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REVIEW

Understanding cancer stem cell heterogeneity and plasticity

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Heterogeneity is an omnipresent feature of mammalian cells *in vitro* and *in vivo*. It has been recently realized that even mouse and human embryonic stem cells under the best culture conditions are heterogeneous containing pluripotent as well as partially committed cells. Somatic stem cells in adult organs are also heterogeneous, containing many subpopulations of self-renewing cells with distinct regenerative capacity. The differentiated progeny of adult stem cells also retain significant developmental plasticity that can be induced by a wide variety of experimental approaches. Like normal stem cells, recent data suggest that cancer stem cells (CSCs) similarly display significant phenotypic and functional heterogeneity, and that the CSC progeny can manifest diverse plasticity. Here, I discuss CSC heterogeneity and plasticity in the context of tumor development and progression, and by comparing with normal stem cell development. Appreciation of cancer cell plasticity entails a revision to the earlier concept that only the tumorigenic subset in the tumor needs to be targeted. By understanding the interrelationship between CSCs and their differentiated progeny, we can hope to develop better therapeutic regimens that can prevent the emergence of tumor cell variants that are able to found a new tumor and distant metastases.

Keywords: cancer stem cells; heterogeneity; plasticity; reprogramming

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Introduction

Stem cells are cells with both self-renewing and differentiation abilities. Embryonic stem (ES) cells are pluripotent and, during development, can give rise to all cell types in the embryo proper. Adult stem cells are multipotent and can generate different lineages of progenitor cells that further differentiate into mature functioning cells. Adult stem cells, committed progenitors, and cells at various stages of differentiation constitute the kaleidoscope of cells in an adult organ and create what we call cellular heterogeneity.

Adult stem cells exist in organs with fast turnovers such as blood, small intestine, and epidermis as well as in organs generally considered ‘post-mitotic’ such as brain, skeletal muscle, and prostate. Owing to their fast turnover nature, hematopoietic stem cells (HSCs) and stem cells in the intestinal and skin epithelia are among the best understood [1-3]. Stem cells and their lineage development in many adult organs with slower turnover rates are not well characterized, and therefore they are frequently termed stem/progenitor cells.

True stem cells with self-renewal capabilities are generally rare and located in a special microenvironment called niche, which is composed of various supporting (epithelial or stromal) cells, extracellular matrix, blood vessels, and nerve fibers [1-3]. Although stem cells can be proliferating or dormant, most primitive stem cells seem to be quiescent [1-4]. Whether dividing or dormant, an important functional characteristic of stem cells is that they possess significant proliferative potential that can be unleashed when needed. However, the most fundamental property of a stem cell is self-renewal, a term often over-used, inaccurately used, or misused, especially in the context of cancer stem cells (CSCs). The relationship

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between self-renewal and differentiation can be viewed using the scheme in Figure 1. Stem cells can have three modes of division: symmetric renewal, asymmetric cell division (ACD), and symmetric stem cell commitment. Self-renewal can either be ACD or symmetric renewal (Figure 1). However, ACD is most frequently used to assess self-renewal because the two daughter cells not only are phenotypically distinct but also take different developmental fates. In reality, the candidate stem cells either genetically tagged or labeled with a fluorescent dye, such as PKH26, are tracked under a time-lapse video microscope. During ACD, the two daughter cells differentially labeled can be identified and temporally followed (Figure 1). Like ACD, the other two division modes can also be recorded (Figure 1). In principle, adult stem cells can generate uni-, bi-, tri-, or even multipotent progenitors that further develop into specialized cells.

Heterogeneity of normal stem/progenitor cells

Most stem cells in adult tissues/organs are identified by cell surface markers. To demonstrate the functionality of the candidate stem cells, marker-purified (or enriched) cells are used in (syngeneic, allogeneic, or xeno-) transplantation assays to assess whether such cells can reconstitute or regenerate the tissues from which the cells are originated. A significant pitfall, often neglected, in such assays is that tissue transplantation represents a major trauma to the recipient animals and will initiate a wound-healing response. Consequently, host-cell contribution may confound the interpretations of stem cell-initiated tissue regeneration. An alternative to surface marker-based stem cell assays is to perform lineage tracing in animals using a reporter driven by ‘stem cell-specific’ gene promoters. Such reporter systems, once established, can definitively characterize true stem cell properties by following their development *in vivo* (i.e., in the animals). One caveat is that the lineage-tracing approach can only be used in lab animals and therefore, the relevance of the results to humans still needs to be validated by transplantation and *in vitro* assays.

In addition to the marker-enrichment and lineage-tracing strategies, stem cells can be identified by virtue of their quiescent and slow-cycling properties. Such cells, called label-retaining cells [5, 6], can now be purified out live in genetic models and used in functional assays [1]. Finally, stem cells can be enriched using the side population (SP) [7] and Aldefluor [8] assays, both of which take advantage of the preferential expression of detoxification molecules (e.g., ABC transporters such as ABCG2 in SP analysis) or enzymes (e.g., ALDH1A1 in Aldefluor assay) in adult stem cells.

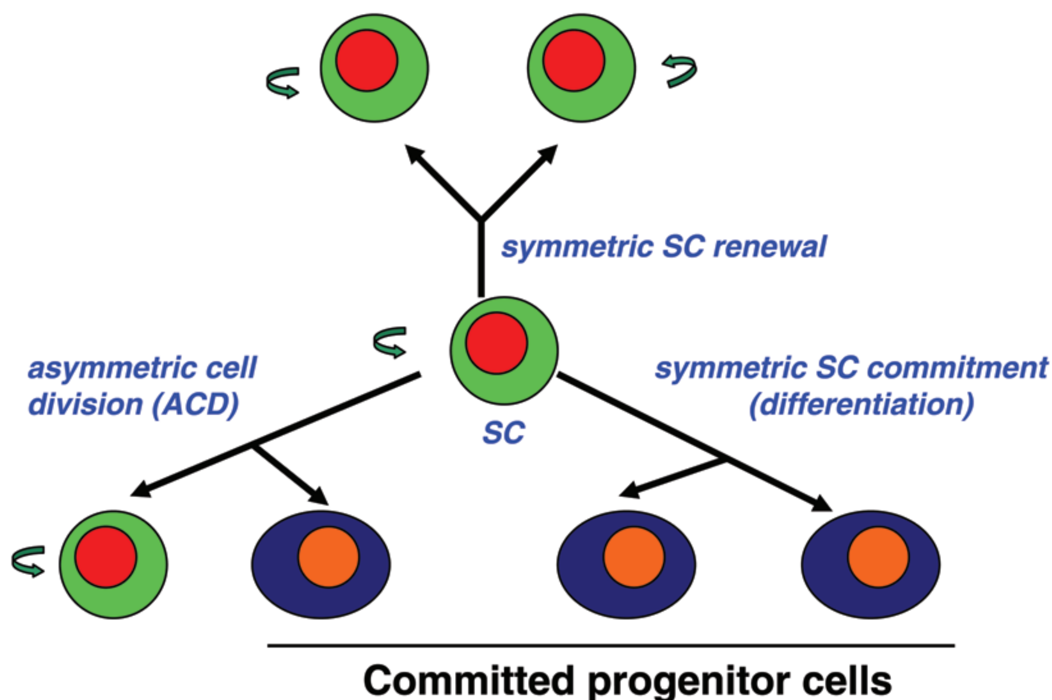

Figure 1 Relationship between stem cell self-renewal and differentiation (or commitment). The stem cell (SC) self-renewal is indicated by curved arrows. Only a uni-potent progenitor cell is depicted. See text for discussion.

Regardless of the approaches used, the 'stem cell' population purified is heterogeneous containing a mixture of true stem cells and more mature progenitors. A good example is human HSCs [9], whose activity is operationally defined by lymphomyeloid engraftment that persists for $\geq$ 20 weeks post transplantation [10]. The human HSCs, which are Lin$^-$CD38$^-$CD45RA$^-$, should better be considered as a hematopoietic stem/progenitor cell pool in which multiple subsets of cells preferentially expressing surface markers, such as CD34 and/or CD90 (Thy-1), (Figure 2) are hierarchically organized. Although it has been known for quite some time that the Lin$^-$CD34$^+$CD38$^-$ blood cells contain HSCs, most CD34$^+$ cells are actually progenitor cells and the HSC activity can be enriched by CD90 [11]. Nevertheless, even the Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$ subpopulation has only ~5% cells possessing long-term hematopoiesis-reconstituting activity compared to ~1% Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD90$^-$ cells having such activity [10]. The HSC activity can be again enriched using the Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$CD49f$^+$ marker profile with 9.5% (1 in 10.5) cells possessing the long-term repopulating property compared to 0.9% (1 in 111.3) Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$CD49f$^-$ cells having such activity [10]. The HSC activity can be further enriched using the 7-marker Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$Rho^{lo}CD49f$^+$ profile with 14% cord blood or 28% bone marrow marker-sorted cells possessing the long-term repopulating property in NOD-*scid-IL2Rγc*$^{-/-}$ or NSG mice [10]. The extraordinary example of human HSCs illustrates that the hematopoietic stem/progenitor cells are quite heterogeneous (Figure 2). It can be imagined that if we have the ability to find the unique markers expressed in the most primitive HSCs and keep fractionating the progenitor pool, we should be able to uncover a very small population of HSCs in which every single cell would fulfill the most stringent HSC definition, i.e., single-cell engraftment, systemic hematopoietic reconstitution, and rescue of the lethally irradiated recipient animal [12].

Similar heterogeneity has also been observed in mouse HSCs. When using the Lin$^{-/lo}$c-Kit^{hi}Sca-1^{hi}Thy1.1^{lo}Flk$^-$ combinatorial markers, ~0.02% (i.e., 1 out of 5 000) mouse bone marrow cells can sustain lifelong self-renewal [9]. However, when single cells bearing markers of Lin$^-$mCD34$^{-/lo}$c-Kit$^+$Sca-1$^+$ were implanted, as many as 21% of the recipient animals sustained long-term lymphohematopoietic reconstitution [12]. Further, when using Lin$^-$c-Kit$^+$Sca-1$^+$CD150$^+$CD48$^-$ as the purification marker, 20%–50% of bone marrow cells bearing the marker profile exhibit long-term reconstituting activity [13].

An example of stem cell heterogeneity in a non-hematopoietic organ is mouse prostatic stem cells, which were first enriched using the GPI-linked surface protein Sca-1 [14, 15]. Cell-labeling experiments using green fluorescence protein-tagged Sca-1$^+$ cells mixed with non-tagged cells demonstrate clonal origins for single prostatic tubules that contain both p63$^+$ basal and AR$^+$ luminal cells [14], suggesting the presence of bipotential progenitors in the Sca-1$^+$ cell population. However, Sca-1$^+$ prostate cells are heterogeneous containing both stem and non-stem cells. Combinatorial marker profiling by adding CD49f (integrin α6) reveals that the Lin$^-$Sca-1$^+$CD49f$^+$ mouse prostate cells constitute ~1% of total prostate epithelial cells, are localized predominantly in the basal layer proximal to the urethra, overlap (~70%) with the basal marker K5, and, importantly, exhibit much enhanced clonal growth and serial (i.e., renewing) sphere-forming potential *in vitro* as well as the ability to regenerate prostatic tubules when transplanted as tissue recombinants under the renal capsules [16]. Again, the regenerated tubules are of clonal origin and contain both basal (e.g., K5$^+$, p63$^+$) and luminal (K8$^+$) cells providing evidence for the existence (and identity) of bipotential normal mouse prostatic basal stem cells. Further

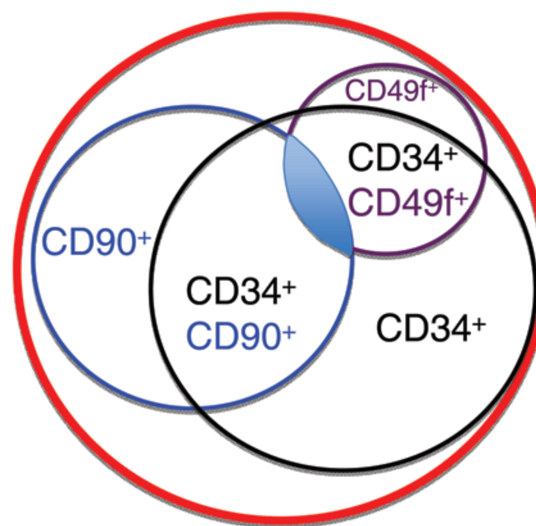

Figure 2 A cartoon depicting the heterogeneous nature of human hematopoietic stem/progenitor cell pool. Illustrated are three subsets (i.e., CD34$^+$, CD90$^+$, and CD49f$^+$) of progenitors inside the Lin-CD38$^-$CD45RA$^-$ population. Combined sorting of triple marker-positive (i.e., CD34$^+$CD90$^+$CD49f$^+$; shaded) blood cells (in either bone marrow or cord blood) greatly enriches HSCs with long-term repopulating activity.

purification using the $Lin^{-}Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$ marker profile reveals multipotent prostate stem cells in the basal compartment [17]. Immunostaining shows that the $CD117^{+}$ (c-Kit, stem cell factor receptor) population is enriched proximal to the urethra, predominantly in the basal layer (~3% of total basal cells). Fractionated $CD117^{+}$ cells combined with rat UGSM (urogenital sinus mesenchyme) and transplanted under the kidney capsules can regenerate prostate-like structures comprising cells of the basal ($K14^{+}$), luminal ($K18^{+}$), and, for the first time, neuroendocrine (synaptophysin^{+}) lineages, thereby demonstrating tripotency [17]. Single cell-reconstitution assays achieved successful engraftments in 14 of 97 single cell transplantations (along with rat UGSM). The single cell-derived outgrowths also contain all three principal prostate cell types (basal, luminal, and neuroendocrine), with luminal cells expressing the critical differentiation-related homeobox protein, Nkx3-1, and the terminal differentiation marker, probasin, suggesting that the reconstituted tubules possessed functional secretory activities [17].

These examples suggest that the term 'stem cells' in most cases is used very loosely and actually refers to a group of heterogeneous cells. It is imperative to bear in mind that stem cells must be functionally assayed by the 'stem cell activity'. To a certain degree, the enrichment of stem cells, or, more precisely, stem cell activity, and separation of stem cells from mature progenitors resemble a biochemical fractionation, in which homogeneous (i.e., high) enzymatic activity can be reconstituted by adopting either more extensive fractionation steps or a more 'specific' fractionation protocol. Stem cell heterogeneity may imply that distinct subsets of stem/progenitor cells are inter-related and thus hierarchically organized or different subpopulations are unrelated. Strictly speaking, however, if all self-renewing blood-generating cells are initially derived from a primitive HSC, all subsets should have the hierarchical relationship. That adult stem cells are heterogeneous should not be surprising as even cultured ES cells, usually thought as being homogeneous, actually contain partially committed neuronal and hematopoietic progenitors [18]. It may be of practical interest to point out that stem cell markers may be unique or conserved, across species and lineages. For example, Sca-1, which enriches both hematopoietic and prostatic epithelial stem cells in mouse, is not expressed in humans. On the other hand, c-Kit appears to enrich both mouse hematopoietic and prostatic epithelial progenitors whereas high levels of CD49f expression appear to be common to human hematopoietic and mouse prostate stem cells.

Plasticity of normal stem cell progeny

It is generally believed that during normal development or homeostasis, stem cells give rise to fast-proliferating progenitor cells that then mature into various types of functional cells (Figure 3A). In other words, this developmental path is usually a one-way 'traffic' under physiological conditions with fully differentiated cells being unable to proliferate or 'dedifferentiate' (Figure 3A). This unidirectionality of development, enforced by epigenetic mechanisms in and post-mitotic nature of most terminally differentiated cells guarantees that different cell types in an organ have distinct identities and play specialized functions. For instance, neurons, skeletal muscle cells, and fibroblasts in adult organs rarely divide. Adult human cardiomyocytes are mostly post-mitotic with a turnover rate of ~1% per year at age 20 and 0.4% per year at age 75. At age 50, 55% of human cardiomyocytes remain from birth and over the first decade of life, cardiomyocytes often undergo a final round of DNA synthesis and nuclear division without cell division, resulting in ~25% of human cardiomyocytes being binucleated [19]. Similarly, the average lifespan of human adipocytes is ~10 years and the number of fat cells in adult humans remains quite constant with an annual turnover rate of only ~10% [20, 21]. The post-mitotic nature of terminally differentiated cells would suggest that dedifferentiation, a process thought to be genetically regulated [22, 23] in which a specialized cell takes on a more primitive state, although representing one potential aspect of plasticity in the stem cell progeny, may not take place prevalently under homeostatic conditions in adult mammalian organs (Figure 3A).

On the other hand, not all mature cells are post-mitotic and differentiated cells may retain the ability to generate more differentiated cells. For example, endothelial cells (ECs), hepatocytes, and Schwann cells are known to retain significant proliferative potential. Certain differentiated cells such as pancreatic endocrine insulin-producing β-cells are able to duplicate themselves under homeostatic conditions [24] (Figure 3B, top). Self-duplication of mature cells represents another potential route of plasticity in the stem cell progeny.

Most cell plasticity occurs in response to injuries or upon experimental manipulations (Figure 3B-3G). One type of cell plasticity involves generation, from differentiated cells, of more differentiated cells of the same or different lineages (Figure 3B-3D). For example, pancreatic β-cell self-duplication increases significantly upon experimentally induced organ damage [24] (Figure 3B, bottom). Genetic deletion of *Pax5* (paired box gene 5) leads to dedifferentiation of mature B cells to uncom-

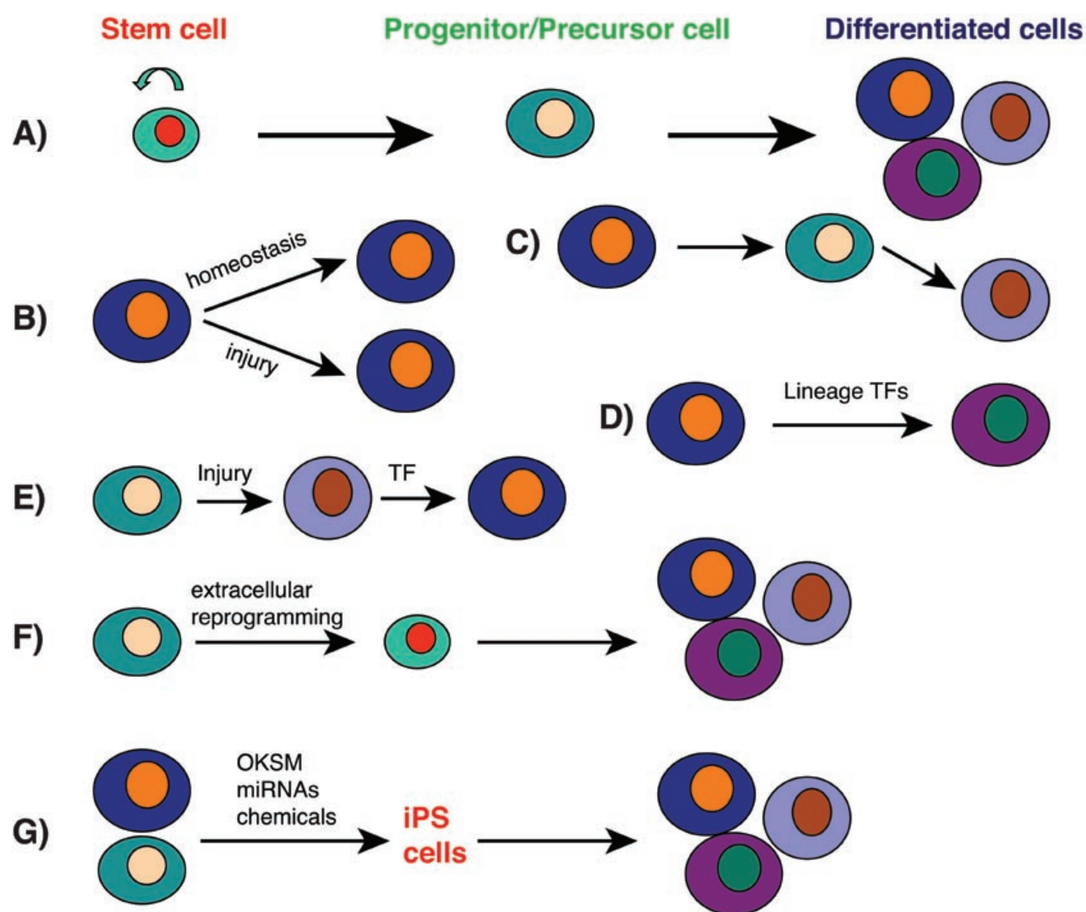

Figure 3 Stem cell development under normal (physiological) conditions **(A)** and different forms of plasticity of the stem cell progeny during injury and upon induction **(B-G)**. In **(A)**, a self-renewing, relatively quiescent stem cell gives rises to a proliferative progenitor cell (sometimes called a precursor cell), which then develops into non-proliferative terminally differentiated cells. Stem cells, progenitors, and differentiated cells are illustrated in different colors and sizes. As an example, this progenitor cell is depicted to generate three different differentiated cells. **(B)** A differentiated cell directly generates another differentiated cell of the same type. The best example is mouse pancreatic β-cells. **(C-D)** Plasticity by which one differentiated cell type is converted **(C)** or directly converts **(D)** to another differentiated cell type. **(E)** A progenitor cell gives rise to a specialized cell type upon injury, which is then transdifferentiated into another specialized cell type by a lineage-specific TF. **(F-G)** Plasticity by which progenitor (or differentiated) cells are reprogrammed to a more primitive cell, which then develops into various specialized cells. See text for individual examples.

mitted early progenitors that then differentiate into T lymphocytes [25], illustrating cellular dedifferentiation induced by manipulating lineage-specific transcription factors (TFs) (Figure 3C). More often and very excitingly, overexpression of lineage-specific TFs is found to be capable of directly reprogramming different cell fates [26-34]. It was first shown that three TFs (i.e., Ngn3, Pdx1, and Mafa) were sufficient to reprogram pancreatic exocrine cells to β cells [27]. Recently, direct reprogramming of fibroblasts into neurons [30-32] or hepatocytes [33, 34] has also been achieved using respective lineage TFs. Of note, in all such experiments [27, 30-34], plasticity is induced and cell fate reprogramming is achieved by directly turning one differentiated cell type to another (Figure 3D), a process often termed cellular transdifferentiation.

Another type of induced plasticity involves progenitor cells (Figure 3E-3G). In response to injury, a population of pancreatic progenitors can generate glucagon-expressing α cells, which then, with ectopic expression of *Pax4*, transdifferentiate into β cells [35]. This example illustrates one special type of cellular transdifferentiation in which injury turns progenitor cells into one specialized cell type, which is transdifferentiated into another specialized cell type upon the action of a lineage-specific TF (Figure 3E). Another example of progenitor cell

plasticity is offered by oligodendrocyte precursor cells, which can be reprogrammed by extracellular signaling molecules into neural stem cells that then develop into astrocytes, oligodendrocytes, and neurons [36]. The ultimate example of cellular reprogramming is induced pluripotent stem (or iPS) cells, which are derived from direct reprogramming of differentiated cells or progenitors by overexpressing pluripotency factors (i.e., OKSM or Oct-4, KLF4, SOX2, and Myc) or by using miRNAs or chemicals [29, 37] (Figure 3G). iPS cells resemble ES cells and thus possess the potential to generate all different types of cells. iPS cells or direct cell reprogramming using lineage-specific TFs hold great promises for generating specific cell types needed for cell therapy. Potential problems are associated with incomplete reprogramming and retention of donor cell memory.

In summary, the stem cell progeny retain significant plasticity that can manifest in response to injuries or upon experimental inductions, which can be employed to generate precious cell types for cell therapy. However, such plasticity may not occur prevalently in normal tissues under homeostatic conditions.

CSCs

Tumorigenesis has been long known to resemble organogenesis and most tumors are heterogeneous containing many phenotypically and functionally different cells. Tumor cell heterogeneity may result from clonal evolution driven by genetic instability and/or from maturation/differentiation of stem-like cells frequently called CSCs or tumor-initiating cells [38]. Recent evidence indicates that clonal evolution and CSC-directed development may not necessarily be mutually exclusive and both mechanisms may cooperate to create tumor cell heterogeneity [39, 40]. Although there are still debates about the CSC concept and some CSCs reported, there is no denying that malignant tumors are immortal at the population level. It will be interesting and important to identify and characterize the immortal subpopulations of cancer cells.

The subject of CSCs is controversial not because CSCs do not exist, but because misconceptions and inadequate assays have caused many confusions and inconsistencies. First, CSC is a functional definition. Just as normal stem cells are measured by the stem cell activity, CSCs should be defined in functional assays by their ability to generate serially transplantable tumors that, at least partially, recapitulate the cellular and histostructural heterogeneity of the parent tumor. Simple marker expression and *in vitro* assays are not sufficient to define any cancer cells as CSCs. Furthermore, many of the CSCs so far reported have not been subjected to series transplantation assays; instead, only tumor incidence and/or tumor weight are used to measure the CSC activity. Such endpoints may well be assaying tumor progenitor rather than CSC activity due to the high proliferative capacity in the progenitor cell population (Figure 4). Serial tumor transplantation assays, especially at low cell doses, should allow the demarcation of long-term CSCs vs fast-proliferating tumor progenitors, both of which possess tumor-initiating capacities.

Second, CSCs may or may not originate from normal stem cells [41]. In fact, because progenitor cells generally represent the largest proliferative pool in an organ they theoretically could represent the best transformation targets (Figure 4). In this scenario, the transformed progenitors acquire self-renewal capacity, become the CSCs, and establish clinical tumors. Unfortunately, in the literature, very often CSCs are said to originate from normal stem cells. A good example for this point comes from analyzing the potential transformation target in basal-like human breast cancers [42]. The authors employed a combination of surface markers to purify out basal stem/progenitor (CD49f^{hi}EpCAM^{-}; expressing p63/CK14/vimentin but not ER/PR), luminal progenitor (CD49f^{+}EpCAM^{+}; expressing high levels of CK8/18/ER/MUC-1/GATA-3), and mature luminal (CD49f^{-}EpCAM^{+}; expressing high levels of ER/PR) cells from normal mammary tissue and preneoplastic specimens from individuals heterozygous for a *BRCA1* mutation. *BRCA1* mutations have been clinically linked to the development of basal-like breast cancers. Surprisingly, the *BRCA1*-mutant samples display a significant reduction in CD49f^{hi}EpCAM^{-} basal stem cells, but a dramatic increase in CD49f^{+}EpCAM^{+} luminal progenitor cells. These observations, together with subsequent gene expression profiling and functional studies, suggest that an aberrant luminal progenitor population may represent the transformation target in *BRCA1*-associated basal breast tumors [42]. Independent studies in mouse genetic models by deleting *Brca1* in either basal stem or luminal progenitor cells verified that only *Brca1* deletion in the luminal progenitor compartment phenotypically and histologically recapitulates the human basal-like breast cancers [43]. Interestingly, mammary epithelial cells derived from *BRCA*$^{mut/+}$ patients give rise to tumors with increased basal differentiation relative to *BRCA*$^{+/+}$ cells [44], suggesting that specific genetic mutations may dictate the phenotypic fate of progenitor cells during tumor development.

Third, the initially transformed cells may not be the cell-of-origin (i.e., CSCs or cancer-initiating cells) for the clinical tumor. The transformed cell may remain dormant for decades without generating a clinically detectable tumor. Recent studies using elegant MADM (mosaic

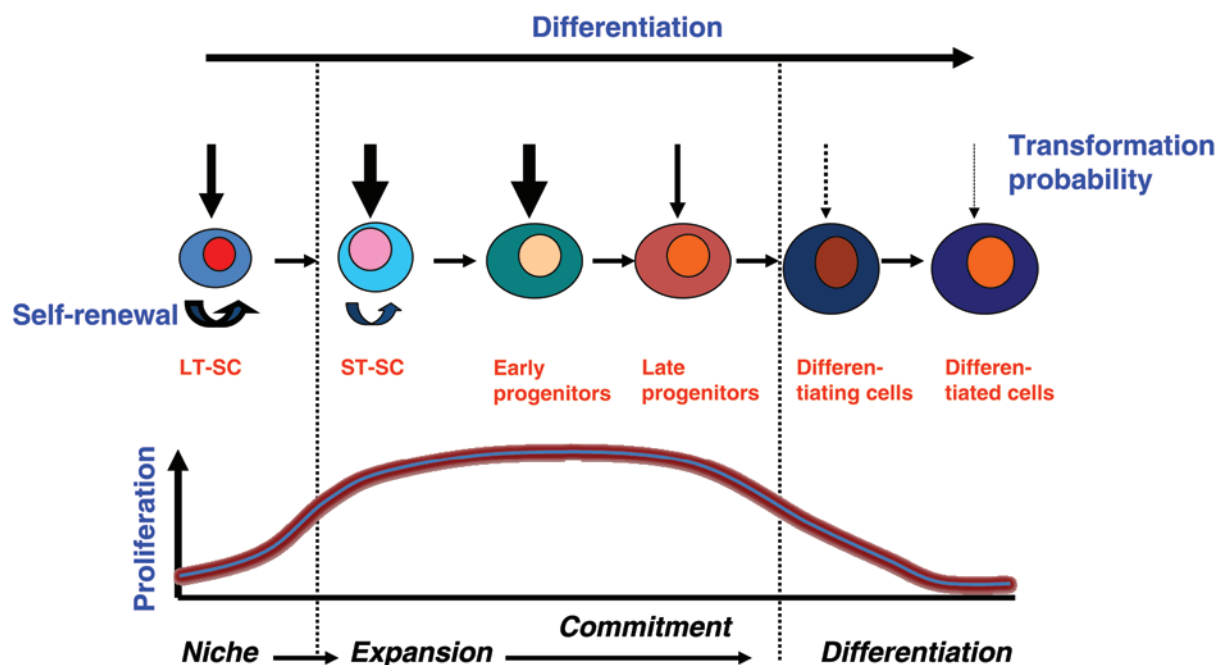

Figure 4 Stem cell proliferation, self-renewal, differentiation, and transformation. Depicted here is a hypothetical long-term stem cell (LT-SC), which has the greatest self-renewal activity and is quiescent in its niche (bottom). LT-SC develops into a short-term stem cell (ST-SC), which shows reduced self-renewal activity but increased proliferation. The ST-SC then gives rise to early progenitor cells that may have lost self-renewal capacity, but probably represent the most proliferative cell population. Early progenitors generate late progenitor cells that begin to commit to differentiate by expressing lineage-specific differentiation markers and these late progenitor cells gradually develop into fully differentiated cells that once again lose proliferative potential (i.e., post-mitotic). From the standpoint of transformation probability, the ST-SC that retains self-renewal activity and progenitor cells that are highly proliferative (demarcated by two vertical thick lines) theoretically could represent the best targets for tumorigenic transformation.

analysis using double markers) mouse models provide strong support for this scenario by showing that the oligodendrocyte precursor cells represent the cell-of-origin for malignant gliomas, although the initial transformation occurs in all neural and glial progenitors [45]. Fourth, as CSCs are in most cases defined, operationally, as tumor-initiating cells and because the current CSC assays have inherent limitations (e.g., relying heavily on xenotransplantations), the reported CSCs may not necessarily be the same as the founding cells that gave rise to patient tumors *in vivo*. This point is important also when considering that *in vivo*, CSCs may differ between different patient tumors and may constantly change as the disease progresses.

Fifth, CSCs may or may not be rare [46, 47], and their relative abundance likely varies with individual patient tumors, tumor type, grade, and treatment status. This is a critical point as it has been mistakenly assumed (and presumed) by many that CSCs must be rare. Also, the abundance of CSCs may not correlate strictly with poor clinical features in every tumor; however, it should theoretically correlate with the levels of differentiation and malignancy. In other words, the more malignant a tumor is, the more stem-like cancer cells it may harbor. A priori, this logic makes sense as more malignant tumors contain more undifferentiated tumor cells, and CSCs are usually less differentiated or undifferentiated. Consistent with the undifferentiated nature of CSCs, early embryonic markers such as SSEA-1, TRA-1-60, and oncofetal protein 5T4 have been employed to enrich CSCs (see discussions below). Sixth, CSCs, though possessing some stem cell properties, are, nevertheless, transformed cells with complex genetic mutations and epigenetic alterations so they should not be equated to normal stem cells with respect to their biological properties. For instance, unlike normal stem cells, CSCs may not be able to undergo normal or complete differentiation. As such, some assays for normal stem cells may not be applicable to CSCs. For example, not all CSCs may be able to undergo multi-lineage differentiation. Seventh, it should be born in mind that mice, despite their wide use and great value in biomedical and cancer research, are different from

humans and normal stem cells in the human and the corresponding mouse organs may be phenotypically different, as illustrated above by HSCs. Consequently, CSCs in human and rodent tumors may also be phenotypically distinct. Frequently, results from the two experimental systems are mixed up, thus creating confusions. Lastly, like normal stem cells, CSCs are heterogeneous and their progeny may also possess plasticity, especially accompanying tumor progression and in response to therapeutic interventions, which I discuss below by focusing on CSCs in human tumors.

CSC heterogeneity

CSCs, now reported in most human tumors, are commonly identified and enriched using strategies for identifying normal stem cells, which include flow cytometry-based sorting using cell surface markers, such as CD44 and CD133, and functional approaches, including SP analysis [7], Aldefluor assay [8, 48], and sphere formation coupled with serial sphere passaging [49]. The CSC-enriched populations prospectively purified using these strategies are then implanted, at various cell doses, in immune-deficient mice to assay their tumor-regenerating capacity, an *in vivo* assay often called limiting dilution assay. The tumor cell subset that can initiate tumor development at low cell numbers is enriched in CSCs, and these cells are further tested for 'self-renewal' capacity in serial tumor transplantations when feasible.

Interestingly, much like the heterogeneity of normal stem cells in an adult organ, CSCs in the same type of human cancer are phenotypically and functionally heterogeneous. Breast CSCs (BCSCs) are the first CSCs to be reported in solid tumors and are among the most intensely studied. Human BCSCs have been enriched in $CD44^{+}CD24^{-/lo}$ [50], SP [51, 52], $ALDH^{+}$ [53, 54], and PKH26 dye-retaining [55] cells. Although the interrelationships between these different subsets of tumorigenic BCSCs remain unclear, these observations [49-55] suggest that human BCSCs are phenotypically diverse. Indeed, recent studies [53-59] indicate that not only CSC marker expression in breast cancer cells is heterogeneous but also there exist many subsets of BCSCs that vary from patient to patient, which may be related to individual tumor's genetic makeup [44]. The enrichment of BCSCs by different approaches also suggests that different phenotypes may identify overlapping tumorigenic populations and consequently, certain combinatorial strategies might further enrich tumor-initiating cells. For example, although there is only a small overlap between $ALDH^{+}$ (i.e., cells expressing high levels of aldehyde dehydroganse (ALDH) activity measured by the Aldefluor assays; sometimes called $ALDH^{bri}$ or $ALDH^{hi}$) and $CD44^{+}CD24^{-/lo}$ cells, the cells bearing two phenotypes (i.e., $ALDH^{+}CD44^{+}$) seem to be more tumorigenic than cells expressing either marker alone [53]. A recent study provides further support to these points. In ERα-negative human breast cancer, xenograft-initiating cells are found in both $CD44^{+}CD24^{-}$ and $CD44^{+}CD24^{+}$ cell populations; however, CSCs are most highly enriched using the combinatorial marker profile $CD44^{+}CD49f^{hi}CD133/2^{hi}$ [59]. BCSCs are among the few CSC types that have been shown to undergo both ACD and symmetric renewal (Figure 1). Using the *ErbB2* breast cancer model and PKH26 dye retention/dilution strategy, Cicalese *et al.*[60] demonstrate higher symmetric renewal divisions in BCSCs than in their normal counterparts. Interestingly, *p53* deficiency in normal mammary stem cells increases symmetric cell divisions, thus predisposing to tumor development [60].

CSCs have been reported in brain tumors, especially in glioblastoma multiforme (GBM) [61-74]. The stem-like GBM cells have been enriched using cell surface molecules, such as CD133 [61, 72], SSEA-1 (stage-specific embryonic antigen-1) [64], EGFR [67, 68], and CD44 [69], or functional assays, including the SP analysis [7, 63, 73] and neurosphere assays [49, 74]. Intriguingly, there are significant uncertainties surrounding the use of CD133 as a marker for brain tumor stem cells (BTSCs). Although CD133 has been widely utilized to enrich BTSCs [61, 72] and molecular profiling has revealed stem cell gene expression patterns associated with $CD133^{+}$ GBM cells [71], tumorigenic cells are found in both $CD133^{+}$ and $CD133^{-}$ cells in some gliomas [62], and some $CD133^{+}$ brain tumor cells may not possess high tumor-initiating capacity, and, in fact, the $CD133^{-}$ cell population from some GBM may actually harbor long-term self-renewing tumor-initiating cells [66]. Similarly, the SP may or may not enrich BTSCs [63, 73]. Furthermore, although it has been reported (or assumed) that there exists a lineage relationship between $CD133^{+}$ and $CD133^{-}$ brain tumor cells, the two populations of cells may have different cells-of-origin [70]. More studies are required to resolve these contradictory findings. Chen *et al.*[66] propose that GBMs contain multiple distinct populations of linearly related $CD133^{-}$ cells representing different stages of differentiation with some $CD133^{-}$ cells generating aggressive tumors containing both $CD133^{+}$ and $CD133^{-}$ cells and some other $CD133^{-}$ cells, perhaps by symmetric stem cell renewal, generating slow-growing circumscribed tumors of $CD133^{-}$ cells. One point is clear and consistent: human malignant gliomas are extremely heterogeneous and harbor multiple distinct pools of self-renewing BTSCs capable of initiating phenotypi-

cally diverse and serially transplantable tumors.

Multiple CSCs have also been reported in human colon cancer [75-86] using cell surface markers CD133 [75-77], CD44 [78, 79] or ABCB5 [85], clonal analysis [80], SP phenotype [84], and Aldefluor assays [82]. As in studies of BTSCs, use of CD133 as a positive selection marker for colon CSCs has generated conflicting results [75-77, 81], but subsequent studies indicate that the AC133 epitope, but not CD133 protein, is differentially and specifically expressed in colon CSCs and its expression is lost upon differentiation [83]. On the other hand, $CD44^+$ cells seem to consistently enrich tumorigenic colon cancer cells and additional subfractionation of the $CD44^+EpCAM^+$ cell population with CD166 further enhances the success rate of tumor engraftments [78]. ALDH has also been used as a marker for colon CSCs, but $ALDH^+$ colon cancer cells are still heterogeneous and further enrichment using CD44 or CD133 significantly increases the tumor-initiating capacity [82].

Similarly, stem-like cancer cells have been reported in human cancers of the prostate [87-100], lung [101-111], and many other organs (liver, pancreas, kidney, bladder, ovary, etc). In prostate cancer, tumor-initiating cells have been identified in xenografts in $CD44^+$ [90, 96], $CD44^+\alpha2\beta1^+$ [91], TRA-1-$60^+CD151^+CD166^+$ [99], and $ALDH^+$ [97, 98] populations as well as in SP [87] and holoclones [94]. The interrelationship between these tumorigenic subsets remains currently unclear except that the $CD44^+\alpha2\beta1^+$ phenotype greatly enriches tumor-initiating cells compared to $CD44^+$ phenotype alone [91]. Although $CD133^+CD44^+\alpha2\beta1^+$ (i.e., triple marker-positive) cells in primary prostate tumors are highly clonogenic [89], whether such cells are endowed with enhanced tumor-initiating capacity is unknown. In fact, one of the major unanswered questions is whether primary human prostate tumors, which do have cells expressing the above-mentioned markers, harbor authentic CSCs, mainly due to the technical difficulties in reconstituting human prostate tumors in immune-deficient mice when using fractionated single tumor cells. Putative lung CSCs have been reported in SP [101] and $CD133^+$ [102-104, 108], $ALDH^+$ [107], $CD44^+$ [109], and oncofetal protein $5T4^+$ [111] cells. Again, the interrelationship between these tumorigenic subpopulations is presently unclear.

How should we understand the complicated picture of CSC heterogeneity? First of all, since normal stem cells are heterogeneous and stem/progenitor cell development is a continuum, it should not come as a surprise that CSCs are heterogeneous, especially when put in the context that CSC activity is only defined, in a rather crude way, as enhanced tumor-initiating capacity. Second, in fact, leukemic stem cells (LSCs) in acute myeloid leukemia (AML), the best-characterized CSCs, are very heterogeneous. The AML LSCs were first reported to bear the $CD34^+CD38^-$ normal HSC marker profile with $CD34^+CD38^+$ and $CD34^-$ fractions containing no clonogenic and leukemia-initiating cells based on engrafting assays in SCID mice [112, 113]. It was found, 10 years later, that true LSCs in the $CD34^+CD38^-$ fraction were very rare and comprised a hierarchy of cells with different self-renewal potential [114]. Recent xenotransplantation studies in NSG mice confirmed the rarity of LSCs (~1 per 0.15-4.1 × 10^6), but revealed more unexpected heterogeneity of SCID leukemia-initiating cells, which were found not only in Lin^-CD38^- fraction but also in $CD34^-$, Lin^+, $CD38^+$, and $CD45RA^+$ fractions [115]. Importantly, the new study [115] indicates that the AML LSCs do not necessarily derive from the normal HSCs as initially hypothesized [112, 113], emphasizing the potential disconnect between CSCs and normal stem cells.

Third, CSC heterogeneity refers mostly to phenotypic heterogeneity and, in most cases, it is unclear whether phenotypically diverse CSC populations are also functionally distinct. To address this relationship, one needs to use the same tumor (cell) system to simultaneously compare the tumorigenic potential of different subsets. In theory, various CSC populations may or may not be hierarchically organized, analogous to how cancer cells in general may be organized. In a hierarchical model, the most ‘primitive’ CSCs would develop into more mature tumor progenitors, which in turn develop into much less tumorigenic cells. These functionally divergent tumorigenic subsets can be dissected by serial transplantations coupled with clonal tracking, as done in analyzing AML LSCs [114]. In this scenario, if the most primitive CSC population is therapeutically targeted, all tumorigenic cells should be eliminated. On the other hand, multiple CSC subsets in a tumor may exist in a tumorigenic pool of undifferentiated cells, have different origins, and are not related to one another lineage-wise. Several pieces of evidence support the co-existence of independent CSC clones. In breast cancer, although the $CD44^+CD24^-$ cell population is enriched with progenitor cells and the $CD24^+$ population is luminally differentiated, in some tumors a lineage relationship does not exist between $CD24^-$ and $CD24^+$ epithelial cells as the two populations harbor different genetic alterations [116, 117]. Similarly, $CD133^+$ and $CD133^-$ glioma cells may have different cells-of-origin [70]. There is evidence that the primary tumor genotypes dictate the overall phenotypes of tumor progenitors [44, 66, 110]. It is conceivable that more mature tumor-initiating cells derived from the primitive CSCs may sustain secondary genetic hits and in turn become new CSCs, and then develop independently of the

original CSCs. In reality, both hierarchical tumor-initiating populations and independently evolving tumorigenic clones may operate to create the heterogeneity of CSCs [80].

Finally, plasticity in CSCs and their progeny will further contribute to cancer cell and CSC heterogeneity [118], which I discuss below.

Intrinsic and induced plasticity in CSC progeny

As discussed earlier, terminally differentiated normal cells, in their natural milieu, generally do not manifest significant plasticity although they possess such potential. During early tumor development and in established tumors under unperturbed conditions, stem-like cancer cells perhaps mainly undergo one-way maturation by developing into tumor progenitors and even differentiated tumor cells (i.e., non-CSCs) (Figure 5A). In other words, developing tumors (or evolving tumor clones) may contain highly tumorigenic undifferentiated CSCs that would develop into phenotypically differentiated tumor cells that functionally possess low or no tumor-regenerating capacity (Figure 5A). Many examples of CSCs discussed in the preceding section in multiple tumor systems have been shown to be generally much less differentiated. For example, although LSC activity has been detected in several cellular compartments, the most tumorigenic subpopulation is still in undifferentiated $Lin^{-}CD34^{+}CD38^{-}$ cells, which can develop into more mature, less tumorigenic cells [112, 113, 115]. Likewise, in marker-independent sphere- (e.g., neurosphere, mammosphere, colonosphere, prostasphere, etc) formation assays, it has been repeatedly demonstrated that a single (undifferentiated) tumor cell can generate a sphere that contains multiple types of lineage-differentiated cells [49, 61, 66, 76, 80, 90, 95, 100, 106]. $CD133^{+}$ colon CSCs can grow exponentially for more than a year as undifferentiated tumor spheres *in vitro* but, upon transplantation *in vivo*, they can generate tumors that histologically recapitulate the morphological and antigenic pattern of the original tumor [76]. These considerations would argue that undifferentiated tumor cells are in general more tumorigenic than their partially differentiated progeny. These discussions also suggest that in untreated (unperturbed) tumors, dedifferentiation of non-CSCs to CSCs may be rather low (Figure 5A). On the other hand, unlike normal organs, tumors lack the normal social control and suffer varying degrees of defects in normal differentiation pathways and, consequently, cancer cells in general display higher intrinsic or spontaneous morphological, phenotypic, and functional plasticity compared to fully differentiated normal cells (Figure 5B). One such plasticity is the phenomenon of 'molecular mimicry' (Figure 5B, left), in which poorly differentiated cancer cells (presumably CSCs or tumor progenitors) in melanoma, prostate cancer, sarcoma, and some other tumors can 'transdifferentiate' into cells resembling ECs, which organize into vessel-like structures that can even allow blood flow [119, 120]. Recent work indicates that defined CSCs in GBM can also transdifferentiate into EC (Figure 5B, right) where both cell types possess the same genetic mutations [121, 122].

There are potentially other forms of spontaneous cancer cell plasticity in addition to transdifferentiation. For example, a subpopulation of reversibly drug-tolerant cancer cells has been detected in drug-sensitive non-small cell lung cancer cell cultures [123]. This subpopulation can arise *de novo* and is maintained by IGF-1 receptor signaling and through epigenetic mechanisms involving the histone demethylase KDM5A (Jarid1A). Interestingly, this subpopulation of drug-tolerant cancer cells overlaps with, but is not identical to, $CD133^{+}$ CSCs [123], suggesting that not all drug-resistant cancer cells are CSCs [124]. It remains to be seen whether such reversibly drug-tolerant cancer cells exist *in vivo* and whether they bear stable and unique phenotypic markers that allow prospective identification. Recently, evidence has been provided [125] or it has been hypothesized [126] that non-CSCs and CSCs can inter-convert and non-CSCs can 'dedifferentiate' into CSCs (Figure 5A). This should not be surprising based on the discussions above. In fact, this phenomenon has been demonstrated in other systems, e.g., the inter-conversions between $ABCG2^{+}$ and $ABCG2^{-}$ prostate and breast cancer cells [87] or between $CD44^{+}$ and $CD44^{-}$ prostate cancer cells [90]. However, as discussed above, the rate of spontaneous conversion of non-CSCs to CSCs is probably very low and the kinetics of dedifferentiation is slow (Figure 5A). It should also be noted that most studies so far that reported on the spontaneous plasticity of non-CSCs have been performed *in vitro*.

Analogous to the induced plasticity in normal stem cell progeny (Figure 3), plasticity of non-CSCs perhaps occurs more prevalently under 'induced' conditions, e.g., accompanying tumor progression *in vivo* (Figure 5C) or as a result of experimental manipulations *in vitro* or therapies *in vivo* (Figure 5D). Hence, to a certain degree, cancer cell plasticity resembles fate reprogramming in differentiated normal cells. As tumors grow, tumor cells increasingly experience hypoxia and microenvironmental changes. Furthermore, there is increasing infiltration of inflammatory cells and accumulation of cytokines, chemokines, and other bioactive molecules such as interleukins (IL-1, IL-6, etc) and TGF-β, which may cause epithelial–mesenchymal transition (EMT). Conceivably,

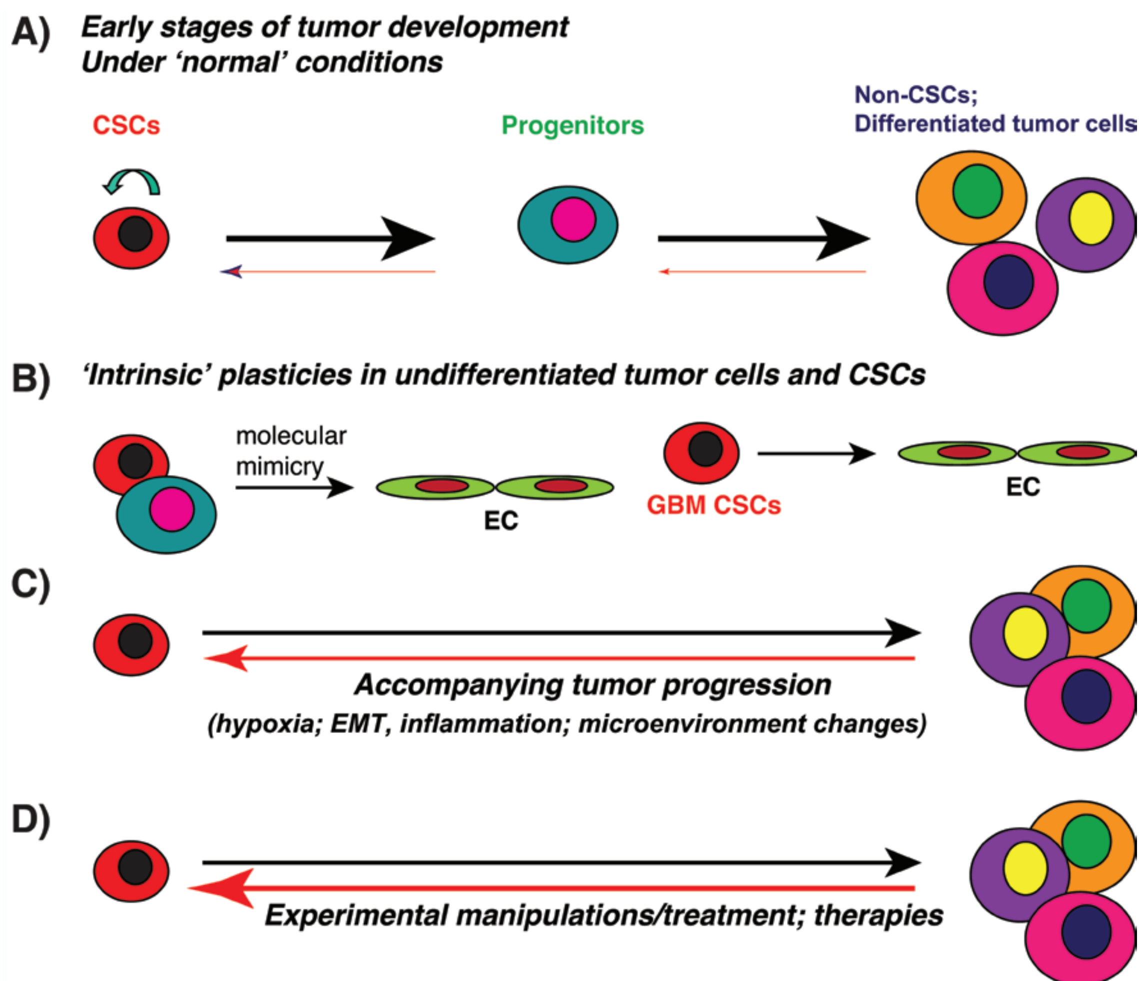

Figure 5 Intrinsic and induced plasticity in CSCs and their progeny. **(A)** In untreated or early-stage tumors, self-renewing CSCs generate rapidly proliferating tumor progenitors, which may in turn develop into differentiated tumor cells or non-CSCs. This hypothetical developmental pathway perhaps represents the major pathway (indicated by thick arrows) although low levels of spontaneous (or intrinsic) dedifferentiation (indicated by thin arrows) may occur. This model predicts that in the untreated or early-stage tumors, most tumor cells will be partially differentiated tumor progenitors and differentiated tumor cells, with undifferentiated cells representing a minority. The great majority of untreated low-grade breast and prostate cancers, for example, fit this model. **(B)** Intrinsic plasticity in CSCs manifested as 'molecular mimicry' or GBM CSC transdifferentiation into endothelial cells (EC). **(C)** During tumor progression, microenvironmental changes, hypoxia, accumulation of inflammatory mediators, together with resultant EMT, may all promote dedifferentiation of non-CSCs (indicated by the thickened reverse arrow). This scenario predicts that in advanced tumors, the undifferentiated, CSC-enriched tumor cells would be in a dynamic equilibrium with more differentiated tumor cells. **(D)** *In vitro* experimental manipulations (e.g., mimicking hypoxic conditions, treating cells with EMT-inducers such as cytokines or anti-cancer drugs, overexpressing oncogenic molecules, etc) or persistent tumor therapy *in vivo* may accentuate dedifferentiation of non-CSCs to stem-like cancer cells (indicated by further thickened reverse arrow) resulting in increased abundance of CSCs.

hypoxia, EMT, inflammatory mediators, and microenvironmental changes can all promote the dedifferentiation of non-CSCs and increase the overall 'stemness' of the tumor (Figure 5C). Recent studies, mostly via *in vitro* experimental manipulations (Figure 5D), provide support to these possibilities. For example, culturing cancer cells under low O_2 tension increases the expression of 'stemness' genes and the percentage of phenotypic CSCs [127-129]. Experimental EMT and inflammatory cytokines IL-6, IL-8, TGFβ, and TNFα can all promote the manifestation of CSC phenotypes and properties [130-135]. In fact, simply overexpressing certain oncogenic molecules (e.g., Nanog, CD44, Twist, hTERT, etc) is sufficient to reprogram primary non-tumorigenic cells or bulk cancer

cells into stem-like cancer cells [136-141]. These latter observations [136-141] are consistent with the observations that such 'stemness' molecules are most frequently expressed in undifferentiated tumor cells. Finally, as CSCs constantly interact with their microenvironment, protumorigenic alterations in the microenvironment (e.g., in stromal cells) will likely affect CSC properties and promote plasticity in CSC progeny [142, 143].

Concluding remarks and perspectives

As CSCs have once been thought as a stable and fixed population of 'unique' cells, the recently reported heterogeneity in CSCs and plasticity in non-CSCs have introduced certain confusions and led some investigators to doubt the presence of CSCs and validity of the CSC concept. It might be easier to understand all these phenomena if we simply perceive CSCs as undifferentiated cells and non-CSCs as more differentiated tumor cells. Thus, undifferentiated cancer cells retain some ability to partially or fully (at least phenotypically) differentiate. This is illustrated by the ability of $Lin^{-}CD34^{+}CD38^{-}$ LSCs to differentiate into $CD38^{+}$ leukemic cells and the ability of single tumor cells to differentiate into various phenotypically mature cells in sphere-development assays (see above). On the other hand, as tumorigenic events would generally disrupt the normal differentiation process, 'differentiated' tumor cells may also possess the ability to dedifferentiate or transdifferentiate, especially accompanying tumor progression and in response to cytotoxic therapies. Most current anti-cancer therapeutics primarily target either differentiated or proliferating cancer cells, and, conceivably, will not be effective against undifferentiated cells that are mostly quiescent. Indeed, multiple lines of evidence have demonstrated that CSCs are more resistant to chemotherapeutics, radiation, and immunotherapy [124, 144, 145] and are endowed with enhanced ability to metastasize [146]. Moreover, many anti-cancer therapies may enrich CSCs [124, 147], perhaps partially by inducing dedifferentiation (Figure 5D). Of great interest, CSCs and non-CSCs may intimately and reciprocally regulate, activate and protect each other. In GBM, the minor subset of glioma cells that carry mutated (i.e., constitutively activated) EGFR behaves like CSCs and preferentially expresses IL-6 and/or LIF (leukemia inhibitory factor), which activate gp130 and wild-type EGFR in the majority of (non-stem) glioma cells and promote their CSC properties [68]. Vice versa, non-CSCs in colon cancers can protect CSCs from the toxicity of chemotherapeutic drugs [148]. These discussions illustrate the potential benefit of combinatorial targeting of both tumor-initiating and differentiated tumor cells [149]. By simultaneously targeting the tumorigenic and non-tumorigenic populations, both cancer cell heterogeneity and plasticity can be overcome (Figure 5C-5D). Encouragingly, recent data suggest that some commonly used clinical drugs such as docetaxel and metformin may actually be able to root out CSCs [124, 150]. By discovering more and novel therapeutics that specifically target undifferentiated and dormant tumor cells, coupled with using CSC gene signatures in guiding clinical treatments [151, 152], we can hope to achieve much improved cure rate in cancer patients.

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