

# 1 Cancer Stem Cells: Similarities and Variations in the Theme of Normal Stem Cells

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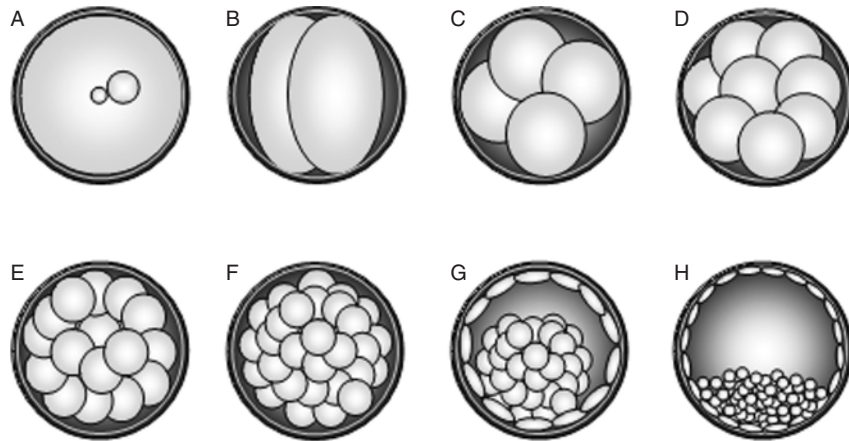
## 1.1 INTRODUCTION

Then we have Beard's "germ-cell" hypothesis, in which he holds that many of the germ-cells in the growing embryo fail to reach their proper position—the generative areas—and settle down and become quiescent in some somatic tissue of the embryo. They may at some later date become active in some way, and so give rise to a cellular proliferation that may imitate the structure in which they grow, so giving rise to new growths.

—*Encyclopedia Britannica*, 1911

Tumors were first likened to embryonic cells by the Scottish embryologist John Beard, who early in the twentieth century proposed a germ cell theory of cancer, inferred from his observations.<sup>[1]</sup> The early blastocyst during its development has two distinct cell groups: the inner cell mass, which gives rise to the embryo, and an outer circle of trophoblastic cells, which enable implantation of the embryo into the uterus and subsequent formation of placenta<sup>[2]</sup> (Fig. 1.1). The trophoblast cells are thus invasive and metastatic, which are also key characteristics of cancer cells. Beard postulated that the presence of pancreatic enzymes in the embryo and the mother actually restricts the invasion of trophoblast cells into surrounding tissues, and claimed that cancer arose from straying by such cells due to a failure of the enzymatic surveillance mechanisms. He went on to demonstrate that injections of the pancreatic proteolytic enzyme trypsin could destroy cancer cells in patients.<sup>[3]</sup>

Although the hypothesis and consequent cancer treatment generated considerable attention, its very nature was such that it could not be confirmed reproducibly and Beard's ideas remained largely unaccepted by his peers. However, his

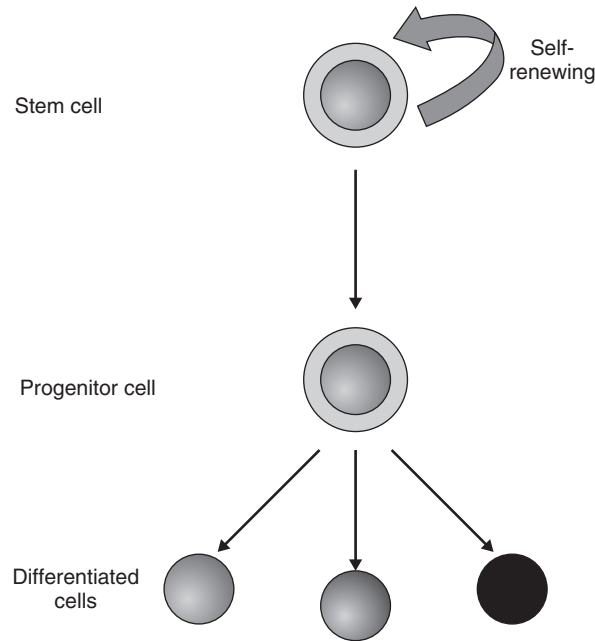


**FIGURE 1.1** Developmental stages from normal early vertebrate embryo development to the blastocyst stage: (A) single-celled zygote; (B) two-celled embryo; (C) four-celled embryo; (D) early morula; (E) compacted morula; (F) late morula; (G) early blastocyst; (H) late blastocyst. (See insert for color representation of figure.)

hypothesis has recently regained prominence, with similar observations that relate cancer cells and disease progression with the characteristics of stem cells. The finding that tumors often express surface antigens (cancer/testis antigens) that are otherwise expressed only at the embryonic–extraembryonic stage<sup>[4]</sup> lends additional support to Beard’s theory. A stem cell is characterized by its unique capacity to differentiate into multiple cellular lineages and to self-renew in an undifferentiated state<sup>[5]</sup> (Fig. 1.2). It has now been shown definitively in certain cancers that a small subset of cells exist in a tumor with similar regenerative and self-renewal mechanisms that enable tumor formation and progression.<sup>[6]</sup> This tiny subset of cells, referred to as *cancer stem cells* (CSCs), is also considered to be more chemoresistant than the bulk of tumor cells and is thus more difficult to target and eradicate.<sup>[7]</sup> Thus, CSCs need to be specifically targeted and eliminated to achieve tumor ablation, a concept that has begun to revolutionize approaches to cancer therapy and drug design. Although distinctly removed from Beard’s original proposal, the theory is currently evolving, with increasing evidence in several types of cancers.

## 1.2 STEM CELLS IN THE LIFE OF AN ORGANISM

The unicellular zygote is recognized to be the first stem cell in a human life and is identified as being totipotent, due to its ability to generate an entire organism.<sup>[8]</sup> However, during further embryonic development, a gradient of decreased regenerative potential is produced and distributed in specific stem cell compartments in a developing embryo. This decrease in regenerative capability leads to a subsequent loss of totipotency but a concurrent retention of pluripotency by stem cells



**FIGURE 1.2** Basic outline of stem cell functioning.

in the embryo. A neonate is equipped with stem cells which for the most part are multipotent (i.e., committed to tissue-specific potential).<sup>[9]</sup> Some stem cells, however, retain a higher level of pluripotency even in the adult (e.g., subsets of bone marrow–derived stem cells<sup>[10]</sup> which are reported to have trans-differentiation potential).

### 1.2.1 Stem Cells in Early Development and Fetal Life

The early embryo generated from the fertilized egg cell exists initially more or less as a “ball of cells” called a *morula* (Fig. 1.1). The first recognized differentiation event in development occurs at the late morula stage, when the outer cell layer of the embryo adopts epithelial features and initiates the formation of a blastocyst that contains two cell types: trophoblast and inner cell mass (ICM). The trophoblast gives rise to the trophoblast, while the ICM undergoes a second differentiative step to form the epiblast and primitive endoderm,<sup>[11,12]</sup> thereby progressing toward the blastocyst stage. The epiblast (sometimes referred to as the primitive ectoderm) further gives rise to the embryo, while the primitive endoderm develops into the extraembryonic endoderm, which provides nutrients and developmental cues to the embryo and contributes to development of the yolk sac. The ICM cells at the blastocyst stage are no longer totipotent but are recognized as being pluripotent.<sup>[8]</sup>

The next recognized landmark during embryonic differentiation is gastrulation, when the epiblast is transformed into the three germ layers of the embryo (i.e., ectoderm, mesoderm, endoderm) and the basic body plan of the animal is established. In mouse, gastrulation is initiated around 6.5 days postcoitum, when the surface ectoderm cells undergo an epithelial–mesenchymal transition, delaminate, and migrate through the primitive streak.<sup>[13]</sup> Ingression of progenitors for the embryonic mesoderm begins around the midstreak stage with the formation of progenitors that will give rise to the lateral plate mesoderm of the upper body as well as cardiac and cranial mesoderm.<sup>[14]</sup> Within the epiblast, nascent mesodermal cells migrate anteriorly and laterally. Early in gastrulation, cells leaving the streak contribute largely to the mesoderm of extraembryonic tissues: the yolk sac, amnion, and allantois.<sup>[15]</sup> Thus, there is a temporal progression of mesoderm commitment. In addition, the site of ingression of progenitors into the primitive streak is regionalized. For example, cells fated to form extraembryonic mesoderm ingress into the posterior portion of the streak, while lateral plate and paraxial mesoderm ingress into the middle and anterior regions, respectively.<sup>[16]</sup> The primitive streak thus functions as a posterior organizing center. How different mesodermal populations are set aside to form specific lineages is not well understood.<sup>[17]</sup>

On transplantation into the proximal epiblast, the distal epiblast cells (which normally differentiate into neuroectoderm and surface ectoderm) colonize the extraembryonic and posterior mesoderm of the embryo. At lower frequencies, cells from the distal epiblast and the extraembryonic ectoderm can be respecified to primordial germ cells,<sup>[18]</sup> which normally arise from cells of the proximal–posterior epiblast. Signals from both the extraembryonic ectoderm and the visceral endoderm are required to establish normal patterning of the underlying epiblast.<sup>[19]</sup> Although it is now evident that reciprocal signaling interactions between the epiblast and extraembryonic tissues play critical roles in the induction and maintenance of embryonic patterning during gastrulation,<sup>[20]</sup> how these complex events are orchestrated is still not fully understood. The remaining events of embryogenesis, a major part of which is organogenesis, rely on the functioning of localized stem cells that together are committed to regenerate specific organs in response to the surrounding microenvironment or niche. Thus, each of these stem cell “nests” will eventually undergo a programmed sequence of events, including cell divisions, migration, and apoptosis, to generate an organ at a specific location with respect to the other embryonic tissues and organs within the embryo.

### 1.2.2 Stem Cells in the Adult Organism

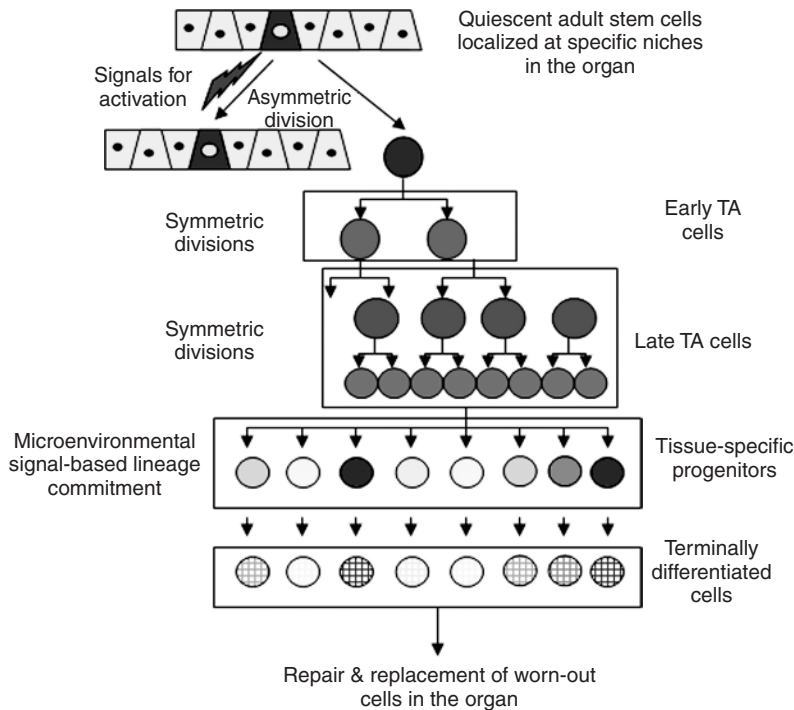
One of the most important functions of stem cells in postembryonic development is to repair and compensate for the loss of damaged tissues in the body. This dynamic process continues throughout life, and the unspecialized stem cells survive in each organ by creating their own niches.<sup>[21,22]</sup> The effective maintenance of a population of healthy stem cells within a particular tissue involves the concurrent operation of multiple genetic and epigenetic factors, along with stringent controls within each niche that create perfect harmony among the different cells of various

organ systems.<sup>[23]</sup> Adult stem cells are thus central to tissue homeostasis and are identified through three distinctive properties:

1. Self-renewal: the ability to undergo division and form new cells with a potential identical to the mother cell<sup>[24,25]</sup>
2. Differentiation: the ability to give rise to a heterogeneous population of cells arranged in a hierarchical manner; includes various tissue-specific lineages, thereby building up the requisite critical mass toward replenishing the tissue of short-lived, differentiated cells
3. Homeostasis: the ability to regulate and balance differentiation and self-renewal in the tissue or organ

This unique combination of properties imparts to stem cells a continuing role during the entire life of an organism and further ensures that almost all developed tissues in the body, from the growing fetus to the adult, harbor stem cells. The property of self-renewal has gained recognition as a defining characteristic of stem cells and involves an asymmetrical cell division into two cell types, with an equal distribution of the gene pool among the daughter cells.<sup>[26,27]</sup> One of the daughter cells returns to the stem cell niche and remains quiescent until further signals for division are received through the microenvironment. The other cell is a progenitor cell committed to a differentiation pathway<sup>[28]</sup> (Fig. 1.2) that initiates the generation of cells toward fulfilling the needs of tissue-specific regeneration. This is achieved through the production of transit-amplifying (TA) cells, which ultimately commit to terminal differentiation (Fig. 1.3). The interposition of the TA population allows requisite cell amplification while eliminating the need for frequent cycling of stem cells, which holds the danger of mutations. Early TA cells are slow-cycling and undergo a few cell divisions, some of which may be asymmetric; late TA cells undergo a state of rapid, yet restricted cell proliferation that effectively builds up the critical mass necessary for differentiation. This arrangement of tissue regenerative processes as a hierarchy thus ensures that tissue-specific stem cells retain a long-term regenerative capacity,<sup>[29]</sup> whereas progenitors characterized by a finite division capacity eventually stop dividing and differentiate along tissue-specific lineages into mature cell types, and on completing their functionality, ultimately undergo apoptosis.<sup>[30]</sup> The process also ensures a long-term genetic stability, as a large majority of the de novo mutations arising during the processes of cell proliferation, commitment, and differentiation in the hierarchy will undergo a process of natural elimination, as all the differentiated cells will ultimately undergo apoptosis. In this way, the property of self-renewal and hierarchical arrangement of stem cell derivatives allows a long-lived to indefinite life span of genetically stable stem cells within an organ.

Stem cell divisions can also occur in a symmetrical manner, which enables the size of the stem cell pool to be increased at certain stages during development or after wounding<sup>[31]</sup>; for example, stem cells within the bone marrow (i.e., the hematopoietic stem cells) maintain relatively rapid division rates in order to meet the demand for a turnover of the large numbers of different blood cells required



**FIGURE 1.3** Stem cell hierarchies in normal tissue regeneration. (From ref. 30.) (See insert for color representation of figure.)

by the body. Stem cells from other tissues, such as those in the skin and colon, maintain a slow but steady or constant growth rate toward replenishing tissue; yet other stem cells, in tissues such as the brain, remain quiescent at most times and are activated only on stimulation by tissue damage or by hormonal exposure resulting in a change in the physiological state. The regulation of stem cells in an adult is thus tightly controlled, to allow for the growth replenishment of tissues and to permit repair of damaged tissues. The properties of stem cells described above also impart to them a continuing regenerative potential: the competence to display a specialized differentiation capability, the ability to give rise to the large number of cell types within the embryo and the adult organism. A decrease in this differential potential is evident as we move from the totipotent single-celled embryo to pluripotent ICM cells: late embryonic and fetal stem cells. Pluripotency is replaced by tissue-specific multipotential capability as somatic cell populations are formed, and subsequently it is retained only in those cells allocated to the germline and certain adult stem cells. This critical balance of self-renewal versus differentiation is achieved by the gene expression programs of stem cells, which regulate transcriptional activation and inactivation mechanisms to allow cells to maintain a

pluripotent state but also permit differentiation into more specialized states. A disruption in such homeostatic mechanisms is believed to lead to the abnormal state of cancer.

### 1.3 CANCER STEM CELLS

Most cancer cells divide rapidly and can be grown indefinitely in culture as immortal cells and express a plasticity of differentiation, similar to embryonic stem (ES) cells and adult stem cells. In fact, before the advances that led to an understanding of the developmental plasticity of ES cells, embryonal carcinoma and teratocarcinoma cells (derived from germ cell tumors and known to differentiate to give rise to cells of many lineages) were used as in vitro models for studies relating to development and differentiation.<sup>[32]</sup> In 1976, Beatrice Mintz and Ralph Brinster showed independently that teratocarcinomas could also give rise to normal chimeric mice.<sup>[33,34]</sup>

The realization of an involvement of stem cells in cancer has been built up over the last several decades—almost a century ago if John Beard is indeed considered “father of cancer stem cell biology.” However, the first direct evidence for the existence of CSCs came from the work of John Dick and his co-workers, who identified the presence of CSCs in acute lymphocytic leukemia through extensive cell cloning and demonstration of their self-renewing capacity—a critical property of all stem cells.<sup>[25]</sup> CSC identification in leukemia has, in fact, changed the way that many scientists view cancer<sup>[35,36]</sup> and has led to their isolation from some solid tumors. CSCs represent only about 1 % of the tumor but appear to be the only cells capable of generating a new tumor in immune compromised mouse models. Whereas stem cells themselves may be difficult to isolate from an already differentiated healthy tissue such as the breast, it has been possible to isolate stem cell–derived clonal tumor cells by engrafting tumor tissues in suitable animal models such as breast tumor–derived cells transplanted in the mammary fat pads of nonobese diabetic/severely compromised immunodeficient mice (NOD/SCID) mice.<sup>[37,38]</sup> Additional studies have presented data indicating that established cell lines also contain a minor population of cells with properties similar to those of stem cells.<sup>[39,40]</sup>

#### 1.3.1 Activation of Stem Cells and Cancer

The modern era has seen the cloning of many genes mutated in the germline of patients in families segregating cancer as a genetic trait. These *tumor suppressor genes* are thought to be critical in preventing the formation of cancer. However, it is unclear why mutations in a gene such as *RB* or *TP53*, expressed in all the cells of a human being, should give rise to tissue-specific cancers such as retinoblastoma or breast cancer. In one report, in which tumors derived from embryonic tissue from tissues under hormonal control or renewal tissues such as skin and gut were distinguished, it is suggested that the action of tumor suppressor genes can be

explained by their differential effects on stem cells derived from varied tissues.<sup>[41]</sup> Further, the oncogenic potential of different resident stem cells may be distinct since the genetic or epigenetic factors vary from person to person and even between organs in the same person. A question thus arises: How do CSCs arise in tissues and progress to give rise to a new organ (i.e., the tumor)?

**Box 1-1 Possible Origins of CSCs in Tissues Leading to the Occurrence of Cancer**

- Transformation of stem cells residing in a tissue, leading to altered growth and differentiation properties
- Transformation of a local pool of early progenitors that reacquire self-renewal properties
- Series of effective mutations that render committed transient-amplifying progenitor or differentiated somatic cells within a tissue immortal (de-differentiation)
- Fusion of circulating bone marrow–derived stem cells with tissue-residing cells

To address this issue among related issues, research over the last decade has attempted to associate cellular mechanisms with mutagenic effects within tissues leading to the emergence of CSCs (Box 1-1). The possibilities that emerge include the following:

1. *Stem cells as targets of transforming mutations.* Through the acquisition of abnormal growth and differentiation properties during its long-lived residence within a tissue, a multipotent tissue stem cell may, following a series of aberrant events, give rise to a CSC. A disruption of the stem cell niche with a shift toward growth-promoting signals rather than growth-inhibiting signals results in dominant stem cell activation rather than the *transient activation* that is required for normal tissue homeostasis. This could occur by hormonal stimulation, recurrent posttissue damage, inflammation, radiation, chemicals, infections, inactivation of tumor suppressor gene(s), and/or activation of oncogene(s). The change in the tissue microenvironment leads to a chronic activation of stem cells and results in their long-term proliferation. Such chronically dividing stem cells could become vulnerable to additional genetic events. The recognized effects of these events include autonomous growth, loss of cell cycle regulation, and resistance to apoptosis, which are all well-understood properties of cancer cells.<sup>[42]</sup> Cancer can thus be thought of as a disease resulting from the abnormal growth of stem cells resulting from their chronic activation followed by genetic insults, culminating in transformation.

2. *Progenitor cells as targets of transforming mutations.* A CSC need not be derived from a bona fide stem cell, but instead, can arise from tissue-specific

early progenitors (i.e., early TA cells following oncogenic transformation). This would retain a minimal number of changes to retain stem cell properties, as they are derived directly from stem cells; and accompanying events that support the transformation process may be identical to those for stem cell transformation.

3. *De-differentiation of committed progenitors or differentiated cells.* In yet other cases, there is evidence that committed progenitors (i.e., late TA cells, or even differentiated cells) may also reacquire the property of self-renewal to give rise to cells with stemlike properties that are pluripotent. The latter is described as a de-differentiation phenomenon that is very commonly reported in plants and also to a certain extent in animals with lower levels of tissue and organ complexities. More recently, the process has also been demonstrated in tissues derived from higher vertebrates such as mice,<sup>[43–45]</sup> wherein transfection of activated oncogenes were shown to transform murine fibroblasts into cells with stem cell–like properties, indicating that non-stem cells can be converted into CSCs.

4. *Fusion of tissue-specific stem cells with circulating bone marrow stem cells.* The finding that fusion of circulating bone marrow–derived stem cells with differentiated tissue cells can create cells with self-renewal capacity leads to yet another speculation on the origin of CSCs.<sup>[46]</sup> This is believed to involve the mobilization of bone marrow–derived cells either at the wrong time and/or their incorporation at the wrong place within other tissues, as a first step toward transformation. Moreover, several CSCs express the pluripotency and self-renewal markers expressed characteristically by hematopoietic stem cells. Whether this reflects a global commonality of expression of these markers or an outcome of the fusion process is not yet very clear.

Of all the possibilities noted above, adult stem cells giving rise to cancer is an attractive hypothesis, given that the classic multistep model of carcinogenesis requires a long-lived cell in which multiple genetic hits can occur. However, regardless of whether the cell of origin is a normal adult stem cell or progenitor or a differentiated tissue cell, and regardless of the mechanism of its emergence, CSCs are defined by their stem cell–like properties (Box 1-2). Moreover, despite the diversity in possible origins of CSCs, all the processes argue for dynamic changes within the tissue leading to transformation.

#### **Box 1-2 Characteristics Shared by Normal and Cancer Stem Cells**

- Capacity for asymmetric divisions (self-renewal), which generates a quiescent stem cell and a committed progenitor and contributes toward developing a critical mass of cells
- Regulation of self-renewal by similar signaling pathways (Wnt, Sonic Hedgehog, and Notch) and at the epigenetic level by Polycomb genes (*BMI-1* and *EZH2*)

- Expression of factors such as Oct4, Nanog, and Sox2 and also nodal and cancer testis-specific antigens (CTAs), which maintain a functional plasticity by promoting pluripotency and immortality
- Capacity to arrange a hierarchy of cellular derivatives that includes progenitors and differentiating cells
- Extended telomeres and telomerase activity that increases the cellular life span
- Expression of ABC transporters, contributing to cellular resistance against specific growth-inhibitory drugs
- Predisposition for growth factor independence through secretion of growth factors and cytokines
- Stimulation of angiogenesis through secretion of angiopoietic factors
- Expression of similar surface receptors (e.g., CXCR4, CD133,  $\alpha 6$  integrin, c-kit, c-met, LIF-R) that are either identified as stem cell markers or are associated with homing and metastases

### 1.3.2 Isolation and Identification of Cancer Stem Cells

Based on the perpetuation of cytogenetic abnormalities during serial transplantation in culture and in animal models, it was reported in 1957 that ascitic fluid possessed possible cancer stem cells.<sup>[47]</sup> Soon after, Kleinsmith and Pierce demonstrated that transplantable teratocarcinomas were derived from a single pluripotent cell.<sup>[48]</sup> Similar to tissue stem cells, CSCs are believed to be capable of asymmetric cell division (i.e., they can give rise to one self-renewing stem cell and one daughter cell committed to differentiation). It is postulated, however, that CSCs are also capable of symmetric division<sup>[6]</sup> (to generate two daughter stem cells), as is the case with normal stem cells when they are required to proliferate rapidly, such as during inflammation or wound healing. All cancers are now believed to be composed of a mixture of self-renewing stem cells, transiently amplifying progenitors, and proliferative cells with a shorter life span that can undergo limited differentiation. Cumulatively, all these factors make the identification and isolation of the initial rare stem cell population within tumors a challenging issue in cancer biology.

Research in the field of stem cell biology has traditionally focused on the hematopoietic system, where detailed lineages have been elucidated and various surface molecules associated with the components of the hierarchy have been identified as specific markers.<sup>[49,50]</sup> Following these well-accepted hierarchies of blood-forming lineages, studies attempted to identify leukemic stem cells using known *cluster of differentiation* (CD) cell surface markers.<sup>[51,52]</sup> Subsequently, in a study of acute myeloid leukemia (AML), Lapidot et al. successfully identified putative leukemia-initiating cells using limiting dilution analysis.<sup>[25]</sup> This study demonstrated that in AML, malignant leukemic stem cells are probably

derived from immature (uncommitted) bone marrow cells, which express a similar cell surface marker profile (i.e., CD34<sup>+</sup>/CD38<sup>-</sup>) and originate from a similar bone marrow niche. Similarly, in multiple myeloma, a subpopulation of CD138<sup>-</sup>/CD34<sup>-</sup> cells were demonstrated to be clonogenic in vitro and capable of propagating tumors upon serial passaging in NOD/SCID mice as compared to CD138<sup>+</sup> cells, which were nontumorigenic.<sup>[53]</sup> More recently, in AML, a population of cells within the CD34<sup>+</sup>/CD38<sup>-</sup> cell fraction have been identified further on the basis of its CD96<sup>+</sup>/CD90<sup>-</sup> expression as being present only in leukemic samples and not in normal bone marrow, constituting a major leap in the specific identification of leukemic stem cells.<sup>[54]</sup>

Apart from the hematopoietic system, the presence of stem cells in tissues with a high cellular turnover, such as skin, gut, testis, and the respiratory tract, has been reported widely. More recently, the presence of stem cells in solid, slow-growing tissues and organs has gained recognition.<sup>[7,28,55]</sup> The specific expression of surface molecules (e.g., CXCR4, CD133, EPCAM, CD44; Table 1.1) by normal stem cells has provided a mechanism to separate out the rare cancer stem cells from within the tumor mass. In solid tumors, Al-Hajj et al. demonstrated a subpopulation of human breast cancer cells also capable of inducing tumors in nude mice.<sup>[35]</sup> Those putative breast CSCs were immunophenotyped as CD44<sup>+</sup>/CD24<sup>lo/-</sup> and were capable of initiating tumorigenesis at a density of merely 200 cells, in contrast to unsorted cells, which generally require a density of at least 10<sup>6</sup> cells per injection. In brain tumors, CD133 expressing candidate CSCs have been isolated that are similar to normal (neural) stem cells. The CD133<sup>+</sup> CSCs were also capable of driving tumorigenesis in mouse models at low cell numbers (about 100 cells).<sup>[56]</sup> Since then, several reports exist of the isolation and identification of stem cells based on the expression of surface markers from other cancers, such as prostate, lung, and colon (Table 1.1).

**TABLE 1.1 Surface Markers Used for Isolation and Identification of Cancer Stem Cells**

Type of Cancer	Markers	Refs.
AML	CD34 <sup>+</sup> , CD38 <sup>-</sup>	57
	CD96 <sup>+</sup> , CD90 <sup>-</sup>	54
Neural	CD133 <sup>+</sup>	56
Mammary	CD44 <sup>+</sup> , Cd24 <sup>-</sup>	35
Prostate	CD133 <sup>+</sup> , β-integrin	58
Lung	Sca1 <sup>+</sup> , CD45 <sup>-</sup> , CD31 <sup>-</sup> , CD34 <sup>+</sup>	59
Hepatocellular	CD133 <sup>+</sup>	60
Pancreas	CD44 <sup>+</sup> , CD24 <sup>+</sup> , ESA <sup>+</sup>	61
Colorectal	EpCAM <sup>high</sup> , CD44 <sup>+</sup>	62
Colon	CD133 <sup>+</sup>	63,64
Liver	CD133 <sup>+</sup>	65

Recently, however, one commentary questioned the reliability of the commonly used methods for identifying CSCs.<sup>[66]</sup> This included concerns regarding enzyme treatments during cell preparation from tumors, isolation based on CD cell surface profiles that are identical to normal tissue stem cells, genomic instability that could result in plasticity in the “stemness” of specific tumor cells, and the routine use of nonphysiologic microenvironments to assess the tumorigenicity of engrafted cells. Despite these objections, however, the CSC hypothesis has gained wide acceptance for the study of such neoplastic progenitors.

### 1.3.3 De Novo Generation of a New Organ (Tumor) by Transformed Stem Cells

Analogies between normal and CSCs in terms of cellular, biochemical, and molecular events are currently being resolved at a basic level, and considerable work is in progress to describe these processes in greater detail during transformation. Nonetheless, similarities and differences in the functioning of normal and cancer stem cells at the tissue level, whereby a consolidation of the events described above contributes toward either maintenance of homeostasis or development of abnormal tumor tissue, are unclear. It is, however, understood that both normal and tumorigenic stem cells give rise to phenotypically heterogeneous populations that exhibit various degrees of proliferation and differentiation capabilities. The heterogeneity in tumor tissues may be attributed to both continuing mutagenesis and aberrant differentiation of cancer cells, the latter being a variation of normal differentiation driven by cancer stem cells. This disrupted differentiation is often accompanied by aberrant expression patterns (e.g., the expression of germ cell markers in epithelial ovarian cancer).<sup>[67]</sup>

Two general hypotheses have been put forth to describe tumor formation based on a microdissection of the events described above. The first, *stochastic cancer stem cell model*, postulates that each population of cells within a heterogeneous tumor has an equal but extremely low tumorigenic potential.<sup>[68]</sup> In such a scenario, tumor progression is a continuous process based on the positive selection of genetically unstable clones that confer a survival advantage on a tumor within its surrounding microenvironment. The stochastic model thus also accounts for the emergence of drug resistance during chemotherapy through selection of cells with genotypes that allow survival from the intended drug insult<sup>[69]</sup> (e.g., DNA damage by platinum drugs). Under this model, however, isolation of tumor progenitors would not be reproducible, as their existence would be random. A recent example is the proposed stochastic model of gastrointestinal stem cells, suggesting that all stem cells in a niche are descendents of a single common ancestral stem cell, and that intestinal crypts expand further clonally by crypt fission, forming two daughter crypts.<sup>[70]</sup> The same mechanism has been postulated to contribute to the expansion of mutated stem cells and CSC clones in the colon and in the entire gastrointestinal tract.

The fact that organ-specific stem cells derive all of the differentiated cells within a given tissue has led to the proposal of a stem cell hierarchical model for

tissue development, maintenance, and repair, as discussed earlier (Fig. 1.3). Deriving from this, the *hierarchical cancer stem cell model* puts forth the suggestion that the tumorigenic potential of tumors is limited to a very small clonogenic population of cells (i.e., the CSCs).<sup>[71]</sup> The large population of cells within a tumor are descendants of these CSCs, do not have self-renewal capacity, and are organized in the form of a hierarchy. Thus, the model posits that not all cells in a tumor are equal and that the tumor-initiating cells are a rare subset with a distinct phenotype. This hierarchical model helps explain why most tumors are heterogeneous despite their clonal origin. This approach also accounts for the perplexities faced by researchers in establishing a permanent cell line from primary tumors or in recreating tumors in animal models.

#### 1.4 SELF-RENEWAL AND DIFFERENTIATION IN CSCS

The steady-state expression of a stem cell in an adult organism is one of quiescence. On receiving specific signals from its microenvironment, it can be induced to undergo asymmetric stem cell division, which generates an identical stem cell (self-renewal) and a progenitor with decreased plasticity (the latter divides further symmetrically to generate several TA cells that are all committed to differentiation). Current knowledge indicates that evolutionary conserved mechanisms regulate this process. Beginning at the late blastocyst stage in embryogenesis and further in adult tissues, timing of self-renewal and regulation of loss of pluripotency are crucial for the orderly functioning of stem cells and the elimination of potentially teratogenic cells. A central question that remains is: How can a single cell divide to produce two progeny cells with different fates?

Many studies examining the mechanisms regulating asymmetrical divisions have focused on mitotic cleavage orientation and the uneven distribution of cell-fate-determining molecules such as Numb and ACBD3 within the two cells resulting from the division of a stem cell.<sup>[71]</sup> In neural stem cells and progenitors, both proteins interact through an essential Numb domain. ACBD3 associates with the Golgi apparatus in neurons and interphase progenitor cells but becomes cytosolic after Golgi fragmentation during mitosis, when Numb activity is needed to distinguish the two daughter cells. Accordingly, cytosolic ACBD3 can act synergistically with Numb to specify cell fates, and its continuing presence during the progenitor cell cycle inhibits neuron production. This represents a mechanism for coupling cell fate specification and cell cycle progression.<sup>[72]</sup> In addition to neuronal stem cells, Numb has also been reported to regulate cell fate in other stem cells (e.g., skeletal muscle satellite cells).<sup>[73]</sup> Similarly, PAR proteins are also suggested to control asymmetric cell division in a wide range of organisms and somatic cell types<sup>[74]</sup>; other asymmetrically segregating proteins include<sup>[75]</sup> CD53, CD133, L-selectin, Lamp-2, and CD71.

Studies of asymmetric division have relied heavily on the lessons learned in the regulation of *Drosophila* germline and somatic stem cells. Early in the cell cycle in larval neural stem cells, the two centrosomes become unequal: One

organizes an aster that stays near the apical cortex for most of the cell cycle, while the other loses microtubule-organizing activity and moves extensively throughout the cell until shortly before mitosis, when it organizes the second mitotic aster near the basal cortex. Upon division, the apical centrosome remains in the stem cell while the other segregates to the differentiating daughter.<sup>[76]</sup> Almost along identical lines, developmentally programmed asymmetric behavior and inheritance of mother and daughter centrosomes underlies the stereotyped spindle orientation and asymmetric outcome of stem cell divisions in the *Drosophila* male germline.<sup>[77]</sup> More recently, in mammalian systems, reports indicate that the fate of daughter cells is determined by their relative orientation within the stem cell niche (e.g., in dividing satellite cells in skeletal muscle, on division the daughter cell attached to the basal lamina remains a stem cell, whereas the daughter that loses contact with the basal lamina up-regulates Myf5 and becomes a committed myogenic cell).<sup>[78]</sup>

Although some of the molecular events associated with asymmetrical divisions as outlined above have been described in normal stem cells, further work is required to identify the similarities and variations of those that determine events in CSC self-renewal.

## 1.5 CSC PLASTICITY AS REGULATED BY INTRINSIC AND EXTRINSIC STEM CELL FACTORS

In contrast to normal stem cells, it has been theorized that CSCs undergo genomic alterations that allow them to escape cell cycle regulation and achieve growth factor, anchorage independence, and resistance to apoptosis, besides contributing to dysregulation of self-renewal and expansion.<sup>[79]</sup> The acquisition of each of these characteristics is complementary to the others and requires a suitable microenvironment in which the transformed CSCs are believed to proliferate and differentiate into an entire tumor.<sup>[80]</sup> This understanding implies that the plasticity gained by CSCs is regulated by a cooperative effect of cell intrinsic (autocrine) factors, which may either involve changes in DNA sequences/copy number of genes or gene silencing through methylation or altered chromatin architecture (genetic and epigenetic effects), together with cell extrinsic (paracrine/derived from the tumor microenvironment) factors.

### 1.5.1 Stem Cell Intrinsic Factors: Genetic and Epigenetic Effects

A disturbed balance in gene regulation of tissue stem cells promoting self-renewal and/or aberrant differentiation is characteristic of cancer.<sup>[81]</sup> However, uncontrolled expansion of stem cells by itself may not produce fully invasive tumors.<sup>[82]</sup> Thereby, proliferating proto-oncogenic stem cells appear to require at least one additional permanent genetic mutation to drive them along a trajectory toward transformation.<sup>[83]</sup> This could be achieved either through oncogene activation or by silencing of tumor suppressor genes, which effectively supplements the

perturbed shift toward self-renewal; continuing mutagenesis would further ensure clone amplification and disease progression. Toward an understanding of such changes in stem cells leading to cancer, several models have been developed based on rapidly self-renewing stem cells in human colon crypts. Thus, in familial adenomatous polyposis the initial germline mutation is in the “gatekeeper” tumor suppressor gene adenomatous polyposis coli. Second hits in this gene involve a stochastic and sequential accumulation of genetic and epigenetic defects that over time are selected for by the position of the earlier mutation. Extensive work indicates that crypts harboring mutated stem cells are clonal units that may develop a selective advantage, eventually leading to niche succession. Aberrant crypt foci are formed that contain several such clonal crypts generated through crypt fission involving the longitudinal division of each crypt into two daughter units. In such models the coexistence of defective progeny and hierarchies consequently generated marks a transition between pre-tumor and tumor progression.<sup>[84]</sup>

Recent studies argue that in a stem cell triggered into division, self-renewal requires the expression of certain “stemness” genes, whereas genes associated with differentiation must be repressed. Conversely, in the progenitor cell committed to differentiate, some of the stemness genes are switched off, whereas specific lineage-associated genes involved in differentiation begin to be expressed. The classical *stem cell self-renewal signature* is defined by the expression of three transcription factors identified primarily in ES cells (i.e., Oct4, Nanog, and Sox2), now known to be crucial for the phenomenon of pluripotency.<sup>[85]</sup> The downstream regulation mediated by these three key molecules in the cell, including their feedback/autoregulatory and feedforward loops are critical in the establishment and maintenance of pluripotency.<sup>[86–88]</sup> Other genes involved in self-renewal besides these three master regulators include *Stella*, *FGF4*, *BMP4*, *Stat3*, *UTF1*, and *Rex1*.<sup>[89,90]</sup> All or combinations of these determinants of pluripotency are almost always detected in malignant cancer tissues. Although not much is known regarding their mechanisms of regulation in CSCs, it is expected that most of their gene targets and therefore, effects of these genes would be similar to those in normal stem cells. Thus, the current understanding of the role of these factors in cancer is derived from the corresponding data in normal stem cells (mostly in embryonic stem cell systems).

Toward counteracting self-renewal and driving cell fate determination, a loss of pluripotency is required which is induced by the activation of several pathways that perturb the levels of the pluripotent regulators above and impose alternate cellular fates, such as those associated with Cdx2 and GCNF function.<sup>[8]</sup> In ES cells, the induction of GCNF expression facilitates differentiation through inhibition of the pluripotent state by repression of *OCT4* and *NANOG* regulatory genes. Other factors involved in regulating Oct4 expression are Oct4 itself, Sox2, SP1, RAR, SF1, COUP TF I and II, and LRH-1.<sup>[91–94]</sup> Oct4, Nanog, Sox2, FGF4, and Stella repression was observed upon retinoic acid-mediated differentiation.<sup>[95]</sup> However, at present the regulation of Sox2 during differentiation remains unclear.

Several members of the Polycomb group (PcG) proteins have been identified as important regulators of cell self-renewal and cell fate determination decisions. These proteins act as transcriptional repressors by regulating chromatin remodeling, especially in association with stem cell function, and are also reported to be altered in various cancers.<sup>[96]</sup> Recently, Lee et al. have identified several genes that were bound by the human Polycomb repressive complex 2 (PRC2) subunit SUZ12, display H3K27 (lysine 27 on histone 3) trimethylation, and were repressed transcriptionally in human ES cells.<sup>[97]</sup> A significant subset of these genes was occupied by Oct4, Sox2, and Nanog. Disruption of H3K27 methylation resulted in de-repression of most of these genes.<sup>[98]</sup> Further data suggest that specific genes are primed for expression in pluripotent cells and that occupancy by opposing epigenetic marks are involved in this process. That this also occurs at many *NANOG*, *OCT4*, and *SOX2* target genes suggests that regions of H3K4 (lysine 4 on histone 3) methylation, within overlapping regions of extended H3K27 trimethylation, are characteristically associated with active and inactive chromatin states, respectively, in ES cells (a detailed version is presented in Chapter 10). These bivalent modifications occupy regions that correspond to developmentally regulated genes usually silenced in ES cells and also tumor suppressor genes. H3K27 methylation deficiency also results in premature expression of repressed genes. On the other hand, increased repression due to persistent H3K27 methylation that is further supplemented with H3K9 (lysine 9 on histone 3) methylation is a feature of transformation and has been reported in several cancer cell lines<sup>[99]</sup> and in embryonal cancer.<sup>[100]</sup> The genetic and epigenetic mechanisms of gene regulation during self-renewal and differentiation, however, represent a striking commonality between normal and cancer stem cells.

### 1.5.2 Stem Cell Extrinsic Effects: Niche Effects and Microenvironmental Signaling

The stem cell niche is loosely defined as stem cells surrounded by other differentiated cells within a tissue at defined locations. The niche consists of heterologous cell types that harbor stem cells and influence their fate through direct contact, thereby functioning to balance the quiescence and activation of stem cells,<sup>[101]</sup> the key to homeostatic regulation of stem cells, yet supporting ongoing tissue regeneration. The niche is thus a physical anchoring site for stem cells and generates factors including certain extracellular matrix (ECM) components and signaling molecules that control stem cell number, proliferation, and fate determination.<sup>[79,102,103]</sup> Examples of stem cell niches include the hair follicle bulge compartment<sup>[104]</sup> and the crypts of Lieberkuhn, located at the base of villi within the intestinal epithelium.<sup>[105]</sup>

A majority of mutagenic agents described to confer a risk for cancer also perturb the normal stem cell niche and tissue homeostasis, besides inducing changes in the DNA of some stem cells and impairing or enhancing some of their characteristic

properties, such as self-renewal or their intrinsic ability for DNA repair. These stem cells with altered DNA may remain in a state of perpetual activation by intercellular communications *in cis* (through ECM proteins) or *in trans* (through autocrine or paracrine factors) produced by niche cells. Such continuous activation further accentuates altered gene expression and protein profiles that drive these stem cells toward differentiation, cell death, and degenerative or transformation pathways. Another function of the tumor niche is the active recruitment of new endothelial and stromal cells into tumors that is essential for developing a pro-angiogenic environment that enhances tumor survival under adverse conditions.<sup>[106]</sup>

Very recently, the stringent regulation imposed on stem cells through their niche have begun to be identified. A normal niche may evolve to become proto-oncogenic, or in fact may be the prime feature in tumorigenesis and therefore represent an oncogenic state. The concept of proto-oncogenic stem cell niches is best exemplified in lung cancer, wherein different histological types of neoplasias have been correlated with stem/progenitor populations at specific tissues in the lung<sup>[107]</sup> (e.g., the putative stem cell populations originating in the bronchiole mucosa in pulmonary neuroendocrine cells of lung-derived neuroepithelial bodies have been proposed as likely originating cells for small cell lung carcinoma<sup>[108–110]</sup>). Similarly, a direct relationship between proximal airway basal progenitors and cells associated with carcinogenesis in murine models for human squamous cell carcinoma is suggested,<sup>[111,112]</sup> while bronchoalveolar cell and central bronchiolar adenocarcinomas are believed to originate from bronchioalveolar stem cells that reside at the bronchioalveolar duct junctions in lung tissues.<sup>[113–115]</sup>

Oncogenic stem cell niches have just recently been described. The retinoblastoma (RB) gene, identified as a tumor suppressor, has been shown to regulate HSCs extrinsically by maintaining the competence of the adult bone marrow to support their growth and normal homeostatic hematopoiesis. Loss of RB expression in the niche and myeloid cells leads to degradation of the osteoblastic niche and consequent displacement of HSCs. The latter then undergo rapid expansion and mobilization to the spleen, promoting myeloid development that ultimately culminates in myeloproliferative disease<sup>[116]</sup> (MPS). Along similar lines, mice deficient for another gene [i.e., the retinoic acid receptor  $\gamma$  (*RAR $\gamma$* )], develop MPS driven by a *RAR $\gamma$* -deficient microenvironment.<sup>[117]</sup> The MPS phenotype in both cases (i.e., loss of RB and *RAR $\gamma$* ) continues through the life span of the mice and is more pronounced in older mice. Moreover, the disease cannot be propagated through successive transplantation of HSCs from MPS-affected mice to normal mice, identifying the disease to be extrinsic to tumor-derived HSCs.

Another common feature of tumor and tissue stem cells is utilization of similar signal pathways that normally control cell fate during early embryogenesis. Such regulatory signal molecules, including components of the Notch, Wnt, and Hedgehog pathways, bone morphogenetic proteins,<sup>[90]</sup> fibroblast growth factor, leukemia inhibitory factor, and transforming growth factor- $\beta$ ,<sup>[118–122]</sup> have been shown to

play roles in controlling stem cell self-renewal and in regulating lineage fate in different systems. In numerous tumors, however, the signaling cascades initiated by these molecules have now been demonstrated to be dysregulated (e.g., in skin, liver, colorectal, and pancreatic cancers, Wnt signaling has been demonstrated to be aberrant<sup>[123,124]</sup>). In ovarian cancer, the Wnt signal transducer  $\beta$ -catenin is over-expressed at an advanced stage of tumor progression.<sup>[125]</sup> The Hedgehog cascade, well known as a regulator of patterning during embryonic development,<sup>[126]</sup> has been associated with breast,<sup>[127]</sup> ovarian,<sup>[128]</sup> and prostate cancers,<sup>[129]</sup> whereas Notch overstimulation has been strongly implicated in T-cell malignancies.<sup>[130]</sup> It has been demonstrated further that various lineage determination molecules within these signaling pathways exhibit a significant degree of crosstalk.<sup>[131]</sup> An important difference in the signals between normal and transformed states is that those in normal tissues are transiently expressed stem cell-activation signals, whereas in cancer, these signals dominate and lead to a state of long-term or permanent activation.<sup>[132]</sup>

The molecules and underlying machinery used by normal stem cells for homing or mobilization and CSCs for invasion and metastasis are also realized to be similar. For example, during HSC activation and mobilization, matrix metalloproteinase-9 (MMP-9) is required for proteolysis of the extracellular matrix components and converting stem cell factor from a membrane-bound form into a free form, which then promotes HSC proliferation and mobilization through a c-Kit receptor. Intriguingly, the molecules of the MMP family are considered as key players in the process of cancer cell metastasis. Additionally, cell surface receptors and the ligands required for their activation, such as SDF1 and CXCR4, are also expressed during normal stem cell homing and mobilization as well as cancer cell metastasis.

## 1.6 CONCLUSIONS AND FUTURE PERSPECTIVES

We have been presenting the widely accepted view that malignancy results from a complex network of interactions between altered cellular genes and numerous exogenous and endogenous factors of a tissue-derived cell. The probability of the accumulation and effects of such changes and interactions is almost certainly highest in long-lived tissue stem cells, but is equally effective in progenitors or differentiated cells that acquire an immortal phenotype. Unfortunately, it is not precisely known how CSCs arise in tissues or which of the resident tissue cells would be more susceptible to the transformation process. Thus, at a very fundamental level, we have yet to determine the extent to which stem cell biology is relevant to all types of human cancers. A detailed update in understanding of stem cell involvement in the major cancers is presented in the following chapters; however, it would be rather naive to believe that CSCs represent a universal modality for emergence and progression. Further studies employing cellular lineage tagging of putative stem and cancer progenitors in tightly regulated transgenic mouse models for specific cancer types are thus sorely needed. Once these cells and

lineage relationships are better understood, one can hope to better understand the process.

Several concerns also remain regarding the strategy of CSC identification using xenotransplantation models that demonstrate the presence of a self-renewing population. Since highly purified, FACS-isolated tumor cells are used in these experiments, the model remains imperfect, as in addition to a horde of other host factors, evolution of a tumor *in situ* involves complex interactions between a stem cell poised on the brink of transformation and its microenvironment. Thus, although most findings make it highly likely that dysregulation of cell fate-determining pathways contributes to the formation of aberrant stem cells and their hierarchies in cancer, identification of crosstalk between the extracellular environment and the regulation of pluripotency at the level of transcription is seriously restricted in the current models.

It is also to be expected that the role of CSCs in migration and metastases would be much different than their role in primary tumors. Such differential regulation of migrating CSCs and those in metastases is also not yet identified. Once additional insights into the biology of candidate CSCs, as well as of those subpopulations that initiate metastases, become apparent with further characterization and validation of their gene and protein expression profiles, as well as the role of these molecules in tumor progression and clinical responses, it will become clear whether engrafting activity (the key feature of identification protocols today) is an accurate reflection of stem cell activity. However, it is certain that advances in our knowledge of the properties of stem cells have definitely culminated in a realization for specific targeting and eradication of CSCs, and that treatment of bulk disease is an insufficient panacea for cancer. The development of a new generation of treatments to target the rare CSCs is thus critical, but poses formidable challenges:

1. Ideally, a therapy should target unique CSC pathways and “turn back the clock” from a state of disease to one of normal tissue and organ homeostasis.
2. A concern in achieving the goal stated above is that normal stem and progenitor cells actually prove to be more sensitive than CSCs to the effects of current chemotherapeutic drugs. This provides a competitive advantage to CSCs and makes their positive selection quite likely, leading to the emergence of drug-resistant clones. Delineation of the effects of the new drug regimes on the evolution of CSCs is thereby imperative.
3. In cases where clinical remission is achieved, the presence of drug-resistant CSCs that have “escaped” chemotherapy would initiate a relapse. This necessitates the development of sensitive methods for detection of residual CSCs for follow-up in patients in remission. The establishment of diagnostic endpoints by which treatment success can be measured is thus required.

A culmination of the understanding of CSC biology will thus aid the development of more effective and targeted therapies to treat this astoundingly complex and devastating disease.

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