human T-cell engraftment
NOD/SCID $B2M^{-/-}$	• Mutation of β2 microglobulin • Lack MHC class I expression	• Very low NK-cell function • No T- or B-cells • Radiosensitive	• Very low innate immunity • Engrafts committed progenitors and HSC	• Reduced life-span (thymic lymphoma) • No human T-cell engraftment • Poor breeders
NOD/SCID IL-$2R\gamma^{-/-}$	• NOD/SCID +	• No NK-cell function	• Highest acceptance of human cells	• T-cell deletion in mouse thymus
	• Mutation of IL-2R common γ chain (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21)	• Further defects in innate immunity • No T- or B-cells • Radiosensitive	• Long lifespan • Develop T-cells, erythrocytes and platelets	• Lack cytokines for the full development of human immune system
NOD/SCID MPSVII	• NOD/SCID + • Spontaneous mutation in beta-glucuronidase gene (GUSB)	• MPS VII disease (stunted growth, neurological defects, liver dysfunction) • Radiosensitive	• Single cell detection of human cells in tissues by GUSB IHC or flow cytometry	• Reduced life-span due to thymic lymphomas and MPSVII disease • No T-lymphocyte engraftment

as foreign and lysed by murine NK-cells. In addition, the SCID model does not support T-cell development in vivo, and transferred human T-cells become profoundly anergic due to incompatability with the murine thymus and a lack of human antigen-presenting cells [34]. Thus, enhanced human cell engraftment and models for the generation of human T-lymphocytes in immune-deficient mice were still sought.

18.2.2 The Nonobese Diabetic SCID Mouse (NOD/SCID)

To circumvent the high NK-cell activity inherent in the SCID mouse, Len Shultz and colleagues at the Jackson Laboratories (Bar Harbour, ME) crossed the SCID mutation onto various strains of inbred mice with known defects in innate immunity. The NOD mouse background, best known as a model of spontaneous autoimmune diabetes, possessed defects in macrophage and NK-cell functions [35, 36]. The result was the generation of the most prevalently used immunodeficient mouse strain, the nonobese diabetic SCID (NOD/SCID) mouse. The combined immunodeficiency produced an improved recipient with excellent capacity to engraft purified human stem cells for the study of hematopoietic development after transplantation. In addition, NOD/SCID mice were excellent breeders, with large and robust litters. However, these mice required housing in a clean barrier facility and required sterile animal handling to prevent untoward infections due to their extreme immune deficiencies.

The NOD/SCID model is by far the most widely used xenograft recipient to date. Sublethally irradiated (300–350 cGy) NOD/SCID mice have been extensively used to demonstrate multilineage human hematiopoietic reconstitution after the transplantation of human BM- or UCB-derived HSCs purified using cell surface markers such as CD34 [2, 3, 22, 37] and CD133 [38–40]; using conserved stem cell functions such as Hoescht dye efflux pumps (side population [SP] cells)[41, 42], and high aldehyde dehydrogenase (ALDH) activity; or a combination of both strategies [43]. Dick and collaborators were the first to identify a novel human hematopoietic stem or progenitor cell, termed the NOD/SCID repopulating cell (SRC), present in the $CD34^+CD38^-$ cell fraction, that was capable of multilineage repopulation in the BM of sublethally irradiated NOD/SCID mice [3, 6]. Using limiting dilution analysis and Poisson statistics, the frequency of this novel cell type was functionally defined in human UCB at 1 SRC in 617 $CD34^+CD38^-$ cells [3], indicating that the $CD34^+CD38^-$ cell population was functionally heterogeneous. The same group was the first to discover a new class of SRC capable of low level engraftment originated from the human $CD34^-$ cell fraction [2], supporting the dogma that a single transplanted murine $CD34^-$ cell could facilitate multilineage reconstitution in vivo [44]. Subsequently, Fujisaki et al. [45] confirmed these findings, and Goodell et al. [42] purified a SP of $CD34^-$ cells that excluded Hoescht 33342 dye and possessed reconstituting capacity. Nakamura et al. [46] demonstrated that $CD34^-$ cells were highly quiescent, and that prestimulation of cultured human $CD34^-$ cells with cytokines prior to transplantation induced CD34 expression and increased their capacity for homing and engraftment. Finally, Dao et al. [47] used serial transplantation to show

the reversibility of CD34 expression within the human stem and progenitor cell compartments.

However, in all these of these studies, differentiated human blood cell production was skewed to the B-lymphoid and myeloid compartments, as the NOD/SCID thymic microenvironment was not able to fully support T-lymphocyte development under normal transplant conditions in vivo [48]. The lack of T-cell specific engraftment is one of the major limitations of the traditional NOD/SCID model, and is potentially due to residual NK-cell activity and incompatibility with the hypertrophic murine thymus in NOD/SCID mice [49]. In addition to the lack of human T-lymphocyte development in vivo, another major drawback of the NOD/SCID model is their shortened lifespan (4–6 months) due to the presence of the *Emv-30* provirus integrated into the NOD genome, which can cause breakthrough of lethal thymic lymphomas in mice with SCID mutation [50]. The duration of experiments that can be performed using 8–10-week old NOD/SCID mice is therefore limited to 8–12 weeks posttransplantation, preventing proper analysis of long-term engraftment. Furthermore, unlike murine donor/murine recipient transplantation systems [44], engraftment and multilineage differentiation after injection of a single human HSC has never been achieved using xenograft models. Therefore, to address the ability of human cells to self-renew using the NOD/SCID model, retroviral or lentiviral marking of transplanted cell clones, the retrieval of human cells from primary recipients, and clonal analysis of short-term vs. long-term hematopoietic propagation after serial transplantation in secondary and tertiary recipients is required [51–53].

18.2.3 The NOD/SCID Beta2 Migroglobulin Null Mouse (NOD/SCID β2M)

Second generation immunodeficient mice have recently been extended from the NOD/SCID background to circumvent the residual NK-cell function, the lack of T-cell engraftment, and the shortened lifespan characteristic of the NOD/SCID strain. The first strain developed was the NOD/SCID β2 microglobulin null mouse (NOD/SCID β2M) characterized in 1997 [54]. These mice lacked MHC class I expression on hematopoietic effector cells due to the targeted deletion of the β2M gene, demonstrated virtually no NK-cell function, and were very permissive for xenograft acceptance [15, 55]. Committed human hematopoietic progenitors ($CD34^+CD38^+$ cells) as well as primitive HSCs ($CD34^+CD38^-$ cells) can engraft NOD/SCID β2M mice [14, 56]. Although early reports described the survival of CD4+ T-cells after adoptive transfer of human peripheral blood mononuclear cells (PBMC) [54], T-lymphocyte development from uncommitted human progenitor cells in NOD/SCID β2M null mice remains inconsistent. Unfortunately, the NOD/SCID β2M null strain has a lifespan that is even shorter than the parental NOD/SCID, potentially due to the duplication of the *Emv30* provirus to additional chromosomes, or perhaps due to more profound NK-cell deficiency. In our experience with this strain, breeding can be problematic because female mice can die from thymomas prior to weaning

of the first or second litters, and pups may require fostering to more stable immune competent mothers. Until the recent development of the NOD/SCID IL-2Rγ null mice, NOD/SCID β2M null mice provided the highest and most reproducible human cell engraftment, but were still limited by the lack of functional T-cell development and severely shortened lifespan.

18.2.4 The NOD/SCID IL-2 Receptor γ Null Mouse (NOD/SCID IL-2Rγ)

The long-standing hurdles of residual NK-cell activity and shortened life-span were finally overcome by the recent production of two related and highly immunodeficient mouse strains: the NOD/Shi-SCID IL-2Rγ null mouse developed in Japan [57], and the NOD/SCID IL-2Rγ null mouse developed at the Jackson Laboratories, USA [20]. Using different NOD/SCID inbred strains as background, these groups used targeted mutation of the IL-2Rγ chain locus, resulting in a truncated version [57] or complete absence of the IL-2R common γ chain protein [58]. The IL-2Rγ chain is a common component of the receptors for multiple cytokines, and is therefore required for high-affinity signaling via IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptor complexes [59]. The lack of functional receptors for the pleiotropic cytokines results in severe impairments in innate immune cell function, and completely prevents T- and B-lymphocyte as well as NK-cell development. Interestingly, the NOD/SCID IL-2Rγ null mouse does not develop thymic lymphomas common to the parental NOD/SCID strain, suggesting cytokine dependence on IL-2R common γ chain signaling in the development of *Emv30* lymphomas. Thus, the NOD/SCID IL-2Rγ null mouse permits reproducible and increased human cell engraftment using smaller numbers of HSCs [20].

NOD/SCID IL-2Rγ null mice have also demonstrated the differentiation of $CD34^+$ stem cells into a complete human immune system, including the generation of platelets and erythrocytes [20], and human T-cell populations [58, 60, 61]. Recently, McDermott et al. compared the overall engraftment and multilineage differentiation of near-limiting doses of lineage-depleted human UCB by direct intrafemoral injection into the parental NOD/SCID mouse or into the NOD/SCID IL-2Rγ null strain. Indeed, the NOD/SCID IL-2Rγ null mice generated moderately higher engraftment levels in the murine BM and improved engraftment in peripheral tissues such as the spleen and T-cells in the thymus. Overall, the NOD/SCID IL-2Rγ null mouse was 3.6-fold more sensitive in detecting human repopulating cells compared with the NOD/SCID strain. Interestingly, the NOD/SCID IL-2Rγ null females exhibited higher engraftment at limiting cell doses [62]. Given the advancements from the parental NOD/SCID strain, there is little doubt that the NOD/SCID IL-2Rγ null mouse will replace the NOD/SCID as the “gold standard” for normal human hematopoietic and cancer stem cell (CSC) research.

The ultimate goal of achieving a xenotransplanted mouse with a fully functional human immune system remains a work in progress. Humanized NOD/SCID IL-2Rγ null mice do not possess fully functional human immune systems with defects in

T-lymphocyte survival and function due to aberrant positive and negative selection in the murine thymus, and a lack of human-specific cytokine stimulation and adhesion molecules resulting in decreased T-cell-dependent antibody responses [58, 63]. Although solutions to these constraints are currently under active investigation [64], humanized mice with or without adoptively transferred human tumor burden represent great promise as future models for preclinical testing of drug- or cell-based therapies prior to advancement into clinical trials.

18.2.5 The NOD/SCID Mucopolysaccharidosis Type VII Mouse (NOD/SCID MPSVII)

Traditional murine xenograft models do not permit easy detection of nonhematopoietic progenitor cells in solid tissues. To overcome this issue, Mark Sands and colleagues have backcrossed the NOD/SCID mouse with the mucopolysacharidosis type VII (MPSVII) mouse [65]. MPSVII is a rare but lethal lysosomal storage disease in humans caused by a deficiency in β-glucuronidase activity, a ubiquitous enzyme found in all human cell types including malignant cells. Thus, the NOD/SCID MPSVII mouse is an immune deficient recipient where transplanted human cells can be efficiently tracked using a colorimetric or fluorescent substrate for GUSB [66]. Individual transplanted cells stand out vividly against the background and GUSB negative murine tissues. This model also enables analysis of cell interactions in situ and detection of cell surface marker coexpression by fluorescence activated cell sorting (FACS) or immunohistochemistry (IHC). We have used this model to document previously unrecognized distribution of nonhematopoietic ($CD45^-$) cell types engrafted in multiple murine tissues after intravenous transplantation of human umbilical cord blood (UCB) ALDH-expressing cells [66]. More recently, we have employed the NOD/SCID MPSVII mouse to track tumor progression and metastasis of single cells to the lung after fat pad injection of ALDH-expressing, tumor-initiating human breast cancer cells [67].

18.3 Models to Study Cancer Stem Cells

A controversial issue in cancer research is the identification of cell types capable of initiating and sustaining the growth of tumors in vivo. Solving this controversy depends on determining whether every cell within a neoplasm is capable of initiating and sustaining tumor growth, or whether only an infrequent subset of cells (so-called “cancer stem cells”) is responsible for maintenance of the tumor. Indeed, the existence of CSCs and the CSC hypothesis was proposed almost 50 years ago [68], and provided a potential explanation for the origin of tumors within humans. Unfortunately, this field had to await the development of modern immune deficient mice to investigate the behavior of human tumor-initiating cells in vivo. It is not surprising that the best evidence supporting the existence of human CSCs has originated from the study of hematologic malignancies [9–12, 69, 70] and various solid tumors [71–75] using NOD/SCID mice. However, recent reports describing increased

tumor-initiation in leukemia [16], and tumor formation by a single human melanoma cell in NOD/SCID IL-2Rγ null mice [76], have rekindled controversy surrounding the CSC hypothesis.

18.3.1 Cancer Stem Cells in Hematopoietic Malignancies

Seminal studies using serial transplantation of human acute myeloid leukemia (AML) cells into SCID [11], or NOD/SCID mice [10], contributed to the hypothesis that only rare cells, termed SCID leukemia-initiating cells (SL-IC), were capable of tumor initiation, maintenance, and self-renewal. In addition, SL-IC could be prospectively purified by selection of $CD34^{+}CD38^{-}$ cells from the AML blast population, and like the same phenotype in normal HSC, the $CD34+CD38^{-}$ cells were the only cells capable of regrowing leukemia in recipient mice [10]. These studies were the first in a series from several groups that compared normal and malignant hematopoiesis [77–80] and ruled out the stochastic possibility that each tumor cell has a low but equal probability of forming new tumors. In contrast, AML could be organized as a hierarchy of distinct, functionally heterogenous cells, most of which have a limited proliferative potential, and only a small subset of which have the ability to initiate and sustain new tumor growth [81]. These studies provided the first direct evidence for the CSC hypothesis. Recently, lentiviral gene marking to track the clonal function of SL-IC following serial transplantation into NOD/SCID mice has shown distinct heterogeneity in the ability to propagate cancer in secondary and tertiary recipients, suggesting the existence of distinct classes of SL-IC with differing self-renewal capacity [8, 12]. Thus, the hierarchal nature of human AML has been exquisitely established using the NOD/SCID model.

The implications of the CSC hypothesis are critical for the design of future treatments for AML and other hematopoietic malignancies. Several studies have shown that in contrast to leukemic blasts studied in vitro, the SL-IC are highly quiescent [82–84], making them difficult to target with traditional chemotherapeutic agents that target highly proliferative blasts. Therefore, survival and subsequent proliferative activation of quiescent leukemic stem cells may explain the rate of relapse associated with AML. Although putative leukemic stem cells share many characteristics with normal HSC, the recent discovery of leukemia specific stem cell markers, such as the IL-3 receptor α chain (CD123) [80], may allow for prospective isolation and elucidation of their unique properties as therapeutic targets. Furthermore, agents and therapeutic approaches can be tested for proof-of-principal in humanized leukemic mice, providing insights into leukemia progression potentially relevant to other human cancers.

18.3.2 Cancer Stem Cells in Solid Tumors

Many of the properties and concepts of CSCs first described in human leukemia have recently been extended to other cancer cell types including solid tumors of the breast [74], brain [72, 73], pancreas [75], and large intestine [71]. Interestingly,

all of these initial discoveries were assayed using the NOD/SCID mouse recipient. Human breast cancer is one of the best-studied examples. Similar to studies with AML, primary breast cancer tumor initiating cells were found to be rare and distinct from the bulk population by the unique $CD44^{+}CD24^{-}$ cell phenotype [74]. Based on the pioneering biochemical work by Sladek and colleagues in the 1990s [85–88], Ginestier et al. used ALDH-activity to identify normal and tumorigenic mammary epithelial cells capable of self-renewal and of generating tumors that recapitulate the heterogeneity of the parental tumor [89]. More recently, we have employed the NOD/SCID MPSVII mouse to track tumor progression and demonstrate enhanced metastastatic ability of ALDH-expressing $CD44^{+}$ tumor-initiating human breast cancer cells [67]. We are currently using this novel model to study angiogenic processes in breast cancer tumor vascularization. Future studies using the NOD/SCID IL-2Rγ null mouse will undoubtedly uncover more detailed information aiding in our understanding of tumorigenesis all the way from initiation to metastasis.

Not all solid tumor subtypes demonstrate the characteristics of CSC hypothesis. In a recent study, Morrison and colleagues have shown that the injection of unfractionated melanoma cells with matrigel scaffold support into the NOD/SCID IL-2Rγ null mouse can increase the detection of tumorigenic melanoma cells in vivo. In fact, single cell transplantation using this model system resulted in tumor establishment in 27% of recipients, demonstrating that not all tumor initiating cells were common in some solid tumors, and that the transplant conditions and immune deficient host greatly influence tumor-initiating cell frequency [76]. Furthermore, the phenotypic heterogeneity between tumorigenic melanoma cells may not be hierarchal in nature [90]. Nonetheless, it is clear that the NOD/SCID IL-2Rγ null should replace the NOD/SCID mouse as the model of choice for human tumor initiating studies.

18.4 Conclusions and Future Perspectives

Based on the sequential improvements in immune deficient animal models, and due to advances in stem cell isolation and propagation technologies, our knowledge surrounding normal and malignant stem cells has grown immensely in recent years. In human AML and perhaps certain types of solid cancers, rare tumor-initiating cells or CSCs are biologically distinct from the bulk of a heterogenous tumor. Current treatment regimes for these diseases rely on the inaccurate principle that tumors are homogeneous mixtures of cells with aberrant proliferative potential. As our understanding of tumor biology advances, new therapies must be designed to target these rare cancer initiating cells as well as proliferative progenitors to effectively eradicate and prevent disease relapse. There is no doubt that proof-of-principal testing of novel therapeutic stategies will benefit from preclinical studies using humanized immune deficient mice as a recipient prior to clinical trials.

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Part V
Clinical and Therapeutic Implications of Cancer Stem Cells

Chapter 19
Cancer Stem Cells and Disease Prognosis

Zeshaan A. Rasheed, Jeanne Kowalski, and William H. Matsui

Abstract Cancer stem cells (CSCs) represent distinct tumor cells defined by their capacity for tumor formation, self-renewal, and differentiation. In addition, the CSC hypothesis has been extended to suggest that specific tumor cell populations are also responsible for distinct clinical scenarios such as initial tumor formation, disease relapse following initial therapy, and cancer progression including the transformation of indolent to aggressive disease in hematologic malignancies and the development of metastatic disease in solid tumors. However, several questions regarding CSCs remain the subject of intense debate, including their actual clinical relevance and/or whether the eradication of CSCs will actually improve patient outcomes. In this chapter, we will review strategies to identify CSCs and evidence that they play a role in disease prognosis, progression, and therapeutic resistance; as well as discuss potential barriers in designing and interpreting clinical trials studying CSC targeting therapies.

Abbreviations

ABCG	ATP-binding cassette subfamily G
ALDH	Aldehyde dehydrogenase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BCR-ABL	Breakpoint cluster region-abelson
CD	Cluster of differentiation

W.H. Matsui (✉)
The Sidney Kimmel Comprehensive Cancer Center,
Johns Hopkins University School of Medicine,
Baltimore, MD, USA
e-mail: matsuwi@jhmi.edu

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CML	Chronic myeloid leukemia
CR	Complete response
CSC	Cancer stem cell
CXCR4	Chemokine receptor 4
EMT	Epithelial-to-mesenchymal transition
HER2	Human epidermal growth factor receptor 2
IGS	Invasiveness gene signature
IHC	Immunohistochemistry
NHL	Non-Hodgkin's lymphoma
OS	Overall survival
RECIST	Response evaluation criteria in solid tumors
SCID	Severe combined immune deficiency
TGF-β	Transforming growth factor beta
TIC	Tumor-initiating cells

19.1 Introduction

Cancer stem cells (CSCs) represent distinct tumor cells defined by their capacity for tumor formation, self-renewal, and differentiation [1]. The CSC concept dates back several decades and was initially proposed to explain observations that only a minority of malignant cells from both hematologic malignancies and solid tumors are tumorigenic [2–4]. In the early 1990s, several groups subsequently demonstrated that phenotypically primitive hematopoietic cells in chronic (CML) and acute myeloid leukemias (AML) were capable of propagating disease in vitro in long-term cultures or in vivo in severe combined immuno deficiency (SCID) mice [5–10]. These important studies established that tumorigenic potential was restricted to distinct cells within an individual tumor. Moreover, these unique functional properties have suggested that the development of strategies capable of inhibiting CSCs may ultimately improve clinical outcomes.

The CSC hypothesis has been extended to suggest that specific tumor cell populations are also responsible for distinct clinical scenarios, such as initial tumor formation, disease relapse following initial therapy, and cancer progression including the transformation of indolent to aggressive disease in hematologic malignancies and the development of metastatic disease in solid tumors. However, several questions regarding CSCs remain the subject of intense debate, including their actual clinical relevance and/or whether the eradication of CSCs will actually improve patient outcomes. In this chapter, we will initially review strategies to identify CSCs and evidence that they play a role in disease progression and therapeutic resistance. We then discuss how the CSC hypothesis may potentially explain why most cancers remain incurable despite numerous advances in anticancer treatments. Finally, we will review studies that provide the initial clinical evidence that CSCs may have prognostic value and discuss potential barriers in designing and interpreting clinical trials studying CSC targeting therapies.

19.2 CSC Identification Strategies and Complexity

The fixed relationship between cancer cell phenotype and function was initially demonstrated through studies in which phenotypically distinct cells were prospectively isolated and then found to give rise to tumor growth in the ectopic setting. Several strategies have been used to identify phenotypic markers capable of discriminating CSCs from nonclonogenic tumor cells. In some diseases, CSCs share markers with normal stem cells and/or progenitors from the corresponding tissue. For example, in AML and CML, leukemia-initiating cells phenotypically resemble normal hematopoietic stem cells [5, 11], and in several types of brain tumors, CSCs express surface antigens that characterize normal neural stem cells [12, 13]. In multiple myeloma, malignant plasma cells constitute the tumor bulk, but CSCs have been found to resemble normal memory B cells that represent a self-renewing compartment in the humoral immune system [14]. Unfortunately, specific surface antigens marking stem cells have not been identified in most normal organs; thus, this strategy cannot be applied to many cancers. The prospective isolation of CSCs from all tumor types has been difficult because of the lack of a universal CSC marker. However, the cell surface antigens CD44 and CD133 have been used to identify CSCs from a number of unrelated malignancies [15–21], suggesting that CSCs from different organs may express shared phenotypes.

The understanding of CSC biology has been further complicated by the identification of multiple CSC phenotypes for a specific tumor type, such as $CD44^{+}CD24^{+}$, $CD133^{+}$, and aldehyde dehydrogenase ($ALDH^{+}$) cells in pancreatic cancer [17, 18, 22]; $CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ cells in breast cancer [15, 23]; $CD133^{+}$ and $SSEA\text{-}1^{+}$ cells in glioblastoma [12, 24]; and $CD20^{+}$, $ABCB5^{+}$, $CD271^{+}$ in melanoma [25–27]. The tumor-initiating cell (TIC) frequency in each of these different populations is markedly higher compared with bulk tumor cells [28], but little is known about how these populations are related to one another. Furthermore, it is unclear whether each CSC population has distinct functional features (such as migratory or invasive potential) in addition to their defining tumor initiating capacity (see later).

19.3 Role of CSCs in Disease Relapse and Progression

CSCs were initially identified through their enhanced tumorigenicity, but growing evidence suggests that CSCs have other functional capabilities that also dictate clinical outcomes. Disease relapse implies that clonogenic CSCs capable of tumor regrowth are relatively drug resistant, and several studies have begun to elucidate the mechanisms involved in this process. In multiple myeloma, CSCs have been found to express several intrinsic properties that promote the resistance of normal stem cells to toxic injury [29]. These include increased expression of membrane-bound drug transporters and intracellular detoxification enzymes that mediate drug

efflux and metabolism, respectively. In addition, CSCs in mantle cell non-Hodgkin's lymphoma (NHL) have been found to be relatively quiescent, and this property may promote drug resistance to cytotoxic agents that are dependent on cell cycle progression for their activity or by decreasing the expression of proteins or pathways inhibited by targeted therapies [29, 30]. In glioblastoma and breast cancer, CSCs have been found to be relatively radioresistant due to the increased activity of DNA repair pathways compared with the tumor bulk [31, 32]. In addition to these in vitro studies, CSCs have been found to be relatively resistant in vivo. Two studies using mouse xenograft models of colorectal and pancreatic cancer showed that tumors are enriched in CSCs following conventional chemotherapy, suggesting that they are relatively drug resistant in vivo [33, 34]. In a clinical study of patients with breast cancer, both the frequency of CSCs and the clonogenic growth potential of tumors were increased after treatment with conventional chemotherapy [35]. Therefore, CSCs have been found to be relatively resistant compared with bulk tumor cells in vitro, in vivo, and in the clinical setting.

CSCs have also been implicated as mediators of disease progression. In pancreatic adenocarcinoma, a subset of CSCs expressing chemokine receptor 4 (CXCR4) were found to be more invasive and mediated metastasis formation in an animal model [17]. In another study, $ALDH^+$ pancreatic CSCs were more frequently identified in metastatic lesions compared with matched primary tumors and were more invasive in vitro [22]. Likewise, $ALDH^+$ prostate CSCs were more frequently found in bone metastases and more invasive in an animal model [36]. A number of studies have recently identified a relationship between CSCs and the epithelial-to-mesenchymal transition (EMT), a proposed mediator of metastatic disease [37]. The link between EMT and CSCs was initially suggested by studies demonstrating that the ectopic expression of the Snail or Twist transcription factors in immortalized mammary epithelial cells induced EMT as well as the capacity to form mammospheres in vitro or tumors in vivo [38]. In pancreatic cancer, studies have similarly found an association between chemoresistance, the mesenchymal phenotype, and CSCs [39]. The link between CSCs and EMT has been further strengthened by multiple findings showing that a number of factors, including TWIST1, ZEB1, ZEB2, the transforming growth factor beta (TGF-β) pathway, and microRNAs are able to regulate both EMT and CSC function [40–42]. CSCs have also been implicated in the progression of hematologic malignancies. During the transition from chronic phase to blast crisis in CML, the CSC phenotype may change as clonogenic precursors in blast crisis express myeloid markers compared to chronic phase leukemic stem cells that lack markers of lineage commitment [43]. Moreover, this transition may be mediated by the activation of specific cellular pathways (such as Wnt and Notch signaling) that confer self-renewal on a previously self-limited cell population [43, 44]. Therefore, the biology of CSCs is likely to be considerably more complex than simply mediating tumor formation and growth and may be dictated by the precise clinical situation studied.

19.4 Clinical Response Does Not Always Correlate with Survival, a Clinical Paradox that May Be Explained by CSCs

The functional studies described earlier suggest that the CSC hypothesis has major implications for basic tumor biology, but it may also provide an explanation for several dilemmas in clinical oncology. The ultimate goal of anticancer therapy is to improve overall survival (OS) across patients with a specific disease. However, in individual patients, the efficacy of a specific therapy is usually judged by monitoring changes in tumor burden, and in several instances disease response has little impact on OS. Three clinical scenarios in which the paradox between tumor response and OS is evident are disease relapse months to years after achieving an initial complete response (CR) (e.g., small cell lung cancer); a failure of immediate treatment to improve survival compared with patients receiving delayed therapy (e.g., indolent NHL); and similar survival rates despite improved response rates for patients treated with high-dose chemotherapy (e.g., multiple myeloma and breast cancer). Studies utilizing syngeneic transplants of murine cancers [2, 45–49] and xenotransplantation of human tumors in immunocompromised mice [25, 28, 50] have shown that CSCs are rare in some diseases, but this may not be universally so in others, such as malignant melanoma. However, it is possible that each of these clinical situations can be explained by the rarity of CSCs since disease response primarily reflects short-term changes in bulk tumor cells, whereas long-term outcomes such as disease relapse and progression may be dictated by resistant and clonogenic CSCs.

Small cell lung cancer is an excellent example in which relapse almost uniformly occurs after achieving a CR. Thirty to 40% of patients with limited- and extensive-stage disease achieve a CR after chemotherapy with or without radiation, but 2-year OS rates are only 40 and 5% for patients with limited-stage and extensive-stage disease, respectively [51]. Likewise, adults with precursor B cell acute lymphoblastic leukemia (ALL) have CR rates of 60–80%, but the 2-year survival is only 35–40% [52]. These findings are not limited to treatments with standard cytotoxic agents, as the treatment of chronic myeloid leukemia (CML) with the Breakpoint cluster region-abelson (BCR-ABL) tyrosine kinase inhibitor imatinib produces 5-year complete cytogenetic response rates of 87% [53], but it does not appear that these responses are durable as relapse occurs in practically all patients when the drug is discontinued [54, 55].

The diagnosis of cancer leads to immediate treatment in most patients. However, in patients with advanced stage indolent follicular NHL, long-term studies have compared immediate treatment with chemotherapy or radiation to delaying treatment until symptoms develop. Even in patients achieving a CR with early treatment, OS rates are equivalent between the two groups [56]. Therefore, a watchful waiting approach is preferred in these patients since the median time to treatment is 2–3 years, but one-third of patients never required therapy (half died of other causes and half remained progression free after 10 years) [56, 57].

Dose intensification can improve response rates in most diseases including the plasma cell malignancy multiple myeloma. Autologous stem cell transplantation can dramatically improve CR rates, but it is unclear whether this approach definitively improves OS, as clinical trials comparing transplantation to standard chemotherapy have produced conflicting results [58]. In patients with high-risk AML based upon the presence of complex cytogenetics, the use of dose-intensive chemotherapeutic regimens can produce better CR rates but minimally impact OS [59]. Likewise, when compared with conventional doses of chemotherapy, high-dose chemotherapy followed by autologous stem cell transplantation soon after a complete or partial remission with conventional chemotherapy does not improve survival in women with metastatic breast cancer [60].

19.5 CSCs Can Be Used as Biomarkers to Predict Patient Outcomes

These scenarios suggest that the inhibition of CSCs will improve long-term outcomes, including OS. However, they fail to provide actual proof that the CSC hypothesis is clinically relevant. To demonstrate that CSCs are clinically important, the ideal study would completely eradicate CSC function that results in improved long-term clinical outcomes, but such studies have yet to be completed. Alternatively, if CSCs truly drive the natural history of tumors, including disease relapse or progression, then the quantification of their frequency or functional capabilities should correlate with clinical outcomes. Accordingly, CSCs may serve as biomarkers to predict long-term clinical outcomes including OS. A number of studies have addressed this possibility and can be broadly categorized as follows: (1) those correlating the frequency of CSCs in pathologic specimens with clinical outcomes; (2) those developing a prognostic gene expression profile based on the isolation of CSCs; and (3) those identifying a prognostic "stem-like" gene expression profile of the bulk tumor population.

Correlation of patient outcomes with the frequency of CSCs has been examined in a number of malignancies using immunohistochemistry (IHC) or functional assays. Abraham et al. used IHC to stain for $CD44^{+}CD24^{-/low}$ cells and found that breast tumors with a high percentage of CSCs were associated with higher rates of distant metastases, although there was no correlation with OS [61]. The frequency of $CD133^{+}$ CSCs assessed by IHC in glioblastoma was also associated with worse progression-free and OS [62]. IHC analysis of ALDH has also been performed in a number of malignances and found to be associated with worse clinical outcomes in patients with breast carcinomas, prostate cancer, pancreatic adenocarcinoma, and AML [22, 23, 63–65].

Functional quantification of CSC activity has also been correlated with disease prognosis. The engraftment of AML in immunocompromised mice has been found to correlate with OS [66]. Similarly, the in vitro quantification of neurosphere formation and in vivo tumor formation in mice has been found to correlate with patient

outcomes [67]. Interestingly, neurosphere formation correlated with worse OS and earlier time to tumor progression, whereas xenograft formation correlated solely with earlier time to tumor progression. Therefore, it is likely that the specific assay itself will be an important factor in predicting outcomes based on CSCs.

The initial study examining the relationship between gene expression of isolated CSCs and patient outcomes was carried out in breast cancer. Here, Liu et al. compared the gene expression profiles of $CD44^{+}CD24^{-/low}$ breast cancer cells and normal breast epithelium [68]. This resulted in the generation of a 186-gene "invasiveness" gene signature (IGS) that was significantly associated with worse overall and metastasis-free survival in patients with localized disease. Interestingly, the IGS was also associated with worse prognosis in patients with medulloblastoma, lung cancer, and prostate cancer. Shipitsin et al. also used a microarray strategy to compare the gene expression of $CD44^{+}CD24^{-/low}$ and $CD44^{-}CD24^{+}$ cells from normal and malignant breast cells [69]. They found that tumors sharing similar gene expression patterns to $CD44^{+}CD24^{-/low}$ cells were associated with worse clinical outcomes. In another study Stevenson et al. used lung adenocarcinoma cells to develop an "embryonic stemness" gene signature that also correlated with OS and resistance to cisplatin [70]. Together these studies indicate that the expression of genes indicative of CSCs may be able to predict clinical outcomes, either because CSCs are increased in these tumors or the overall tumor takes on CSC characteristics.

19.6 Development of CSC Targeting Strategies

CSCs may be resistant to conventional therapies; however, several cellular pathways, such as Hedgehog and Notch signaling, that are required for normal embryonic development have been found to regulate the function and maintenance of CSCs [44, 71, 72]. A number of preclinical studies have identified novel agents capable of targeting these pathways and abolishing the function of CSCs in pancreatic adenocarcinoma, breast cancer, glioblastoma, and some B cell malignancies [30, 34, 73–76]. Several clinical trials based on these preclinical findings are now underway, and the improvement in clinical outcomes, especially long-term endpoints such as OS, may provide the most definitive proof that the CSC hypothesis is clinically relevant.

19.6.1 Designing and Interpreting Clinical Trials to Evaluate CSC Targeting Therapies

Although results from clinical trials explicitly targeting CSCs are not yet available, several clinical experiences may provide insights into the potential outcomes and responses observed. In breast cancer, HER2/*neu* has been found to play a role in CSC

self-renewal [35, 77], and a study examining lapatinib, a tyrosine kinase inhibitor of HER2/*neu* signaling, has been found to increase OS without significantly improving response rates [78]. Therefore, it is likely that changes in tumor bulk will be inadequate endpoints in CSC targeting trials. In CML, tyrosine kinase inhibitors such as imatinib have emerged as the standard of care due to their relative safety and ability to produce rapid disease responses [53], but prior to the introduction of these agents, most patients were treated with alpha-interferon. Interestingly, responses to interferon are typically slow with a median time to the best response of nearly 2 years [79]. However, CRs achieved by interferon may be durable following discontinuation of the drug, in stark contrast to imatinib [55, 79]. Laboratory studies have provided an explanation for these findings as interferon can eradicate CML CSCs, whereas imatinib primarily inhibits differentiated tumor cells and progenitors [79–81].

Several endpoints are available to measure efficacy in clinical trials, including tumor response rate (i.e., tumor regression based on Response Evaluation Criteria in Solid Tumors [RECIST]), progression-free survival, metastasis-free survival, relapse-free survival, and OS. The ultimate goal of drug development is to improve OS, but undertaking trials that measure this endpoint are not always feasible because of the large sample sizes and long periods of follow-up that are required. Therefore, Phase II trials in oncology have traditionally relied on short-term measurements of tumor response that are meant to act as surrogates for long-term patient outcomes. Since CSCs represent a minority of all tumor cells in most diseases studied thus far, a major challenge in these CSC targeting trials is the ability to detect efficacy against cells that represent a small proportion of the total tumor burden. However, the use of endpoints that reflect the functional capabilities of CSCs should be used. For example, a potential clinical trial could first “debulk” patients with conventional therapy then examine the activity of a CSC-targeting agent by measuring time to relapse and OS.

Since CSCs may have other properties besides tumorigenic potential, their specific functions must also be considered to ensure that CSC targeting therapies are studied in the proper clinical context using the appropriate endpoints. If the primary function of a particular CSC is to mediate metastasis, then the optimal clinical scenario to test a CSC targeting agent might be when a patient presents with localized (non-metastatic) disease, following induction therapy with conventional chemotherapy and/or radiotherapy, or in the context of adjuvant therapy. In these settings, it would be most appropriate to measure relapse-free or metastasis-free survival in addition to OS. Alternatively, if the predominant property of a particular CSC is therapeutic resistance, then a novel anti-CSC therapy might be studied in combination with a known cytoreductive therapy. In this setting, tumor response rates may be appropriate primary endpoints along with progression free survival.

Another important consideration in developing clinical trials is to identify surrogate endpoints capable of detecting the inhibition or reduction of CSCs early in the course of treatment, since these may be informative in early phase trials inadequately powered to evaluate survival. Several novel functional assays have been developed during the efforts to identify and characterize CSCs, and it is possible that these assays can act as surrogates within CSC targeting clinical trials. Huff et al. have

demonstrated that the serial measurement of in vitro clonogenic tumor growth from patients with multiple myeloma correlates with response to therapy and predicted clinical relapse [82]. Therefore, serial functional assessment of CSCs may provide a dynamic endpoint to monitor CSC-based clinical trials.

19.7 Conclusions

The ever-increasing number of reports regarding CSCs has significantly increased the complexity of our understanding of both their basic and clinical biology. In addition to their defining characteristic of tumorigenicity, a number of properties such as drug resistance and migratory and invasive potential are now attributed to CSCs that suggest a primary role in disease relapse and progression. Although these findings have driven attempts to develop novel therapies, they fail to prove that the CSC hypothesis is clinically relevant. An observed improvement in OS resulting from the documented inhibition of CSCs would be the most definitive means of demonstrating that CSCs are clinically important, but challenges exist in identifying the proper endpoints to clinically assess therapies targeting a minority (in most cancers) of tumor cells. Therefore, the development of novel clinical trial designs and biomarker strategies is likely to be just as (or even more) important than the development of the drugs themselves.

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Chapter 20
Mechanisms of Radioresistance in Cancer Stem Cells

Cleo Y-F Lee and Maximilian Diehn

Abstract Cancer stem cells (CSCs) have recently been identified and characterized in many types of solid tumors and may contribute to treatment failure since they have been shown to be relatively resistant to conventional therapies. Recent data suggest that both intrinsic and extrinsic determinants confer radioresistance to CSCs through a variety of mechanisms including high DNA repair capabilities, lower cellular reactive oxygen species (ROS) levels, induced autophagy, activation of survival signaling pathways, and the influence of the microenvironment in hypoxic regions of tumors. In this chapter, we review long-established mechanisms for tumor radioresistance and describe recent findings indicating that CSCs may also contribute to treatment failure following radiotherapy. In addition, we examine the mechanisms that appear to govern radioresistance in CSCs and discuss potential approaches to overcoming them.

Abbreviations

ALDH	Aldehyde dehydrogenase
AML	Acute myelogenous leukemia
ATM	Ataxia telangiectasia mutated
CSC	Cancer stem cell
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
GSH	Glutathione

M. Diehn (✉)
Stanford Cancer Center, CA, USA

Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford, CA, USA

Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA, USA
e-mail: diehn@stanford.edu

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GSI	Gamma-secretase
HIF	Hypoxia-inducible factor
HR	Homologous recombination
hTERT	Human telomerase reverse transcriptase
Lin	Lineage
LSC	Leukemia stem cell
MMTV	Murine mammary tumor virus
mRNA	Messenger ribonucleic acid
NHEJ	Nonhomologous end-joining
NICD	Notch intracellular domain
NOD	Nonobese diabetes
NTC	Nontumorigenic cell
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
shRNA	Short hairpin ribonucleic acid
TSC	Tumor stem-like cell
Wnt	Wingless type
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4

20.1 The Cancer Stem Cell Theory

In recent years, application of the principles and techniques of normal stem cell biology to the study of cancer has led to the identification and isolation of cancer stem cells (CSCs) from many human malignances. CSCs have the capacity to both proliferate indefinitely (self-renew) and to give rise to phenotypically distinct daughter cells that are unable to form new tumors (i.e., nontumorigenic cells [NTCs]) (Fig. 20.1a). In many tumors, CSCs appear to make up a minority subpopulation of cancer cells, although this is not an absolute requirement of the CSC hypothesis [1]. The existence of CSCs has critical implications for cancer therapy; hence, elimination of CSCs is likely critical for complete disease eradication (Fig. 20.1b).

CSCs were first identified and characterized in hematopoietic malignancies [2, 3]. In acute myelogenous leukemia (AML), only the $CD34^{+}CD38^{-}$ leukemia stem cell (LSC) subpopoulation can lead to the engraftment of human leukemia in immunodeficient mice, whereas the $CD34^{-}CD38^{+}$ population, which represents the majority of leukemic blasts, is unable to transplant the disease [3, 4]. Subsequently, CSCs were discovered in many solid tumors including cancers of the brain [5], breast [6], head and neck [7], liver [8], pancreas [9], colon [10], bladder [11], and prostate [12]. CSCs in solid tumors were first documented in human breast cancer, where the $CD44^{+}CD24^{-/low}Lineage^{-}$ subpopulation was shown to be able to reconstitute human breast cancer in immunocompromised mice. Importantly, the resulting xenografts recapitulated the morphology and phenotypic heterogeneity of the parental tumor [6]. As few as 100 $CD44^{+}CD24^{-/low}Lin^{-}$ cells were able to cause tumor formation

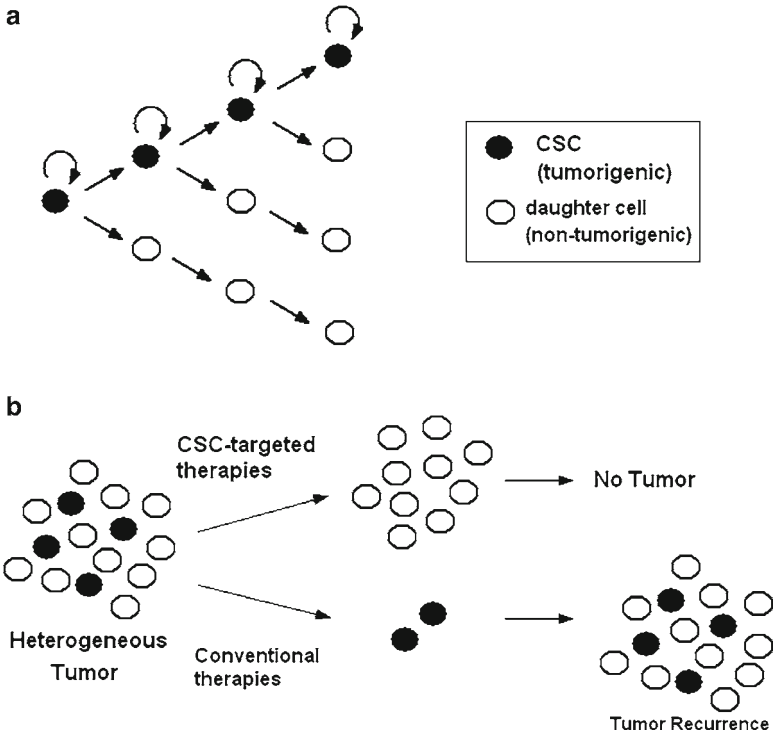

Fig. 20.1 The cancer stem cell hypothesis and its implications for therapy. (**a**) The cancer stem cell hypothesis argues that a specific subpopulation of tumor cells, called cancer stem cells (CSCs), are the only tumor cells that can self-renew and proliferate indefinitely. CSCs can also give rise to more differentiated and phenotypically distinct daughter cells, often called nontumorigenic cancer cells (NTCs), which can only undergo limited divisions and therefore cannot form new tumors. (**b**) CSCs in many tumors appear to preferentially survive conventional therapies such as chemotherapy and radiotherapy. These treatments appear to be more effective at eliminating NTCs, which comprise the bulk of many tumors. CSCs that survive therapy are able to reform the tumor by giving rise to more CSCs and NTCs. Ideally, a CSC-specific targeted therapy would eliminate this subset of cells and result in permanent tumor control

in mice after transplantation, whereas tens of thousands of the remaining NTCs could not. Importantly, secondary xenograft tumors displayed the same immunophenotypic diversity of the primary tumor after retransplantation of CSCs. More recently, aldehyde dehydrogenase (ALDH) was reported to be a marker of stem/progenitor cells in normal human breast tissue and breast cancer, and as few as 20 $CD44^{+}CD24^{-/low}Lin^{-}$ALDH-positive cells have been reported to form tumors in NOD/SCID mice [13]. This subpopulation of cells also posessed enhanced malignant and metastatic ability as demonstrated in several breast cancer cell lines [14]. Taken together, these data document the existence of a hierarchical organization within many human tumors, and CSCs occupy the top of this hierarchy.

20.2 The 4 R's of Radiobiology

Radiotherapy is one of the most commonly employed and effective cancer therapies currently used in the clinic. Ionizing radiation causes cell killing when cells fail to repair the damaged components that are critical for the survival of the cell. Of the several potential molecular targets of radiation damage, deoxyribonucleic acid (DNA) has been shown to represent the critical target and DNA damage is closely correlated with cell lethality [15, 16]. Cells that are inherently deficient in DNA repair pathways or inhibited in their ability to repair DNA damage exhibit distinctively high radiosensitivity [17, 18]. There are two types of radiation-induced DNA damage: direct and indirect interactions. In the direct interaction, ionizing photons interact directly with the DNA molecule itself to cause damage [19], whereas in the indirect interaction (which predominates), water molecules are ionized to form free radicals that are highly reactive and capable of causing DNA damage [20]. In general, the success of fractionated courses of radiation therapy for cancer depends on four factors ("the 4 R's"): repair of radiation-induced DNA damage, repopulation of cells, redistribution of cells in the cell cycle, and reoxygenation.

20.2.1 Repair

Both direct and indirect ionizing radiation can cause single-stranded or double-stranded breaks in DNA. On the one hand, single-stranded DNA breaks are relatively easy to repair since the broken strand can be repaired using the other strand as a template. Hence, single-stranded breaks are not primarily related to cell death by radiation. On the other hand, double-stranded DNA breaks are considered the most important contributors to radiation cytotoxicity and these are more difficult to repair [21]. There are two main DNA repair pathways for double strand breaks in mammalian cells: homologous recombination (HR), which occurs primarily during S and G2 phases and requires an intact sister chromatid; and nonhomologous end-joining (NHEJ), which is of significantly lower fidelity since it functions by indiscriminately rejoining broken double-stranded DNA lesions. NHEJ is thought to be the more commonly employed repair pathway. The first event to occur in NHEJ after radiation-induced DNA damage is the recognition of Ku70/80 end binding proteins to the DNA lesions followed by the recruitment and phosphorylation of histone H2AX by ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) repair proteins, resulting in the rejoining of the broken ends by X-ray repair complementing defective repair in Chinese hamster cells 4 (XRCC4) and Ligase 4 [22].

20.2.2 Redistribution

The radiosensitivity of cells changes as they progress through the different stages of the cell cycle. Cells in late S-phase are relatively resistant to radiation, whereas cells

in G2/M are preferentially killed during fractionated radiation therapy [23, 24]. Resistant cells in the S-phase can redistribute to other phases of the cell cycle in between fractions of radiation therapy to become more radiosensitive.

20.2.3 Repopulation

Repopulation of tumor cells is thought to be the most common reason for treatment failure of conventional fractionated radiation therapy [25, 26]. Radiation therapy is currently most frequently administered in daily fractions of 1.8–2.0 Gray for courses extending up to 7 or 8 weeks. The reason for this fractionated scheduling is to allow normal cells to repopulate after surviving radiation therapy, thereby reducing undesired toxicities [27]. However, at the same time, fractionated treatment regimens also give the surviving tumor cells a chance to divide, thus increasing the number of tumor cells that need to be eliminated and therefore reducing the effectiveness of a given total dose of radiation therapy.

20.2.4 Reoxygenation

Oxygen is the most potent modifier of radiation sensitivity, and tumor cells in hypoxic niches are 2–3 times more radioresistant than nonhypoxic cells [28]. High levels of hypoxia have been correlated with poor prognosis and accompanying local recurrence or systemic dissemination of disease in human tumors [29–32]. During fractionated radiotherapy, tumors can become reoxygenated through various mechanisms. As the tumor reduces in size during the course of radiotherapy, tumor cells previously located within hypoxic regions may now be juxtaposed to nearby blood vessels as a result of reorganization of the surrounding vasculature and may thus be exposed to higher oxygen tensions [33]. This reoxygenation process occurs between fractions of radiotherapy and renders the tumor cells more radiosensitive to subsequent fractions.

20.3 Radioresistance in Cancer Stem Cells

Even before the relatively recent focus on CSCs, radiobiologists studied clonogenic tumor cells and their radiation resistance properties through the use of local control assays. Since by definition, CSCs are the only cells that can lead to significant regrowth of tumors, in vivo local control assays test the ability of CSCs to survive a given therapy. Using such assays, it was shown that CSC content and intrinsic radiosensitivity measured by in vivo and in vitro clonogenic assays directly correlated with tumor radioresistance [34–37]. These data suggested that CSC radioresistance

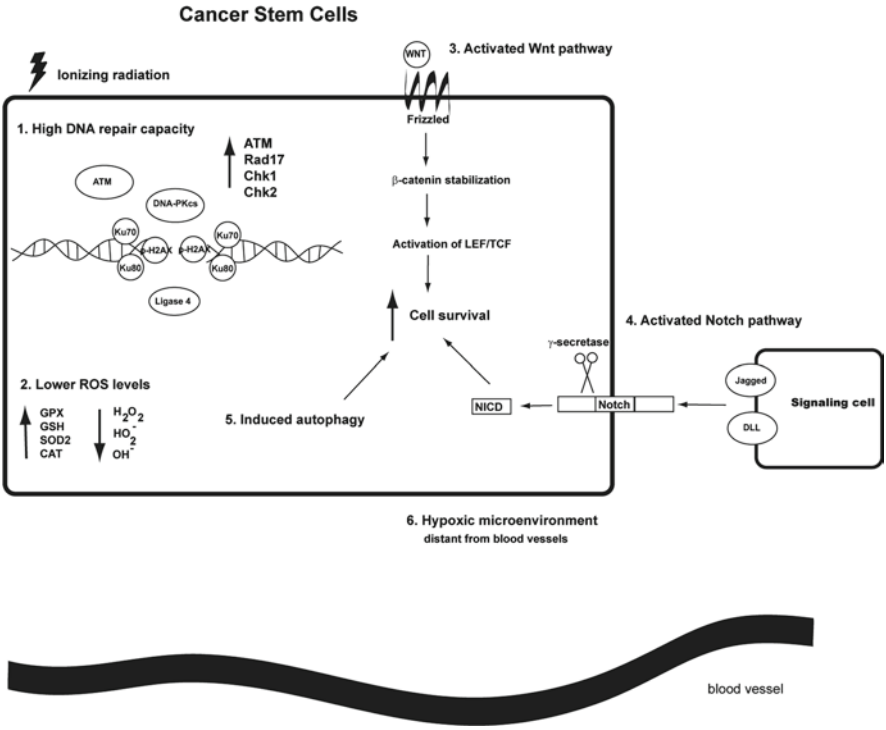

Fig. 20.2 Mechanisms of radioresistance in cancer stem cells. (*1*) CSCs display enhanced DNA repair activity, at least in part due to enhanced activation of the DNA damage checkpoint. (*2*) CSCs contain lower ROS levels and overexpress ROS scavengers, resulting in less DNA damage after ionizing radiation. (*3*) CSCs can induce autophagy to promote survival. (*4*) The canonical Wnt/β-catenin signaling pathway and (*5*) the Notch signaling pathway are overactivated in CSCs, leading to upregulated transcription of genes responsible for cell proliferation and survival. (*6*) CSCs may be located in hypoxic niches within tumors which would contribute to resistance to radiation

properties may significantly contribute to overall tumor radioresistance. However, most of these studies employed long-term cell lines or xenografts, and it remains unclear how faithfully these reflect the properties of CSCs in the clinical setting.

With recent advances allowing prospective isolation of CSCs from many types of tumors, the issue of CSC radioresistance is being explored in more detail. These studies have important clinical implications, since any CSCs that survive radiation could lead to tumor relapse. Thus far, experimental data have demonstrated that CSCs in multiple types of tumors are relatively radioresistant compared with their NTC counterparts. Several mechanisms appear to be involved, including higher DNA repair capacity, lower reactive oxygen species (ROS) levels, activation of Wnt and Notch signaling pathways, induced autophagy, and the possible existence of a hypoxic CSC niche (Fig. 20.2).

20.3.1 Enhanced DNA Repair in CSCs

One recurring mechanism of radioresistance found in CSCs is increased DNA repair capacity. Bao et al. demonstrated that glioma stem cells appear to be relatively radioresistance due to preferential activation of the DNA damage checkpoint responses and increased DNA repair ability [38]. The authors found that tumor cells expressing the glioma CSC marker CD133 were enriched after radiation in vitro and in vivo. $CD133^+$ tumor cells activated the DNA damage checkpoint and repaired DNA damage more effectively than $CD133^-$ tumor cells from both human glioma xenografts and primary glioblastoma specimens. Activating phosphorylation of the ATM, RAD17, CHK1, and CHK2 proteins was significantly higher in $CD133^+$ tumor cells than in $CD133^-$ cells. These observations were further confirmed recently in atypical teratoid/rhabdoid tumor (AT/RT), which is a rare and aggressive pediatric brain tumor [39]. Increased phosphorylation of p-ATM, p-RAD17, and p-CHK2 was observed in $CD133^+$ tumor cells when compared with $CD133^-$ cells after ionizing radiation, indicating that $CD133^+$ AT/RT cells exhibited greater checkpoint activation in response to DNA damage. Similarly, $CD133^+$ Daoy medulloblastoma cells from an established cell line showed consistently more effective repair of sublethal DNA damage and were more resistant to radiation than the $CD133^-$ cells [40]. In addition, RAD51, a protein involved in strand pairing during HR of DNA DSBs, has recently been reported to be overexpressed in $ALDH^+$ CSCs in breast cancer cell lines, suggesting that Chk1-dependent HR may play an important role in DNA repair in CSCs [41]. Recently, using the p53 null mammary tumor mode, Zhang et al. demonstrated that $Lin^-CD29^{Hi}CD24^{Hi}$ CSCs are intrinsically more radioresistant than corresponding NTC subpopulations ($Lin^-CD29^{Hi}CD24^{Lo}$, $Lin^-CD29^{Lo}CD24^{Hi}$, and $Lin^-CD29^{Lo}CD24^{Lo}$) as evidenced by the increased DNA damage repair responses within the CSCs [42]. Taken together, these results suggest that CSCs in at least some tumors are relatively resistant to ionizing radiation through enhanced DNA damage repair compared with NTCs. These data suggest that targeting the DNA damage checkpoint response pathway could sensitize CSCs to radiation therapy.

Although there is evidence for enhanced DNA repair in CSCs from some tumors, CSC or CSC-like cells did not show similar capabilities in all systems. For example, Phillips et al. examined radioresistance of CSC-like cells from a breast cancer cell line and did not find clear evidence of enhanced DNA repair rates [43]. Similarly, CSCs from primary murine breast tumors arising in MMTV-*Wnt*-1 mice do not appear to have enhanced DNA repair capabilities [44]. Ropolo et al. examined five stem and nonstem glioma cell lines and reported enhanced activation of CHK1 and CHK2 kinases in unirradiated $CD133^+$ cells when compared with $CD133^-$ cells [45], suggesting an elevated basal activity of DNA damage responses in $CD133^+$ cells. However, they found no change in DNA base excision, single-strand break repair, or resolution of pH2AX nuclear foci in $CD133^+$ cells in vitro. In addition, McCord et al. showed that $CD133^+$ glioblastoma tumor stem-like cells (TSCs) from recently established neurosphere cultures were not always more radioresistant than

the $CD133^-$ cells from the same cultures, suggesting that the relative radioresistance of $CD133^+$ cells may depend on the tumor from which they are isolated. Furthermore, they found $CD133^+$ cells were more radiosensitive when compared with three established glioma cell lines as evidenced by the defective DNA damage response using neutral comet assay and the reduced presence of H2AX and RADd51 foci in $CD133^+$ TSCs [46]. These data suggest that enhanced DNA repair is not a universal property of CSCs from all tumors.

20.3.2 Low ROS Levels in CSCs

Another mechanism of radioresistance in solid tumors is the maintenance of lower levels of ROS and enhanced levels of ROS scavengers, since the major mechanism of cell killing by ionizing radiation is through ROS mediators [21]. Our group has recently demonstrated that normal mammary epithelial stem cells contain lower concentrations of ROS than their more mature progeny cells, and that the CSC subpopulation in human and murine breast tumors maintains lower ROS levels than NTCs [44]. CSCs from breast tumors arising in MMTV-*Wnt-1* mice, characterized by the $Thy^+CD24^+Lin^-$ immunophenotype, developed less DNA damage after radiation. Furthermore, we found a twofold enrichment of CSCs when compared with NTCs after in vivo irradiation. Both human and mouse breast CSCs displayed increased expression of genes involved in free radical scavenging and single-cell qRT-PCR analysis showed a significant overexpression of glutathione (GSH) biosynthesis genes such as Gclm and Gss by CSCs compared with NTCs. GSH is a critical intracellular free radical scavenger. Similar findings were reported in a study by Phillips et al. using the CSC-like cells from the MCF-7 cell line [43]. Specifically, they demonstrated that $CD24^{-/low}CD44^+$-enriched breast CSC-like cells, propagated as mammosphere cultures, were more resistant to radiation than NTCs, which were grown as monolayer cultures, and that CSC-like cells were enriched during fractionated courses of radiation [43]. Lower concentrations of ROS and minimal phosphorylation of H2AX after irradiation were observed in the CSC-like cells. Similarly, $Sca1^+$ progenitor cells from the COMMA-Dβ-geo cell line developed less H2AX foci immediately after irradiation, suggesting differences in initial levels of DNA damage [47]. These results indicate that CSCs in some tumors develop lower levels of DNA damage immediately following radiation, which appears to be at least in part due to enhanced expression of ROS scavengers.

20.3.3 Activation of Wnt Signaling Pathway in CSCs

It has been shown that radiotherapy resistance can also be mediated through activation of the Wnt/β-catenin signaling pathway, which has been implicated in stem cell survival [48, 49]. Woodward et al. reported an enrichment of $Sca1^+$ progenitor cells

in mammary epithelial cells isolated from MMTV-*Wnt-1* transgenic mice after in vivo radiation and observed that β-catenin was selectively activated in $Sca1^+$ cells but not in $Sca1^-$ cells in response to radiation [50]. Furthermore, real-time PCR analysis revealed elevated messenger ribonucleic acid (mRNA) levels of survivin, a bifunctional member of the inhibitor of apoptosis gene family, in $Sca1^+$ after radiation, suggesting that overexpression of β-catenin may enhance cell survival through the regulation of survivin [51, 52]. These observations indicate that disrupting the Wnt/β-catenin pathway may radiosensitize CSCs.

20.3.4 Hypoxic Microenvironment/Niche

The tumor microenvironment is dynamic and heterogeneous, with ever-changing pH, oxygen concentrations, nutrient supplies, and growth factor levels [53], which may profoundly influence the activity of CSCs. Prolonged or chronic hypoxia is regarded as "diffusion-limited" hypoxia in which tumor cells are located far away from the vascular supply, whereas acute hypoxia in tumors is considered as "perfusion-limited" hypoxia, in which tumor oxygenation fluctuates over time [54–56]. Hypoxia in tumors is a well-established poor prognostic indicator as it promotes aggressive transformation of tumor cells leading to enhanced metastatic potential [57–59], and it is well recognized that hypoxic tumor cells are more radioresistant than nonhypoxic cells [60]. Hypoxia has been shown to promote an immature stem cell-like phenotype in neuroblastoma cells and a dedifferentiated phenotype in ductal breast carcinoma in situ, thereby reverting tumor cells to become more "stem cell-like" [61]. Another recent study showed that a highly tumorigenic subpopulation of cells from several solid tumor cell lines, including neuroblastoma, rhabdomyosarcoma, and small-cell lung cancer, are preferentially located in hypoxic regions of solid tumors [62]. Furthermore, CSCs in medulloblastoma cells cultured under hypoxic conditions have been reported to upregulate the expression of the CSC marker CD133 [63]. However, CSCs may not reside in hypoxic niches in all tumors. Calabrese et al. showed that $Nestin^+CD133^+$ brain CSCs are located in a perivascular niche where they directly interact with endothelial cells [64]. Thus, in some (but not all) tumors, CSCs may be enriched in hypoxic regions, which could contribute to their in vivo radioresistance.

Besides the direct protection of CSCs residing in hypoxic niches by the lack of oxygen, hypoxia could also influence CSC maintenance through the regulation of hypoxia-inducible factors (HIFs) [65]. Li et al. recently demonstrated that HIF2α and multiple HIF-regulated genes are preferentially expressed in glioma CSCs when compared with NTCs and normal neural progenitors [66], and that targeting HIFs in glioma CSCs using lentivirus-mediated shRNAs inhibited self-renewal, proliferation, and survival of CSCs in vitro and reduced the tumorigenic potential of CSCs in vivo. These results suggest that HIFs play an important role in CSCs and may contribute to tumor resistance to radiation.

20.3.5 Notch Pathway Activation in CSCs

The Notch signaling pathway plays an important role in cell fate determination in neural, hematopoietic, and embryonic stem cells as well as in CSCs [67]. In mammals, there are four Notch receptors (Notch 1–4) and five ligands (Jagged-1 and -2, and Delta-like-1, -3, and -4) [68]. Upon activation through ligand binding, Notch receptors undergo sequential proteolytic cleavage resulting in the release and nuclear translocation of the intracellular domains of Notch receptors (NICDs), which subsequently leads to activation of Notch-regulated transcription [69]. The γ-secretase complex is required for Notch activation [70, 71], and inhibitors of γ-secretase (GSIs) have been used to inhibit Notch signaling in vitro and in vivo. A recent study by Wang et al. demonstrated evidence for Notch-mediated radioresistance in glioma CSCs [72]. It was shown that inhibition of the Notch signaling pathway by GSIs rendered glioma CSCs more sensitive to radiation, and that GSIs impaired clonogenic survival of glioma CSCs but not NTCs through the downregulation of Akt activity.

20.3.6 Induced Autophagy

Autophagy is a "self-cannibalizing" process involving the degradation of long-lived proteins and cytoplasmic organelles, which are then recycled to macromolecules to maintain cellular homeostasis [73]. It has been shown to be a method of cell death distinct from apoptosis that can contribute to the killing of cancer cells by cytotoxic therapies. Somewhat counter-intuitively, autophagy also appears to lead to radioresistance in some systems, potentially by acting as a stress response system. Induced autophagy may be a mechanism that contributes to radioresistance in CSCs as demonstrated by Lomonaco et al. in $CD133^+$ glioma stem cells [74]. In this study, a greater degree of autophagy was induced in $CD133^+$ glioma stem cells when compared with $CD133^-$ cells, and higher levels of autophagy-related proteins such as LC3, ATG5 and ATG12 were expressed in $CD133^+$ cells. Inhibition of autophagy by bafilomycin A1, which inhibits autophagy by disrupting fusion between the autophagosome and lysosome, and targeting beclin1 and ATG5 genes by shRNAs enhanced radiosensitivity of $CD133^+$ cells to radiation. Although these data will need to be confirmed in other studies, they suggest that induced autophagy may contribute to radioresistance of CSCs.

20.4 Overcoming Radioresistance in CSCs

Recently gained insights into CSC radioresistance mechanisms provide novel opportunities for the development of CSC-specific radiation sensitizers. A number of such strategies have shown promising results in preclinical models. For instance, CSCs can be radiosensitized by targeting DNA damage repair pathways as demonstrated

by Bao et al. in gliomas [38]. By pretreating tumor cells with debromohymenialdisine (DBH), an inhibitor of checkpoint kinases CHK1 and CHK2 [75], resistance of $CD133^+$ tumor cells to radiation was disrupted. Our group recently demonstrated that pharmacological inhibition of free radical scavengers by pretreating mouse mammary CSCs with the GSH synthesis inhibitor L-S,R-buthionine sulphoximine (BSO) radiosensitized these cells, suggesting that targeting of ROS defenses in CSCs could be a useful therapeutic strategy.

Another potential approach to overcoming radioresistance in CSCs is through modulation of the Akt signaling pathway. In p53 null mammary tumors, it has recently been shown that inhibiting the Akt pathway with perifosine significantly reduces the self-renewal ability of $Lin^-CD29^HCD24^H$ CSCs and sensitizes them to radiation treatment in vitro [42]. Perifosine appears to block DNA damage responses in CSCs, indicating that inhibitors of Akt pathway could be utilized to selectively radiosensitize CSCs.

On the one hand, inhibition of the Notch signaling pathway is also a promising approach to sensitize CSCs to radiation therapy. Mammosphere-forming cells derived from ductal carcinoma in situ have been shown to exhibit diminished self-renewal capacity when the Notch signaling pathway is inhibited [76]. On the other hand, activation of Notch pathway through overexpression of NICD1 promoted the formation of neurospheres in a glioma cell line [77]. Blockade of the Notch pathway using pharmacologic GSIs has also been reported to preferentially reduce the $CD133^+$ stem-like cell subpopulation in medulloblastoma cell lines by decreasing their tumorigenic capacities [78]. More recently, Wang et al. showed that GSIs significantly reduced the clonogenic ability and increased cell death of $CD133^+$ glioma CSCs after radiation treatment [72]. Additionally, overexpression of the constitutively active intracellular domains of Notch1 or Notch2 (NICD1 or NICD2) abolished the radiosensitizing effect of GSIs. In a xenograft mouse model, knockdown of Notch1 or Notch2 using shRNAs dramatically decreased tumor incidence, indicating that glioma CSCs were dependent on Notch signaling and could be radiosensitized by inhibition of the Notch pathway.

Oncolytic virotherapy is a newly emerging approach to specifically target and kill tumor cells, and similar targeting strategies could also be applied to targeting CSCs. It has recently been shown that radioresistant esophageal CSC-like cells exhibited high telomerase activity and that a tumor-specific replicating adenoviral vector Ad/TRAIL-E1, which carries the TRAIL and E1A genes under the control of the human telomerase reverse transcriptase (hTERT) promoter, preferentially targeted and eliminated these esophageal CSCs without causing collateral damage to normal tissues in a xenograft mouse model [79].

Development of anti-CSC therapies should take into consideration the maintenance and preservation of normal stem cell functions. It will be important to identify molecular pathways that are distinctively different between CSC and normal stem cells so that anticancer drugs or radiosensitizers could be utilized to target CSCs specifically without causing a significant adverse effect on normal stem cells. Along these lines, it has been shown that after phosphatase and tensin homolog (PTEN) deletion, LSCs were able to reconstitute irradiated mice while normal hematopoietic

stem cells could not, and that rapamycin treatment not only eliminated the LSC population but also restored normal hematopoietic stem cell function [80]. These data demonstrated that mechanistic differences between CSCs and normal stem cells can be utilized to target CSCs specifically while leaving normal stem cells unharmed.

20.5 Conclusions

A number of mechanisms contributing to radioresistance in CSCs have recently been uncovered, including high DNA repair capacity, overexpression of free radical scavengers, activation of WNT and Notch signaling pathways, induced autophagy, and preferential presence in hypoxic regions of tumors. Taken together, these studies suggest that the particular resistance mechanism(s) present in CSCs of a given tumor may vary. Thus, it remains to be seen which mechanisms are the most common and if there are possible interactions between some of them.

Further investigation of the mechanisms and signaling pathways involved in CSC resistance to radiation therapy will allow the development of novel, CSC-targeted radiosensitizers. As such, future clinical protocols should incorporate characterization of CSCs and design patient-specific treatment regimens to improve the effectiveness of anticancer therapies. In addition, CSC-specific endpoints for treatment response need to be established, since currently tumor response is most often determined by a reduction in the size of a tumor. Since CSCs appear to be a minority population in many tumors, gross shrinkage will mostly reflect killing of NTCs rather than CSCs. Assessing the response of CSCs to anticancer therapies would be greatly aided by the development of molecular imaging tools that can locate and track CSCs in vivo [81]. Such technology would also be particularly helpful in the optimization of radiotherapy planning, as it might allow a “dose painting” approach to be utilized that could deliver higher doses to CSC-rich regions of tumors [82, 83]. In summary, developing a deeper understanding of CSC biology and the mechanisms governing radioresistance in these cell subpopulations will allow the development of CSC-specific radiosensitizers and will hopefully lead to improved treatment outcomes for cancer patients.

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Chapter 21
The Role of ABC Transporters in Cancer Stem Cell Drug Resistance

Vera S. Donnenberg, Ludovic Zimmerlin, and Albert D. Donnenberg

Abstract There can be no cure for epithelial cancer until there is a significant therapeutic index that separates the most therapy-resistant cells within tumors, and the cells that mediate functions essential to life. The cancer stem cell paradigm explains tumor heterogeneity and has led to the hypothesis that therapy resistance originates in the mechanisms by which normal tissue stem cells protect themselves from toxic insults. However, therapeutic index is not merely the ratio of cancer stem cell kill to normal stem cell kill. It is further compounded by the fact that a small number of clonogenic cancer cells need only to survive to perpetuate the neoplasm, whereas vital tissue functions cannot be disrupted for long without lethal consequences. In this chapter, we review the concepts of tumor grade and therapeutic index; maximal tolerated dose; innate vs. acquired multiple drug resistance (MDR); tumor heterogeneity; and the role of ABC transporters in multipotent, therapy resistant, clonogenic cancer stem-like cells in the context of therapeutic use of MDR inhibitors to change the maximal tolerated dose of currently used antineoplastic agents.

Abbreviations

ABC	ATP binding cassette transporters
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
CD	Cluster of differentiation
CFTR	Cystic fibrosis transmembrane conductance regulator
CSA	Cyclosporin A

V.S. Donnenberg (✉)
Department of Cardiovascular Surgery, Division of Hematology/Oncology, University of Pittsburgh Cancer Institute and University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
e-mail: donnenbergvs@upmc.edu

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ED_{50}	Effective $Dose_{50}$
EpCAM	Epithelial cell adhesion molecule
Hh	Hedgehog
LD_{50}	Lethal $Dose_{50}$
MDR	Multiple drug resistance
MHC	Major histocompatibility complex
MRD	Minimal residual disease
MRP	Multidrug resistance protein
MTD	Maximum tolerated dose
NCI	National Cancer Institute
PI	Propidium iodide
R123	Rhodamine 123
SHh	Sonic hedgehog
SP	Side population
SP1	Sphingosine-1-phosphate
TIL	Tumor infiltrating lymphocytes

21.1 Introduction

Lack of success in the eradication of epithelial cancers may be attributed to the failure to identify tumor specific targets, together with the tumor's ability to hijack many normal stem cell-like properties, including dormancy, self-renewal, self-protection, and telomere maintenance. Despite the success of current therapies in eliminating bulky disease and rapidly proliferating cells, the same therapies often spare this self-renewing, self protected stem-like compartment, missing the tumor reservoir responsible for recurrent disease and metastasis. Depending on the aggressiveness of the particular neoplasm, the resistant compartment may be dormant or proliferative. In the first case, long-term remission may be obtained, but in the latter, extreme drug resistance is encountered at the time of diagnosis. Recent advances in the understanding of ABC transporter expression, activity, and regulation in normal tissue stem cells as well as in tumors provide a basis for revisiting the process of oncogenesis, tumor heterogeneity, and drug resistance in the context of anticancer therapy.

ABC transporters (ATP binding cassette transporters) are highly conserved and represent a major protective mechanism for highly differentiated barrier tissues as well as adult tissue stem cells. Multiple drug resistance (MDR) mediated by their activity [1] was initially discovered in tumors and was recognized as an impediment to cancer therapy [2, 3]. Further, the significant redundancy in substrate specificity among the individual transporters, the significant homology between eukaryotic and mammalian transporters, and the lack of a lethal phenotype with specific ABC knock-outs demonstrates the essential nature and redundancy of these proteins for cellular transport and trafficking [4–6].

More recently, intrinsic MDR transport has been shown to constitute a normal physiologic protective mechanism shared by long-lived stem cells in a variety of tissues [7–12]. Survival of tissue stem cells is essential for tissue maintenance and repair, and constitutive (as opposed to substrate-induced) MDR activity is thought to be one of several protective mechanisms by which normal tissue stem cells guard themselves from toxic insults, including damage caused by antineoplastic therapy [13]. The notion that the cancer-initiating cell may retain or reacquire constitutive drug resistance predicts the persistence of a therapy resistant stem cell-like fraction following apparently successful cytotoxic cancer therapy, even in the face of marked shrinkage or disappearance of measurable tumor.

Understanding the central role played by MDR transporters in the protection and self-renewal of normal and cancer stem cells may allow us to identify differences that can be exploited therapeutically. Recognizing that normal stem cells in individual tissues differ with respect to damage tolerance and degree of multipotentiality may translate into differential drug susceptibility and metastatic potentials of cancer stem cells, depending on the tissue of origin. For example, hematopoietic stem cells responsible for bone marrow maintenance and regeneration after toxic injury are far more susceptible to radiotherapy [14, 15] and chemotherapy than their epithelial counterparts, despite constitutive MDR expression among the most primitive and multipotent hematopoietic progenitors [7, 8].

In this chapter, we will discuss the role of ABC transporters as a barrier to therapy as well as their role in transport of physiological biomolecules involved in signaling, differentiation, proliferation, and migration.

21.2 Discovery of MDR Transporters

Resistance to chemotherapy was recognized as an impediment to efficacious cancer treatment in the earliest stages of anticancer drug development [16]. Further, cancer cell lines selected for resistance to specific compounds also demonstrated “cross-resistance” to a broad spectrum of structurally unrelated agents [2, 3]. The advent of molecular approaches enabled the isolation of the genes for MDR. Roninson and colleagues isolated two genes, now recognized as the hamster homologs of the human MDR1 and MDR2 genes [17–19]. In 1986, Gros and colleagues transfected the *MDR1* gene (P-glycoprotein, [ABCB1]) into drug sensitive hamster cells and showed that gene duplication or mutations were not required for the acquisition of the multidrug resistant phenotype [20]. ABCB1 did not account for all forms of MDR and additional transporters were identified, among them ABCG2, first described as mitoxantrone resistance [21, 22] breast cancer resistance protein (BCRP). ABCC1, also known as multiple resistance protein 1 [23, 24], was also recognized early as a transporter with activity over a range of substrates. Today, the ABC transporter family is the largest of all transmembrane protein families, utilizing

an ATP-dependent mechanism to transport a wide range of xenobiotics and cellular products against the concentration gradient. The ABC-A family predominantly transports lipids; the ABC-B family is responsible for transport of intracellular peptides (major histocompatibility complex [MHC] class I antigen presentation) as well as xenobiotics (ABCB1, P-glycoprotein); the ABC-C family contains transporters mediating drug/xenobiotic efflux as well as the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7 [25]; and the ABC-D family encodes for peroxisomal half transporters. The ABC-E and -F families, lacking the transmembrane domain, are implicated in mRNA translation [26].

21.3 Role of MDR Transporters in Normal Tissue Stem Cells

Although hematopoiesis remains the leading paradigm for tissue differentiation and replacement, the study of adult tissue stem cells has gained momentum with the emergence of regenerative medicine. Although little is known concerning the role of MDR transporters in adult tissue stem cells, we hypothesize that they follow the hematopoietic paradigm, affording resting stem cells protection against toxic insults (which would damage cycling progenitor cells and mature tissue) as well as transport and trafficking of endogenous molecules. As in the hematopoietic system, where some highly differentiated cell subsets (such as T lymphocytes) also have the ability to upregulate transporter activity when exposed to substrates [27–29], *induced* activity may also play an important role in differentiated nonhematopoietic cells. In the absence of tissue stem cell specific markers, the activity of these transporters has been used to obtain enriched populations of adult stem cells in a variety of tissues [8, 30, 31]. The efflux or exclusion of fluorescent MDR substrates such as rhodamine 123 (R123, ABCB1 substrate, R123dull/dim phenotype) and Hoechst 33342 (ABCG2 and to a lesser degree ABCB1 substrate; side population [SP] phenotype) are frequently used in fluorescence activated cell sorting of tissue stem cells [8–12]. The caveat is that MDR activity is not limited to stem cells [1], and conversely, not all adult tissue stem cells have constitutive MDR expression at a given time [32] (Fig. 21.1).

21.4 Measurement of MDR Transporter Activity

The critical proof of constitutive MDR transporter activity in primitive hematopoietic stem cells came from Goodell et al. who showed that 10% of Sca-1+, lineage negative murine bone marrow cells (a phenotype used to define early hematopoietic stem cells) were also Hoechst 33342 dim [8]. This subset was termed the SP, after the distinctive flow cytometric profile resulting from DNA-bound quenched dye vs. membrane associated unquenched dye [33] (Fig. 21.2). Compared with whole bone marrow, SP cells (0.1% of whole bone marrow cells) were 1,000-fold enriched in their ability to repopulate lethally irradiated mice. Sorrentino and colleagues elegantly worked out the details, showing that transfection of ABCB1 into normal murine

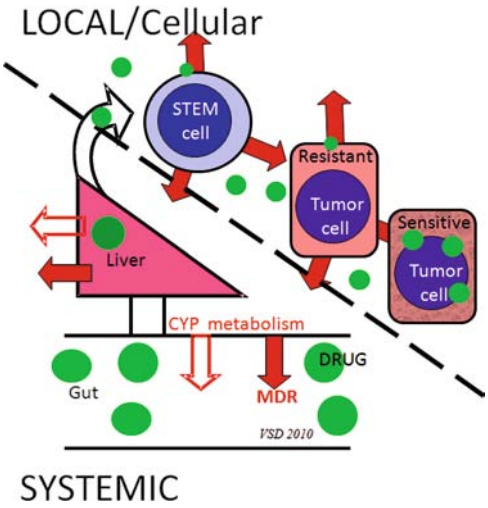

Fig. 21.1 Systemic and local ABC transporter expression. ABC transporters have multiple functions in complex tissues and in single cells. In tissues they prevent absorption of substrate xenobiotics from the gut into the systemic circulation, from the systemic circulation into the brain (blood brain barrier), and from the maternal circulation to the fetal circulation (placenta). Transporters protect the liver by moving xenobiotics into the systemic circulation. At the level of the single cell, ABC transporter activity may be constitutive or induced by exposure to substrates. Constitutive activity protects a proportion of tissue stem cells and tumor cells from xenobiotics

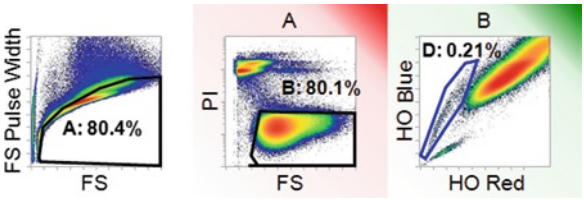

Fig. 21.2 Cells with MDR activity form a distinctive "side population (SP)" profile when incubated with the MDR substrate Hoechst 33342. The breast cancer cell line MCF7 was incubated for 90 min with 8 μM Hoechst 33342. Doublets and nonviable (PI staining) events were eliminated from the analysis. MDR activity transports the dye out of the cell against the concentration gradient, preventing intracellular dye accumulation and DNA binding. The peak emission of unbound Hoechst 33342 is in the *blue* range. When bound to double stranded DNA at high concentration, quenching results in a shift in the emission spectrum from *blue* toward *red*

marrow increased the SP phenotype [34]; ABCG2 knockout abrogated the SP phenotype [35]; and both ABCB1 and ABCG2 are constitutively active in cells with SP phenotype [36]. A caveat worth mentioning that bears on the comparison of sorted SP+ and SP negative populations is that SP negative but not SP+ cells accumulate significant levels of Hoechst 33342, a toxic fluorescent reporter and DNA intercalator, possibly confounding clonogenicity assays performed after SP sorting [37, 38]. The detailed step by step instructions for SP phenotyping of dissociated epithelial

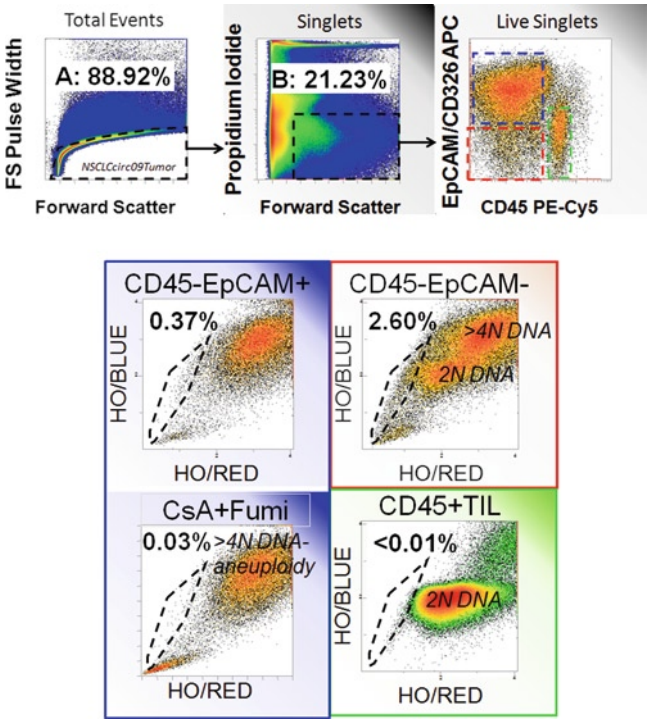

Fig. 21.3 Detection of SP activity in subpopulations of an untreated freshly excised nonsmall cell lung tumor. The *top row* shows the gating strategy used prior to detection of Hoechst 33342 transport. The freshly excised tumor was processed by mechanical and enzymatic disaggregation and prepared for flow cytometry. Five-hundred thousand events were acquired. *Top row* (*left to right*): (*A*) forward light scatter pulse analysis is used to eliminate cell clusters and retain singlet cells the DNA stain propidium iodide (PI) is used to eliminate permeable (PI+), nonviable cells; (*B*) CD45 (hematopoietic) vs. CD326/EpCAM is used to distinguish tumor cells negative and positive for the epithelial differentiation marker EpCAM and CD45 bright EpCAM negative tumor infiltrating lymphocytes (TILs). These regions are used for subsequent analysis of SP phenotype in EpCAM+ tumor (*blue frame*), EpCAM negative tumor (*red frame*), and TILs (*green frame*). The SP is most prevalent in the EpCAM negative tumor fraction, and absent in TILs. Inhibition of MDR activity by addition of CSA and fumitremorgin results in disappearance of the SP

tissues are summarized in [38]. Figure 21.3 illustrates measurement of MDR activity in three cellular subsets in a freshly isolated disaggregated lung tumor.

21.5 Therapeutic Index and Maximal Tolerated Dose of Antineoplastic Drugs

According to the National Cancer Institute (NCI) Dictionary of Cancer Terms, a *Complete Response* (also called a complete remission) is “the disappearance of all signs of cancer in response to treatment. This does not always mean the cancer

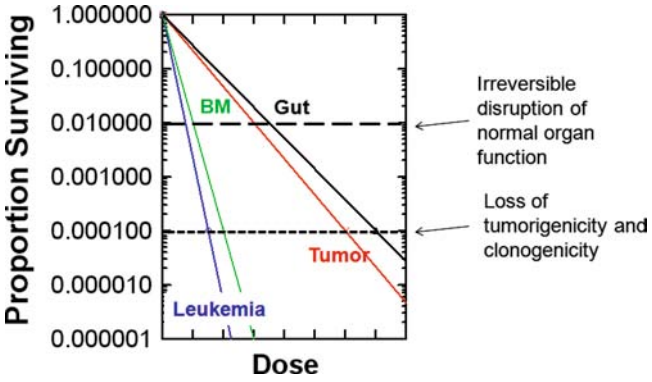

Fig. 21.4 Therapeutic Index refined in terms of lethal tissue damage vs. eradication of clonogenic tumor cells. Hypothetical cytotoxic dose response curves are given for bone marrow stem cells (BM), leukemia, gastrointestinal stem cells (gut), and in the most therapy resistant clonogenic cells within an epithelial tumor. The *dashed lines* indicate 2 and 4 logs of kill. Making the conservative assumptions that: (1) the most resistant cells within a tumor are at least as therapy-sensitive as gut stem cells; (2) normal tissue function will be irreversibly disrupted at a dose that destroys 99.9% of stem cells (*dashed line*); and (3) tumor eradication requires at least 6 logs of kill of the most resistant cell; it follows that it is virtually impossible for a nonselective cytotoxic agent to eradicate tumor while sparing vital tissue function

has been cured". This working definition has proven useful for the evaluation and comparison of antineoplastic regimens. However, the interpretation that an effective dose$_{50}$ (ED$_{50}$) is one that results in complete response as defined above in 50% of subjects takes liberties with the notion of therapeutic index (the lethal dose$_{50}$ [LD$_{50}$] divided by the ED$_{50}$), as it is understood in nononcologic applications. The theory that the maximally tolerated dose is the most effective drug dose is unique to oncology and emphasizes the tradeoff between toxicity and efficacy inherent to cytotoxic therapy. Using the NCI effectiveness criterion of complete response, an antineoplastic agent or regimen with a high therapeutic index is the one in which the dose required to achieve a complete remission is much lower than the dose which will cause death by acute organ failure or by irreparable loss of the body's ability to repair damaged essential tissue. By this criterion, we already have many agents with high therapeutic indices. Taking a more traditional view of therapeutic index, in which the ED$_{50}$ is defined in terms of the dose required to eradicate tumorigenic cells from 50% of subjects, therapeutic index would pit retention of vital organ function against mere survival of clonogenic tumor cells [39] (Fig. 21.4). This is a much more difficult standard, as a damaged organ such as bone marrow, liver, or gut has a finite amount of time to repair itself before life-threatening complications ensue. In contrast, surviving occult tumor cells have the lifetime of the patient to reestablish the genetic, epigenetic, or niche effects required for reactivation of their invasive phenotype [40].

21.6 Attempts to Use MDR Inhibitors Therapeutically to Change the MTD of Antineoplastic Agents

The discovery of the molecular mechanism of cross-resistance led immediately to attempts to block MDR transporters with putative reversal agents. Although reversal of MDR in vitro was easily attained with a variety of inhibitors [41], reversing MDR in the clinical setting has been unsuccessfully pursued for almost three decades [42–45]. Convincing evidence that an MDR reversal agent could increase the intratumor concentration of a chemotherapeutic agent was provided by Bates [46], who used the imaging agent Tc-99m sestamibi, an ABCB1 substrate, to measure MDR activity in vivo and demonstrated the efficacy of the nonimmunosuppressive cyclosporine analog PSC 833 (Valspodar) to reverse MDR activity in vivo. During the coadministration of the reversal agent, tumor visualization was markedly enhanced due to inhibition of MDR-mediated sestamibi efflux, suggesting that intratumor chemotherapeutic concentrations were likewise increased. Despite the success of highly specific ABC transporter inhibitors (PSC 833, GF120918, VX-710 (Biricodar), and LY335979 [47]) to increase the intratumor concentrations of antineoplastic agents, the same in vivo studies failed to show any survival benefit by the inclusion of second or even third generation reversal agents [43, 46, 48–54]. The failure of the reversal agents to specifically alter the pharmacokinetics of antineoplastic drugs administered systemically can be explained in part by the multiple and redundant cellular mechanisms of resistance, not only within the ABC transporter family but also including other resistance-related proteins expressed in solid tumors (e.g., glutathione S-transferase, metallothionein, O6-alkylguanine-DNA-alkyltransferase, thymidylate synthase, dihydrofolate reductase, heat shock proteins), as well as other factors contributing indirectly to resistance such as vascularization [55]. Perhaps the most critical factor is the inherent lack of differential activity of reversal agents on MDR positive tumor cells and normal tissue stem cells [13, 56, 57]. The take home message of these studies is that MDR reversal agents increase the plasma concentration of a variety of antineoplastic agents, but not their therapeutic index (reviewed in [13, 57, 58]). Taken together, systemic administration of an efficacious broad spectrum reversal agent would render tumor and normal tissue stem cells equally susceptible to chemotherapeutic agents, offering no net gain in therapeutic index.

21.7 Are ABC Transporters Essential to the Tumor? The Cancer Stem Cell as a Multipotent, Therapy Resistant, Clonogenic Cell

The cancer stem cell concept has had several incarnations, the first dating from the 1800s. In 1858, Rudolf Virchow coined the embryonic rest theory, postulating that cancer arises from embryo-like cells. Julius Cohnheim (1875) elaborated on this

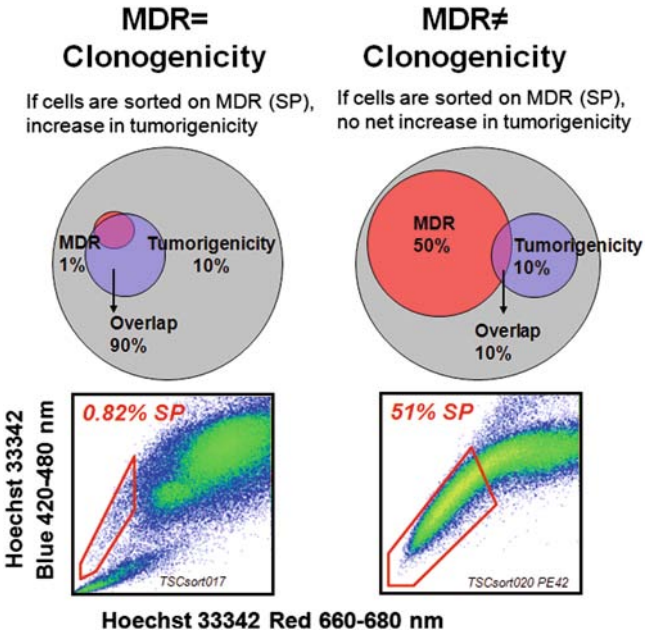

Fig. 21.5 MDR activity (SP phenotype) and tumorigenicity in two untreated nonsmall cell lung cancers. In the first case, MDR activity is rare and is correlated with clonogenicity with the MDR+ fraction comprising a small subset of tumorigenic cells. In the second case, the MDR+ fraction is prevalent and is uncorrelated with tumorigenicity. In both cases, an MDR+ clonogenic population is present, which would predictably be therapy resistant

idea, proposing that tumors derive from residual embryonic cells, which lie dormant through development and become cancerous upon activation (reviewed by Sell [59]). Fiala revived this notion in 1968 [60], hypothesizing the cancer-initiating cell to be a "stem cell unable to differentiate." Interest in the relationships between cancer and our modern notion of tissue stem cells was rekindled by Weissman [61], who first isolated the multipotential hematopoietic progenitor cell [62]. One of the defining characteristics of adult tissue stem cells is the fact that, at any given time, a proportion of them are constitutively resistant to xenobiotics, including most chemotherapeutic agents. Understanding the central role that MDR transporters play in protecting normal adult tissue stem cells allows us to extrapolate to the clonogenic, drug resistant cell subpopulations within a tumor and add new insights that may prove relevant to explaining treatment failure, late recurrence, metastasis, and tissue-specific differences in cancer incidence.

Our own experience leads us to believe that, as in normal tissues, clonogenicity (self-renewal or tumorigenicity), and tumor resistance to therapy are correlated but physiologically uncoupled (Fig. 21.5). Therapies that target proliferating cells, enzymes, growth receptors, adherence molecules, or signaling molecules in metabolically active cancer cells may be highly effective at debulking tumors and dispatching tumorigenic cells without the stem cell-like property of self-protection, but

they will consistently fail to eradicate the tumor cell fraction that constitutively shares protective mechanisms with normal stem cell counterparts. Working in a human breast cancer xenograft model, we demonstrated that MDR activity is not an intrinsic characteristic of a unique tumor stem cell compartment. Both $CD45^{-}CD44^{+}CD90^{+}ABCG2^{+}$ cells and $CD45^{-}CD44^{+}CD90^{+}ABCG2^{-}$ cells were tumorigenic and serially transplantable. Further, the $ABCG2^{+}$ phenotype appears to be inducible in $ABCG2^{-}$ clonogenic tumor cells, since tumors derived from the $ABCG2^{-}$ fraction gave rise to a substantial proportion of $CD90^{+}$ $ABCG2^{+}$ cells. We interpret this data to indicate that, in the absence of drug-induced selective pressure, there is a bidirectional or stochastic relationship between drug resistance and drug sensitivity among clonogenic tumor cells. This relationship is contrary to a rigid hierarchical model in which protected stem cells give rise to sensitive progenitor cells, but is consistent with observations that conditionally differentiated cells, such as those found in the normal airway [9], liver [63], and pancreas [64] can assume a self-renewing and self-protected phenotype after injury. Cancers that respond to therapy initially may appear to acquire drug resistance during the course of treatment, while other cancers may appear to be intrinsically resistant. Our interpretation of the cancer stem cell paradigm stipulates that at any given time a proportion of self-renewing stem-like tumor cells has innate drug resistance by virtue of its stem cell-like phenotype [13, 65, 66]. Acquired drug resistance in more differentiated cancer cells, through gene amplification or rearrangement, may contribute to an aggressive phenotype, but is not the primary reason for cancer recurrence or spread after therapy. Taken together, it seems that the multiple drug-resistant clonogenic tumor cell is a moving target, since resistance and tumorigenicity are uncoupled and resistance can be modulated in the absence of therapy mediated selective pressure. Thus, it is unlikely that any one phenotypically defined subset will encompass all tumorigenic, therapy resistant SP-like cells, which have the potential to metastasize and reactivate [67].

21.8 Other Physiologic Roles of ABC Transporters

For decades we have focused on ABC transporter drug efflux activities to determine the cause of treatment failure. However, significant correlative evidence suggests that in addition to their roles in drug transport, the same transporters are involved in intracellular and extracellular molecule trafficking (reviewed in [58]). Although confounded by the fact that ABC transporters confer resistance of cancer cells to apoptosis in the context of antineoplastic therapy, several studies provide evidence that ABC transporters promote cell survival independent of their ability to limit cytotoxic drug exposure. The earliest report from Johnstone et al. demonstrated that ABC transporter inhibition sensitized cells to FAS-induced cell death via FAS–FAS ligand interaction [68, 69]. Further, increased expression of ABCB1 (P-glycoprotein) delayed apoptosis in normal as well as malignant cells [70, 71] through the sphingomyelin-ceramide pathway. In neuroblastoma, down-regulation

of ABCC1 (multidrug resistance protein [MRP]) augmented cell death [72]. It has also been documented that ABC transporters play roles in the migration of dendritic cells, most likely mediated by transport of leukotriene C4 and D4 [73–75]. Interaction of ABCC1 and CD44 has been shown to enhance migration of several cancer cell lines [76], and similar studies demonstrated that inhibition of ABCC1 and ABCC4 reduces migration of neuroblastoma cell lines [58]. Both ABCC4 [75] and ABCC11 [77] participate in secretion of leukotriene LTC4, amplifying tumor-associated inflammation and possibly affecting cell migration. Furthermore, the ABCC family members also efflux prostaglandins, raising the possibility that prostaglandin signaling may be enhanced, providing protumorigenic PGE_2 and PDG_2 to their G-protein coupled receptors [78–80].

Sphingosine-1-phosphate (SP1), a potently angiogenic molecule, is a substrate of ABCA1 and ABCC1, and its inhibition results in significant down modulation of SP1 efflux from mast cells [81], thus linking MDR and vascular index in poor outcome tumors. The Hedgehog (Hh) signaling pathway, a highly conserved key mediator of embryonic morphogenesis, was recently linked to ABCG2 and ABCB1 regulation [82–84]. In vitro studies in myeloma and prostate cancer cell lines [85, 86] demonstrated that pharmacologic inhibition of the Hh pathway resulted in increased sensitivity to cytotoxic agents mediated through downregulation of MDR transport. Conversely, stimulation of cancer cell lines with sonic hedgehog (SHh) increased the expression of both ABCB1 and G2 transporters. These findings provide preliminary evidence that constitutive Hh pathway activation in clonogenic tumor cells may play a critical role in tumor growth, resistance to therapy, and metastasis. Therefore, in cancers that utilize the Hh pathway, selective MDR inhibition may increase the therapeutic index of existing agents by mechanisms distinct from interference with drug transport. Taken together, the multiple roles of ABC transporters in apoptosis resistance, motility, vascularization, and inflammation justify a continued effort to selectively target these molecules in cancer cells.

21.9 Innate vs. Acquired Multiple Drug Resistance in Tumor Cells

The discovery of the first MDR transporter began with the finding of gene amplification in hamster cells selected in vitro for drug resistance [17]. Removal of the drug resulted in the outgrowth of cells without amplified MDR genes and loss of the multiple-resistant phenotype. However, in vivo drug resistance is not dependent on prior drug exposure [87]. Current understanding of the regulation of MDR activity in stem cells and their progeny allows reconciliation of these findings [58]. Drug resistance, mediated by constitutive ABC transporter activity in a proportion of cells, is an innate characteristic of the resting tumor stem-like cell. In aggressive neoplasms, constitutive activity can also be present in highly proliferative tumor cells. Although studies performed in cell lines [19] suggested drug resistance resulted from the juxtaposition of MDR and active genes through gene rearrangement

[88, 89] or chromosomal activation [90, 91], such transforming events are not essential for acquisition of the MDR phenotype in vivo. Like its normal counterpart, a proportion of stem-like cancer cells express constitutive MDR activity, which is evident prior to drug-imposed selective pressure. Even when therapy succeeds in eliminating the bulk of proliferating cells, this population may survive as occult disease and reactivate months or years later. Additionally, selective pressure imposed by chemotherapy leads both to mutation and secondary genetic changes, including MDR upregulation in the bulky tumor [13, 92]. If these changes occur in self-renewing proliferating cells, the result is an aggressive therapy-resistant tumor. Therefore, MDR activity, whether constitutive in resting tumor cells, induced in active tumor cells, or amplified by genetic alterations, remains a major barrier to therapy.

21.10 Tumor Grade and Therapeutic Index

According to the NCI, "Tumor grade is a system used to classify cancer cells in terms of how abnormal they look under a microscope and how quickly the tumor is likely to grow and spread. Many factors are considered when determining tumor grade, including the structure and growth pattern of the cells. The specific factors used to determine tumor grade vary with each type of cancer." Like normal tissues, tumors are heterogeneous with respect to stromal cells, vascular cells, infiltrating immune cells, and the differentiation state of the epithelial cells themselves. In low-grade cancers, many of the tumor cells may be nonclonogenic progeny of clonogenic cells. A tumor cell does not have to derive from a mutated stem cell to acquire stem cell attributes such as self-protection and self-renewal. Mutation and epigenetic reprogramming can produce any phenotype that offers a selective advantage to the cancer cell, including expression or reexpression of stem cell associated genes. Our interpretation of the cancer stem cell paradigm posits that a fraction of cancer cells evade therapy and reactivate because they have retained or reexpressed multiple mechanisms by which adult tissue stem cells protect themselves from environmental stressors. As such, *stemness* may be central not only to the tumor's seemingly limitless capacity for growth, but also for the fundamental lack of therapeutic index separating essential tissue stem cells and the most therapy-resistant tumor cells [13]. The stem cell paradigm makes intuitive sense in low-grade well differentiated tumors [65], where the majority of tumor cells are nonclonogenic progeny with distorted but recognizable architectural features and protein expression reflecting their tissue of origin [93]. In such cases, analysis of dissociated tumor examining millions of cells reveals a minor subpopulation of proliferating progenitor cells and, rarer still, a population of stem cell marker positive resting cells [66], a subset of which will also express constitutive MDR and detoxifying enzyme activity (e.g., aldehyde dehydrogenase). Low-grade tumors are often slow growing, but the retention of a dysregulated differentiation pattern ensures a low therapeutic index between the stem-like cells in the tumor and normal tissue stem cells. This differentiation

process is less well-defined in high grade neoplasms, which retain stem cell-like self-renewal while acquiring the proliferative capacity of the progenitor. In the absence of stem-like self-protection, these cancers may be highly responsive to therapy, as is the case with certain high grade non-Hodgkin's lymphomas [94]. If, however, these proliferating cells retain or have acquired the self-protective mechanisms of the tissue stem cell, the result is highly aggressive, therapy resistant disease.

21.11 Implications for Therapy

Unlike bone marrow or mobilized peripheral blood stem cell rescue following dose intensive therapy in hematopoietic malignancies, no analogous ability now exists to rescue nonhematopoietic tissue stem cells following stem cell ablative therapy. Given the similarities between the sensitivities of stem-like cells within a tumor and normal tissue stem cells, the inescapable conclusion is that systemic cytotoxic therapies are doomed to fail, because regimens that spare resting normal stem cells will also likely spare resting tumor stem-like cells. Successful therapies will require the identification of biological and immunological differences between the tumor and the normal stem cells [13, 39, 40].

Considering the parameters that comprise therapeutic index for antineoplastic therapy, we are struck by the similarities between the protective mechanisms constitutively expressed in normal tissue stem cells and those retained or acquired by tumor cell subsets. Sensitizing tumor cells with MDR reversal agents has been tested in Phase I clinical trials [54], but has been unsuccessful precisely because it decreased the ED_{50} but fails to improve the therapeutic index. The identical scenario will be true if therapies are targeted against other shared protective mechanisms (i.e., stem cell niche interactions, detoxifying enzymes) [95]. The most fertile approaches for increasing the true therapeutic index, particularly for metastatic or recurrent cancers, may require investigation of the properties of the cells which survive therapy, whether frankly resistant tumor or minimal residual disease (MRD). It is by the comparison of these cells and their normal tissue counterparts that unique therapeutic targets may be discovered and modalities developed to drive a wedge between residual tumor and normal tissue stem cells. Several paradigms exist in which stem-like attributes of tumors may be specifically targeted. These include the following: (1) Physically targeting reversal agents to tumor cells (antibodies, prodrugs processed by tumor cells); (2) Taking advantage of tumor associated leaky vasculature to selectively deliver therapeutic compounds; (3) Mobilizing stem-like cells out of their protective niche, increasing their susceptibility to therapy; and (4) Finding targets unique to the tumor that are upstream in the regulation of MDR.

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Chapter 22
Resistance to Endocrine Therapy in Breast Cancer: Are Breast Cancer Stem Cells Implicated?

Ciara S. O'Brien, Sacha J. Howell, Gillian Farnie, and Robert B. Clarke

Abstract From a developmental point of view, tumors can be seen as aberrant versions of their tissue of origin. For example, tumors often partially retain differentiation markers of their tissue of origin and there is evidence that they contain cancer stem cells (CSCs) that drive tumorigenesis. This chapter summarizes current evidence that breast CSCs may partly explain endocrine resistance in breast cancer. In normal breast, the stem cells are known to possess a basal phenotype and to be mainly estrogen receptor-α-negative (ER^-). If the hierarchy in breast cancer reflects this, the breast CSC may be endocrine resistant because it expresses very little ER and can only respond to treatment by virtue of paracrine influences of neighboring, differentiated ER^+ tumor cells. As we learn more about CSCs, differentiation, and the expression and function of the ER in these cells in diverse breast tumor subtypes, it is hoped that our understanding will lead to new modalities to overcome the problem of endocrine resistance in the clinic.

Abbreviations

ALDH1	Aldehyde dehydrogenase 1
AML	Acute myeloid leukemia
ASCO	American Society of Clinical Oncology
AZA	5-aza-2′-deoxycytidine
BrdU	5-bromo-2-deoxyuridine
CD	Cluster of differentiation
CK	Cytokeratin

R.B. Clarke (✉)
Breast Biology Group, School of Cancer and Enabling Sciences,
Paterson Institute for Cancer Research, University of Manchester,
Wilmslow Road, Manchester, M21 9WP, UK
e-mail: robert.clarke@manchester.ac.uk

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CSC	Cancer stem-like cells
DCIS	Ductal carcinoma in situ
DNMT	DNA methyltransferase
DTC	Disseminated tumor cells
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor α
ESA	Epithelial specific antigen
FACS	Fluorescence activated cell sorting
GSI	Gamma secretase inhibitor
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HMEC	Human mammary epithelial cells
IGFR	Insulin-like growth factor
IKK	I kappa B kinase
IL	Interleukin
Lin	Lineage
LN	Lymph node
LTED	Long-term estrogen deprived
MAPK	Mitogen-activated protein (MAP) kinase
MDSK	Methylation specific digital karyotyping
MMP	Matrix metalloproteinase
MVD	Microvessel density
NOD/SCID	Nonobese diabetic/severe combined immune deficiency
NS	Neurosphere
PgR	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
PROCR	Protein C receptor
RANKL	Receptor activator for nuclear factor κ B ligand
SAGE	Serial analysis of gene expression
TAM-R	Tamoxifen treated
TGF-β	Transforming growth factor beta
TSA	Trichostatin A
VEGF	Vascular endothelial growth factor
VPA	Valproate

22.1 Introduction

From a developmental point of view, tumors can be seen as aberrant versions of their tissue of origin. Certainly, tumors often partially retain differentiation markers of their tissue of origin. In normal development, adult tissues such as the

mammary epithelium are derived from tissue-specific stem cells, which can be identified by specific cell surface markers and enriched using antibodies and flow cytometry before transplantation into new host animals to confirm that they can regenerate mammary epithelial tissue [1, 2]. In human leukemia, an infrequent population of stem-like cells with a surface-marker phenotype similar to normal hematopoietic stem cells has been shown to transfer the disease into immune-deficient mice, supporting the idea that these cancers contain their own stem cell population [3]. Accumulating evidence supports the concept that epithelial and other solid tumors are aberrantly developed tissues containing a developmental hierarchy that includes cancer stem-like cells (CSCs) and more differentiated progenitor cells. The frequency of this CSC population has been hotly disputed, ranging from very infrequent in leukemia (0.02%) to very frequent (10–25%) in some transgenic models of lymphoma and human melanomas [3–5]. It appears that CSC frequency in breast tumors may very well depend on tumor grade, stage, and molecular subtype [6–8]. A significant role for the stromal microenvironment in determining CSC frequency also appears to be emerging [5]. However, there is no doubt that the evidence that CSCs are responsible for tumorigenesis and cancer recurrence is becoming increasingly solid and needs to be considered for therapeutic decision-making in the clinic. Indeed, analysis of drug compounds which may target cancer stem cells therapeutically has already begun [9, 10]. In terms of clinical trials of novel therapies, it will be important to determine biomarkers for breast CSCs so that their successful targeting can be assessed. In this chapter, we will address the likely contribution of CSCs in resistance to breast cancer treatment, in particular endocrine therapies, and explore the potential for targeting CSCs to resensitize them to treatment.

22.2 Cancer Stem-Like Cells (CSCs)

There is now a large body of evidence to show that leukemia originates from an infrequent leukemic stem-like cell. The first evidence for such CSCs described a small but variable proportion of human acute myeloid leukemia (AML) cells, which could be identified and purified with the cell surface markers $CD34^{+}CD38^{-}$. These cells were found to be the only cells capable of transferring AML from human patients to NOD/SCID mice, providing evidence that not all AML cells have in vivo clonogenic capacity and that only the small subset of CSCs was capable of regenerating the cancer [3]. Many groups have extrapolated the CSC hypothesis from the hematopoietic system to solid cancers, and although the evidence for CSCs in solid cancers is in its infancy compared with the hematopoietic field, the body of supporting data is growing rapidly. Cells with CSC characteristics from human brain tumors (glioblastomas) were first isolated using a clonogenic sphere culture technique to produce the so-called neurospheres (NSs) [11, 12]. These NS cells are highly enriched for the cell surface marker CD133 and nestin (a neural stem cell marker),

have a marked capacity for proliferation and self-renewal, and are capable of in vitro differentiation into phenotypes identical to the tumor in situ. CSC populations have also been found in other solid tumors, including prostate, colon and breast cancers [13–16].

In the breast, Al-Hajj et al. was the first to identify a subpopulation of human breast cancer cells which initiated tumors in immune-deficient NOD/SCID mice [13]. They reported using a set of cell surface markers to sort cells with an increased tumorigenic capacity. Cells that were $CD44^{+}CD24^{lo}ESA^{+}$ and $Lineage^{-}$ (cells lacking markers CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) isolated from one primary breast cancer and eight metastases were able to form heterogeneous tumors 8 out of 9 times. The tumors contained not only the $CD44^{+}CD24^{lo}ESA^{+}Lineage^{-}$ tumor initiating cells but also the phenotypically diverse nontumorigenic cells that comprise the bulk of tumors. As few as 200 $CD44^{+}CD24^{lo}ESA^{+}Lineage^{-}$ cells transplanted into NOD/SCID mice could form tumors with 100% efficiency, while no tumors formed using 200 cells from the $CD44^{-}CD24^{+}ESA^{-}$ cell population. A subsequent study by Ponti et al. carried out on 16 breast lesions (13 primary invasive carcinomas, 1 recurrent carcinoma, and 2 fibroadenomas) using the sphere culture technique resulted in the production of 3 long-term primary cultures which had self renewing capacity and could differentiate into the different breast lineages [17]. Almost all sphere derived cells were found to be $CD44^{+}CD24^{lo}$; however, cells with self renewal capacity only accounted for 10–20% of the total cell number, showing that only a subgroup within the $CD44^{+}CD24^{lo}$ sorted cells had self renewal capacity. This is consistent with only 1 in 200 cells being capable of initiating a tumor in the previous study. Tumor initiating capacity was measured in a long-term sphere culture of the MCF7 breast cancer cell line, termed MCF-S. $CD44^{+}/CD24^{lo}$ cells from parental MCF7 cells were implanted into the mammary fat pad of SCID mice, and only gave rise to tumors when at least one million cells were implanted. However, $CD44^{+}CD24^{lo}$ MCF-S cells gave rise to tumors with smaller numbers of cells (10^{5}, 10^{4}, and 10^{3}) with at least a 60% success rate. Thus, both the mammosphere culture system and the cell surface marker selection enriched for tumor initiating cells in this study, including breast cancers expressing estrogen receptor-α (ER). However, the enriched rather than pure CSC population that these methods produce and recent data suggesting that the regulation of CD24 is dynamic both in vitro and in vivo [18] has highlighted the need for additional markers to further purify the *de facto* CSC.

One such marker is aldehyde dehydrogenase 1 (ALDH1), the cellular activity of which can be demonstrated using the fluorescent substrate Aldefluor and flow cytometric analysis [19]. ALDH1 activity has been shown to identify a stem/progenitor population in both human hematopoietic tissue and in the normal mammary gland. Using primary human breast cancer samples cultivated as xenografts prior to disaggregation and sorting, Ginestier et al. demonstrated that only Aldefluor-positive cells could generate tumors in NOD/SCID mice. When combined with fluorescence activated cell sorting (FACS) analysis for CD44/CD24/Lin, the $Aldefluor^{+}CD44^{+}CD24^{lo}Lin^{-}$ population of cancer cells could reliably form tumors

with as few as 20 cells in the innoculum, whereas 50,000 Aldefluor^{-}CD44^{+}CD24^{lo}Lin^{-} cells failed to form tumors [20]. However, recent data suggest that the Aldefluor assay may be less effective at discriminating CSC in breast cancer cell lines of luminal type [21].

There is also emerging evidence that some breast cancer cell lines will provide valuable and reliable models of tumor hierarchies containing CSCs with both cell sorting and xenografting being demonstrated from infrequent cell populations expressing markers such as CD44, Cytokeratin 5/6 (CK5/6), and ALDH1 [21–23]. A common theme of many investigations into CSCs is that they have inherent resistance to chemotherapy and radiotherapy. This is proposed to be due to mechanisms such as more efficient DNA damage checkpoints and survival pathways compared with more differentiated tumor cell populations [24, 25]. Endocrine therapy remains a pivotal treatment for breast cancers which express the ER. However, despite initial response to endocrine therapy, 25% of patients with early breast cancer and all patients with metastatic disease will eventually relapse [26]. In the following sections, we will focus on how breast CSCs may have inherent resistance to endocrine therapies for a variety of reasons including their basal-like phenotype and the pathways that determine their stem cell-like behavior.

22.3 Steroid Hormones and the Cellular Hierarchy of the Normal Breast

The rudimentary mammary gland matures at puberty and functionally differentiates during pregnancy, lactation, and menopause due to the influence of epidermal growth factors and steroid hormones such as estrogens and progesterones [27–30]. This developmental plasticity at tissue level suggests a stem cell population within the mammary gland, which renews and differentiates to form a cellular hierarchy according to highly regulated functional cues. Human embryonic post mortem studies show absent expression of ER before 30 weeks gestation, although rudimentary mammary development commences from week 12 [31]. Moreover, ER knockout mice show no development of the breast beyond the rudimentary ductal structures of early gestation [32]. By contrast, in the mature human mammary gland, 10–20% of luminal epithelial cells coexpress ER and the progesterone receptor (PgR) [33, 34]. Progesterone receptor (PgR) expression is positively regulated by ER. Interestingly, ER^{+} cells in mature mammary glands of both mice and humans do not actively divide but are in proximity to mitotic cells [33, 34]. This would suggest a model whereby ER expression in the normal mammary gland is closely linked to a differentiated cell phenotype with limited replicative capacity.

Recent studies in mice and humans have suggested a more complicated role for the progesterone receptor in the postpubertal mammary gland. Beleut et al. [35] observed progesterone to be the main driver of alveolar proliferation in the adult murine mammary epithelium. Progesterone but not estrogen stimulation in

ovariectomized mice led to two waves of cellular proliferation, measured by BrdU incorporation labeling. The first small proliferative peak occurred in PgR^+ cells within the first 24 h and was driven by cyclin D1. This was followed by a second and larger wave of proliferation in PgR^- cells, which was dependent on RANKL (receptor activator for nuclear factor κ B ligand). The authors postulated that PgR^+ cells may play a progenitor role in the postpubertal mammary gland, which is in contrast to the rudimentary mammary epithelium during early development [35].

The mouse mammary stem cell population characterized by expression of the markers $CD29^{hi}$(β1 integrin)/$CD24^+$/Lin^- [1] consists of less than 0.01% cells expressing ER [36, 37]. Interestingly, epidermal growth factor receptor (EGFR) was found to be expressed in $CD29^{hi}$(β1 integrin)/$CD24^+$/Lin^- cells, although expression of PR and erbB2/HER2 receptor was absent [36]. A further murine study defined the cellular hierarchy further, separating the luminal compartment by expression of Sca1, CD133, CD24, and ER [38]. ER rich $CD133^+$/$Sca1^+$/$CD24^{hi}$ cells were weakly proliferative whereas the milk-protein rich, ER low population of $CD133^-$/$Sca1^-$/$CD24^{hi}$ cells showed high proliferative capacity.

In a study using normal human tissue derived from mammoplasties, Raouf et al. [39] defined bipotent progenitor cells, luminal committed progenitor cells, and differentiated luminal cells by surface marker expression and subsequently assessed gene expression in each population. The cell sorting methods enriched for primitive bipotent cells ($EpCAM^+$/$CD49f^{hi}$/CALLA $(CD10)^+$/$Thy1^+$/$CD133^-$) at a purity of 45 ± 3% (containing 57% of all bipotent cells) and luminal-restricted progenitors ($EpCAM^+$/$CD49f^+$/$MUC1^+$/$CD133^+$/$CD10^-$/$Thy1^-$) at a purity of 32 ± 3% (containing 96% of all luminal progenitor cells). Transcriptional profiling revealed ER^{lo}/PgR^{hi} expression in the bipotent cell population compared to ER^{hi}/PgR^{lo} expression in the luminal committed progenitor population of the normal human breast, in an analogous manner to recent murine data [35]. These findings concur with the work of Shipitsin et al. [40], who determined ER^{lo} expression of the stem cell population; albeit defined by an alternative cell marker methodology ($CD44^+$/$PROCR^+$/$CD24^{lo}$).

Finally, in exciting recent work Asselin-Labat et al. [37] have investigated the role of steroid hormones in murine mammary stem cell function. Whilst demonstrating the adult mammary stem cell to be ER^- and PgR^-, they observed that sensitivity to steroid hormones still remained, as ovariectomy diminished the stem cell pool, whereas pregnancy transiently increased stem cell number by 11-fold in a RANKL-dependent manner. This links the increased breast cancer risk associated with pregnancy and cumulative estrogen exposure with the mammary stem cell field.

22.4 Breast Cancer Stem Cells and Endocrine Resistance

In contrast to the normal mammary gland, actively dividing ER^+ cells are prominent in breast hyperplasia and breast cancers. The levels of ER and PR expression are predictive of treatment response rates to endocrine therapy and distinguish luminal

A tumors, which are highly ER^+ and PR^+, from luminal B and HER-2 tumors, which have lower ER expression, do not express PR, and coexpress other growth factor receptors such as EGFR and erbB2 [41, 42]. Intrinsic and acquired resistance to endocrine therapy remains a significant cause of disease relapse and mortality in ER^+ breast cancers [43, 44].

22.4.1 EGFR Pathway

Enhanced interaction between estrogen receptor signaling and growth factor tyrosine kinase pathways such as EGFR, HER2/erbB2, and IGFR mediates resistance to endocrine therapy. For example, EGFR1 expression is inversely correlated with that of the ER and coexpression of both receptors confers relative resistance to endocrine therapy compared with tumors not expressing EGFR1 [42, 45]. A similar inverse expression relationship occurs between ER and erbB2/HER2. Tamoxifen-resistant MCF7 breast cancer cells show a five to tenfold increase in mRNA and protein expression of erbB2/HER2 and the EGFR receptor compared with sensitive MCF7 cells [46]. Similarly, resistance to fulvestrant and aromatase inhibitors can also be mediated by upregulation of the erbB2 pathway [47]. Long-term stimulation of the EGFR and HER2/erbB2 pathways in endocrine resistant cancer cells down-regulates the ER. Ligand-independent activation of ER may be mediated by growth factor or intracellular kinase phosphorylation of the AF1 domain of ER, for example at serine 118 or 167 [48, 49] by mitogen-activated protein (MAP) kinase (MAPK), phosphoinositide 3-kinase (PI3K), AKT and Src kinase [48, 50, 51], thus allowing expression of estrogen regulated gene products despite endocrine therapy. In two models of acquired endocrine resistance (long-term estrogen deprived [LTED] and tamoxifen treated [TAM-R] cells), treatment with the dual EGFR inhibitor Lapatinib restored endocrine sensitivity [52]. In TAM-R cells, Lapatinib treatment led to reactivation of ER activity. In contrast, in LTED cells, which were exquisitely sensitive to estrogen stimulation at baseline, Lapatinib suppressed ER transcriptional activity. Intriguingly, the recent EGF30008 trial [53] demonstrated a significant improvement in progression free survival in $HER2^-$ patients with low ER expression, who had relapsed within 6 months of tamoxifen discontinuation with dual treatment with letrozole (an aromatase inhibitor) combined with Lapatinib (13.6 months) compared with letrozole alone (6.7 months $p < 0.005$).

The acquisition of enhanced EGFR/erbB2 pathway signaling in ER^+ breast cancer with tamoxifen resistance potentially results from selection of a more stem-like phenotype. Expression of EGFR has been demonstrated in stem cells of the normal mammary gland in mice and humans [36, 54]. This is in contrast to ER, which is predominately expressed in more differentiated luminal cells [34, 38, 40]. In malignant CSCs, Farnie et al. [6] showed activation of the EGFR pathway in ductal carcinoma in situ (DCIS) of the breast. Inhibition with gefitinib, an EGFR pathway inhibitor, significantly reduced mammosphere formation in vitro.

There is also emerging evidence for a role of the HER-2 pathway in the function of CSCs. In one series of 491 breast cancer patients, expression of erbB2/HER2 and presence of ALDH1$^+$ CSCs were positively correlated [55]. Recently, a report showed erbB2/HER2 over-expression enriched for normal and malignant stem cells in mammosphere and Aldefluor assays and increased in vitro clonogenicity and tumorigenicity in immunocompromised mice [56]. Separately, the CSC population of four HER2$^+$ breast cancer cell lines have been demonstrated to express more HER-2 mRNA and protein compared with the non-CSC cell population, regulated at the level of transcription. In a clinical study in HER2 over-expressing large primary breast cancers, lapatinib (Tykerb; a dual EGFR/HER-2 tyrosine kinase inhibitor) reduced the CD44$^+$/CD24^{lo} CSC fraction and mammosphere forming efficiency of the residual tumor, although this did not reach formal statistical significance [57]. Notably, treatment with chemotherapy alone increased the proportion of CSCs in the residual breast cancer [57, 58]. Thus the well-described upregulation of the EGFR/HER2 pathway in endocrine resistant breast cancer may in fact reflect an enrichment of a CSC phenotype.

22.4.2 Notch Pathway

An intriguing interaction is emerging between the Notch pathway, CSCs, and endocrine treatment in breast cancer. The Notch pathway has been implicated in cell fate delineation in the normal human mammary gland [39, 59] and regulation of CSCs in DCIS [6] and invasive carcinoma of the breast [60, 61]. For example, Farnie et al. observed that inhibition of the Notch pathway by the gamma secretase inhibitor (GSI) DAPT or a Notch 4 neutralizing antibody significantly reduced mammosphere formation in primary human DCIS in vitro [6]. Similarly, recent work by Harrison et al. [62] demonstrated an eightfold increase in Notch 4 activity in CSC-enriched populations in breast cancer cell lines and invasive human breast cancers, compared with more differentiated tumor cells. Pharmacological and genetic inhibition of Notch 4 and Notch 1 receptor signaling significantly reduced both CSC activity in vitro and overall tumor formation in vivo.

Breast cancer of luminal type has been shown to express low levels of Notch and ErbB2 but high levels of ER compared with basal breast cancers, which show the opposite pattern [63]. This inverse relationship between the expression of ER, ErbB2, and Notch activity in breast cancer may provide clues regarding the regulation of CSCs and endocrine resistance, although the mechanisms underlying these interactions remain to be elucidated. However, this cross talk appears to be relevant therapeutically, as Osipo et al. recently demonstrated a two to sixfold increase in Notch 1 activity in MCF7, BT474, and SKBR3 cell lines after treatment with trastuzumab or lapatinib. Such treatment induced nuclear accumulation of Notch 1 intracellular domain and increased expression of Notch downstream targets including Hes 1, 5, and Hey 1. Inhibition of the Notch pathway led to resensitization to trastuzumab, and the combination of Notch antagonism and trastuzumab inhibited growth in both trastuzumab-sensitive and -resistant cell lines [64]. This is analogous

to data reporting resensitization to docetaxel and doxorubicin chemotherapy in breast cancer after RNAi mediated knockdown of Notch 1 [65].

Estrogen signaling also down-regulates Notch signaling. Rizzo et al. demonstrated estradiol-induced reduction in the expression and activation of Notch 4 and Notch 1 in T47D and MCF7 cell lines. This reduction in Notch activity could be abrogated by tamoxifen and fulvestrant [63]. In a mouse xenotransplantation assay using the BT474 cell line, tumors were treated with tamoxifen alone or in combination with a GSI. Combination therapy was significantly superior to the use of tamoxifen alone, and the authors concluded that tamoxifen antagonism of the estrogen stimulus leads to reactivation of the Notch signaling pathway and promotion of proliferation and survival. More recently, a potential mechanism of direct transcriptional crosstalk between Notch 1 and ER target genes has been described via a nuclear I kappa B kinase (IKK)-dependent pathway [66]. However, further investigations will have to be carried out to determine whether this effect is on a cellular population level or specifically mediated by the CSC population.

22.4.3 Cellular Hierarchy of Breast Cancer and ER Expression

One mechanism of resistance to ER targeted endocrine therapy may be the presence of an ER^-, treatment-resistant CSC population with the capacity to differentiate and produce treatment sensitive ER^+ luminal cancer cells. One prediction that follows from this proposed mechanism is that after endocrine treatment, there would remain a resistant population of $ER^{-/lo}$ progenitor-like cells to seed relapse and metastases despite endocrine therapy.

In primary human breast cancer samples, Shipitsin et al. [40] used transcriptional profiling to characterize $CD44^+/PROCR^+$ stem cells and $CD24^+$ luminal type cells from the same donor. This group showed that $CD44^+/PROCR^+$ cells in breast cancers were enriched for stem cell markers and for gene expression related to cell motility and angiogenesis. Interestingly, malignant $CD44^+$ cells were ER^{lo} in a similar manner to $CD44^+$ cells from normal mammoplasty specimens in this report and in a study by Fillmore and Kupperwasser [22, 40].

A recent study has also demonstrated the presence of rare steroid receptor negative $CD44^+$ cells present in the ER^+ breast cancer cell line T47D [23]. The size of this $CD44^+/CK5^+/ER^-/PgR^-$ population did not proportionally increase with expansion of the rest of the tumor population, and this infrequent ER^- cell type was observed in both in vitro clonogenic and in vivo tumorigenic assays, whereas the bulk of the tumor consisted of proliferative $CD44^-/CK5^-/ER^+/PgR^+$ cells. Notably, an intermediate $CK5^-/ER^-/PgR^+$ cell population was demonstrable in in-vitro colony assays when treated with progesterone. These defined populations within an ER^+ cell line appear to mimic the cellular hierarchy of steroid receptor transcript expression in the normal breast as shown by Connie Eaves' [39] and Cathrin Brisken's groups [35]. It is intriguing to speculate that there may be a role for the PgR in a putative progenitor cell population in cancer.

Such findings might be consistent with a model in which an ER^- stem cell generates a cellular hierarchy at a metastatic site comparable to the hierarchy of the primary tumor. Endocrine therapy in resistant patients may enrich for the CSC population in an analogous manner to the effects of chemotherapy or radiotherapy [57, 58, 67], leading to eventual relapse. Certainly recent data suggests residual cancer cells after letrozole therapy are enriched in the $CD44^{hi}/CD24^{lo}$ phenotype, have enhanced mammosphere forming capacity, and express mesenchymal type markers including vimentin and MMP2 [25]. In contrast, ER^+ breast cancers with a gene expression signature similar to mammosphere derived gene sets were more frequently low grade ($p < 0.001$) and of luminal A subtype ($p < 0.001$), with a superior overall survival rate over 10 years (HR 0.24 95% CI 0.11–0.52) [68]. From this study, the authors contend that survival from ER^+ breast cancer is largely governed by cellular proliferation rather than cancer stem cell activity. However, as the mammospheres in this study were harvested on day 7, it is possible that the cancer stem cell gene expression signature may be obscured by that of the larger population of differentiated daughter cells within the mammosphere colony at this time.

22.4.4 Cancer Stem Cells, Mesenchymal Phenotype, and Endocrine Resistance

Recent work by Weinberg's group [69] has linked the mesenchymal cell phenotype to stem cells in normal tissue and to CSCs. Immortalized human mammary epithelial cells (HMECs) induced to undergo epithelial-to-mesenchymal transition (EMT) exhibited stem cell markers and had increased capacity to form mammospheres enriched in stem cells. Similarly, stem cells isolated from normal and cancerous human and mouse mammary glands demonstrated markers of a mesenchymal phenotype normally apparent in EMT. This included up-regulation of the transcription factors Snail and Slug and also the transforming growth factor beta (TGF-β) signaling pathway, which has been previously implicated in stem cell function [40].

Metastatic potential has long been associated with the loss of markers of the epithelial cell phenotype and the acquisition of basal/mesenchymal properties. Interestingly, recent analysis of a panel of breast cancer cell lines of luminal, intermediate, and basal phenotypes has shown a significantly increased fraction of CSCs (defined by $CD44^+/CD24^{lo}/ESA^+$ expression) in basal type breast cancers compared with hormone sensitive luminal cancers (2.5 vs. 0.5% $p < 0.0001$) [22]. Furthermore, a positive correlation was shown between CSC number and cell line tumorigenicity in vivo [22].

Endocrine resistant ER^+ breast cancers are reported to gain a more basal phenotype, for example, reduction in E-cadherin expression [70] and enhanced motility and invasion by upregulation of Src kinase [71, 72], NF-κB [73] and CD44 [74, 75]. As ER negatively regulates the expression of the key transcription factors regulating EMT such as Snail and Slug [76, 77], a functionally redundant ER in endocrine resistant breast cancer might therefore promote a more mesenchymal stem-cell-like

phenotype. Neoadjuvant letrozole therapy in a series of 36 patients [25] induced a comparative mesenchymal phenotype and claudin low signature [78] of cells remaining after systemic therapy. The residual cells showed enhanced expression of vimentin, fibronectin, and Snail; whereas expression of E-cadherin was diminished consistent with the acquisition of mesenchymal characteristics postendocrine therapy.

22.4.5 Epigenetic Regulation of the Cellular Hierarchy

Gene-expression profiling of breast cancer has demonstrated at least six distinct molecular subtypes, including basal, erbB2, luminal B, luminal A, normal-like and claudin-low [41, 78, 79]. These subtypes probably represent a differentiation spectrum comparable to the developmental hierarchy of the breast, with poorly differentiated ER-negative basal type at one extreme to well-differentiated luminal A type at the other. As such, these subtypes may derive from a cell of origin at a different stage of the developmental hierarchy [80] and reflect the hormone and growth factor sensitivity of that distinct cell. Prolonged endocrine therapy may lead to the reacquisition of a more primitive cancer cell phenotype with intrinsic resistance to hormone manipulation.

A recent study elegantly demonstrates that targeted epigenetic modification of the genome has an important role to play in cell-fate determination in the cellular hierarchy of the human mammary gland and breast cancer [81]. Using MDSK (methylation specific digital karyotyping) and SAGE (serial analysis of gene expression) techniques, adult mammary stem cells ($CD44^+$) and breast cancer stem cells ($CD44^+$) were compared and contrasted to more lineage committed ($CD24^+$) cells. Normal adult mammary SCs and CSCs showed comparable genomic hypo-methylation of transcription factors implicated in stem cell function such as HOXA10, FOXC1, and TCF3 compared with the more highly methylated progenitor and lineage committed cells. Forced expression of FOXC1 in differentiated mammary cells, where FOXC1 is normally methylated, led to the reacquisition of a progenitor-like phenotype. This suggests an important role for epigenetic modification in cell fate specification and function of normal and cancer stem cells, which in the future may be amenable to therapeutic targeting.

Acquired endocrine resistance may thus result from an alteration in cancer phenotype between the primary tumor and the metastases to a more stem-like hormone insensitive cellular identity; however, the evidence for this remains circumstantial. In one series of 200 patients, 19.5% of metastases were found to be ER^- in the presence of an ER^+ primary breast cancer, and these findings have been replicated in another smaller study [82]. Fehm et al. have shown that in 88 patients with ER^+ primary breast cancers, 76 had only ER^- disseminated tumor cells (DTC) in the bone marrow [83]. These data raise the possibility that the ER^- CSC is responsible for tumor metastasis and that cell surface phenotype of such cells facilitates communication with a stromal niche that enables intravasation and metastatic growth. It is worth noting that ER is lost completely in only 20% of metastases from ER^+

primary cancers, suggesting that the ER^- DTCs isolated by Fehm et al. may undergo differentiation into tumors that can be subsequently defined as ER^+. Up to half of metastatic tumors, which continue to express ER, show no functional inhibition by endocrine agents. Interestingly, aberrant methylation of ER and PgR promoters has been observed in up to 40% of hormone receptor negative breast cancers [84, 85], and epigenetic modifications have been shown in tamoxifen resistance [86]. Forced reexpression of ER by therapeutic demethylation may thus lead to the intriguing possibility of reacquisition of endocrine sensitivity in these malignancies and we will discuss this possibility further later in this review.

22.4.6 The Stem Cell Niche and Its Influence on Resistance to Endocrine Therapy

CSCs are associated with an increased invasive and metastatic/migratory phenotype [87–89]. Cells isolated as CSCs by virtue of $ALDH1^+$ and/or $CD44^+/CD24^{lo}$ demonstrated increased metastasis from primary tumors in NOD/SCID/IL2γ receptor null mice [87]. Such augmented invasive and metastatic phenotypes are also seen in endocrine resistant breast cancer cell lines [50, 90, 91]. These cell lines exhibit over-expression of EGFR and the c-MET receptor through which they derive proliferative and migratory/invasive signals from stromal derived ligand secretion. Significantly, such resistant cells also overexpress CD44, the adhesion of which to bone marrow derived endothelial cells is enhanced by stromal derived HGF in vitro [75, 92]. Thus, adaptive endocrine resistance in cell lines is associated with a metastatic and stem cell-like phenotype.

Using human breast cancer cell lines in a murine model, it was demonstrated that CD44 was sparsely expressed in primary tumor cells but homogeneously overexpressed in cells transiting the lymphatics and populating lymph nodes (LNs) [93]. The authors hypothesized that CD44 expression targeted tumor cells for metastasis to, and uptake in the LN, although induction of CD44 expression by interaction of the epithelial cells with the LN stromal cells is also a possibility. The CD44 expressing cells were relatively insensitive to the effects of estradiol and estradiol withdrawal despite ER expression levels comparable to those seen in the primary tumors [93, 94]. The same group have also recently shown that a small subpopulation of the cells expressing CD44 express CK5 but not ER or PgR, and are resistant to both endocrine and chemotherapy [95]. Thus, the LN and stromal microenvironments may be responsible for maintenance of the CSC phenotype and suppression of estrogen sensitivity in such cells.

Supporting the former hypotheses are recent data from Farmer et al. demonstrating a stroma-related gene signature in primary breast cancers [96]. This signature was associated with the presence of a reactive stroma, and predicted for resistance to neo-adjuvant chemotherapy. Importantly, the stroma-related signature demonstrated a pattern of expression similar to that of mammospheres, suggesting that the

stroma may support the CSC phenotype and promote resistance to therapy. As the signature was only tested in ER negative tumors, the relevance to luminal tumors and endocrine resistance is unknown; however, such analyses are eagerly awaited.

Another emerging target that is likely to have an impact on CSCs is anti-angiogenic therapy, since evidence is accumulating that both tissue stem cells and CSCs preferentially associate with blood vessels. For example, in oligodendrogliomas and glioblastomas, there is a direct correlation between nestin positive CSCs and microvessel density (MVD) [97]. This study also reported that CSCs preferentially associate with $CD34^+$ capillaries in vivo (in tumor sections) and endothelial vascular tubes in a basement membrane (Matrigel) culture assay in vitro compared with non-CSCs. In a prior report, this association had been shown to be secondary to CSC secretion of vascular endothelial growth factor (VEGF), which directly stimulates endothelial cell growth [98]. Currently, there is little data to support or refute the existence of a vascular niche for the breast CSC and further investigation is required.

22.4.7 Differentiation Agents and Endocrine Treatment

There is evidence to show that histone deacetlylation and DNA methylation plays a key role in inactivation of ER gene expression. In ER^- breast cancer cells, studies have demonstrated that the ER CpG promoter is occupied by abundant HDAC1 and HDAC2 [99, 100]. Similarly, DNA methylation has also been reported to be enhanced in ER^- breast cancer cells [101]. Investigation of de novo ER gene methylation in vitro showed DNA methyltransferase 1 (DNMT1) levels were significantly elevated in ER^- breast cancer cell lines compared with their ER^+ counterparts [85]. Furthermore, recent research into cell type specific DNA methylation patterns revealed that progenitors were hypomethylated compared with differentiated cells in the human normal breast and breast cancer [81]. The role of epigenetic modification in regulation of ER expression and cell fate in breast cancer may provide a therapeutic targeting strategy for ER^- breast cancer patients.

Epigenetic therapies such as histone deacetylase (HDAC) and DNMT inhibitors have shown considerable promise in the treatment of hematological malignancies [102], and trials are ongoing in solid cancers. Cell line studies have shown that functional ER gene expression can be induced by pharmacological administration of the DNMT inhibitor 5-aza-2′-deoxycytidine (AZA) and a HDAC inhibitor trichostatin A (TSA) [103–105]. Furthermore, combination AZA and TSA treatment acts synergistically to induce reexpression of ER in ER^- breast cancer cells [106]. A recent preclinical xenograft model has demonstrated that ER^- MDA-MB-435 cells treated with AZA and TSA reexpressed functional ER, which by itself caused a significant reduction in tumor growth. In addition, after ovarian ablation to mimic endocrine treatment, there was a further reduction in tumor growth [107]. Finally, using the clinically available HDAC inhibitor sodium valproate (VPA), Fortunati et al. [108] were also able to

restore ER transcriptional activity to MDA-MB-231 cells and furthermore convey tamoxifen sensitivity to the previously tamoxifen insensitive HDAC treated cells using in vitro assays.

An inverse relationship between ER and EGFR expression has been well documented in breast cancer cell lines. Using the HDAC inhibitor vorinostat, the ER^- cell lines MDA-MB-231 and MDA-MB-468 cells exhibit ER gene expression and reduced EGFR expression. Reduction in EGFR expression led to reduced EGFR signaling and PAK1 expression levels [109]. Interestingly, immunohistochemical analysis of PAK1 showed significantly increased expression in breast cancers from hormone resistant patients [110–112].

HDAC inhibitors are being used in a number of ongoing clinical trials including a Phase II trial evaluating vorinostat in ER positive patients with metastatic breast cancer who failed prior aromatase inhibitor therapy and up to three chemotherapy regimes [113]. A report of preliminary findings presented at the American Society of Clinical Oncology (ASCO) 2008 meeting showed that out of the 17 enrolled patients, 21% had a partial response and 29% had stable disease after treatment with vorinostat 400 mg daily for 3 of 4 weeks and tamoxifen 20 mg daily, continuously. These findings suggest that the addition of an HDAC inhibitor to tamoxifen in patients who have failed prior aromatase inhibitors or adjuvant tamoxifen may restore hormone sensitivity. The in vitro studies would also suggest that HDAC inhibitors in combination with endocrine inhibitors may be highly applicable to ER^- breast cancers as well.

22.5 Concluding Remarks

In this chapter, we have summarized current evidence that supports our understanding of CSCs to explain endocrine resistance in breast cancer. The biology of breast CSCs is becoming better characterized, and the data suggest that they may be resistant to several forms of cancer therapy through diverse mechanisms. In terms of responsiveness to endocrine therapy, we can learn about CSC biology and hierarchies in breast cancer (Fig. 22.1) by examining what is known about the developmental hierarchy of the normal breast epithelium. In normal breast, the stem cells are known to possess a basal phenotype and to be mainly ER^-. If the hierarchy in breast cancer reflects this, the breast CSC may be endocrine resistant because it expresses very little ER and can only respond to treatment by virtue of paracrine influences of neighboring, differentiated ER^+ tumor cells. Normal breast epithelial stem cells are highly dependent on EGFR and other growth factor receptors, and it may be that the observed increased growth factor receptor expression in resistant breast cancers reflects an increased proportion of stem-like cells selected by endocrine therapies. There is evidence from a number of studies that breast CSCs are ER^-, which would support this view. CSCs also express mesenchymal proteins, which are suppressed by ER expression, further indicating the mutual exclusion between ER^+ cells and CSCs. It is likely that this is regulated at the epigenetic level, and differences in DNA methylation and chromatin organization can be observed

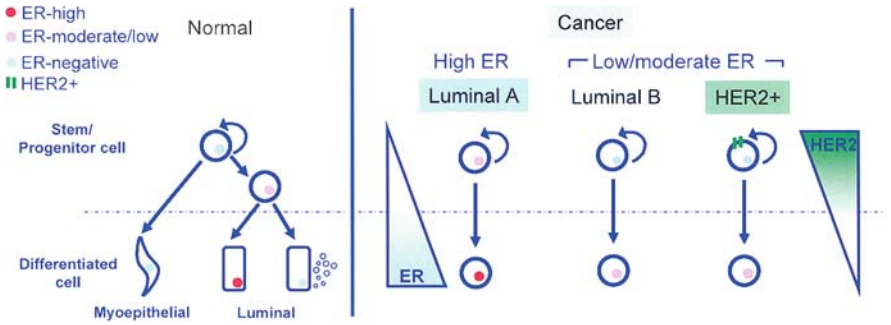

Fig. 22.1 Hypothetical cellular hierarchy of normal and malignant breast illustrating putative differential estrogen receptor α (ER) expression. In the normal breast, an ER^- stem/progenitor cell either differentiates into an ER^- myoepithelial cell lineage or via a ER moderate/low expressing progenitor will produce the luminal lineage which is either ER^+ (nonmilk secreting) or ER^- (milk secreting). Three different breast cancers are illustrated showing the Luminal A high ER^+ tumors differentiating from ER low/moderate stem/progenitor cells. The Luminal B and $HER2^+$ low to moderate ER tumors both differentiate from an ER^- stem progenitor population. In the $HER2^+$ tumors, the stem/progenitor populations are highly $HER2^+$

between breast CSCs and more differentiated populations. This may in turn be regulated extrinsically by the influence of stromal elements including the stem cell niche/microenvironment associated with the vasculature, the LNs and the bone marrow to which breast cancer cells often metastasize. It is known that epigenetic programming can be remodeled by using drugs, particularly those that change the methylation and chromatin patterns of the DNA. Such drugs can effectively differentiate the cells, including potentially the CSCs, leading to a reduction in growth factor receptors and an increase in ER^+ cells, which may overcome resistance to endocrine agents in combination therapy. Such combinations are currently in clinical trials and their outcome is eagerly anticipated. As we learn more about CSCs, differentiation, and the expression and functional activity of the ER in these cells in diverse tumor subtypes, it is hoped that our understanding will lead to new modalities to overcome the problem of endocrine resistance in the clinic.

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Chapter 23
Future Directions: Cancer Stem Cells as Therapeutic Targets

Alysha K. Croker and Alison L. Allan

Abstract Although cancer therapies are becoming steadily more effective, the reality is that none of our current therapies are effective at curing the disease. Almost all cancers will inevitably relapse, and the relapsed tumor will usually be more aggressive and more resistant to current cancer therapies. The cancer stem cell (CSC) hypothesis suggests that a small population of CSCs is inherently resistant to many forms of cancer therapy, and is therefore the cause of tumor relapse. In this chapter, we review the implications of the CSC hypothesis as it relates to therapy resistance. We discuss normal stem cell pathways that are up-regulated in cancers as a way to target these cancers therapeutically, as well as the idea of differentiation therapy in the context of CSCs.

Abbreviations

AATP	AML-associated translocation product
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
Ara-C	Cytarabine
ATP	Adenosine triphosphate
ATRA	All-*trans* retinoic acid

A.L. Allan (✉)
Departments of Oncology and Anatomy & Cell Biology,
Schulich School of Medicine and Dentistry, University of Western Ontario,
London, ON, Canada

London Regional Cancer Program, London Health Sciences Centre,
London, ON, Canada
e-mail: alison.allan@lhsc.on.ca

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Bcl-2	B-cell lymphoma-2
BCRP1	Breast cancer resistance protein-1
C/EBP	CCAAT enhancer binding protein
CAK	Cyclin-dependent kinase activating kinase
CD	Cluster of differentiation
CML	Chronic myeloid leukemia
CSC	Cancer stem cell
CXCR4	C-X-C chemokine receptor type 4
DEAB	Diethylaminobenzaldehyde
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERα	Estrogen receptor-α
GBM	Glioblastoma
Gli	Glioma-associated oncogene homolog 1
GSI	Gamma (γ) secretase inhibitor
Gy	Gray
HER-2	Human epidermal growth receptor-2
Hh	Hedgehog
HSC	Hematopoietic stem cell
LSC	Leukemic stem cell
MAT-1	Menage-a-trois-1
Mcl-1	Myeloid cell leukemia-1
MDR1	Multidrug resistance protein 1
MGMT	O6-methylguanine DNA methyltransferase
NAD	Nicotinamide adenine dinucleotide
N-CoR	Nuclear receptor corepressor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
PI3K	Phosphoinositol-3-kinase
PLZF	Promyelocytic leukemia zinc finger
PML	Promyelocytic leukemia
PTCH1	Patched homolog-1
RA	Retinoic acid
RAR	Retinoic acid receptor
ROS	Reactive oxygen species
RT	Radiation therapy
RXR	Retinoic X receptor
SC	Stem cell
SCF	Stem cell factor
SHH	Sonic hedgehog
SMO	Smoothened
SMRT	Silencing mediator for retinoid and thyroid hormone receptor
STAT	Signal transducer and activator of transcription
VEGF	Vascular endothelial growth factor

23.1 Introduction

It is estimated that North Americans have an approximate 40% risk of developing cancer in their lifetimes [1]. With many cancers, early detection and timely treatment with existing therapies can successfully reduce morbidity and mortality, such that a little less than half of patients diagnosed with cancer will actually die of the disease [1]. Depending on the type and severity of the cancer, current therapeutic options include surgery, radiation therapy (RT), and systemic therapies such as cytotoxic chemotherapy and/or hormonal therapy [2, 3]. More recently, several promising molecular targeted agents have been approved for use in the clinic, including targeting of Her-2 with Herceptin® (trastuzumab; breast cancer) [4], targeting of vascular endothelial growth factor (VEGF) with Avastin® (bevacizumab; colorectal and lung cancer) [5, 6], targeting of the epidermal growth factor receptor (EGFR) with Iressa® (gefitinib; lung cancer) [7], targeting of the EGFR and Her-2 with Tykerb® (Lapatinib; breast cancer) [8, 9], and targeting the BCR-ABL oncoprotein with Gleevec® (Imatinib; chronic myelogenous leukemia) [10–12].

Despite these promising advances, therapy failure due to resistant cancer cells remains a devastating reality, especially in the metastatic setting. For example, even after complete remission in response to therapy, less than 20% of patients with metastatic breast cancer will remain disease-free for more than 5 years [13]. There are a number of mechanisms that attempt to explain therapy resistance. In response to therapy, cells can increase their expression of drug pumps or detoxification enzymes to either pump out or detoxify chemotherapeutic agents. Additionally, cancer cells have been shown to increase their activation of DNA repair pathways, activate antiapoptotic or pro-survival pathways (i.e., nuclear factor kappa-light-chain-enhancer of activated B cells [NFκB], PI3K/Akt), and/or disrupt apoptotic signaling pathways (i.e., p53) [14].

The classical theory of therapy resistance involves cells developing acquired immunity following a particular therapy [15, 16] (Fig. 23.1). For example, if a patient received the chemotherapeutic agent paclitaxel, the initial therapy may shrink the bulk of the tumor because the paclitaxel-naive cancer cells would succumb to the therapy; however, because the cancer cells are inherently highly mutagenic, a subset of these cells could mutate in response to the paclitaxel therapy, thus becoming resistant to the mechanisms by which paclitaxel kills cancer cells. It is impossible to tell whether this mutation happens in one cell or multiple cells, or how the cells become resistant to the therapy. In this way, it is possible that the cells that are resistant to paclitaxel may still be sensitive to a different type of chemotherapeutic agent because of the different mechanisms of action of the two chemotherapeutic drugs (i.e., doxorubicin acts by intercalating into DNA, while paclitaxel stabilizes microtubules) [15, 17]. However, it is also entirely possible that after exposure to one chemotherapeutic, cells may have developed a way to combat not only just the mechanism of action of that particular drug, but also those of many other chemotherapeutics and thus become multidrug resistant. In this case, many patients would fail no matter what chemotherapeutic agent was used, and the same may hold true with targeted therapies.

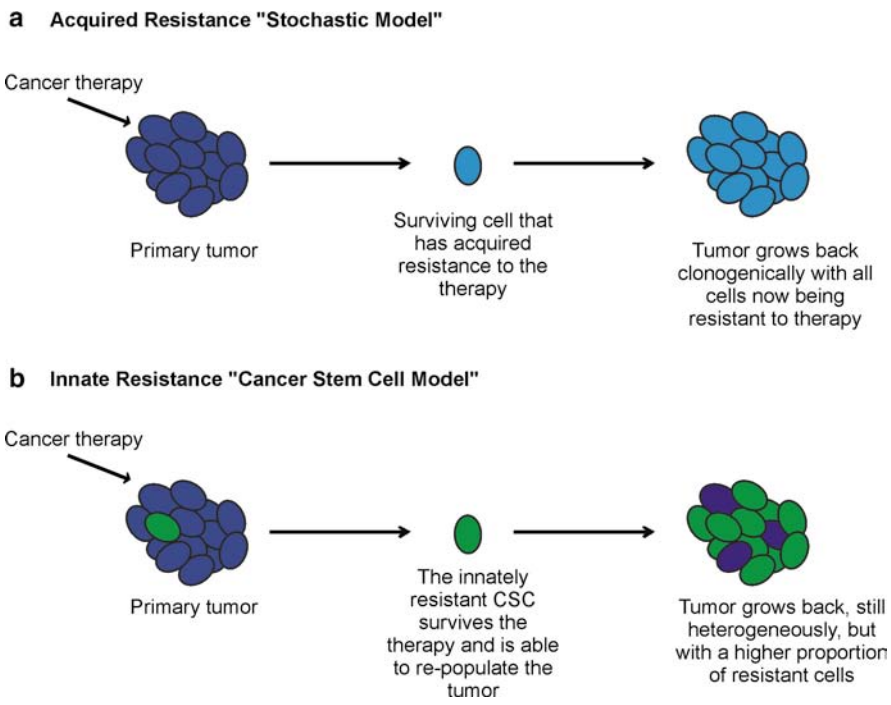

Fig. 23.1 Therapeutic implications of the CSC hypothesis. (**a**) In the traditional tumor model, when tumors are treated with chemotherapy, radiation therapy (RT), or both, tumor relapse is explained by cells mutating and acquiring resistance to the therapy, which allows them to survive and repopulate a new, resistant tumor. (**b**) The CSC hypothesis suggests that there is a small population of CSCs within tumors that are inherently resistant to cancer therapy. When tumors are treated with chemotherapy, RT, or perhaps even targeted therapy, the bulk of the tumor will shrink because the non-CSCs will die off, leaving behind the resistant CSCs that can easily repopulate the tumor, this time with a higher proportion of CSCs, thus rendering it even more resistant to therapy [19]

Another school of thought involves the idea of innate resistance [18, 19] (Fig. 23.1). This hypothesis posits that there is a small subset of cells that are inherently resistant to both chemotherapeutics and RT. As in the above example, treating cells with paclitaxel would cause the majority of the cancer cells to die, but the subset of cells that were inherently resistant to the therapy would survive and repopulate the tumor with resistant cells, causing the tumor to become more resistant to any kind of therapy. In this way, it would be imperative to identify which cells cause the therapy resistance so that they can be destroyed before they propagate to make up the bulk of the tumor. In this scenario, destroying the resistant cells would be the only way to eradicate the patient's disease.

Targeting resistant cancer cells remains the biggest barrier we face in successfully treating cancer. The most frustrating thing about therapy resistance is the cancer cells' remarkable ability to adapt to new environments. However, the first step in

any kind of long and difficult campaign is to identify the proper questions that will lead to useful answers. A good place to start may be asking which cells are the resistant cells in a tumor. If it is possible to know which cells may become and/or are inherently resistant to therapy before the therapy even begins, then perhaps the therapy could be altered, or those resistant cells specifically targeted, in order to sensitize the cancer to cytotoxic therapy, radiation, or targeted therapy. The emergence of the cancer stem cell (CSC) hypothesis (predicting that a small subpopulation of "stem-like" cells are responsible for initiating and maintaining cancer growth) may hold promise in terms of new approaches to cancer therapy. This hypothesis also suggests that CSCs may be the resistant cells in a tumor because they retain normal stem cell (SC) self-protection mechanisms. If this is the case, then how can we target these therapy resistant CSCs? Does it matter whether cells are intrinsically resistant or whether this therapy resistance develops over time by natural selection and mutation in response to a particular form of therapy? How can we target multiple drug resistance pathways at the same time? These questions and others will be the focus of this chapter, which will address the therapeutic implications of CSCs in the context of future directions for cancer therapy.

23.2 Therapeutic Implications of CSCs

The CSC hypothesis could have broad therapeutic implications. Since normal SCs are resiliently resistant to many forms of cellular insult including traditional chemotherapy and radiotherapy, then it would make sense that the CSC population within a tumor would also be at least somewhat inherently resistant to many cancer therapies [20, 21]. If this is true, then cancer relapse could easily be explained as follows: When a tumor is initially treated with chemotherapy or radiotherapy, this would cause the bulk of the tumor to shrink, since the non-CSCs which make up the bulk of the tumor would die. However, the treatment would leave behind a small population of resistant CSCs, which could easily repopulate the tumor and be responsible for tumor relapse.

23.2.1 Cytotoxic Therapy

Cancer cells most often become resistant to chemotherapeutic agents by inducing expression of ATP-dependent drug pumps, which actively transport toxic substances out of cells, and can lead to multidrug resistance in many cancer cells [22–24]. There is evidence to suggest that the CSC compartment of many tumors inherently expresses high levels of drug resistance proteins compared with the rest of the cells in the tumor. For example, $CD133^{+}$ CSCs within glioblastomas (GBM) were shown to be more resistant to chemotherapy, likely due to the high expression of breast cancer resistance protein-1 (BCRP1) and O6-methylguanine DNA

methyltransferase (MGMT) that was observed in the CSC population [23]. In another GBM study, researchers found that the CSCs within the tumor had both increased levels of multidrug resistance protein 1 (MDR1) and increased resistance to chemotherapy [24]. In pancreatic tumors, it was observed that $CD44^+$ CSCs were responsible for gemcitabine resistance. Furthermore, $CD44^+$ breast cancer cells have been shown to preferentially survive chemotherapy compared with the non stem-like cancer cells [22]. Interestingly, it has been shown that the CD44 receptor actually interacts with MDR-1, indicating that CD44 itself may actively contribute to drug resistance of CSCs in various tumor types [25].

In addition to a decreased sensitivity to chemotherapeutic agents due to a high expression of drug resistance genes, expression of both Hedgehog (Hh) and Bmi-1 have been identified in breast CSCs [26]. In many types of normal SCs, Hh signaling is essential for promoting SC self-renewal and proliferation. Hh signaling also increases Bmi-1 expression, and Bmi-1 has been shown to play an important role in the regulation of self-renewal of haematopoietic SCs and neuronal SCs [27]. The cytotoxic agent cyclopamine exerts its effect on cancer cells by binding to and inhibiting Smoothened (Smo), which inhibits the growth of tumors with activated Hh signaling [28, 29]. Studies have demonstrated that xenograft tumors resulting from injection of mice with DU-145 and PC-3 prostate cancer cells can be virtually eliminated by treatment with cyclopamine [20, 30]. It is possible that this is a result of the drug being able to inhibit CSC self-renewal, and hence the overall growth of the tumor.

In brain cancer, intracranial GBM and medulloblastoma xenografts treated with a C-X-C chemokine receptor type 4 (CXCR4) antagonist (AMD3100) showed reduced cell growth and increased tumor cell apoptosis [31]. Furthermore, Jin et al. were able to show that by targeting CD44, leukemic stem cells (LSCs) can be eliminated in an acute myeloid leukemia (AML) model. The authors hypothesized that this result was due to interference with transport to SC-supportive microenvironmental niches and/or alteration of CSC fate toward differentiation [32]. Other studies have shown that treatment of prostate and breast cancer cell lines with a siRNA against CD44 can decrease cancer cell adhesion to bone marrow endothelial cells [33]. This could reduce cellular ability to migrate and invade tissues, and further supports the idea of using a CD44 blocker to target CSCs in cancer therapy.

23.2.2 Radiation Therapy

A serious clinical problem associated with fractionated RT is accelerated repopulation, or the increase in rate of growth as a result of time between treatments. During accelerated repopulation, each day of a treatment gap reduces the efficacy of RT by about 0.6 Gy, making it one of the major reasons for local failure of RT [34, 35]. This may potentially be explained by the CSC compartment within tumors. CSCs are believed to be resistant to RT by preferentially up-regulating their DNA proofreading mechanisms to avoid cellular death due to DNA damage [35, 36].

Studies have shown that treating a tumor with radiation can deplete the non-CSC population and increase the CSC population by 3- to 5-fold, thus rendering the tumor even more aggressive and resistant to treatment [36]. It is also possible that CSCs may tend to be located in the hypoxic regions of tumors, which would affect their sensitivity to radiation via the oxygen enhancement ratio. It is more likely that radioresistance is a general property of CSCs, because of their ability to more efficiently repair their DNA than non-CSCs [34].

In breast cancer model systems, $CD44^{+}CD24^{-/low}$ CSCs from MCF-7 and MDA-MB-231 cancer cell lines were isolated and subjected to a single dose of radiation [35]. The CSCs were observed to be more radioresistant, had fewer or no double stranded DNA breaks (or they were quickly repaired), and had a 50% lower dose-dependent formation of reactive oxygen species (ROS) in response to the radiation. In addition, the increase in the CSC population was associated with the activation of Notch-1 (important in specifying cell fate during development), so it is possible that CSCs may activate this developmental pathway in response to radiation [35, 37]. Another elegant study by Bao et al. demonstrated that glioma cells expressing CD133 showed preferential survival following radiation treatment when compared with $CD133^{-}$ cells (non-CSCs) [36]. Interestingly, even after radiation of up to 5 Gy, the $CD133^{+}$ CSCs retained a similar tumor formation ability and multilineage differentiation potential as the nonirradiated CSCs. The CSCs also demonstrated reduced apoptosis relative to non-CSCs, and this was supported by a decrease in caspase-3 activation and increased activation of the DNA checkpoint proteins Rad17, Chk1, and Chk2 in response to DNA damage by radiation [36]. Diehn et al. demonstrated that the CSC population in various tumor types contained an enhanced antioxidant defence system, which resulted in these cells experiencing lower levels of intracellular ROS and decreased DNA damage following RT [38]. Thus, in the face of radiotherapy, CSCs appear to survive better, repair their DNA more efficiently, and begin to self-renew to increase the CSC population within the tumor [34–38]. Ultimately, this may allow the tumor to become even more radioresistant. It may be reasonable to suggest that targeted therapies could be beneficial in preventing the expansion of the CSC pool following radiation. Similarly, therapies targeting DNA checkpoint proteins may sensitize CSCs to radiation, resulting in a cancer that is potentially less resistant to radiation because the cells will no longer be able to proofread their DNA at such a superior rate.

23.3 Targeting Stem Cell Pathways

There is increasing evidence that stem-like cancer cells are responsible not only for tumor initiation and progression, but also for therapy resistance. For example, AML is a well-characterized disease of blocked differentiation and apoptosis, with cells blocked at the myeloid progenitor stage. This block in differentiation leads to a lack of functionally differentiated hematopoietic cells, leading to pancytopenia [39–42]. Not surprisingly, many of the signaling pathways that have been identified as being

able to maintain normal hematopoietic stem cells (HSCs) ex vivo have also been identified as being deregulated in leukemia; most notably the Wnt/β-catenin, STAT3 and 5, Notch-1, and myeloid cell leukemia-1 (Mcl-1) signaling pathways [41, 43–45]. If activation of normal SC machinery in cancer cells contributes to or causes therapy resistance by inducing the CSC phenotype, then perhaps by inhibiting the normal SC pathways that are activated in cancer, we might be able to eliminate the CSC population, thereby sensitizing the cancer to conventional chemotherapy and RT. This section will discuss potential pathways that may be targeted for this purpose.

23.3.1 Hedgehog Signaling

Hh signaling is an ancient, highly conserved developmental pathway that has critical functions in embryonic development, particularly in relation to the epithelial-to-mesenchymal (EMT) transition [46]. Upon binding a Hh ligand, Patched homolog-1 (PTCH1) is internalized and inactivated so that the endogenous agonist of Smo accumulates in the cytoplasm and activates Smo [47]. This results in the translocation of Smo from the endosome membrane to the plasma membrane. Smo then activates the intracellular signaling molecule Fused, causing the release of the Gli family of transcription factors (Gli1-3), which can then translocate into the nucleus and activate gene transcription. Transcriptional targets of Gli-1 include genes that control cell adhesion, cell cycle, signal transduction, vascularization, and apoptosis [47] (Fig. 23.2a).

In the adult, Hh signaling is important for maintaining and regulating SCs in many normal tissues [46–48]. For example, in haematopoiesis, Hh signaling has been shown to play a role in the regulation of stem and progenitor cell expansion as evidenced by experiments showing that the loss of Smo impairs HSC self-renewal [48]. Furthermore, downstream Hh signaling leads to entry into the cell cycle, inhibition of apoptosis, maintenance of self-renewal, regulation of tissue SC differentiation, and modulation of tissue polarity [48]. Sonic Hedgehog (SHH) has been shown to promote proliferation by opposing signals for growth arrest. For example, in retinogenesis, Hh activation accelerates G_1 and G_2 phases of the cell cycle without affecting the duration of S or M phases, whereas Hh inhibition leads to cells spending more time in the G_1 and G_2 phases [47]. In fact, there is evidence suggesting that SHH signaling may act upstream of other pathways that regulate SC self-renewal. For example, it was shown that the activation of SHH pathway is required for Notch signaling during retinal development, and SHH signaling has been shown to act upstream of Notch during arterial endothelial cell differentiation in mice [47].

Because of the role that Hh signaling plays in SC self-renewal and proliferation (as well as other normal SC features), it is possible that Hh signaling may be an interesting therapeutic target in many cancers, as blocking Hh signaling may cause a reduction in the CSC population. In fact, it has been estimated that up to 1/3 of all tumors have aberrant Hh signaling, and constitutive activation of the SHH signaling pathway has been shown to lead to some cancers [46]. Moreover, SHH-neutralizing

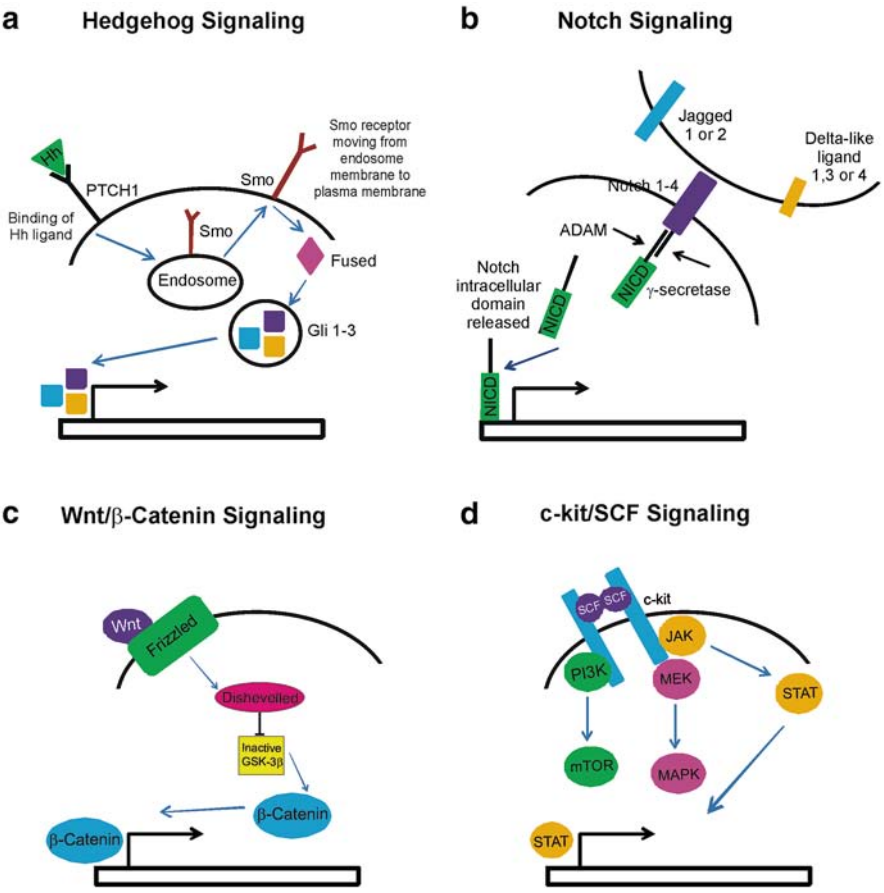

Fig. 23.2 Stem cell signaling pathways identified in cancer. (**a**) Hedgehog pathway. Binding of the Hh ligand to the PTCH1 receptor causes Smo to be released from the endosome membrane so that it can be expressed at the plasma membrane. Smo then activates the intracellular signaling molecule fused, causing the release of the Gli family of transcription factors (Gli1-3) which can then translocate to the nucleus and activate gene transcription. (**b**) Notch pathway. Notch ligands bind to either jagged or delta-like membrane receptors, which leads to cleavage of the receptor by ADAM and γ-secretase, resulting in the release of the notch intracellular domain (NICD). The NICD can then translocate to the nucleus where it recruits various co-activators and co-repressors leading to activation of target genes. (**c**) Wnt/β-catenin pathway. Wnt binds the Frizzled receptor, which causes a signaling cascade ending with β-catenin translocating to the nucleus and activating transcription. (**d**) C-kit/SCF pathway. When stem cell factor (SCF) binds kit, this causes a homodimerization of two kit molecules, which in turn, structurally changes the receptor resulting in activation of the kit kinase domain. Phosphorylated tyrosine residues on kit serve as binding sites for various cell signaling proteins, including members of the PI3K/Akt, MAPK and the JAK/STAT pathways [47, 55, 65, 68]

antibodies have been shown to inhibit tumor cell growth while exogenously added SHH ligand was shown to stimulate tumor cell growth [47]. There was also an interesting negative relationship with estrogen receptor-α (ERα) and Gli-1 expression, where low levels of Gli-1 were found in breast cancers expressing high levels of ERα [46]. Strongly ERα$^+$ breast cancers are generally not very aggressive, and are usually highly treatable [49]. This suggests that Gli-1 expression in breast cancer may lead to a more aggressive tumor. In fact, in vitro studies have demonstrated that stable transfection of Gli-1 into ERα, estrogen-dependent cell lines increased cell growth in estrogen-deficient medium through induction of cell cycle progression [46]. Furthermore, inhibition of Hh signaling was shown to not only inhibit the expansion of LSCs, but also to induce apoptosis in the LSC compartment [50, 51]. In fact, loss of Smo was able to completely abrogate the transplantability of chronic myeloid leukemia (CML) LSCs; whereas constitutive activation of Smo increased the numbers of LSCs and accelerated leukemic disease [51]. Interestingly, high levels of Smo were found specifically in LSCs and not in normal HSCs, suggesting that inhibiting Smo may be an excellent therapeutic target to eliminate the LSC pool [50]. Dierks et al. found that the loss of Smo in regular hematopoiesis had no significant impact on the regeneration of hematopoiesis except for the nearly complete loss of CD8$^+$ T-cells and reduction in the number of short-term repopulating HSCs; however, the long-term repopulating cells in the bone marrow seem to be independent of Hh signaling [50].

In addition to affecting the CSC population, it seems that Hh signaling can also promote both chemo- and radio-resistance in the CSC compartment of leukemia and various solid tumors [48, 52, 53]. For example, it has been shown that Hh promotes cancer cell survival via Bcl-2 [52]. The Gli-1 and Gli-2 transcription factors actually bind to sequences in the Bcl-2 promoter in epithelial cells and induce transcription. When medulloblastoma cells were treated with cyclopamine, a Hh antagonist, lower levels of Bcl-2 were noted, as well as increased cellular apoptosis [52]. It has also been shown that inhibition of Hh signaling significantly decreased drug resistance in CD34$^+$ LSCs to cytarabine (Ara-C), the chemotherapeutic of choice for many leukemias [48]. Furthermore, SHH signaling not only opposes apoptosis, but also actively promotes multidrug resistance by regulating drug trafficking. Studies done by Sims-Mourtada et al. demonstrated that stimulation of cells with SHH ligand resulted in increased expression of both MDR1 and BCRP, whereas blockade of SHH activation by cyclopamine or a Gli-1 specific siRNA resulted in decreased expression of these transporters [53]. Finally, it seems that Hh signaling also plays a role in cancer metastasis. Feldmann et al. were able to show that Hh-dependent pancreatic tumor cells treated with cyclopamine showed a greater than 500-fold reduction in the number of invading and migrating cells, which correlated to a significantly lower rate of metastasis in vivo. Furthermore, cyclopamine treatment also caused a reduction in the aldehyde dehydrogenase (ALDH)^{hi} CSC population, and a significant decrease in therapy resistance [54].

It seems that targeting the Hh pathway may provide therapeutic benefit on many levels. First, inhibiting Hh signaling can decrease or even kill the CSC population within both solid tumors and leukemia [46, 47, 50, 51]. In doing so, these cancers may become more sensitive to therapy simply due to the decrease in the number of

stem-like resistant cancer cells. Furthermore, Hh signaling has been shown to actively promote therapy resistance by increasing expressing of drug resistance proteins, so inhibiting this pathway may, again, enhance the effectiveness of current chemotherapeutics [48, 50–53]. Finally, the role that Hh signaling plays in cancer metastasis is interesting and important since all our current cancer therapies fail in the metastatic setting [54]. In this way, Hh signaling inhibitors may prove to be extremely valuable players in combination with conventional therapies to eradicate many cancers.

23.3.2 Notch Signaling

The Notch signaling pathway is another interesting therapeutic target because of the role it plays in proliferation, apoptosis, and maintenance of the SC state in many normal tissues [55, 56]. Ligands bind to either Jagged or Delta-like membrane receptors, which leads to cleavage of the receptor. This results in the release of the Notch intracellular domain (NICD), which translocates to the nucleus where it recruits various coactivators and corepressors leading to activation of target genes, and finally, degradation of the Notch IC domain [55] (Fig. 23.2b). Interestingly, it has been shown that the inappropriate activation of Notch results in signals that stimulate proliferation, restrict differentiation, and prevent apoptosis, potentially leading to cancer [57]. Further studies actually demonstrated that Notch signaling plays a critical role in GBM CSC self-renewal mediated by endothelial cells, and inhibiting Notch signaling results in a significant reduction in not only the number of CSCs, but also decreass their tumorigenicity [55]. Furthermore, inhibition of Notch-1 or Notch-2 inhibited xenograft tumor formation of GBM cells [56]. Notch signaling has also been shown to play a role in radioresistance and in promoting a hypoxia-resistant phenotype [56–58], and it has been shown that γ-secretase inhibitors (GSIs) (which inhibit Notch signaling) impair cell growth and survival after radiation [56]. Interestingly, Notch does not protect tumor cells from radiation by altering the DNA damage response, but instead by promoting radioresistance through the prosurvival regulation of PI3K/Akt and Mcl-1 [56].

In breast cancer, there is an interesting relationship between Notch and HER2 expression, where HER2-overexpressing cells display activated Notch signaling, and inhibition of Notch signaling using siRNA or a GSI results in down-regulation of HER2 expression [59, 60]. This also results in decreased tumorsphere-forming ability, suggesting that inhibiting Notch and down-regulating HER2 decreases the CSC population [60]. This would make sense since it has been shown that HER2 over-expression increases the stem/progenitor population of both normal and malignant mammary cells [61]. In this study, Korkaya et al. found that HER2 over-expression increased the normal mammary SC pool and caused a 4- to 5-fold increase in $ALDH^+$ breast cancer cells (CSCs). Furthermore, HER2 over-expression increased the expression of numerous SC-related genes (i.e., Oct3/4, Notch1/2, Jag1, and Gli1). Finally, Herceptin, which is used to successfully treat HER2 over-expressing tumors, reduced the $ALDH^+$ CSC population by approximately 50% [61].

23.3.3 Wnt/β-Catenin Signaling

The Wnt/β-catenin pathway plays a well characterized role during embryogenesis, and is implicated in the survival of normal SCs [62–65]. Similarly, the Wnt/β-catenin pathway has been shown to play a role in maintaining the CSC population in CML, as well as a variety of solid tumors including breast, melanoma, colon, and liver [62, 63]. In mouse mammary progenitor cells, treatment with ionizing radiation caused an increase in the progenitor pool, a phenomena that was enhanced by β-catenin stabilization in these cells. Furthermore, the radioresistance was mediated through the Wnt/β-catenin pathway, indicating that this pathway may also be involved in radioresistance [64]. It has also been shown that activated Wnt/β-catenin in lung adenocarcinoma enhanced tumor cell proliferation, clone formation, drug resistance, and the up-regulation of Oct-4, a primitive SC marker [63] (Fig. 23.2c).

23.3.4 Snail/Slug Signaling

Snail and Slug are zinc-finger transcription factors that have been shown to play an important role in wound healing, SC protection from DNA damage, and regulation of EMT [66]. The stem cell factor (SCF) and c-Kit pathways, which are essential for the formation of HSCs and other SCs during embryonic development, have been shown to activate Slug [67, 68]. In this way, the SCF/c-Kit/Slug pathway was shown to be absolutely necessary for SC survival in the bone marrow following lethal irradiation in a mouse model [67]. Similarly, Slug has been shown to mediate radioprotection and enhance survival of progenitor cells in ovarian cancer through activation of the SCF/c-Kit pathways, and an increase in both Snail and Slug expression is seen in ovarian cells treated with chemotherapy, indicating that these proteins may be involved in chemoprotection of these cells [66]. Further evidence of this was seen in studies done by Catalano et al., which demonstrated that the SCF/c-Kit/Slug pathway actively mediates multidrug resistance in malignant mesothelioma cells [14]. When c-Kit expression was knocked down, this increased tumor cell sensitivity to various chemotherapeutic agents in multidrug resistant sublines, and forced expression of SCF/c-Kit signaling in a way that was sufficient to lead to multidrug resistance in parental lines. These processes were shown to be mediated by Slug [14] (Fig. 23.2d).

23.4 Differentiation Therapy

If it is true that the CSC pool within a tumor is responsible for therapy resistance and that this is because of the cells' inherent SC nature, then the idea of differentiation therapy is an interesting one. Differentiation therapy would not kill the CSCs directly, but it does have the potential to restrain their self-renewal capacity, and perhaps increase the efficacy of more conventional therapies (such as chemotherapy),

which are often most effective in differentiated cells. Furthermore, differentiation agents often have less toxicity than conventional cancer treatments [69, 70].

23.4.1 Retinoid Signaling and Differentiation Therapy

The retinoic acid receptor (RAR) signaling pathway is a well-characterized differentiation pathway in many developmental systems, although it is best described in the hematopoietic system [44, 45, 71–73]. Briefly, aldehyde dehyogenase (ALDH) catalyzes the reaction of retinol to retinoic acid (RA), which then binds to a RAR (usually RARα or RARβ). RA binding causes a conformational change to the RAR, which facilitates the release of a repressor protein and binding of an enhancer protein (i.e., C/EBPα and C/EBPβ), and allows transcription of RARα/β and targeting of differentiation proteins [74–80] (Fig. 23.3). The transcription factor acute myelogenous leukemia-2 (AML2) has also been shown to play a role in HSC differentiation and is regulated by RA through RARα-dependent signaling [81].

Blockage of this pathway using either RAR or retinoic X receptor (RXR) inhibitors has been shown to inhibit HSC differentiation [75, 78, 82]. Furthermore, the inhibition of ALDH has been shown to delay the differentiation of HSCs, probably by decreasing the amount of available RA [83, 84], although it has also been shown that ALDH can maintain $CD34^+$ HSC activity and prevent apoptosis through RA signaling [74]. This may have something to do with retinoic acid receptor-γ (RARγ), a member of the RAR family (commonly involved in SC differentiation), as it has been shown to be important in maintaining the HSC phenotype. In fact, when RARγ was silenced, it led to a reduction of HSCs in vivo [84, 85]. RA is not isotype specific, so it will bind to RARα, RARβ, and RARγ with the same ease [86]. In this way, the type and number of RARs expressed in specific cells may contribute to how the cells respond to RA. This demonstrates the complicated nature of retinoic signaling and highlights the importance of gaining a better understanding of this process.

23.4.2 Acute Promyelocytic Leukemia (APL): The Differentiation Therapy Success Story

Many AMLs are initiated by translocation events that fuse proteins involved in cell differentiation, apoptosis/cell survival, cell cycle control, and DNA-binding proteins [87–90]. The AML-associated translocation products (AATPs) have been shown to activate Wnt signaling by increasing γ-catenin expression, leading to SC self-renewal and accelerated cell cycle progression [90], indicating that these fusion proteins may work to give AML cells their blocked differentiation phenotype. Further support of this is found in the observation that many of the AATPs involve RARα (i.e., PML:RARα and PLZF:RARα), which plays a major role in myeloid differentiation of HSCs [91–94].

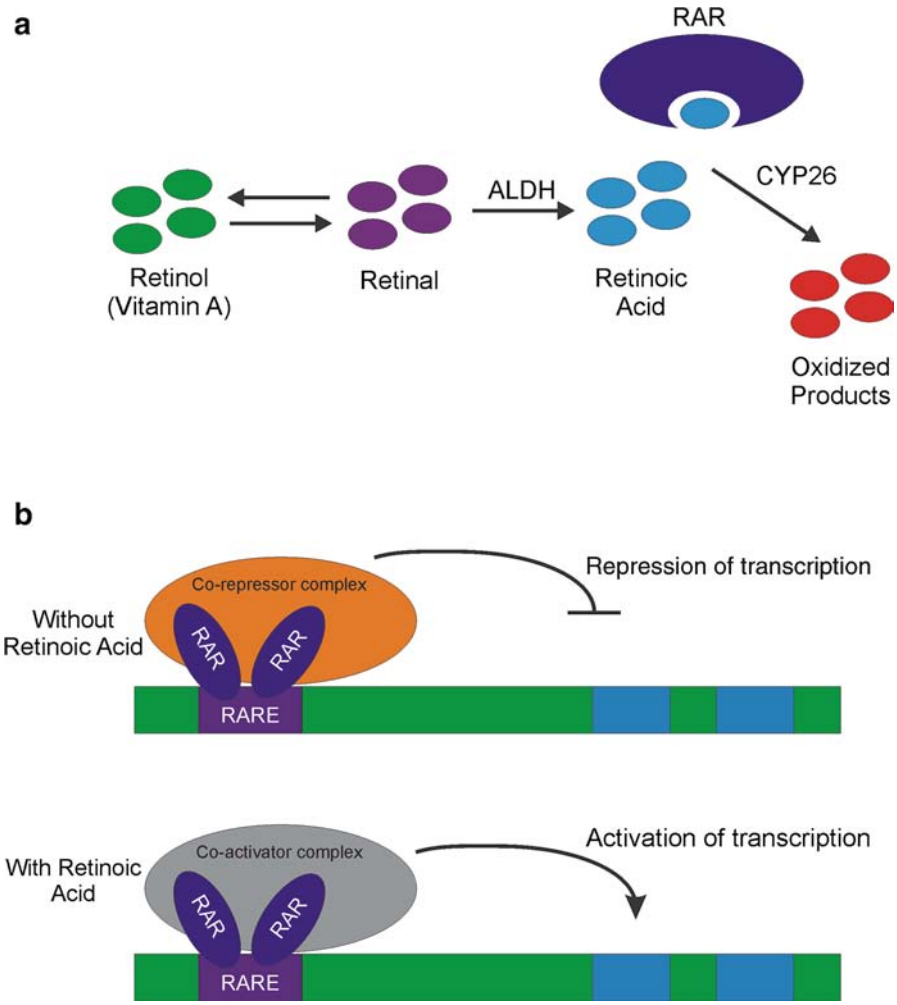

Fig. 23.3 The retinoic acid (RA) signaling pathway. (**a**) The metabolic pathway for synthesis and degradation of endogenous RA. ALDH synthesizes RA by oxidizing retinal. RA is then free to interact with the RARs. Endogenous RA is degraded by CYP26 enzymes. (**b**) RARs, positioned on the retinoic acid response element (RARE), mediate the effects of RA. In the absence of the RA ligand, the RAR dimer is bound to DNA and co-repressors, which induces transcriptional repression of RAR-induced gene expression through histone deacetylation. Binding of RA induces a conformational change, allowing the binding of co-activators and leading to activation of transcription [78]

The differentiation agent all-*trans* retinoic acid (ATRA) is used clinically in combination with chemotherapy to treat acute promyelocytic leukemia (APL) because of its ability to down-regulate ALDH expression [86, 95, 96]. The increase in intracellular RA resulting from ATRA treatment suppresses levels of ALDH1A1 and 3A1,

driving differentiation of the malignant promyelocytes into mature neutrophils and causing enhanced sensitivity to chemotherapy [97, 98]. PML:RARα AML cells exert their pathogenic activity by recruiting histone deacetylases through nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT), activating the cyclin-dependent kinase activating kinase (CAK) complex to hyperphosphorylate RARα, and blocking terminal differentiation and apoptosis [99, 100]. Treatment with ATRA induces ubiquitination-proteolysis of Menage-a-trois-1 (MAT-1), which results in a decrease of CAK-induced phosphorylation of RARα, promoting granulocytic differentiation [76, 100]. ATRA has therefore been used to treat these patients with great success [101–103].

Given that RARα plays such an important role in normal myeloid cell differentiation, it would make sense to hypothesize that ATRA therapy should work across all AMLs to differentiate malignant myeloid cells. However, in reality, this is not the case. All non-PML:RARα AMLs are insensitive to ATRA treatment, including PLZF:RARα, whose AATP actually involves the RARα [104–107]. It was originally assumed that this outcome may have been due to mutations in RARα that abolished the RA binding site, but studies investigating RARα in hundreds of AML cell lines and patient samples found that not only were RARα mutations extremely infrequent, but also RARα hypermethylation was also rare, indicating that most RARα should be fully functional in leukemic disease [108]. It is possible that instead of an RARα culprit, there is instead an upregulation of RARγ in AML patients. It has been shown that RARγ can interact with p85 (phosphoinositol-3-kinase [PI3K] signaling), leading to activation of Akt and NFκB, and playing a critical role in self-renewal and cell survival [109]. ATRA is not isotype specific, so it will bind RARα, RARβ, and RARγ at the same rate [86]. However, it is currently unclear why ATRA can only successfully differentiate PML:RARα AML cells.

23.4.3 Differentiation Therapy in Solid Tumors

Much less is known about the effect of differentiation therapy in solid tumors; however, there is considerable interest in ATRA treatment in breast cancer because of the way ATRA can inhibit cell growth. When MCF-7 breast cancer cells were treated with ATRA, cells accumulated in G_1 phase and by day 10, approximately 50% of the MCF-7 cells had died [110]. Interestingly, the ATRA-mediated growth inhibition in breast cancer cells correlated with the presence of functional estrogen receptors, and the ATRA actions were enhanced by the use of Tamoxifen. Furthermore, studies by Ginestier et al. show that when breast cancer cells are treated with ATRA, the cells demonstrated a decrease in both primary and secondary tumorsphere formation, whereas cells treated with diethylaminobenzaldehyde (DEAB, which specifically blocks ALDH activity, thus potentially blocking the production of RA) had an increase in both primary and secondary tumorsphere formation [111]. This indicates that activation of RA signaling decreases the CSC population, perhaps by “differentiating” the CSCs. In support of this, it was shown

that a portion of genes over-expressed in the $ALDH^+$ (CSC) population were also highly expressed in DEAB treated cells, whereas $ALDH^-$ (non-CSC) genes were highly expressed in ATRA treated cells, indicating that retinoid signaling plays a role in the control of breast CSC differentiation. In addition, several other gene sets related to the carcinogenesis process, metastatic activation, or drug-resistance were down-regulated by ATRA treatment [111].

Given the promising preclinical results with ATRA treatment in breast cancer, a Phase I/II clinical trial was initiated to investigate the use of ATRA in human breast cancer patients. In a single institution Phase II study, 17 patients with hormone refractory, metastatic breast cancer were administered 150 mg/m^2 oral ATRA. Of those 17, only one patient experienced a partial response, which lasted only 4 months. Three other patients experienced stable disease for anywhere between 2 and 4 months [112]. In a different Phase I/II study, breast cancer patients with measurable disease or evaluable nonmeasurable disease were given differing doses of ATRA (70–230 mg/m^2/day) on alternating weeks during Tamoxifen treatment. Of the seven patients with measurable disease, two experienced a partial response to the combination therapy of ATRA and Tamoxifen. Of the 18 patients with evaluable, nonmeasurable disease, 7 experienced a partial response for 6 months or more [113]. These results indicate that ATRA may not be effective as a single agent, but may enhance the effects of chemotherapy or hormonal therapy in the treatment of breast cancer. At any rate, it is clear that ATRA is an interesting therapeutic avenue to explore, but much more knowledge of the RA signaling pathway in both normal and malignant scenarios is required.

23.4.4 ALDH as a Therapeutic Target

It has been reported that the difficulty in successfully eradicating CSCs originates from the expression of many CSC self-protection mechanisms, including drug transporters and ALDH expression [114–118]. ALDH activity renders cells exquisitely resistant to cyclophosphamide therapy, as it has been shown that the transfer of ALDH into $CD34^+$ HSCs conferred resistance to cyclophosphamide, and that cyclophosphamide-resistant AML clones have higher expression of ALDH than the nonresistant clones [119]. Cyclophosphamide is a prodrug, which is activated by the liver to become aldolphosphamide, and eventually phosphoramide mustard, which is the actual cross-linking metabolite of cyclophosphamide. ALDH catalyzes the NAD-dependent oxidation of aldolphosphamide to carboxyphosphamide, which is a harmless metabolite [99, 120–123] (Fig. 23.4). As many clinicians use chemotherapeutics other than cyclophosphamide to treat both leukemia and solid tumors, it would be important to know whether ALDH conferred cellular resistance to other chemotherapeutics, or even to RT. Until recently, it was only assumed that this was true based on the fact that ALDH1 expression increases in primary breast cancer tumors following neoadjuvant chemotherapy [124], and that high ALDH1 expression

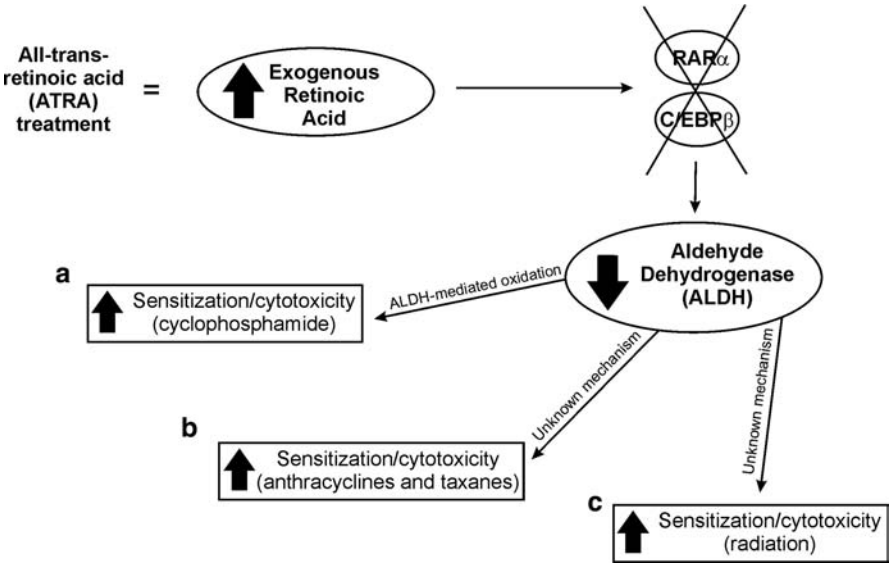

Fig. 23.4 Inhibition of ALDH as a strategy for overcoming treatment resistance. When cells are treated with ATRA, this increases the levels of exogenous RA, which in turn causes ALDH down-regulation by decreasing the amount of the RARα and C/EBPβ transcription factor complex. (**a**) This decrease in ALDH causes a direct sensitization of cells to cyclophosphamide. Cyclophosphamide is a pro-drug, which is activated by the liver to become aldolphosphamide, and eventually phosphoramide mustard, which is the actual cross-linking metabolite of cyclophosphamide. ALDH catalyzes the NAD-dependent oxidation of aldolphosphamide to carboxyphosphamide, which is a harmless metabolite. (**b** and **c**) A decrease in ALDH causes a sensitization of breast cancer cells to both taxanes and anthracyclines (**b**) and radiation (**c**) by an as yet unknown mechanism [97, 98, 126]

correlates with poor patient outcome [125]. Recently, however, studies performed in our lab [126] have shown that when resistant $ALDH^{hi}CD44^{+}$ breast cancer cells are pretreated with DEAB (to block ALDH activity) or ATRA (to indirectly down-regulate ALDH), a significant initial sensitization to doxorubicin, paclitaxel, and RT was observed, in many cases to a level equivalent to that of the nonresistant $ALDH^{low}CD44^{-}$ cells. However, only DEAB pretreatment was able to also reduce the long-term regrowth/colony-forming ability of chemotherapy- or radiation-treated $ALDH^{hi}CD44^{+}$ cells, whereas cells pretreated with ATRA were able to regrow just as well as non-ATRA treated cells. These results indicate that specifically blocking ALDH is key for sensitizing resistant $ALDH^{hi}CD44^{+}$ cells to therapy (Fig. 23.4). As discussed earlier, ATRA can down-regulate ALDH through an indirect route, but in our breast cancer cells, we saw that this ALDH down-regulation was short-lived as cells began re-expressing ALDH as early as 48 h following treatment. Therefore, a more direct targeted approach, such as DEAB inhibition of ALDH or ALDH-specific siRNA, may be necessary [126].

23.5 Concluding Remarks

It is true that there have been giant leaps forward in designing and testing successful novel cancer therapeutics that target the disease more successfully, and even (in some cases) with fewer side effects. It is clear that the more we learn about the biology of both solid tumors and leukemia, the better and more targeted our therapies will become. Half the battle in combating cancer is known which cell(s) to target, since there is usually great heterogeneity within cancers. More and more evidence is accumulating to support the idea that CSCs play a major role in not only initiating and sustaining primary tumors, but also in facilitating metastasis to distant organs [125, 127–137]. In this way, it is logical to assume that these CSCs might make excellent therapeutic targets since, if we can get rid of the CSC population, then we could theoretically be able to get rid of the tumor. However, targeting the CSC population using conventional therapies has proven extremely challenging since it has been shown that CSCs preferentially survive both chemotherapy and/or RT compared to their non-CSC counterparts [20, 21, 24, 35, 36, 38, 126, 138]. Interestingly, normal SCs are also highly capable of protecting themselves from cellular insult, much more so than their differentiated progeny, via a number of different mechanisms including up-regulating multidrug resistance pumps, DNA protection mechanisms, and even just via their quiescent nature [21]. In this way, perhaps by amalgamating what we know of normal SC biology and applying it to the cancer field, we will be better poised to tackle the problem of therapy resistance.

This has led to work investigating whether blocking either normal SC properties or pathways could lead to CSC sensitization to therapy, and this has been met with moderate success. Drugs have been developed to target different stages of multiple SC pathways including Notch [55–58], Hh [46, 52, 54], Wnt/β-catenin [62–64], and c-kit/SCF/Snail/Slug signaling [14, 66]. Drugs that target SC self-protection mechanisms have also been used with some interest, including drugs that target MDR-1, BCRP, and ALDH to try and sensitize tumors to conventional therapy [20, 23, 24, 47, 50, 51, 53, 115]. Finally, the idea of "differentiation therapy" has been used to successfully treat APL patients by differentiating the LSC population, thereby rendering the disease more sensitive to conventional chemotherapy (cytarabine) [48, 86, 96]. Further work is needed to determine whether differentiation therapy will be as successful in solid tumors.

Although focusing in on the CSC population is a huge leap forward in identifying the cells that need to be targeted in cancer, it is important not to forget that the CSC population itself may, in fact, be a heterogeneous population. Therefore, it will be crucial to better study and understand the true nature of the CSC population. By doing this, we will be able to better answer important questions about whether there is a true CSC hierarchy within cancers, with some of the most primitive cancer cells, quiescent and elusive, maintaining the cancer and surviving the cancer therapies. Furthermore, are all CSCs inherently resistant to cancer therapy, and are all CSCs equally able to metastasize? Interesting work done by Hermann et al. found that only a small subpopulation of pancreatic CSCs (with a $CD133^{+}CXCR4^{+}$ phenotype) was able to successfully metastasize, and that if CXCR4 was blocked, the cells were no longer able to metastasize [131]. This kind of research has enormously implications for cancer therapy.

For pathologists and clinicians, it would be very interesting if, along with normal staging and tumor analysis, the CSC population could also be analyzed to identify the best possible therapeutic course of action. If it is true that tumors are driven by a small population of CSCs, then treating the tumor based on the characteristics of the CSC population, rather than the characteristics of the bulk of the tumor, would be more helpful in successfully eradicating the disease. By analyzing CSCs, it would also be easier to identify which SC signaling pathways (if any) are activated, which may also help guide therapeutic decisions.

We live in a very exciting time where identifying novel cancer therapeutic targets and drugs is encouraged and supported [15]. With this freedom and support, however, it is essential that impartiality be maintained in order for scientists to conduct good scientific research. Millions to billions of dollars are invested in each new drug that makes its way through clinical trials. Without carefully designed preclinical and Phase I/II trials, the likelihood of a drug being successful in huge, expensive Phase III clinical trials is not very high. Currently, there are more Phase III trials that fail than succeed, and it seems that many compounds are being recycled instead of new compounds developed. Although it is difficult to let go of a favorite compound, if it has failed to show benefit in a clinical trial, it is imperative to move on. If not, targets that are not going to be effective will continue to be pushed down the pipeline, and the results will inevitably be disappointing. This current approach negatively affects not only just the bottom line, but also the patients who are impatiently waiting on a therapy that will successfully treat their cancer.

Over the short term, agents that target/inhibit SC signaling pathways combined with conventional chemotherapy and RT will probably be our most effective means of battling cancer. Blocking the SC properties of the CSC population would, in theory, make the cells more sensitive to conventional therapy. As we learn more about both normal SCs and CSCs, and how they avoid or resist our therapies, we will be better suited to identify more useful therapeutic targets. In this way, we are heading toward a time when we have therapies that will make cancer a chronic disease, rather than an acute, life-threatening disease.

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Part VI
Final Thoughts

Chapter 24
Final Thoughts: Complexity and Controversy Surrounding the "Cancer Stem Cell" Paradigm

Craig Gedye, Richard P. Hill and Laurie Ailles

Abstract Many patients die of cancers that are metastatic at presentation, or relapse after treatment with curative intent. Cancers are known to contain heterogeneous populations of cells. The cancer stem cell (CSC) hypothesis posits the intriguing possibility that cancer cells are hierarchically organized, such that an identifiable subgroup of these cells may cause metastatic spread, treatment failure, and relapse. These "CSCs" should then become the focus of our research and treatment efforts. Although there is increasing evidence to support this hypothesis, it remains controversial due to increasing complexities in the data reported. We will discuss these maturing data under the framework of the scientific method itself; how we formulate and conceptualize the hypothesis, how we experimentally test the hypothesis, and how we analyze our experimental data. Whether tumor heterogeneity is ultimately determined to be hierarchical or stochastic, interrogating the CSC hypothesis will lead to novel mechanistic insights and improved outcomes for patients with cancer.

Abbreviations

ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
BLAST	Basic local alignment search tool
BRCA	Breast cancer susceptibility gene
CD	Cluster of differentiation
CSC	Cancer stem cell

L. Ailles (✉)
Ontario Cancer Institute, Campbell Family Institute of Cancer Research;
and Departments of Medical Biophysics, University of Toronto, Toronto, ON, Canada
e-mail: lailles@uhnresearch.ca

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EHS	Engelbreth-Holm-Swarm
EMT	Epithelial-to-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ESA	Epithelial specific antigen
GM-CSF	Granulocyte macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
IGH	Immunoglobulin heavy chain
IL	Interleukin
iPS	Induced pluripotent stem cells
LIC	Leukemia-initiating cell
NCBI	National Center for Biotechnology Information
NGFR	Nerve growth factor receptor
NK	Natural killer
NOD/SCID	Nonobese diabetic/severe combined immune deficiency
NSG	NOD/SCID/IL2Rγ−/−
PDGFR	Platelet-derived growth factor receptor
PECAM1	Platelet endothelial cell adhesion molecule 1
SCF	Stem cell factor
Shh	Sonic hedgehog
TGF-β	Transforming growth factor beta
TIC	Tumor-initiating cells

24.1 The Cancer Stem Cell Hypothesis: What It Is and What It Isn't

The cancer stem cell (CSC) hypothesis postulates that a hierarchy exists within cancers such that only some cancer cells have the ability to self-renew, extensively proliferate, and recapitulate the phenotype of the original tumor. This has the obvious clinical implication that perhaps only a subset of cells are the most relevant target for treating cancer patients. The hypothesis has grown from many observations; Paget's recognition that cancers seed into organs that provide a fertile soil; [1] large numbers of cells needed to transplant spontaneously arising murine tumors into syngeneic mice; [2] Hamburger and Salmon's demonstration that only a fraction of cells from freshly excised human cancers are clonogenic [3], and from frankly unethical experiments which demonstrated that millions of human cancer cells were required to form tumors when injected into palliative cancer patients [4]. Although the increasingly complex evidence supporting the CSC hypothesis has been discussed in detail throughout this book, the concept of a "CSC" in solid tumors remains controversial [5, 6]. There are many interdependent concepts that inform this controversy, and we will discuss these under the framework of the scientific method; how the hypothesis is posed, how the experiments to test the hypothesis are performed, and how the experiments are analysed.

24.1.1 What It Is

CSCs are defined based upon their functional properties. CSCs have a selective ability to initiate tumors in immunocompromised mice (with the implication that the remaining non-CSC cells cannot initiate tumors in mice); they have the ability to recapitulate the heterogeneity of the primary tumor (i.e., to give rise to both more CSCs and to the non-CSC cancer cells within the tumor); and they can be prospectively isolated based on a variety of biomarkers. Since these properties are typically assayed by performing tumorigenicity assays in immunocompromised mice, many in the field now prefer to use terms such as "cancer-initiating cell," or "tumor-initiating cell" (TIC) to more accurately describe the functional assay used to define them. The obvious clinical implications of the CSC hypothesis are that we may not have to eliminate every cell within a tumor to eliminate the cancer. This attractive concept had early support from radiation therapy studies in syngeneic transplantable spontaneous rodent tumors, where the dose of radiation required to cure early generation transplants was inversely proportional to the number of cells required to transplant the tumor [7]. These studies suggested that the proportion of CSCs within these tumors was very low (≤1%) but more importantly implied that not every cell within a tumor needs to be eliminated in order to achieve a cure [7].

24.1.2 What It Isn't

The CSC hypothesis does not state that CSCs necessarily arise from normal somatic stem cells. In tissues that undergo rapid regeneration throughout the lifetime of an organism (such as the myeloid component of the blood or the epithelial lining of the gastro-intestinal tract or the skin), the somatic stem cell is the most logical candidate for the accumulation of a sufficient number of mutations to cause malignant transformation due to its long lifespan relative to its downstream progeny. However, this does not preclude the accumulation of "premalignant events" in the somatic stem cell, with the final transforming event occurring in a downstream, short-lived progenitor, or even terminally differentiated cell. Alternatively, although perhaps less likely, a rare event may occur within a short-lived progenitor or terminally differentiated cell that confers self-renewal upon that cell, thus lengthening its lifespan to allow for the accumulation of the required additional events; indeed in some cases, a single event may be sufficient to cause transformation (e.g., MLL fusion genes in myeloid progenitors [8, 9]). In both of these scenarios, the cell of origin would then be a nonstem cell that has acquired the characteristics of CSCs described earlier. In other tissues which do not have rapid turnover of terminally differentiated cells (such as the brain), there is no reason to assume that terminally differentiated, post-mitotic cells cannot acquire mutations that could lead to their "de-differentiation" back to a proliferative, less differentiated state, culminating in cancer. Indeed, the ability to transform terminally differentiated cells into induced pluripotent stem

(iPS) cells [10] supports the concept that such events may be possible. Although this can occur through artificial manipulation of cells in a culture dish, it is unclear how likely is it for the perfect combination of genetic and epigenetic events to converge within a single differentiated cell within the lifetime of a human. Overall it seems likely that tumors of a particular type may arise from different cells of origin in different patients, including somatic stem cells, progenitors, and terminally differentiated cells, and that these events may correlate with other tumor characteristics such as tumor grade, aggressiveness, response to therapy, and TIC frequency.

The CSC hypothesis does not imply that a fully normal differentiation program is intact within cancers, such that the non-CSCs within the tumor resemble the normal differentiated cell phenotype. Although this appears to be the case in some tumors (i.e., well-differentiated squamous cell carcinomas contain cells with a terminally differentiated squamous cell phenotype, based on histological resemblance to keratinocytes as well as expression of normal differentiation markers such as involucrin [11]), in other tumor types the non-CSC compartment in no way resembles the normal differentiated cells (i.e., blasts in acute myeloid leukemia [AML]).

24.2 The Hypothesis

Although we strive for rationality and objectivity, it is human nature to remain vulnerable to perceptual or psychological pitfalls that can lead us to sheepishly say in hindsight "if I hadn't believed it, I never would have seen it" [12]. These cognitive biases face us in every walk of life, including the field of scientific research.

In the first instance "pareidolia," or the tendency to perceive a random stimulus as organized or significant (Fig. 24.1) may impact our interpretation of tumor heterogeneity by priming us to accept the more organized hierarchical CSC model, rather than a more stochastic, context-dependent model of epigenetic heterogeneity in cancer. The "survivorship bias" [13] may also confound our perception, in that because only a small number of cells seem to be required to cause relapse, metastasis or treatment failure, we assume that these surviving cells must *a priori* have "special properties" that render them resistant. "Inattentional blindness" [14] is a cognitive bias made famous by counting basketball passes that may also lead us to over-interpret the ability of the CSC hypothesis to account for tumor heterogeneity. For example, while we focus on the contribution of epigenetic mechanisms to tumor heterogeneity, we may not be paying attention to genetic clonal evolution that is likely to be occurring in parallel, or to heterogeneity within the nonmalignant cell compartments within a tumor (see below). Finally, the "anchoring" or confirmation bias, where we rely too heavily on past models or information when faced with a novel situation [15], may influence our assumption that "CSCs" represent an aberrant or flawed version of the normal somatic stem cell hierarchy. Anchoring may be a particular challenge surrounding the CSC hypothesis since it is a very polarizing idea with important implications for how much of cancer research and new drug development studies are currently performed; one seems to either intuitively accept or reject the idea. Interestingly, it has been

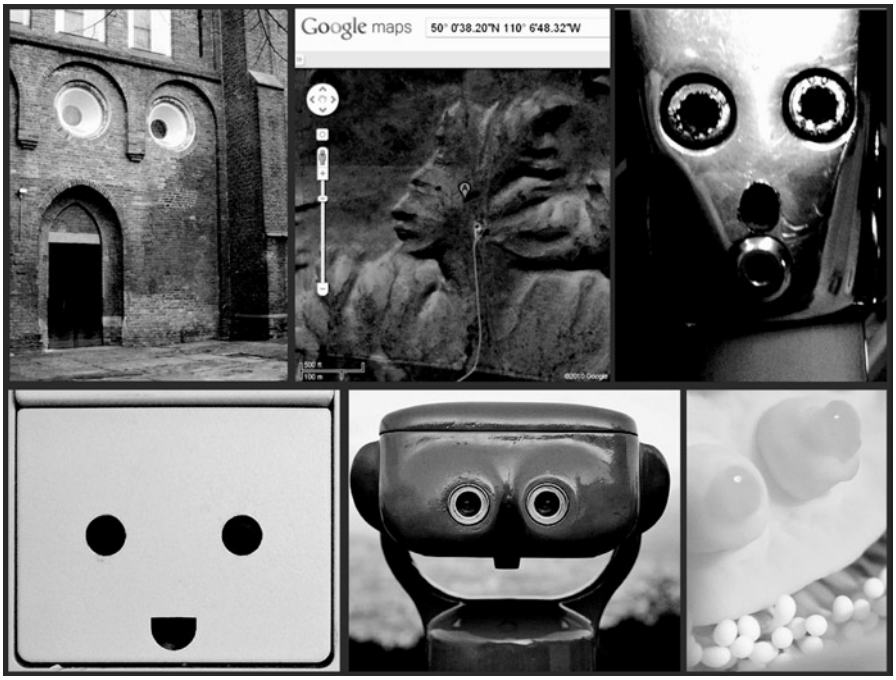

Fig. 24.1 Pareidolia describes the cognitive bias of perceiving significance in random stimuli. The classic example of this is seeing shapes in clouds, but our tendency to see order amongst the chaos can alter our perception in all walks of life. Images gratefully used under creative commons and fair use. Credits: http://www.flickr.com/photos/eworm/3401596917/, http://maps.google.com/maps?f=q&hl=en&q=50%C2%B0+0%2738.20%22N+110%C2%B0+6%2748.32%22W&ie=UTF8&t=h&om=1&ll=50.010139,-110.10689&spn=0.00684,0.024548&z=16NB, http://www.flickr.com/photos/marcoannunziata/3205402229/,http://www.flickr.com/photos/28481088@N00/1049198442/, http://www.flickr.com/photos/martin_borjesson/3704268639/, http://www.flickr.com/photos/martin_borjesson/4339608509/in/set-72157605305172306/

argued that the normal somatic stem cell model may not be inviolate. Lander [16] illustrated that an apparently hierarchical lineage of stem cells, transit-amplifying cells, and differentiated cells could be controlled by the signaling of a single secreted growth factor. This is an example of an emergent phenomenon, where complex, apparently organized systems can become established based on just a few simple feedback rules [17].

We must emphasize that all these biases can also apply to arguments against the CSC hypothesis. In particular, the confirmation bias may make it very difficult to change one's thinking in light of a new, radically different hypothesis, especially when it may call into question the validity of previous bodies of work. Although difficult, it is essential that we as scientists remain aware of our cognitive biases so as not to skew our interpretation of data in favor of one hypothesis over another, but rather to use rigorous scientific methods and unbiased data analysis and interpretation to reach the correct conclusion, and thus help patients.

Finally, we should recognize that the CSC hypothesis is based upon inductive reasoning, and as Popper proposed, inductive statements are by definition difficult to verify and must be tested by falsifiability [18]. Thus, as the CSC field matures, and we try to account for the increasing number of conflicting observations and interpretations being reported, future experiments may be best informed by attempts to *disprove* the CSC hypothesis, and by constantly reflecting on the relevance of these data with regard to cancer patients' clinical and pathological outcomes.

24.3 The Experiment

Having visited some of the perceptual biases that may distort our conception of tumoral heterogeneity, let us consider some of the technical and methodological challenges. As the CSC hypothesis is grounded upon the "gold standard" of prospective isolation and serial xenotransplantation assays [19], we will review these concerns in the context of the steps required to perform these experiments. The serial xenotransplantation assay (Fig. 24.2a) has been employed to demonstrate serial self-renewal in subpopulations of cancer cells, such that if a marker is able to fractionate cells with tumorigenic potential from cells with little or no ability to form tumors, then these marker-positive cells are functionally defined as TICs. In addition, the xenografts formed should continue to express the putative CSC marker, and marker-positive cells from these xenografts should continue to proliferate extensively and retain all the tumor-initiating ability in subsequent mice. Finally, the resulting xenografts should recapitulate the phenotypic heterogeneity of the patient tumor.

24.3.1 The Seeds: Do We Use the Wrong Cancer Cells, Processed in the Wrong Way?

24.3.1.1 Practical Limitations to Isolating Cell Subsets from Solid Tumors

The first technical problem that confounds attempts to quantify TICs in cancers relates to the source of the cancer cells used, and their preparation and handling. For example, in solid tumors we collect tumor tissues from patients undergoing surgery who have kindly consented to share their tissue for research. As the tumor must also undergo formal histological examination (particularly of the tumor margins) to confirm the diagnosis and provide prognostic information, we can most often only receive a small sample of the tumor that typically comes from deep within the lesion. Thus, we may unavoidably be introducing a sampling bias, as cancer cells from different parts of the same tumor may have different properties [20]. For example, there is evidence that self-renewal and CSC marker expression are lower and proliferative potential higher in glioma cells at the "presumed" periphery of a tumor compared with tumor cells at the "presumed" center of the tumor [21]. Having taken a

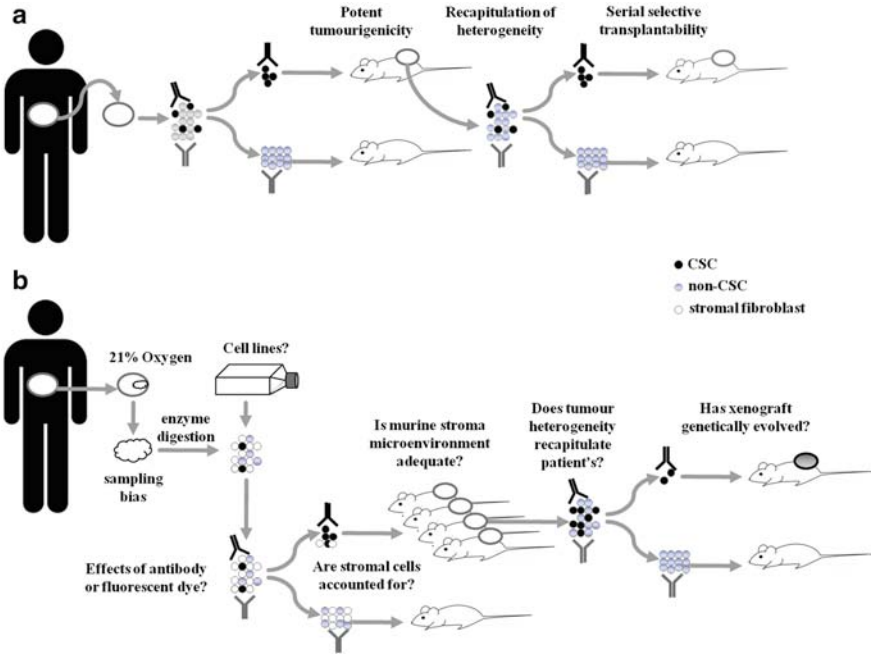

Fig. 24.2 (**a**) The CSC hypothesis is based on the "gold standard" serial xenotransplantation assay, where CSC are defined by their potent tumorigenicity (only CSC form xenografts), recapitulation of tumor heterogeneity (CSC-derived xenografts contain marker-positive CSC and non-CSC) and serial selective transplantability (only marker-positive CSC from xenografts initiate secondary xenografts). (**b**) A number of methodological challenges confound this assay, including sampling bias at the time of pathology cut-up, enzymatic loss of cell surface antigens, use of non-fidelitous cancer cell lines, processing under "hyperoxic" 21% oxygen conditions, incomplete characterization of putative CSC marker epitopes, antibody-dependent effects, numerical and functional contamination by stromal cells, a potentially unsupportive murine microenvironment, suboptimal injection niche and incomplete phenotypic and genotypic assessment of resulting xenograft tumors

sample from patients' tumors, we then use mechanical dissociation and enzymatic digestion to generate a single cell suspension. Although it is known that enzymes such as collagenase and dispase can alter the detection of some cell surface markers [22], it is not known if they can influence markers associated with putative CSCs, or conversely if important markers are being lost. Furthermore, during the entire processing and cell sorting procedure, the cells are exposed to ambient (~21%) oxygen concentrations, rather than the physiological and hypoxic concentrations (1–5%) found in tumors in vivo [23]. There is abundant evidence [24, 25] that oxygen can regulate and alter gene expression in tumor cells, and more recent evidence that oxygen directly impacts CSC biology [26–29].

Having generated a single-cell suspension, we most often employ labeled antibodies to perform cell sorting for exclusion of nonmalignant cells and identification of subpopulations of neoplastic cells within the tumor that we propose to be CSCs.

But what are we actually separating? Some of the CSC markers that are commonly employed are complex, with multiple isoforms (e.g., CD44 [30]) that may have different biological associations i.e., CD44 variants and metastasis [31]) or multiple glycosylation states with uncharacterised epitopes (e.g., AC133 in CD133 [32]). The antibodies used to separate cells may themselves influence the underlying biology. For example, a recent report suggests that CD38 monoclonal antibodies, but not CD38 antibody Fab fragments can deplete leukemia-initiating cells, presumably because of antibody-dependent cell killing [33]. Putative CSC markers themselves may not be expressed in all patients' cancers [34, 35], although this inter-patient heterogeneity is increasingly being recognized and dissected [36]. The use of intracellular markers of "stemness" such as the side-population of Hoechst stained cells and Aldefluor staining for aldehyde dehydrogenase (ALDH) activity are also employed in the search for CSCs. These too must be examined critically as Hoechst dyes may be toxic to cells [37], and although promising, ALDH activity may not segregate stem-like cells in every tumor type [38].

Finally, though many publications in the CSC field are based on primary patient cancer samples or passaged xenografts, there are also many studies that use commercial cancer cell lines, which may have acquired different properties associated with the ability to grow in culture. Human cancer cells cultured for extended periods of time in animal serum rapidly acquire in vitro mutations, which irreversibly corrupt their genotype and phenotype compared with the patient's tumor [39, 40], and an increasing body of evidence suggests that commercially available serum-grown cell lines are poor models for tumor heterogeneity [41, 42]. Studies involving the use of these types of cell lines may yield interesting preclinical results, but these should be validated in human cancer samples to ensure clinical relevance.

24.3.1.2 Do We Know Which Cells Are Actually Being Assayed?

In addition to cancer cells, solid tumors also contain nonmalignant stromal fibroblasts, infiltrating immune cells and blood vessels. Many authors have accounted for these stromal components in CSC studies by depleting the lineage-positive cells, i.e., labeling and removing these cells during the cell sorting process. Markers such as CD31 (PECAM1) and CD45 have been used to account for endothelial cells and haematopoietic cells, respectively [11, 43]. Some groups have attempted to account for the presence of stromal fibroblasts (e.g., staining for CD140b/PDGFR-β as a fibroblast marker in breast cancer [43]), but fibroblasts have an ambiguous and incompletely defined cell surface phenotype, and no single marker can as yet capture their diversity across all human tissues and tumors [44]. In fact, although CD140b/PDGFR-β has not been studied extensively in breast cancer, it does not appear to be ubiquitously expressed by breast-cancer associated fibroblasts [45], and may be expressed by tumor cells in some breast cancers [46, 47]. Accounting for stromal fibroblasts may be particularly important in cancers dominated by a dense desmoplastic reaction, where fibroblasts may numerically outnumber tumor cells, for example in colorectal and pancreatic cancers (Fig. 24.3a, b). Being unable to account for

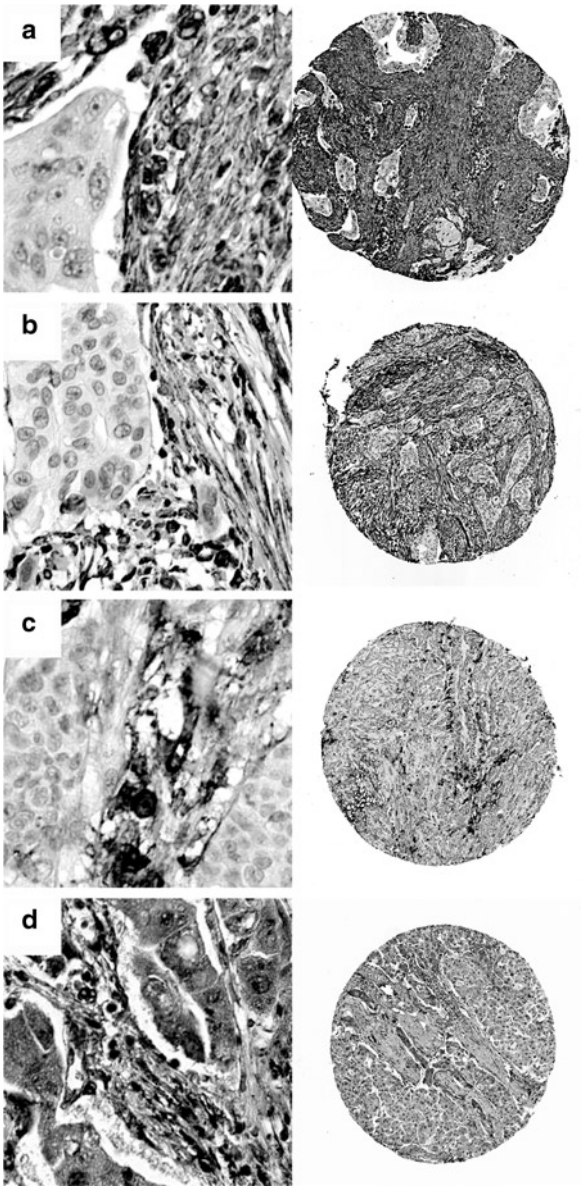

Fig. 24.3 Stromal cells can be numerous, express "CSC markers" and may numerically and biologically confound TIC assays. Vimentin-positive stromal cells (here staining black) can numerically dominate malignant epithelial cells (see low magnification at *right*) in cancers such as colorectal (**a**) and pancreatic carcinoma (**b**). Stromal cells can also express putative CSC markers such as (**c**) CD44-positive stromal cells staining black here in breast carcinoma; and (**d**) CD90-positive stromal and malignant cells both moderately staining (*black*) in hepatocellular carcinoma, thus potentially confounding the interpretation of xenotransplant-initiating cell assays. Images courtesy of the Human Protein Atlas (**a**) http://www.proteinatlas.org/cancer_unit.php?antibody_id=1762&mainannotation_id=337759, (**b**) http://www.proteinatlas.org/cancer_unit.php?antibody_id=1762&mainannotation_id=337946, (**c**) http://www.proteinatlas.org/cancer_unit.php?antibody_id=5785&mainannotation_id=669879, (**d**) http://www.proteinatlas.org/cancer_unit.php?antibody_id=3733&mainannotation_id=492749; http://www.proteinatlas.org [159]

fibroblasts may numerically under-estimate the TIC frequency of a cancer, conceivably by up to tenfold. In addition, stromal cells may express markers that are shared with the putative CSC themselves. For example, CD44 as a marker of CSC in breast cancer is confounded by the presence of $CD44^+$ stromal cells in many breast cancer samples (Fig. 24.3c). This is not confined to the CD44 marker; for example, the interpretation of $CD90^+$ hepatocellular carcinoma CSCs [48, 49] may be confounded by $CD90^+$ fibroblasts (Fig. 24.3d). If stromal cells are contaminating one or both of the CSC and non-CSC fractions, we may instead be measuring the tumorigenic potential of cancer cells and stromal cells vs. cancer cells alone. This contamination may be particularly confounding if cosorted tumor cells and stromal cells express the same marker (i.e., CD44 or CD90). A method to overcome this problem might be to include markers specific for malignant epithelial cells. In their early studies, Al-Hajj et al. employed ESA/EpCAM to identify tumor cells from which to fractionate $CD44^+/CD24^-/lineage^-$ CSC in breast carcinoma [43]. However, subsequent studies have demonstrated that ESA/EpCAM has variable expression and may not stain all cancer cells in epithelial cancers; for example, approximately two-thirds of breast cancers had low intensity and infrequent epithelial cell adhesion molecule (EpCAM) staining in a study of 205 breast cancer patient samples [50]. Accounting for stromal cells such as fibroblasts remains a challenging practical concern while studying human tumor heterogeneity.

24.3.2 The Soil: Stroma and Microenvironment

24.3.2.1 Which Cells Are Absent?

Once a single cell suspension has been generated and candidate CSC and non-CSC populations have been purified, they are assayed for tumor-initiating ability in immunocompromised mice. Paradoxically, efforts to efficiently deplete all of the stromal components prior to assaying a cell population for TIC activity may also confound results by excluding cell populations that may contribute to the growth of the cancer cells in vivo (i.e., fibroblasts [51] and inflammatory cells [52, 53]), and thus also lead to an underestimation of true TIC frequency [54]. Early work from the Bissell lab documented that virus-initiated tumors in avian models could form only at a site of wound inflammation and healing [55]. Compelling evidence for the influence of stromal cells in tumorigenicity also comes from careful studies in spontaneously arising transplantable murine tumor models. First demonstrated by Révész [56] and confirmed by other workers in a wider panel of murine tumors [57], it was shown that the presence of lethally irradiated tumor cells can have a profound effect on absolute and relative tumorigenicity, increasing the TIC frequency by several orders of magnitude. In another report, the number of viable tumor cells in tumor fragments was estimated, and either enzymatically digested single cell suspensions or undigested tumor fragments were transplanted into syngeneic or allogeneic recipients. In three different tumor models, the number of cells in suspension required to form tumors vastly outnumbered the calculated number in the tumor

fragments [58], suggesting that extracellular matrices and/or other 3-D architectural elements are important for tumor initiation.

There are recent reports that stromal cells may influence the behavior of populations functionally defined as CSC in human cancers. These include $CD133^+$ pancreatic cancer cells [59], prostate CSCs in a conditional *Pten* deletion mouse model [60], the influence of the vascular niche on glioblastoma multiforme CSC [61], and the influence of mesenchymal stem cells on mammosphere formation and stem-like cells in breast cancer [62]. Thus, the ideal assay would be a situation in which the putative CSC population is efficiently purified, and then recombined with the "microenvironmental components" to assess their true ability to initiate murine xenografts.

The ability to form xenografts is dependent on the grade and type [63] of tumor, and also on the anatomical niche of implantation [64]. Orthotopic implantation of human tumor xenografts would seem to be the ideal, but apart from the obvious niche for breast [43] and brain cancers [65], most other CSC publications have utilized the heterotopic subcutaneous injection site. Whether this can influence TIC frequency per se has not yet been reported, but injection of melanoma cell lines into the subdermal space rather than subcutaneously is associated with a more physiological model of melanoma with a higher propensity for metastasis [66]. Similarly injection of renal carcinoma cell lines underneath the renal capsule is associated with a higher rate of metastasis compared with subcutaneous injection [67, 68]. This suggests the possibility that some cancers may be more independent of cell–cell signaling and are more "niche-permissive" (e.g., melanoma), while other cancers may be more dependent on stromal cell paracrine and endocrine signaling. In these latter cancers (e.g., leukemia, medulloblastoma), larger doses of closely apposed human cells may be required to provide sufficient paracrine signals. Alternatively, the propensity of human tumors to engraft into a xenogenic environment may simply parallel the relative "niche-fastidiousness" of their putative cells-of-origin. For example, while haematopoietic stem cells require a very specific niche in bone marrow [69], melanocyte precursors spread much more widely from the neural crest and are found throughout the body [70] where they retain self-renewal capacity in adult life [71]. One observation supporting this hypothesis is the clinical presentation of different cancers; for example, a myeloid sarcoma or chloroma (a solid mass of leukemic blasts outside the spleen or marrow) is a rare finding in patients with AML [72], but melanoma can metastasize to almost any organ in the body including the small intestine [73].

24.3.2.2 Mouse Models Matter

In addition to considering the location, purity, and identity of the population(s) of cells that are injected, let us consider the host animal itself. One of the most compelling challenges to the relevance of the CSC hypothesis has recently come from a study of tumorigenic cells in melanoma: Quintana et al. [74] injected melanoma cells with high concentration Matrigel (a heterogeneous mixture of basement membrane proteins secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells) into severely immunocompromised mice; the NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) strain which entirely lacks natural killer (NK) cell activity (compared with the previous "gold standard"

nonobese diabetic/severe combined immune deficiency (NOD/SCID) strain in which low NK cell activity is present). Matrigel [75, 76] and tissue components such as brain extract [77] are well known to enhance tumor transplantability. Whether this is due to tumor cell aggregation due to the formation of a gel that maintains the injected cells in proximity, or due to growth factors embedded in the matrix remains unclear, but it does not appear to be due to collagen or laminin components of the matrix alone [78]. The rationale was that these conditions would be more permissive for human primary xenograft formation. Under these conditions, the authors found that tumorigenic cells in melanoma were relatively common, such that around 1 in 4 melanoma cells implanted subcutaneously could form a xenograft. This contrasted with much lower tumorigenic frequencies from melanoma implanted in parallel in NOD/SCID mice (~1/111,000), and in a previous report (~1/1,000,000) [79]. Subsequent data demonstrated that when marker positive and negative sorted cell populations (using several markers, including CD133, p75/NGFR, and others) were implanted in NSG mice, both populations gave rise to a mixed xenograft, expressing both marker-positive and marker-negative cells; [80] i.e., no selection (and hence no hierarchy) could be demonstrated. A criticism of this work was that it used cells from passaged xenografts and from advanced stage III and IV melanoma specimens; however, this more recent data show that single melanoma cells are highly tumorigenic (TIC frequency 29%) even when directly isolated from early stage patient samples [80]. Thus with sufficient optimization of the xenotransplant model, tumorigenic cells in melanoma were found to be common, rather than rare as had been previously proposed.

The CSC hypothesis does not assume that CSCs must be rare. The hypothesis initially arose in part from the observation that large numbers of cells are required to initiate tumors, suggesting that only a rare subset has this ability. However, even in cases where cancers have high TIC frequencies such as melanoma, this may simply represent a more “shallow” hierarchy, i.e., where few malignant cells exhibit a terminally differentiated phenotype (Fig. 24.4b). One could imagine that the depth of the hierarchy will depend on many parameters, including the depth of the hierarchy in the normal tissue from which the cancer arose, where the cell from which the cancer arose is situated in the hierarchy, and the nature of the underlying mutations (e.g., mutations that block differentiation may lead to a higher frequency of TIC than mutations that increase self-renewal, or inhibit cell death). We and others [81] recently reported limiting dilution tumorigenicity experiments performed side-by-side in NOD/SCID and NSG mice, showing that the TIC frequency remains rare in non-small cell lung carcinoma, pancreatic carcinoma, and head and neck squamous cell carcinoma, using the same methods as Quintana et al. This suggests that the phenomenon observed in melanoma does not necessarily apply to other solid tumors, but does not rule out the possibility that the heavily immunocompromised NSG mouse model is still lacking other essential microenvironmental components that, if present, would radically alter the readout of these assays in other tumor types.

While some have taken this work from the Morrison lab as a harbinger of doom for the CSC hypothesis, it may be that cancers can follow two models of heterogeneity. In some cancers, a hierarchy may not exist and tumor heterogeneity is best

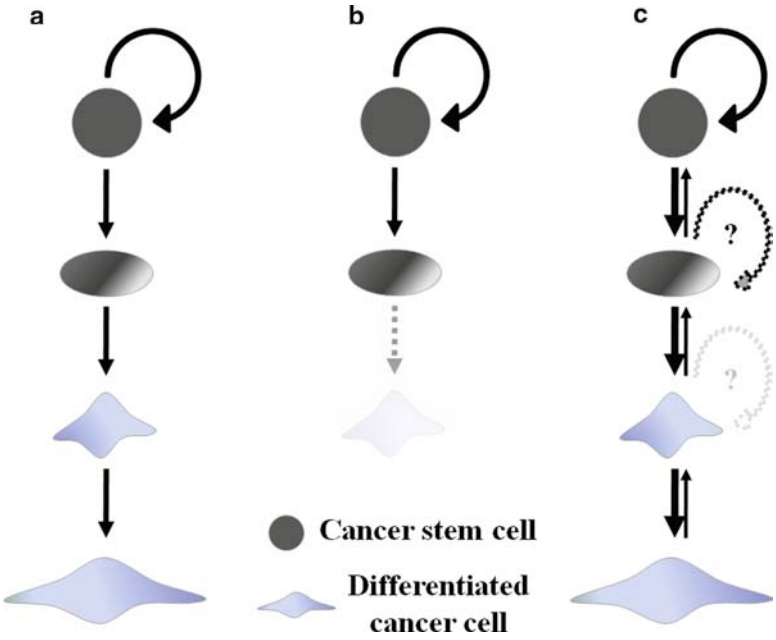

Fig. 24.4 (**a**) The classical schema of a hierarchically CSC driven cancer structure posits that CSC may asymmetrically divide to give rise to a progressively differentiating daughter cell (*straight arrows*), or symmetrically self-renew to give rise to a sister CSC (*circular arrow*). This is supported by in vitro data in lung carcinoma cell lines showing that during asymmetric cell division, the template DNA cosegregates with the putative CD133 lung cancer stem cell marker [160]. (**b**) TIC need not be rare however for the CSC hypothesis to account for tumor heterogeneity. For example, some cancers may have a broad range of differentiated cell phenotypes (i.e., chronic myeloid leukemia) and a "deep" hierarchy, whereas other cancers may have a much more limited spectrum of differentiation (i.e., anaplastic carcinomas or melanoma), with a corresponding "shallow" hierarchy. (**c**) A central tenet of the CSC hypothesis is that CSC can differentiate to give rise to non-CSC, but the reverse should not occur. An alternate model for tumor heterogeneity is epigenetic reversibility, where cancer cells with a more differentiated phenotype can de-differentiate "up the hierarchy" to a more primitive cell phenotype. Whether these more differentiated cancer cells could also undergo symmetric self-renewal is not known

modeled as reversible (Fig. 24.4c). However, in other cancers, a CSC/hierarchical model may still be valid, for example in acute and chronic myeloid leukemia in humans [82] and in animal models of cancer [83, 84].

24.3.2.3 The Difference Between Mice and Men

While much attention has been given to the degree of immunosuppression of the mouse strain (e.g., nude, SCID, NOD/SCID, NSG, or $Rag^{-/-}\gamma c^{-/-}$), less attention has been focused on whether the full repertoire of growth factors and cytokines that can

Table 24.1 Growth factors reported to be noncross-reactive between mice and humans

	Human ligand active on mouse receptor	Mouse ligand active on human receptor	Protein homology (%)
HGF [85–87]	Y	N	91
SCF [96]	N	Y	83
EPO [96]	Y	N	80
M-CSF [96]	Y	N	71
GM-CSF [96, 167]	N	N	56
IL-2 [96, 168–170]	Y	N	64
IL-3 [96]	N	N	45
IL4 [171]	N	N	29
IL-6 [96]	Y	N	42
LIF [95, 172]	Y	N	79
FGF7 [173]	N	?	94

This manually curated subset is presumably only representative. Note that cross-reactivity is lost at 94% homology, with a median of 75% overall

modulate growth in human cancer cells are cross-reactive between mice and humans. In several cases, they are known to be non-cross-reactive (Table 24.1), and this has obvious implications for the quantitative study of human cancer cell tumorigenic potential in mice. For example, scatter factor/hepatocyte growth factor (HGF) has been shown in several studies to be non-cross reactive in mice and humans; [85, 86] specifically murine HGF does not activate the human HGF receptor, MET. MET signaling occurs in almost all solid tumors [87], and while this is sometimes autocrine, HGF is also secreted by stromal cells in human cancers and influences many behaviors including invasion [88], motility, and proliferation [87]. For example, Vande Woude's group showed that various cancer cell lines proliferated more rapidly in human HGF-transgenic SCID mice than in control SCID mice, though a melanoma cell line did not. Although the MET/HGF pathway is overexpressed in melanoma [89] and may be involved in melanomagenesis [90], it is either autocrine or dispensable in established melanoma [91]. It is possible that this "tolerance" of the absence of human HGF contributes to the high tumorigenic frequency of melanoma compared with other cancers.

Other growth factors implicated in CSC pathobiology such as leukemic inhibitory factor [92, 93] and erythropoietin [94] are also noncross-reactive between mouse and humans [95, 96]. Reported non-cross-reactive factors are summarized in Table 24.1. This may identify only a fraction of potential mismatches since a survey of signaling protein murine-to-human homology by the Protein Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI) Homologene suggests many more potentially noncross-reactive signaling pathways (Table 24.2). This murine-human discrepancy may also help account for the high failure rate of "promising new treatments" in the transition from preclinical models to early clinical trials [97, 98].

Table 24.2 Protein homology of growth factor ligands and receptors in mice compared to humans (NCBI homologene and protein BLAST)

Ligands		Receptors		Ligands		Receptors		Ligands		Receptors	
HGF	91%	90%	MET	TGFB1	90%					69%	IL1R1
MST1	81%	76%	MST1R	TGFB2	95%			IL1A	62%	62%	IL1R2
IGF1	83%	96%	IGF1R	TGFB3	98%			IL1B	70%	62%	IL2RA
IGF2	84%	82%	IGF2R	BMP2	92%			IL2	64%	59%	IL2RB
FGF1	95%	98%	FGFR1	BMP3	81%			IL3	29%	71%	IL2RG
FGF2	94%	97%	FGFR2	BMP4	98%			IL4	45%	35%	IL3RA
FGF7	94%	93%	FGFR3	BMP5	93%			IL5	72%	57%	IL3RB
FGF10	91%	91%	FGFR4	BMP6	92%			IL6	42%	54%	IL4R
EGF	68%	90%	EGFR	BMP7	98%			IL7	70%	69%	IL5RA
TGF-a	93%	88%	ERBB2	BMP8a	86%			IL9	57%	56%	IL6RA
HB-EGF	81%	91%	ERBB3	BMP10	86%			IL10	73%	78%	IL6ST
AREG	72%	97%	ERBB4	BMP15	65%			IL11	88%	65%	IL7R
DLL1	88%	90%	NOTCH1	GDF1	69%	98%	ACVR1	IL12A	60%	62%	IL9R
DLL3	83%	92%	NOTCH2	GDF2	80%	98%	ACVR1B	IL12B	70%	57%	IL10RA
DLL4	86%	90%	NOTCH3	GDF3	71%	97%	ACVR1C	IL13	61%	69%	IL10RB
JAG1	96%	80%	NOTCH4	GDF5	92%	99%	ACVR2A	IL14	91%	83%	IL11RA
JAG2	89%			GDF6	89%	99%	ACVR2B	IL15	73%	57%	IL12RB1
SHH	87%			GDF7	85%	98%	BMPR1a	IL16	82%	68%	IL12RB2
IHH	95%	96%	PTCH	GDF8	96%	98%	BMPR1b	IL17A	63%	75%	IL13RA1
DHH	97%			GDF9	74%	97%	BMPR2	IL17B	88%	60%	IL13RA2
BDNF	98%	93%	LNGFR	GDF10	84%	93%	BAMBI	IL17C	78%	55%	IL15RA
NT-3	96%	94%	NTRK2	GDF11	100%	53%	CFC1	IL17D	78%	70%	IL17RA
NT-4	91%			GDF15	62%			IL18	66%	75%	IL17RB
M-CSF	71%	76%	CSF1R	INHA	80%			IL19	70%	65%	IL18R1
GM-CSF	56%	37%	CSF2RA	INHBA	97%			IL20	76%	65%	IL20RA
TNFA	80%	66%	TNFRSF1A	INHBB	97%			IL21	63%	75%	IL20RB
LTA	73%	64%	TNFRSF1B	CHRD	87%					65%	IL21R

(continued)

Table 24.2 (continued)

Ligands		Receptors		Ligands		Receptors		Ligands		Receptors	
CXCL1	68%			CER1	69%			CCL1	43%		
CXCL2	70%			NODAL	81%			CCL2	68%		
CXCL3	65%			LEFTY1	82%			CCL3	75%	81%	CCR1
CXCL4	72%	70%	CXCR1	THBS1	95%			CCL4	76%	80%	CCR2
CXCL6	62%	71%	CXCR2	NOG	99%			CCL5	80%	70%	CCR3
CXCL7	55%	87%	CXCR3	GREM1	98%			CCL7	64%	86%	CCR4
CXCL9	72%	91%	CXCR4	FST	98%			CCL11	59%	82%	CCR5
CXCL10	68%	84%	CXCR5	WNT3	99%	95%	FZD1	CCL17	64%	75%	CCR6
CXCL11	68%	75%	CXCR6	WNT3a	96%	100%	FZD2	CCL19	78%	87%	CCR7
CXCL12	95%	93%	CXCR7	WNT5a	99%	98%	FZD3	CCL20	64%	71%	CCR8
CXCL13	48%			WNT5b	94%	97%	FZD4	CCL22	63%	86%	CCR9
CXCL14	95%			WIF1	95%	97%	FZD5	CCL24	59%	88%	CCR10
CXCL16	51%			DKK1	83%	84%	FZD6	CCL25	54%		
CXCL17	71%			DKK2	95%	97%	FZD7	CCL26	50%		
VEGFA	91%			DKK3	76%	97%	FZD8	IFNA1	63%	51%	IFNAR1
VEGFB	87%	82%	VEGFR1	SFRP1	95%	96%	FZD9	IFNB1	50%	50%	IFNAR2
VEGFC	86%	87%	VEGFR2	SFRP2	98%			IFNG	42%	54%	IFNGR1
VEGFD	85%	88%	VEGFR3	PDGFa	92%					61%	IFNGR2
PIGF	88%			PDGFb	89%	92%	PDGFRa	FLT3L	73%	86%	FLT3
LIF	79%	75%	LIF-R	PDGFc	87%	86%	PDGFRb	GDNF	93%	94%	GFRA1
SCF	83%	83%	KIT	PDGFd	85%			EPO	80%	81%	EPOR

White = 100% homology, 50% greyscale = 94% homology, black = 75% homology (minimum and median homology respectively for noncross-reactive factors from Table 24.1). Human factors with no murine homolog: CCL8, CCL14, CCL16, CCL18, CCL21, CCL23, CCL27, CXCL5, CXCL8/IL8. Murine factors with no human homolog: Ccl6, Ccl9, Ccl10, Ccl12, Cxcl15. Ccl2 is the murine homolog of CCL10. Cxcl1 is the murine homolog of CXCL2

24.3.2.4 In Vitro Assays for "Cancer Stem Cells"?

For many tumors, surrogate in vitro assays for CSCs have been employed, including brain [99], breast [100], and colon [101] cancers. The microenvironment for cancer cells growing in a culture dish is obviously very different to that within the primary tumor, but in some cases it has been demonstrated that "tumorspheres" initiated under the appropriate conditions from single cells can go on to initiate tumors that recapitulate the heterogeneity of the primary tumor; [102] and such systems may have potential as surrogate in vitro assays for CSCs. This is exemplified by a publication demonstrating that glioma cell lines established in bovine serum mutated into cell lines with a bland, differentiated morphology and a phenotype indistinguishable from glioma cell lines that have been in culture for decades [39]. In contrast, matching ex vivo cells cultured de novo as "tumorspheres" in a defined serum-free media formulation [103] generated lines with richer morphological heterogeneity, expressed markers of a more primitive stem-like phenotype, formed tumors that were diffusely invasive as typically seen in glioma patients, and most importantly maintained the genotype of the original patient's tumor sample. These cell lines grown in defined media have subsequently been employed to demonstrate the efficacy of targeting the glioma TIC niche [61], and the Notch [104], Shh [105], and TGF-β [106, 107] signaling pathways. These culture conditions have been applied in many other tumor types [108–110], although as yet, comprehensive validation has not been repeated in other cancers. It is extremely important that such in vitro assays be carefully validated, and not to assume that a tumorsphere equals a CSC [111].

24.4 The Analysis

In addition to our cognitive biases and experimental challenges, we also unfortunately add to the complexity and controversy surrounding the CSC hypothesis when we analyse and interpret our findings.

24.4.1 The Numbers Game

There are significant numerical discrepancies that may affect analysis of CSC experiments. Firstly, many studies ignore interpatient heterogeneity by pooling the measured TIC frequencies of cancers from different patients. In almost all cancers, there is considerable interpatient variability and heterogeneity in driver mutations [112], transcriptional phenotype [113], and clinical behavior and outcome [114], and one would predict a range of TIC frequencies depending on these variables. More broadly, there is a discrepancy between reported TIC frequencies (i.e., one in thousands) and the percentage of marker positive cells (up to 30% in some cancers [65]).

A more serious numerical objection to the CSC hypothesis that has been previously raised [5, 6] is the lack of correlation between the absolute numbers of cancer

cells with tumorigenic potential in unsorted tumor cell suspensions and the absolute number of cells with tumorigenic potential in marker-positive and marker-negative sorted cell fractions. For example, if the tumor-initiating frequency in unsorted cells indicates that there are n cells capable of tumor-initiation within a tumor derived single cell suspension, and x of those n cells are found in the marker-positive population, then $n-x=y$ of the TIC ought to be found in the marker-negative population. However, this is generally not the case, and it has been argued that these analyses are most consistent with the presence of an inhibitor [115], i.e., either the presence of marker-negative cells inhibits the tumor initiating capacity of marker-positive cells, or that tumorigenic potential is context-dependent rather than an intrinsic property of a specific tumor cell subset. Indeed, if the appropriate calculations are done, in some cases the absolute number of TIC is actually higher in the "non-CSC" fraction than in the "CSC-enriched" fraction [116].

Another unresolved numerical issue within the CSC field is the use of the posthoc rationalization that xenograft tumors formed after injection of non-CSC (i.e., marker-negative cells) must be due to contamination of the non-CSC cells by (marker-positive) CSC. By performing flow cytometry purity checks on sorted populations and appropriate statistical calculations, it should be possible to calculate the probability that contamination of non-CSC with CSC has in fact occurred. This has not been published in a CSC study to date. Finally, we may be using entirely the wrong mathematical models to discuss heterogeneity in cancer. For example, dynamic models [117] and statistical approaches employed in evolutionary and population biology may be more appropriate [118].

24.4.2 Measuring Xenograft Heterogeneity

One of the tenets of the CSC hypothesis is that tumors initiated by the CSC subset must recapitulate the heterogeneity of the primary tumor. Most CSC publications show histological images to demonstrate morphological similarity, and flow cytometry and/or immunohistochemistry to demonstrate regeneration of the marker-positive and marker-negative populations, but extensive phenotypic characterization has not commonly been presented. This is of considerable importance as it would seem likely that different genomic subgroups of cancers may have different hierarchical phenotypes [36] with different "CSC" populations, for example as has recently been demonstrated in mouse lung cancer models [119]. Furthermore, genomic heterogeneity is likely to remain in active flux during the serial xenotransplantation process [120], as cancer genomes are known to be unstable. The relationship between stemness and differentiation may therefore change over time. Studies performed in transplantable murine tumors [121, 122] showed that dynamic changes are possible in cancer cell genomes or epigenomes, at relatively high rates (~10^{-5} per cell per generation). If such frequencies apply to human cancers, there may be 1,000 of such events per day in clinically apparent tumors, especially in cancers where DNA repair defects are a feature of their particular genotype (i.e., mismatch repair genes and BRCA1/2 mutations).

Considering the genotype of xenograft tumors raises other questions regarding tumor heterogeneity and the CSC hypothesis. For example, does the xenografting process itself maintain the genomic heterogeneity of the parent tumor or does the "most flexible" clone outcompete it's less xeno-capable peers? The comprehensive genomic sequencing of a basal breast carcinoma, a subsequent metastases and a xenograft established from the primary tumor [123], suggested that a small population of cells within the primary tumor gave rise to the metastases and the xenograft.

Genetic heterogeneity alone may not fully account for the functional and antigenic heterogeneity present in all human malignancies. For example, ultradeep sequencing of the immunoglobulin heavy chain (IGH) locus in patients with chronic lymphocytic leukemia has revealed phylogenetic structures indicative of clonal evolution within the malignant cell compartment. Intriguingly, patients with early stage disease had more complex clonal structures whereas patients with advanced disease seemed to have undergone selection to a dominant clone [124]. Clonal dominance was also suggested by copy-number variation analysis in end-stage metastatic prostate cancer [125], while methylation analysis of topologically distinct biopsies from primary colorectal cancers also suggests that heterogeneity of clinically apparent cancers can "almost always" be accounted for by a single clonal expansion [126].

There are conflicting data, however. For example, distinct genetic clones within an individual patient's cancer can be associated with morphologically and functionally distinct phenotypes, as elegantly demonstrated in a recent study of metaplastic breast cancers [127], where lobular and squamous histological subtypes could be found in adjacent parts of a primary breast cancer, each with unique focal genomic amplifications and immunohistochemical staining patterns. Sampling from one part of the tumor may not allow us to accurately encompass the heterogeneity seen throughout such a patient's cancer, and genetic selection occurring during the xenograft process would complicate this further [123].

Parallel assessment of genetic and epigenetic heterogeneity is now, however, becoming technically possible and early data are being presented [128]. Such parallel analysis of two likely mechanisms of heterogeneity within cancers will provide powerful information to improve patient outcomes. Indeed it is highly likely that both genetic and epigenetic heterogeneity can evolve simultaneously, as has recently been eloquently argued [120]. With increasingly detailed sequence data becoming available for different tumor types [129], it is possible to compare genomic complexity with tumorigenic frequency in various cancers (Fig. 24.5). Though this dataset is obviously limited, it will be intriguing to see if there truly is a correlation between the number of coding mutations and the frequency of tumorigenic cells as more genome and TIC frequency data becomes available.

Finally, though the CSC hierarchical model must involve epigenetic regulation, little attention to date has been paid to known epigenetic mechanisms such as DNA methylation, histone acetylation, microRNA, and noncoding RNA. As both the tumor heterogeneity and epigenetics fields mature, we expect new evidence to inform our understanding of the validity of the CSC hypothesis.

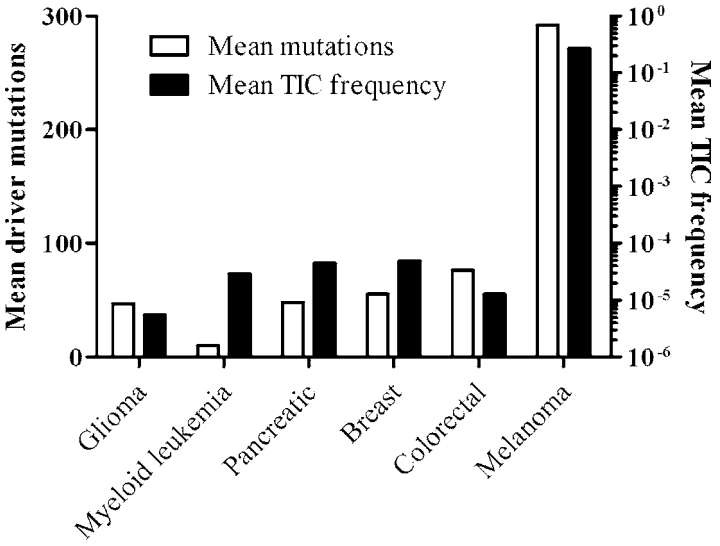

Fig. 24.5 Is genomic complexity sufficient to account for differences in TIC frequency across different tumors? Intriguingly in these early data sets, the mean number of mutations per tumor [123, 161–164] appears to be associated with the mean TIC frequency in that tumor type; [43, 65, 74, 110, 116, 165, 166] this raises the hypothesis that TIC frequency may be primarily determined by "genetic flexibility" for xenograft proliferation and survival. When genomic information and TIC frequencies are available from the same patient's tumor this hypothesis can be properly tested

24.4.3 Reversibility Is Not Compatible with an Irreversible Hierarchical Model

Perhaps most importantly, studies of TICs in melanoma [130] and ovarian cancer [131] have shown evidence consistent with "phenotypic reversibility"; where both marker-positive or marker-negative cells can give rise to tumors which express a mixture of marker-positive and marker-negative cells (Fig. 24.4c). This reversible cell surface phenotype argues against the presence of a hierarchy and instead suggests a context-dependent or stochastic model. Irreversible loss of the CSC marker in non-CSC cells is a central tenet of the CSC hypothesis, whereas phenotypic reversibility is more compatible with other models to explain epigenetic heterogeneity, such as epithelial-to-mesenchymal transition (EMT). In either case, the number of different patients' cancers that will need to be evaluated to statistically test these possibilities is substantially larger than current studies have been reported. For example, many CSC publications to date have studied on average ten separate cases with roughly six to ten events (injected doses) per case. These studies are akin to case series or phase I clinical trials; hypothesis-generating but insufficiently powered to test if tumor heterogeneity is stochastic or hierarchical.

24.5 The Next Iteration of the CSC Hypothesis

Marston Bates said that "research is the process of going up alleys to see if they are blind." Despite the complexities and controversies outlined above, we believe it will be of great value to continue to interrogate the CSC hypothesis. Although hypotheses can never be conclusively proven to be true in a Popperian world-view, comprehensively disproving the CSC hypothesis in a given cancer would require extremely fastidious and detailed knowledge of the intratumoral heterogeneity in individual patients' cancers. Even if the CSC hypothesis is false, that is if epigenetic heterogeneity is not organized in an irreversible hierarchy, it would seem likely that the molecular pathways and signaling mechanisms being identified in CSC studies (e.g., Notch [104], Shh [105], and TGF-β [106, 107]) will continue to be relevant when studying epigenetic heterogeneity in human cancers. Perhaps the best example of this potential duality is in breast cancer heterogeneity. One of the earliest reports of CSCs in solid tumors described the $CD44^+/CD24^-$ subpopulation in breast cancer as being highly enriched for breast CSCs [43]. This work generated considerable discussion and controversy, and led to work by other groups that sought to refute the concept [132, 133], instead postulating the heterogeneity in breast cancer was reversibly regulated. A number of subsequent publications have strengthened the connection between $CD44^+/CD24^-$ breast cancer cells and EMT [134–136], invasion [137], and mechanisms of treatment resistance such as immunoevasion [138–141], chemo-resistance [142, 143], and radiation resistance [144, 145]. Our attempts to interrogate complex phenomena with incomplete models by indirect observation may leave us like the Blind Men and the Elephant; [146] using limited sensory data to get partial knowledge, but all approaching the same truth from a multiplicity of perspectives. Finally, as we have outlined above, the act of attempting to investigate the state and behavior of cell fractions may influence the state and behavior of those cells, such that it is impossible to accurately predict their future behavior; the biological equivalent of Heisenberg's uncertainty principle. This has been eloquently discussed at the level of the genome of a single cell [147], and by extension we imagine it would be no less intractable in populations of cells within a tissue or cancer.

A number of methodological improvements are also likely to assist in supporting or refuting the CSC hypothesis. For example, tracking of each individual cancer cell within a xenograft population would help us to define if human solid tumors are organized hierarchically or stochastically. Perhaps the most compelling evidence for the CSC hypothesis in human cancer is the application of this concept with clonal analysis of leukemic-initiating cells (LIC) by lentiviral insertion site tracking, which demonstrated a hierarchy of short-term and long-term LIC in AML [82].

Optimizing mouse strains to better model the human microenvironment in xenografts would also help us to more accurately address the question of whether TICs are rare or common in human cancer. Examples of this endeavor include the study of TIC in myeloma engrafted in mice implanted with human fetal bone [148] and the finding that AML xenograft efficiency in NSG mice can be substantially improved by constitutive expression of human stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-3 [149].

We should also seek to directly measure the mechanisms that underpin epigenetic heterogeneity to help discover to what extent this is reversible and how this differs between tumor tissues and their normal counterparts. Epigenome-wide arrays of DNA methylation [150] and microRNA, and global histone modification [151] can now be more easily assessed on small samples, and this will provide more definitive evidence of the relevance of CSC-marker-positive populations in cancer [152]. Simultaneous assessment of genomic heterogeneity and epigenetic heterogeneity within ex vivo human cancers will help us understand the relative contribution of these two mechanisms to tumoral heterogeneity.

Finally, we must address the discrepancies in the analysis of CSC experimental results. We must attempt to account for the numerical discrepancies outlined above, for example by calculating the "recovery" of TIC from sorted and unsorted tumor fractions, which will allow us to rigorously assess if our data best support a hierarchal or stochastic model. The number of individual tumor cases required to provide sufficiently robust statistics will need to be much greater than most studies to date. We must also consistently and frequently return and question the clinical relevance of data obtained in CSC studies. Even if we eventually discover that prominin-1/CD133 is not a hierarchical marker in colorectal cancer, it is useful and encouraging that its expression is an increasingly well validated prognostic marker [153–155] and is associated with mechanisms of relapse and resistance [101, 156, 157]. Likewise, even if $CD44^{+}/CD24^{-}$ breast cancer cells are not "breast CSCs," this subpopulation has identified a gene signature of "invasiveness" potential that is extraordinarily predictive for overall survival in breast cancer patients [158].

Regarding hypotheses, Enrico Fermi stated that "there are two possible outcomes: if the result confirms the hypothesis, then you've made a measurement. If the result is contrary to the hypothesis, then you've made a discovery." Despite the complexities and controversies outlined here, better definition of genetic and epigenetic contributions to tumor heterogeneity in individual patients will either collect more measurements that refine our knowledge, or discover more controversy that makes us question our hypotheses. Cancer patients must surely benefit in either case.

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