Cancer Stem Cells: Basic Concepts and Therapeutic Implications

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Abstract

Different mechanisms contribute to intratumor heterogeneity, including genetic mutations, the microenvironment, and the existence of subpopulations of cancer cells with increased renewal capacity and the ability to recapitulate the heterogeneity found in primary tumors, which are referred to as cancer stem cells (CSCs). In this review, we discuss how the concept of CSCs has been defined, what assays are currently used to define the functional properties of CSCs, what intrinsic and extrinsic mechanisms regulate CSC functions, how plastic CSCs are, and the importance of epithelial-to-mesenchymal transition in conferring CSC properties. Finally, we discuss the mechanisms by which CSCs may resist medical therapy and contribute to tumor relapse.

INTRODUCTION

Cancer is caused by an accumulation of genetic, epigenetic, and transcriptional alterations conferring key properties to cancer cells—including sustained proliferation, invasion, metastasis, replicative immortality, and angiogenesis, as well as the ability to evade growth suppression and apoptosis, collectively described as the hallmarks of cancer cells (1). Despite arising initially from a single mutated cell, almost all tumors become very heterogeneous, expressing different markers and containing proliferative and more differentiated cells. Tumor heterogeneity may be responsible for tumor progression, metastasis, resistance to therapy, and relapse (2, 3).

Tumor heterogeneity was first noticed and described by pathologists more than a century ago (4). This histological heterogeneity is accompanied by heterogeneous expression of different markers among cancer cells, termed intratumoral heterogeneity. In addition, there is an important heterogeneity among the tumors arising in different patients with a given cancer, which is known as intertumoral heterogeneity (2, 3).

Different mechanisms have been proposed to account for intratumoral and intertumoral heterogeneities; these mechanisms include the genomic landscape of individual tumors and their clonal evolution, the existence of different populations of cancer cells with cancer stem cells (CSCs) residing at the top of the hierarchy, and the influence of the tumor microenvironment (2, 3, 5–7) (**Figure 1**).

In this review, we provide a historical perspective on the concept of CSCs; the experimental evidence for the existence of tumor heterogeneity with distinct populations of tumor cells exhibiting different functional properties; and the ways in which tumor heterogeneity influences tumor progression, metastasis, and response to medical therapy.

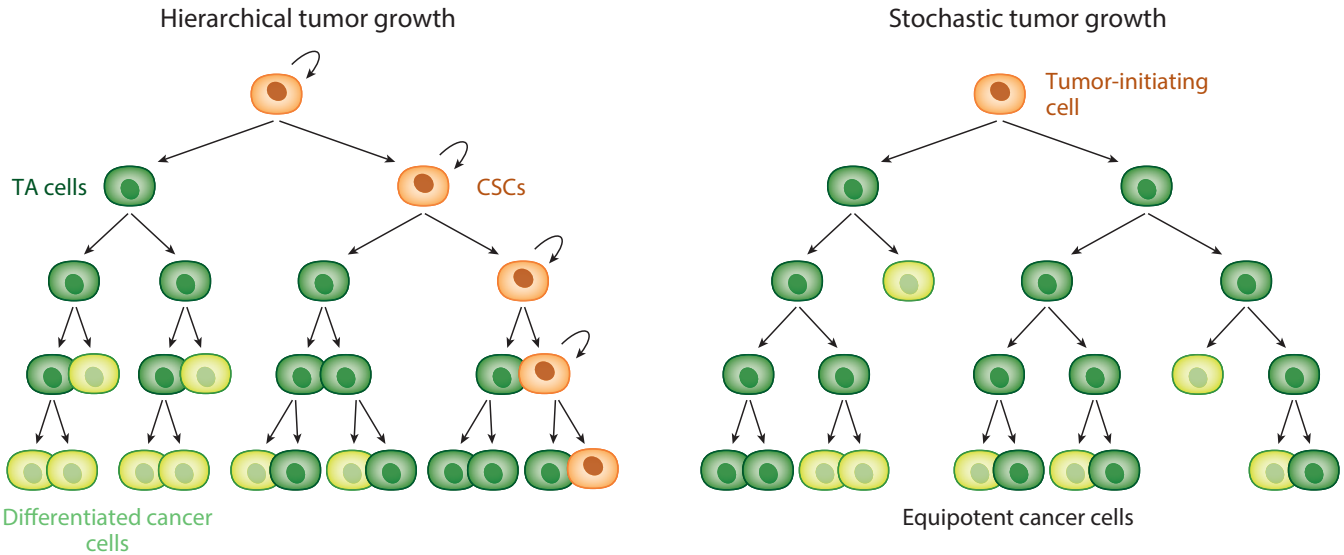


Figure 1

Models of tumor growth. According to the hierarchical model of tumor growth (*left*), only a subset of cancer cells known as CSCs present extensive self-renewal properties, whereas TA cells have limited proliferative capacity and eventually differentiate into nonproliferative differentiated cancer cells. The stochastic model of tumor growth (*right*) posits that all cancer cells are equipotent and can either self-renew or give rise to nonproliferative cells in a stochastic manner. Abbreviations: CSC, cancer stem cell; TA, transit-amplifying.

GENETIC HETEROGENEITY AND CLONAL EVOLUTION

The classical model of tumor evolution follows a Darwinian process, whereby additional somatic mutations confer selective advantages to more fit clones (8). Next-generation sequencing technology allows for assessing genetic tumor heterogeneity in unprecedented detail (6, 9–11).

The mutational landscapes of large cohorts of different types of human cancers have been published in the last five years (12, 13). Multiregion sequencing of primary tumors has revealed extensive intratumor heterogeneity in certain cancers, suggesting a high level of genetic heterogeneity and a branched clonal evolution (14–16). Interestingly, a shift in the mutational processes between the early (common trunk mutations) and late (branched mutations) events, contributing further to the genomic heterogeneity, has been observed; moreover, temporal sampling of primary tumors and postsurgical relapses showed that particular subclones are associated with tumor relapse (17).

By sequencing a breast cancer tumor at very high density and applying principles of evolutionary population genetics, researchers identified a most recent common ancestor in breast cancer evolution (18). Interestingly, this common ancestor appeared very early during tumor formation and passively accumulated mutations without expansion. Additional mutations driving branched expansion with the appearance of a dominant clone occurred later during tumor progression. This introduced a model of long-lived early lineage for cancer genome evolution, which parallels the concept of CSCs (18).

In all these studies, there was a predominant clone in every sequenced tumor region, further suggesting a selective advantage conferred by independent genetic events allowing a clone to outcompete other minor clones in a Darwinian process (19); however, the relationship and dynamics among different clones within a tumor remain poorly described (20–22). Functional studies in mice grafted with subclones from a breast cancer cell line expressing factors implicated in tumor growth (such as Ccl5, Il-11, and Loxl3) showed that a minor cell population could drive tumor growth in a non-cell-autonomous manner (23). The minor clone does not necessarily grow proportionally with the tumor growth but induces a tumor-promoting environment for other clones. In this model, subclonal cooperation together with clonal interference (i.e., competition between highly fitted clones) stabilizes tumor heterogeneity (23). In the *MMTV-Wnt1* murine breast cancer model, some tumors are hierarchically organized but others are biclonal with lineage-restricted basal *hras* mutant and luminal *hras* wild-type subclones (24). In these biclonal tumors, there is an evidence of subclonal cooperation to promote tumor maintenance and propagation. Interestingly, regardless of whether the tumors fit a hierarchical or a biclonal pattern, they show classical mixed-lineage histology, suggesting that different mechanisms could account for intratumoral heterogeneity within the same types of tumors.

THE CANCER STEM CELL CONCEPT

In many adult tissues, stem cells (SCs) are responsible for tissue homeostasis and regeneration (25). Upon division, SCs can give rise to transit-amplifying (TA) cell populations, which after several rounds of divisions will terminally differentiate and eventually be lost from the tissue. In addition, adult SCs are activated following injuries, rapidly expand, and contribute actively to tissue repair (25). There is no unique marker of tissue-specific SCs, and SCs are usually defined by their functional properties, namely by their capacity for long-term self-renewal (as opposed to progenitors, which can usually self-renew for shorter periods) and their capacity to differentiate into one or multiple cell lineages. Unipotent SCs differentiate into only a single lineage (e.g., spermatogonial SC), whereas multipotent SCs differentiate into multiple cell lineages (e.g., intestinal

SCs, which give rise to enterocytes and goblet, Paneth, and enteroendocrine cells). Given that all cells in normal tissues are genetically identical, the hierarchical organization of tissues is regulated by intrinsic mechanisms, such as the expression of specific transcription factors, or by extrinsic factors, such as the microenvironment, or by a combination of both.

Inspired by the concept of adult hematopoietic stem cells (HSCs), Dick and colleagues (26) have shown that in human acute leukemia, not all leukemic cells are able to propagate the leukemia when transplanted into immunodeficient mice. Leukemic cells with blast characteristics were not able to propagate the disease, whereas leukemic cells expressing the same markers as normal adult HSCs ($CD34^+CD38^-$) were much more efficient at propagating the leukemia and were termed leukemia-initiating cells, leukemic SCs (LSCs), or CSCs (27). The researchers estimated the frequency of LSCs to be approximately 1 in 250,000. Similarly to normal tissue SCs, CSCs are defined by their functional properties and should be able to self-renew and propagate the tumor over an extended period and recapitulate the different cell lineages found in the primary tumors. By combining transplantation assays and transcriptional profiling, Dick and colleagues found that LSCs express genes similar to those expressed by HSCs, which correlate with disease outcome, suggesting that LSCs are regulated by molecular mechanisms similar to those of their normal counterparts (28).

CANCER STEM CELLS IN SOLID TUMORS

Inspired by the pioneering work of Lapidot, Bonnet, and Dick, many other groups have now demonstrated that in solid tumors only a fraction of cancer cells present the capacity to reform secondary tumors following their transplantation into immunodeficient mice. The first demonstration that a human solid tumor contains cells with increased tumor-propagating potential was in breast cancer, in which $CD44^+CD24^{-/low}$ cells have higher ability to generate tumors upon transplantation into immunodeficient mice (29). In ductal and inflammatory breast cancers, $ALDH1^+CD44^+CD24^-$ expression enriches for tumor-propagating cells (TPCs) and mediates metastasis, with ALDH1 expression correlating with poor prognosis in patients with breast cancers (30, 31). In certain types of brain tumors, glioblastoma multiforme (GBM) and neuroblastoma, CD133 expression enriches for TPCs (32). Colorectal cancer also contains CSCs expressing CD133 (33, 34). Many other solid tumors, including pancreatic cancer (35, 36), squamous cell carcinomas (SCCs) (37), colon cancer (38), and melanoma (39–41), have been shown to contain subpopulations of tumor cells with greater ability to propagate the tumors in xenotransplantation assays. In solid tumors, the frequency of TPCs seems to be low and varies from 1 per 100,000 to 1 per 1,000 cells (42).

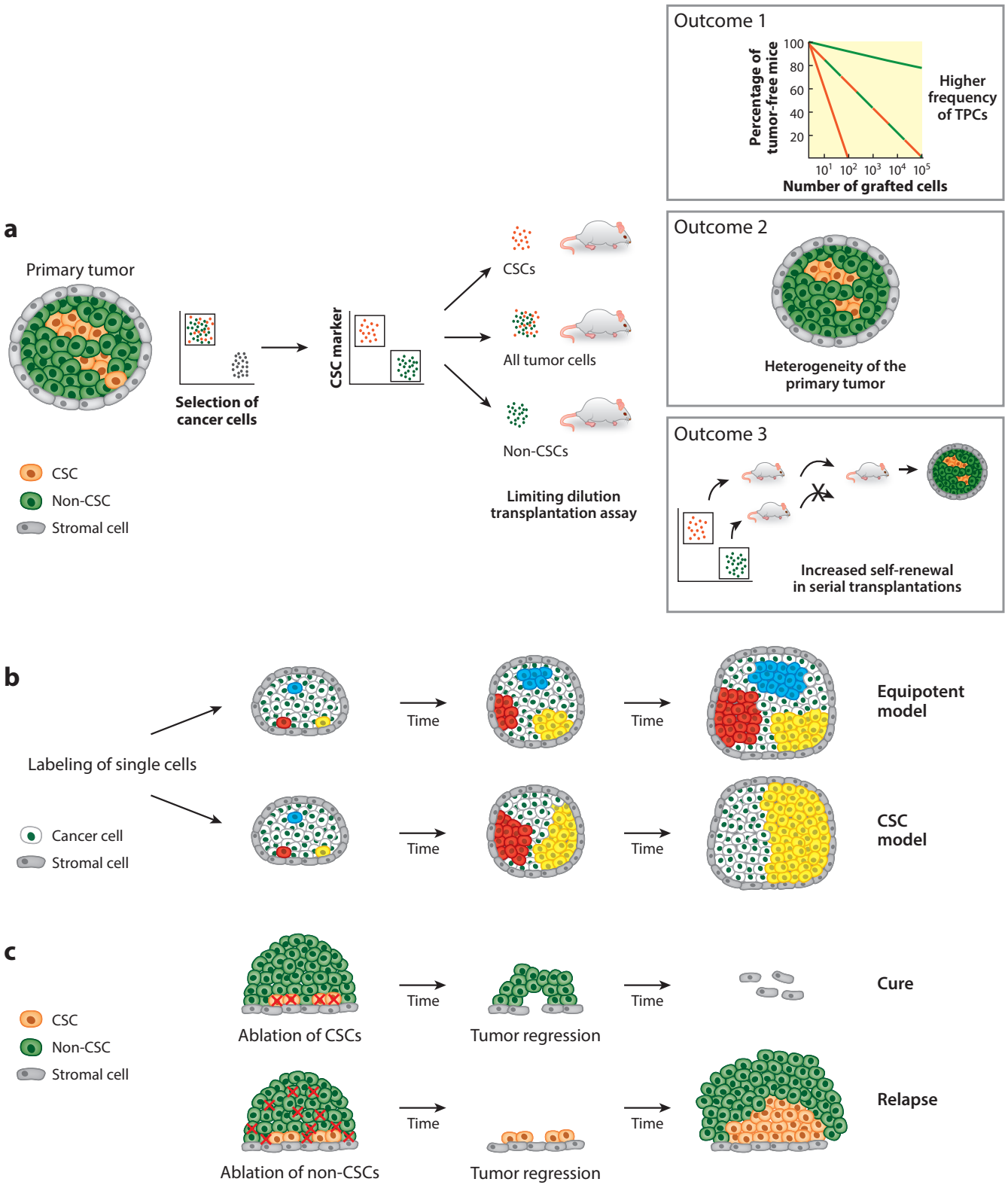
All these studies used a common experimental approach to study TPCs, which included dissociation of tumors into single cells, labeling of cells with different markers, fluorescence-activated cell sorting (FACS) isolation of specific cancer subpopulations expressing a combination of particular markers, and their xenotransplantation into immunodeficient mice (**Figure 2*a***). This assay presents inherent technical and biological limitations, including mechanical dissociation and enzymatic digestion to isolate the cells from the tumors, labeling with antibodies and FACS isolation requiring the existence of one marker or a combination of specific markers, the degree of immunodeficiency of the recipient mice, the injection of the tumor cells into an unrelated tissue (heterotopic site) rather than their injection into the organ from which they were derived (orthotopic transplantation), usage of tumor-derived extracellular matrix (Matrigel) to embed the transplanted cells, in vitro expansion of tumor cells before tumor cell fractionation and transplantation, and the different species between stromal and tumor cells, potentially leading to the inability of the secreted molecules to bind to human or mouse receptors. All these parameters could be major sources of

technical biases affecting the capacity of certain cells to form tumors, and thus CSC frequencies may be underestimated or overestimated in these experimental conditions. In melanoma and lung squamous cell carcinoma, for example, xenotransplantation of cancer cells into more severely immunocompromised NOD/SCID/IL2RG$^{-/-}$ mice increases the frequency of tumor-forming cells compared with NOD/SCID mice by several orders of magnitude, and when late-stage melanoma cells combined with Matrigel are transplanted into NOD/SCID/IL2RG$^{-/-}$ mice, up to one in four melanoma cells forms secondary tumors (43), suggesting that at a late stage of tumor progression, all melanoma cells become equipotent in generating secondary tumors. In certain cases, the success of tumor engraftment requires additional factors. In benign skin tumors, addition of stromal cells—either cancer-associated fibroblasts (CAFs) or endothelial cells (ECs)—is required for tumor propagation (44). Depending on the tumor genotypes, some malignant peripheral nerve sheath tumors require the addition of laminin for successful engraftment (45). These findings indicate that transplantation assays need to be adapted and optimized for each tumor subtype, possibly by cotransplantation of different components of the tumor stroma, such as CAFs or ECs, or by the addition of exogenous components of the CSC niche.

Importantly, transplantation assays provide information about what a tumor cell can do in a particular experimental setting but do not tell us what the tumor cells actually do within their native microenvironment. It is still unclear whether the cancer cell population that presents the greatest tumor propagation efficiency in transplantation assays is the same population of cancer cells that contributes to tumor growth, progression, metastasis, and resistance to therapy in vivo. Also, transplantation assays usually do not study the dynamics of different populations of cancer cells and do not consider clonal cooperation or competition that is observed in primary tumors. Further, the tumor-propagating capacity is usually not restricted to a single tumor population, and these tumor populations can sometimes be plastic (39, 40, 43, 46). In addition, the frequency of TPCs varies from one patient to another, and the validity of one marker in a given group of patients may not be applicable to another group. For example, CD133 enriches only for TPCs in a subgroup of patients with GBM, whereas other tumors are maintained by Nestin^{+}CD133^{-} or Tbr2^{+}CD133^{-} TPCs (47). Collectively, these differences observed across different studies may also be a consequence of some tumors being hierarchically organized and others not, or of the cellular hierarchy changing with tumor progression or metastasis and affecting the ability of TPCs to propagate into immunodeficient mice.

Whether transplantation assays select particular clones of cancer cells to grow and whether the dynamics of the clonal evolution and genetic heterogeneity after xenotransplantation reflect clonal dynamics in the primary tumors remain unclear. In breast cancer, deep-genome sequencing and single-cell sequencing from serial xenotransplants and corresponding primary tumors have shown clonal selection in all examined xenotransplants. In several cases, xenotransplants contained dominant clones that had very low frequency in the primary tumor (21). Interestingly, when tumor populations were transplanted into different mice, reproducible expansions of initially minor clones were observed, suggesting a deterministic genetic mechanism of clonal fitness in this assay. In T cell acute lymphoblastic leukemia, xenotransplants arose from minor subclones existing in patients at the time of diagnosis and bore a stronger genetic resemblance to relapse samples than to the bulk of the tumor at diagnosis, suggesting that xenotransplantation assays select aggressive and resistant clones that could be responsible for disease relapse (48, 49). Further assessment of the biological significance of clonal selection in xenotransplants is needed.

Likewise, CSCs have been identified in murine models of solid tumors through transplantation assays. Their identification is particularly interesting for several reasons. Mouse cancer cells do not induce a xenogeneic immune reaction in transplantation assays. Tissue-specific SCs and their markers are more accurately characterized in mice (25). Mouse cancer models may be more



reproducible, in particular when the same oncogene drivers are used, compared with the great diversity of genetic heterogeneity found in human cancers. Finally, the power of mouse genetics allows researchers to mark or ablate certain tumor subpopulations, or to assess the role of particular genes in the regulation of CSC function. In mice with chemically and genetically induced skin SCC, tumor cells expressing CD34, a marker of hair follicle SCs (50), present increased self-renewal potential compared with $CD34^-$ cells (44, 51, 52). $CD34^+$ CSCs are heterogeneous, and a subpopulation of CD34-expressing Sox2 represents the most potent TPCs in mouse skin SCCs (53). The tumor-propagating frequency of these skin SCC CSCs correlates with the invasiveness of the tumors and is augmented with serial transplantation, whereas the degree of immunodeficiency does not affect the engraftment and growth of secondary tumors (44). In different mouse models of lung cancer, each tumor genotype has TPCs with a unique cell surface phenotype. $Sca1^+$ cells are enriched for TPCs in $Kras^{G12D}/p53^{-/-}$ tumors, whereas TPCs are $Sca1^-$ in *Egfr* mutant tumors (54). In a mouse model of medulloblastoma mediated by the activation of the Shh pathway, TPCs are $CD133^-CD15^+$ (55). Similarly, CD61 enriches for TPCs in *MMTV-Wnt1* and *Balb/c-*$p53^{+/-}$ but not in *MMTV-neu* mouse models of breast cancer (56).

LINEAGE TRACING AND CLONAL DYNAMICS OF CANCER STEM CELLS

Although transplantation assays assess the potential of the tumor cells, they do not necessarily assess the fate of these cells within their natural microenvironment. In normal tissues, the fate of the cells is best assessed by a technical approach known as lineage tracing (57), in which one particular cell type and all its progeny are permanently marked with a reporter gene (such as a fluorescent protein). If a differentiated cell is initially marked, then the labeled cells will be lost with the tissue turnover, whereas if a TA cell is initially marked, the labeled cell population will initially expand but eventually disappear. In contrast, if an SC is initially marked, the labeled cells will remain long term, and the part of the tissue that is maintained by the SCs will be permanently labeled (**Figure 2*b***). Lineage-tracing experiments have demonstrated that there could be a great difference between the potential and the fate of a given cell (25). Hair follicle stem cells (HFSCs) can give rise to all epidermal lineages upon transplantation (50, 58), but lineage tracing shows that during homeostasis, HFSCs give rise only to hair follicle lineages (59); however, upon wounding, HFSCs rapidly migrate to the damaged epidermis and give rise to all epidermal lineages (59), suggesting

Figure 2

Functional assays to characterize CSCs. (*a*) Transplantation assays rely on the dissociation of tumors into single cells and on FACS isolation of different tumor subpopulations followed by their transplantation into immunodeficient mice. These assays have several predicted outcomes in cases of enrichment for CSCs. Transplantation of limiting dilutions allows the estimation of the proportion of TPCs in each tumor subpopulation (Outcome 1). CSCs should be more enriched in TPCs compared with non-CSCs or all tumor cells. Secondary tumors should recapitulate the tumor heterogeneity of the primary tumor (Outcome 2). CSCs should present higher self-renewal properties compared with non-CSCs in serial transplantations (Outcome 3). (*b*) Clonal analysis using lineage-tracing experiments relies on the labeling of single cancer cells using the Cre recombinase system with a reporter gene, DNA barcoding, or lentiviral transduction. The fate of the labeled cells is then followed during cancer progression. If the tumor grows in a hierarchical manner, labeled CSCs will show long-term renewal and important clonal expansion, whereas TA cells will show limited proliferative potential and finally differentiate into nonproliferative cells. If the tumor growth is mediated by equipotent cancer cells, all labeled cells will participate equally in tumor growth. (*c*) Lineage ablation allows for specific elimination of a subpopulation of cancer cells using targeted expression of suicide genes. In tumors that are maintained by CSCs, if CSCs are eliminated, the remaining non-CSCs will not be capable of sustaining tumor growth, inducing tumor regression. If non-CSCs are ablated, CSCs will sustain tumor growth, and no long-term regression will be observed. Abbreviations: CSC, cancer stem cell; FACS, fluorescence-activated cell sorting; TA, transit-amplifying; TPC, tumor-propagating cell.

that the transplantation assays mimic a wounding situation. Similarly, although mammary basal cells are multipotent in transplantation assays (60), the same cells are unipotent by lineage tracing (61, 62), again demonstrating the difference between the fate of epithelial SCs within their natural environment and their potential in transplantation assays.

Lineage-tracing studies coupled with clonal analysis in murine models of skin and intestinal tumors provide strong evidence for a hierarchical organization of growth in solid tumors in vivo. Our group has used chemically induced skin tumors combined with inducible clonal tracing using *K14CREER/Rosa-YFP* mice (63). The Cre recombinase was induced with a very low dose of tamoxifen, allowing the labeling of single tumor cells. Only a small fraction of the induced tumor cells survived long term; the others were lost through terminal differentiation. The remaining clones filled up a vast part of the tumors, producing hundreds if not thousands of cells within several weeks, consistent with the initial labeling of tumor SCs (**Figure 2*b***). Mathematical modeling of these clonal fate data supports a hierarchical organization of the tumor growth with a CSC population at the top of the hierarchy that divides rapidly and asymmetrically, where one CSC gives rise on average to another CSC and a committed cancer progenitor, which will eventually be lost through terminal differentiation. Interestingly, it seems that at the clonal level, CSCs balance symmetric and asymmetric division in a stochastic manner (63) reminiscent of the mechanisms regulating homeostasis in the normal epidermis (64, 65).

Concomitantly, Clevers and colleagues (66) used *Lgr5CREER* mice to activate Wnt signaling, together with the *Rosa-Confetti* reporter in mouse intestinal crypt SCs, which led to adenoma formation labeled with only one of the four colors of the confetti reporter system. The adenoma, like the normal intestine, contains a population of tumor cells expressing the SC marker Lgr5. By readministering tamoxifen to mice bearing adenomas, the researchers induced an inversion of the *Rosa-Confetti* construct, leading to the expression of another color of the confetti reporter within a clone of fluorescently labeled cells, a technique known as retracing. The retraced Lgr5 tumor cells rapidly expanded and filled up a significant part of the tumor only a few weeks following the initial labeling, consistent with the notion that Lgr5 marks a subpopulation of CSCs within intestinal adenoma cells representing up to 20% of tumor cells and able to generate all the cell types within the adenoma. Using a novel lineage-tracing technique leading to continuous clonal labeling, Winton and colleagues (67) confirmed the appearance of large dominant clones within adenomas sustained by CSCs, but they suggest that only a small fraction of Lgr5-expressing cells are acting as tumor SCs in this model. Using lineage tracing in an *MMTV-PyMT* mouse model of breast tumors, Van Rheenen and colleagues (68) observed the clonal dynamics of tumor cells over time using intravital imaging. Interestingly, similarly to what has been observed in skin and intestinal tumors, some clones initially expanded and then contracted or disappeared, whereas other clones rapidly expanded and became dominant within 2 weeks of observation, consistent with important tumor heterogeneity and the existence of CSCs in this model. Taken together, these studies of distinct mouse models of tumors demonstrate that, in vivo within their natural environment, tumors are heterogeneous and contain cells that act as CSCs and contribute substantially to tumor growth, irrespective of the oncogene used and the tissue from which these tumors arise (63, 65–68). Further studies will be needed to define the commonalities and differences between CSC dynamics within different tumor types and the clonal dynamic that accompanies tumor progression and metastasis.

Genetic lineage tracing using inducible recombination has not yet been performed in human cancers; however, analysis of spontaneous mutations in mitochondrial DNA as well as of the methylation pattern of nonexpressed genes has allowed reconstruction of clonal lineages in normal colonic crypts and adenomas from human patients carrying familial adenomatous polyposis *apc* mutations (69, 70). These studies show the existence of important tumor heterogeneity

with rapidly growing clones, supporting the existence of quickly dividing CSCs that fuel tumor growth (69). Another method to perform genetic clonal tracing in human samples is the use of viral integration sites to follow the dynamics of TPCs following their xenotransplantation into immunodeficient mice. Using this technique, Kreso & Dick (71) showed that functional variability in tumor propagation potential exists within a uniform genetic lineage and that intraclonal heterogeneity contributes to tumor growth and response to therapy in colorectal cancers. Recently, retroviral transduction of human cells was used to introduce vectors harboring random sequence tags or barcodes. With this technique, a pool of normal SCs or CSCs is exposed to a library of viral vectors, allowing the integration into the genome of each transfected cell of a uniquely identifiable heritable sequence. By coupling barcoding with sequencing, researchers can perform clonal analysis of xenotransplants to assess the clonal dynamics in normal SCs and CSCs (72–74). Xenotransplants of barcoded breast cancer cells show that distinct clones are predominant at different stages, suggesting the existence of distinct clonal dynamics at different stages of tumor progression (75).

LINEAGE ABLATION OF CANCER STEM CELLS

Another powerful method to study the importance of a particular lineage during development, homeostasis, and cancer is genetic or laser-induced cell lineage ablation (25).

In theory, lineage ablation of differentiated tumor cells should have no impact on tumor growth and maintenance, whereas lineage ablation of CSCs should lead to tumor regression (**Figure 2*c***). In the normal brain, SCs are located within the subventricular zone and express the SC marker Nestin (76). Parada and colleagues (76) introduced, in a genetically engineered mouse model of GBM, a transgene expressing *GFP* and the *Herpes Simplex Virus tyrosine kinase* (*Hsv-tk*) suicide gene under the *Nestin* promoter. They found that $GFP^+Nestin^+$ marks a restricted population of slow-dividing tumor SCs. Lineage ablation of $Nestin^+$ slow-cycling CSCs by ganciclovir administration delays tumor progression. Administration of the drug temozolomide (TMZ) preferentially targets rapidly cycling tumor cells, whereas $Nestin^+$ CSCs reenter the cell cycle after TMZ treatment and contribute to tumor relapse. Lineage ablation of the $Nestin^+$ tumor cells by ganciclovir administration and sequential administration of TMZ improve the survival rates of mice with GBM, demonstrating the importance of $Nestin^+$ CSCs in mediating tumor relapse following therapy (76).

Although in the case of brain tumors, CSCs express markers of their SC of origin, in other tumors, CSCs express new markers that were not initially expressed in the cell of origin. For example, in the normal intestine, Dclk1 marks differentiated cells, and intestinal cells labeled by Dclk1CREER are lost within 2 weeks. In contrast, in polyps and adenomas, Dclk1 marks a population of CSCs that rapidly expand and drive tumor growth (77). Lineage ablation of $Dclk1^+$ cells using diphtheria toxin induces tumor regression without damaging the normal intestine, showing that Dclk1is a marker specifically expressed by CSCs and providing a means to specifically target CSCs without impairing normal SC function (77). Likewise, Sox2 is expressed in different types of normal SCs (78). Sox2 is not present in the normal skin epidermis but appears during the early stage of skin tumor initiation and is expressed in a heterogeneous manner in both mouse and human SCCs (53). Sox2 marks a subpopulation of $CD34^+$ tumor cells that present the highest rate of tumor engraftment following their transplantation into immunodeficient mice. Lineage ablation of Sox2-expressing cells in mouse skin tumors rapidly induces tumor regression in both benign and malignant squamous skin tumors, demonstrating the essential role of Sox2 CSCs in tumor maintenance within their natural microenvironment and that targeting a small subpopulation of tumor cells is sufficient to eradicate established tumors (53).

PLASTICITY AND REVERSIBILITY OF STEM CELLS AND CANCER STEM CELLS

In normal tissues, progenitors may present a certain degree of plasticity and reversibility to an SC-like state. Several studies have shown that differentiated cells or progenitor cells that are normally committed to terminal differentiation can reacquire SC features following injury (25). The intestinal crypt offers beautiful examples of cellular plasticity in response to lineage ablation and tissue injuries. Ablation of Lgr5 SCs using mice expressing diphtheria toxin receptors in Lgr5 cells did not result in intestinal atrophy due to dedifferentiation of progenitors normally committed to terminal differentiation into an SC-like state, making them capable of sustaining intestinal homeostasis (79). Likewise, radiation induced massive apoptosis in the crypt, including in Lgr5 SCs, but also promoted dedifferentiation of committed progenitors ($Dll1^+$ cells), which repopulated the empty SC niche and acquired SC properties, including long-term self-renewal capacity and multilineage differentiation potential (80, 81), thus demonstrating the important plasticity of these progenitors. However, the intestinal progenitor plasticity is not sufficient to ensure tissue regeneration in response to damage in the absence of Lgr5 SCs, demonstrating the essential role of Lgr5 SCs during tissue repair (82).

Genetic lineage tracing combined with the expression of oncogenes or deletion of tumor suppressor genes in different cell lineages of the same tissue has demonstrated that in general, only long-lived SCs, and not TA cells, are capable of forming tumors when targeted with oncogenic hits (83). In the skin epidermis, targeting short-lived but rapidly proliferating matrix cells of the hair follicle with $Kras^{G12D}$ and *p53* deletion does not lead to tumor formation, whereas expression of the same oncogenic hits in hair follicle SCs rapidly leads to tumor formation (84, 85). Likewise, Wnt activation alone through APC deletion in TA crypt cells leads to the formation of a few microadenomas, whereas the same oncogene hit in Lgr5 SCs rapidly leads to adenoma formation (86). However, combining Wnt activation with $Kras^{G12D}$ or activated *Nfkb* in TA cells can reinduce SC potential in these otherwise committed cells and lead to tumor formation (87). Although these data demonstrate that it is possible with the combination of multiple oncogenic hits to induce dedifferentiation of TA cells, it is unlikely that in vivo TA cells, usually exhibiting a half-life of less than a week, will be able to sequentially acquire two key oncogenic events required to initiate tumor formation; therefore, in normal physiology, most likely the first oncogenic mutation still needs to occur in the long-lived SC.

A pillar in the CSC field is the isolation of distinct populations of cancer cells based on the expression of a combination of cell markers, and the assessment of their ability to reform a tumor exhibiting the same heterogeneity as the primary tumor (88). In theory, if CSCs follow a linear and rigid hierarchical organization in this assay, CSCs should give rise to tumor populations with more limited or without growth potential, or with more limited differentiation potential, that do not recapitulate the tumor heterogeneity of the primary tumors, whereas non-CSCs should not be able to revert back to a CSC-like state. Very often, this ideal situation is not experimentally observed, and a certain degree of tumor cell plasticity occurs. Human breast cancer cell lines, which are a priori clonal, have been shown to transition stochastically and reversibly, although with different probabilities, between distinct cell states (mesenchymal, basal, and luminal) to reach an equilibrium containing equal proportions of the different cell populations that were presented in the initial cancer cell line (89). The acquisition of mesenchymal fate has been shown to confer enhanced tumor-propagating potential in this model (89). If tumor cells are completely plastic and every cell type, irrespective of its initial marker expression, gives rise to secondary tumors that recapitulate the heterogeneity of the primary tumor, with the expression of the same set of markers, then no strong conclusion can be drawn about the tumors' putative hierarchical organization. In

melanoma, many cellular markers can be reversibly expressed by tumorigenic cells (90), including $CD271^-$ cells that give rise to $CD271^+$ cells, although the latter have been shown to be enriched for tumorigenic cells, in particular in mice with lower degrees of immunodeficiency (40, 41). In mouse skin SCCs, although cells expressing high levels of CD34 present enhanced self-renewal potential (51, 52), $CD34^-$ cells can upon transplantation reform secondary tumors presenting both $CD34^+$ and $CD34^-$ cell types (44, 91). It has been further shown that $Sox2^+CD34^+$ cells present the highest tumor-propagating potential in primary skin SCCs, followed by $Sox2^+CD34^-$ cells, whereas $Sox2^-CD34^+$ and $Sox2^-CD34^-$ are the least clonogenic cell types (53), possibly explaining the presence of CSCs in both $CD34^+$ and $CD34^-$ populations (44, 91). Interestingly, although the $Sox2^-$ cells were extremely inefficient at reforming secondary tumors, the rare tumors formed following the transplantation of several hundred thousand $Sox2^-$ tumor cells expressed proportions of $Sox2^+$ cells similar to those of the parental tumors, suggesting that $Sox2^-$ cells can be reprogrammed to generate $Sox2^+$ cells only rarely, as this transition occurs at a very low frequency (53). In melanoma, *Jarid1b* marks a subpopulation of slow-cycling cells that is required for continuous tumor growth, and knockdown of *Jarid1b* leads to tumor exhaustion. Nevertheless, single *Jarid1b*-negative melanoma cells can be tumorigenic and reexpress *Jarid1b*, implying that non-CSCs can reenter a CSC state in transplantation assays (92). Consistent with the reversibility and plasticity of non-CSC populations, human basal breast cancer cells can readily switch from a non-CSC to a CSC state. Non-CSCs maintain the *Zeb1* promoter in a bivalent chromatin configuration that contains both active and repressive epigenetic marks, enabling the non-CSCs to respond to signals from the microenvironment—such as Tgf-β—which leads to the conversion of bivalent to active chromatin marks and promotes the conversion of non-CSCs into tumor cells with SC properties (93).

The plasticity between CSCs and non-CSCs, whether spontaneously occurring or selected under certain conditions such as therapy, makes the concept of CSCs dynamic rather than rigid and unidirectional. This poses biological and clinical challenges, as it renders the targeting of the CSC population insufficient to cure the disease, and necessitates the identification and understanding of the mechanisms involved in the reversion that may occur after the elimination of the CSC population.

REGULATION OF CANCER STEM CELLS BY EPITHELIAL-TO-MESENCHYMAL TRANSITION

One of the most important processes associated with tumor heterogeneity and cancer stemness is the epithelial-to-mesenchymal transition (EMT).

EMT refers to the cellular process occurring when an epithelial cell loses adhesion with its neighbors and adopts a mesenchymal morphology, allowing the cell to migrate over long distances. EMT naturally occurs during mesoderm formation at the gastrulation or the neural crest delamination stage (94). Upon arrival at their destination, progenitor cells that underwent EMT during embryonic development reacquire epithelial features in a process known as mesenchymal-to-epithelial transition (MET). EMT is controlled by environmental factors, signaling pathways, transcription factors (TFs) (such as Snail1, Twist1, and Zeb1), micro-RNAs (miRNAs), and other mechanisms that promote the loss of epithelial and adhesive characteristics and the acquisition of invasive and migratory properties (95, 96). The expression of EMT TFs in primary tumors has been linked to tumor invasion, metastasis, and poor prognosis (97).

Besides these proinvasive and metastatic functions, EMT promotes stemness of cancer cells. Consistent with this notion, Twist1 overexpression in human mammary cancer cells gradually confers SC features in vitro and increases tumor-propagating potential in vivo (98, 99). Studies

have confirmed that overexpression of EMT TFs in many other epithelial cancer cells induces SC properties in vitro and in vivo by transplantation assays (100). EMT TF expression may be dynamically regulated during tumor progression. Twist1 primes a subset of mammary epithelial cells for SC-like properties, which emerge only when Twist1 has been downregulated, whereas sustained Twist1 expression locks tumor cells into their EMT state (101).

Transition from the epithelial to the mesenchymal state is not always irreversible. Several studies have recently demonstrated that overexpression of TFs promoting EMT should be downregulated to promote metastatic colonization. For example, transient but not sustained overexpression of Twist1 promotes lung metastasis in SCCs (102). Prrx1 is a potent EMT inducer promoting cell migration and invasion; however, Prrx1 downregulation increases metastasis and tumor stemness in cancer cell lines (102). Likewise, overexpression of Snail1 in PC3 prostate cancer cell lines inhibits metastasis and tumor propagation, and knockdown of EMT TFs in mesenchymal cancer cell lines promotes the expression of self-renewal genes (103). Together, these studies suggest that EMT, metastasis, and tumor stemness may be regulated by distinct mechanisms and indicate that EMT TFs should be downregulated during metastatic colonization.

Recent studies have revealed that EMT TFs play an essential role during the early steps of tumorigenesis. Twist and Zeb overexpression following *p53* and *Rb* loss of function prevents apoptosis and oncogene-induced senescence, suggesting that Twist1 regulates tumor initiation as well as tumor maintenance (100, 104). In primary tumors in vivo, overexpression of Twist1 promotes the conversion of benign skin papillomas into malignant SCC (102, 105). Interestingly, Twist1 is also expressed at the early time points of tumorigenesis, in preneoplastic and benign skin tumors, and its expression is increased during malignant progression (106). Deletion of both alleles of *Twist1* completely suppresses skin tumor initiation, including the formation of benign tumors, whereas deletion of one allele does not prevent benign tumor formation but prevents their progression into invasive carcinoma, demonstrating that low levels of Twist1 are required for the early steps of tumor initiation and that higher levels of Twist1 are required for malignant progression. Interestingly, in benign tumors, Twist1 is essential for tumor maintenance and propagation but does not regulate EMT, demonstrating that tumor stemness and EMT can be dissociated (106). Conditional deletion of Twist1 at different time points during tumorigenesis has revealed that different molecular mechanisms are employed between Twist1-mediated prevention of apoptosis and Twist1-mediated tumor stemness and EMT. Twist1 inhibition of apoptosis is regulated by a *p53*-dependent mechanism, whereas Twist1 promotion of renewal and tumor stemness is regulated by a *p53*-independent mechanism. Molecular profiling has revealed that Twist1 controls tumor stemness by regulating the expression of cell surface receptors and key metabolic genes (106).

Further studies are needed to elucidate the mechanisms regulating EMT and MET during tumorigenesis and metastatic spreading, the factors that regulate the maintenance of and conversion between these two cellular states, the contribution of intrinsic and extrinsic factors in the regulation of the transition, the extent of EMT reversibility, the role of epigenetic factors, and the global change in the chromatin landscape accompanying the switch and regulating its plasticity.

MICRO-RNA AND EPIGENETIC REGULATION OF CANCER STEM CELLS

In addition to the genetic diversity and differential expression of genes and proteins among tumor cells, noncoding RNAs and specific epigenetic marks further regulate functional tumor heterogeneity.

MiRNAs regulate different aspects of tumorigenesis, from tumor initiation to the maintenance of established tumors (107). MiRNAs also contribute to tumor heterogeneity, in part by regulating EMT (108).

The *miR-200* family members regulate EMT in cancer cells. MiR-200 represses EMT by targeting EMT-related TFs Zeb1 and Zeb2 (109, 110). Zeb1 directly suppresses transcription of the miRNA-200 family members, creating a feed-forward loop that stabilizes EMT and promotes invasion of cancer cells (111).

MiR-200 family members inhibit tumor formation in breast CSCs and duct formation in normal mammary SCs (112). Interestingly, p53 induces the expression of *miR-200* by directly binding to *miR-200c* promoter, whereas *p53* inactivation decreases *miR-200* and increases Zeb1 and Zeb2, promoting EMT, invasion, and metastasis (113, 114). p53 also induces the expression of *miR-34*, which represses EMT in epithelial cancer cells by targeting Snail, providing a link between p53 and EMT (115). Interestingly, *miR-34* inhibits induced pluripotent stem cell (iPS) reprogramming by targeting pluripotency genes—including *Nanog*, *Sox2*, and *Mycn* (116)—some of which are critical regulators of cancer functions (53). MiR-200 family members also inhibit *Bmi-1*, a polycomb-group gene that is essential for colorectal CSC renewal and tumor formation (117). Twist1 promotes Bmi-1 expression, and together they induce EMT and promote tumor initiation (118). Together, these data provide compelling evidence that miRNAs are essential regulators of tumorigenesis, linking p53 activation, EMT, and tumor stemness.

Let-7 is another miRNA that has been shown to regulate EMT and CSCs (119). In breast cancer cell lines, *Let-7* is downregulated in tumor-initiating cells and its knockdown increases self-renewal and sphere formation (120). Lin28, an RNA-binding protein, blocks Let-7 biogenesis and thus inhibits differentiation and sustains self-renewal in embryonic SCs (121). Lin28 overexpression is associated with *Let-7* repression in human cancers and correlates with advanced diseases and poor prognosis (122). Downstream of Lin28a and -b, *Let-7* represses a network of genes involved in cell growth, metabolism, and self-renewal, as well as oncogenes—such as insulin-PI3K-mTOR pathway, Ras, Myc, Hmga2, Igf2, Igf2bps, and Hmga1—and mRNAs encoding metabolic enzymes, ribosomal peptides, and cell-cycle regulators to promote stemness and tissue repair (123). In SCC, the *Lin28/Let-7* axis induces the expression of stemness genes such as Sox2 and enhances their reprogramming into CSCs (124). In skin SCC, Lin28 is also a direct Sox2 target gene (53).

There is now growing evidence that CSCs are also regulated by epigenetic mechanisms such as histone modifications and DNA methylation (125). Histones are chromatin-binding proteins that, depending on methylation or acetylation on lysine residues, pack the DNA into a repressive or an active state. In hepatocyte cell lines stimulated by Tgf-β, EMT is accompanied by a remodeling of specific chromatin domains consisting of a reduction in H3K9me2 marks and an increase in H3K4me3 and H3K36me3 marks (126), which are associated with resistance to chemotherapy (126) and are controlled by lysine-specific demethylase-1 (Lsd1) (126). Genome-wide profiling of histone methylation and DNA methylation of prostate cancer cell lines analyzed at different stages of EMT progression shows dynamic changes in histone markers (but not in DNA methylation), with increased H3K4me3 marks and decreased H3K27me3 marks in the upregulated genes and vice versa in the downregulated genes (127). In the MLL-AF9 subtype of acute myeloid leukemia (AML), *Kdm1a* (also known as *Lsd1*), a lysine histone (H3K4 and H3K9) demethylase, is required for sustaining clonogenic potential (128). Interestingly, *Kdm1a* knockdown does not affect normal HSC clonogenic and repopulation properties, and selectively targets LSCs (128).

Ezh2 is the catalytic subunit of polycomb repressor complex 2 (PRC2), which trimethylates histone H3 at lysine 27 (H3K27me), leading to gene silencing and chromatin remodeling. Several studies have demonstrated the importance of Ezh2 during cancer initiation and progression. Ezh2 silences E-cadherin expression via H3K27 trimethylation (129). In tumor cells, Snail1 recruits and directly interacts with PRC2 subunits Ezh2 and Suz12 at E-cadherin promoter, leading to H3K27 trimethylation that suppresses *E-cadherin* transcription (130). Silencing of Ezh2 or its pharmacological inhibition impairs in vitro properties of GBM CSCs as well as their TPC potential

in vivo (131). Further, Ezh2 confers resistance to radiation therapy of GBM cells (132), and its inhibition sensitizes *brg1* and *egfr* mutant lung tumors to etoposide chemotherapy (133), providing evidence that it constitutes a promising therapeutic target (134).

Histone acetylation regulates transcription activation in cancer cells, and histone deacetylase inhibitors target leukemia SCs (135), increase radiosensitivity of prostate CSCs (136), and sensitize SCC of the head and neck to treatment by gefitinib (a tyrosine kinase inhibitor) by reverting EMT (137). The importance of epigenetic regulation of EMT and CSCs translates into a number of therapeutic inhibitors in clinical trials, including Ezh2 and Lsd1 inhibitors (138).

DNA methylation on CpG promoter regions has been associated with transcription repression. For example, promotion of EMT in breast cancer cells is accompanied by DNA hypermethylation of the E-cadherin promoter, suggesting that DNA methylation may further help to lock cells in their epithelial or mesenchymal state (139). On a genome-wide scale, EMT is accompanied by changes in DNA methylation patterns, on both promoters and gene bodies of EMT-related genes (140).

Future studies will be required to clarify the causes versus the consequences of these epigenetic changes that accompany the different cancer cell states and how pharmacological activation or inhibition of these epigenetic regulators can provide additional benefit for anticancer therapy.

EXTRINSIC REGULATION OF CANCER STEM CELLS

Several studies have shown that, like their normal tissue counterparts, CSCs reside in particular tumor microenvironment niches that play an important role in regulating their proliferation, renewal, differentiation, and stemness. CSC regulation by their niche operates through cell-cell interaction, secreted factors, cell-matrix interaction, and the biophysical properties of the niche, such as hypoxia.

Endothelial Cells

The importance of the vascular niche in tumor maintenance is reflected by the efficacy of antiangiogenic therapies such as the anti-Vegfa bevacizumab in GBM, colorectal carcinoma, non-small-cell carcinoma, and renal cell carcinoma (141). Besides providing oxygen and nutrients, blood vessels interact with CSCs and regulate their properties. In brain tumors, Nestin$^+$ and CD133$^+$ CSCs are located in a perivascular niche. Physical interactions between CSCs and ECs promote their renewal in vitro and tumor-propagating potential in vivo through the secretion of paracrine factors by ECs (142). In a mouse model of skin SCC, CD34$^+$ CSCs are located in close contact with the perivascular niche owing to the high expression of Vegf by the CSCs (52). In this model, blocking Vegfa reduces the pool of CSCs, in parallel with the reduction of tumor angiogenesis. In addition, *Vegfa* deletion in normal epidermal cells prevents tumor initiation, and *Vegfa* deletion in established tumors induces tumor regression. Vegfa controls tumor maintenance and stemness in a non-cell-autonomous manner by promoting the formation of the perivascular niche and in a cell-autonomous manner via an autocrine or paracrine loop that promotes proliferation, apoptosis, and tumor stemness by a Neuropilin-1 (Nrp1)- and Flt1-dependent mechanism (52, 143). The importance of the Vegfa-Nrp1 loop in regulating tumor stemness is also observed in GBM, where CD133$^+$ CSCs are located in the perivascular niche and rely on signaling through the Vegfa-Nrp1 axis that promotes self-renewal and tumorigenicity (144). ECs stimulate EMT of ALDH$^+$CD44$^+$ CSCs of human head and neck SCCs via the secretion of Egf (145). In a mouse model of Pdgf-induced glioma, nitric oxide secreted by the ECs promotes CSC features by

activating Notch signaling (146). ECs also secrete Il-8 and stimulate CSCs to upregulate the Il-8 receptors Cxcr1 and Cxcr2 to further promote CSC maintenance in vitro and tumor propagation in SCID mice (147). CSCs are generally attached to their perivascular niche by adhesion molecules. In a murine model of *Bcr-Abl1*$^+$ chronic myeloid leukemia (CML), the expression of Selectins by the leukemia SCs is required for their engraftment in the bone marrow niche (148). Interestingly, the inhibition of Selectins causes a greater alteration of leukemia SC homing to the bone marrow niche compared with normal HSCs. In B cell lymphoma, tumorigenic cells, by secreting Fgf4, stimulate the expression of Jag1 in ECs. By a feed-forward loop, endothelial Jag1 activates Notch2 on lymphoma cells and promotes their tumor-propagating potential and chemoresistance (149). In GBM, CD44 expression promotes CSC features and radioresistance by binding to its ligand osteopontin in the perivascular niche. CD44-osteopontin binding releases the intracellular domain of CD44 via γ-secretase activity, which enhances Hif-2α activity through a Cbp/p300-dependent mechanism (150). Together, these data demonstrate the important role of the perivascular niche in regulating various properties of CSCs and the importance of tumor cells in recruiting and regulating ECs.

Hypoxia

The rapid proliferation of cancer cells and aberrant angiogenesis lead to hypoxic regions within tumors (151). Although cancer cells, like normal cells, need oxygen to survive, a hypoxic environment promotes CSC properties. Hypoxia increases the tumorigenic potential of many cancer cell lines from different tissues, including neuroblastoma, rhabdomyosarcoma, and non-small-cell lung carcinoma (152). Hypoxia-inducible factors Hif1a and Hif2a are expressed by glioma CSCs and correlated with poor prognosis in brain tumors, whereas their knockdown decreases self-renewal and tumor propagation of CD133$^+$ brain CSCs (153, 154). Blocking Hif1a in hematological malignancies inhibits in vitro clonogenic properties and in vivo tumor-propagating potential of leukemic and lymphoma CSCs (155). In these malignancies, Hif1a promotes stemness by enhancing Notch-Hes1 expression. Hypoxia can also stimulate resistance to therapy. In CML, hypoxia-induced expression of Hif1a in the bone marrow protects leukemia SCs from imatinib mesylate therapy, despite persistent inhibition of Bcr-Abl1, by impairing therapy-induced apoptosis (156). In triple-negative breast cancer, enriched with CSCs, Hif1a expression is associated with poor prognosis, and inhibition of Hif1a in breast cancer cell lines abrogates their resistance to therapy (157). Further, hypoxia promotes CSCs by inducing EMT through epigenetic regulation in breast cancers (158). Hypoxia inhibits oxygen-dependent H3K27me3 demethylase Kdm6a/b, which results in silencing the miRNA-processing enzyme DICER, reducing the Zeb1 inhibitor miR-200s and stimulating EMT and CSC phenotypes (158).

Inflammation

The inflammatory component of the tumor microenvironment regulates tumor initiation, progression, and CSC properties (159). Inflammation promotes both the acquisition of a CSC phenotype and its maintenance by stimulating EMT. In a mouse model of Kras-driven pancreatic cancer, EMT was detected at the first stage of tumor initiation as well as in the invasive stage and was promoted by inflammation (160). Human mammary cancer cells that undergo EMT and acquire CSC features express the surface markers CD90 (also known as Thy1) and Epha4, which enable them to physically interact with tumor-associated macrophages (TAMs) and to form a metastatic niche (161). In the CSC niche, TAMs are M2 polarized and activate the Nfκb

pathway, leading to the secretion of cytokines, including Il-6, Il-8, and Csf2. These cytokines maintain tumor stemness via paracrine mechanisms (161). Colorectal cancer cells exposed to Il-6 repress miR-34, which in turn allows the expression of EMT transcription factors, invasion, and metastasis (162). In transformed mammary cells, Il-6 activates Lin28 transcription via Nfκb, which reduces *Let-7* levels, leading to EMT and enhanced CSC features (123, 163). Il-6 also suppresses *miR-200c* by the activation of p65/RelA and Jnk2, promoting cell transformation (164), as well as by the derepression of *Zeb1* (165). $Her2^+$ breast cancer cells that develop resistance to trastuzumab therapy are enriched in CSCs and show EMT features as well as a major upregulation of Il-6. Blocking IL-6 receptors reduced the tumor-propagating potential of these cells, further suggesting that Il-6 controls CSCs through an autocrine loop. TAMs secrete the milk fat globule–epidermal growth factor 8, which cooperates with Il-6 to promote stemness and resistance to therapy in cancer cells (166). Highly aggressive amelanotic melanomas with histological features of EMT are highly infiltrated by leukocytes and show features of Tgf-β pathway signature (167). Tgf-β is one of the most potent and well-characterized EMT inducers. It is secreted mainly by infiltrating myeloid cells and acts in a paracrine manner on cancer cells by generating CSCs and increasing their metastatic potential (168–170). Tgf-β also acts on the tumor immune cells to drive their polarization toward an immunomodulatory phenotype (171); moreover, in mammary CSCs, canonical and noncanonical Wnt signaling act together with Tgf-β in a paracrine and autocrine manner to trigger EMT programming (170). In mouse SCCs, Tgf-β-responding cells are a subpopulation of slow-cycling CSCs, exhibiting EMT and resistance to chemotherapy (172).

Fibroblasts and Myofibroblasts

Like their normal tissue counterparts, CAFs and other stromal cells synthesize the extracellular matrix, supporting the functions of cancer cells via cell-cell and paracrine interactions (173). CAFs are a central component of the CSC niche. Paradoxically, conditional ablation of myofibroblasts in a mouse model of pancreatic ductal carcinoma enhances hypoxia, increases EMT transcription factors, and enriches the tumors in $CD44^+CD133^+$ CSCs (174). Myofibroblast-depleted tumors also show increased infiltration with $CD4^+Foxp3^+$ regulatory T lymphocytes, which decrease immune surveillance and enhance tumor growth and invasiveness. CAFs are activated by signals secreted by cancer cells and respond by promoting EMT and upregulating matrix metalloproteinases (MMPs), thus increasing invasiveness (175). Interestingly, knockout of all four tissue inhibitor of metalloproteinase (*Timp*) genes in fibroblasts induces the acquisition of a CAF-like state that stimulates cancer cell motility and the expression of CSC markers through the secretion of exosomes enriched in the metalloproteinase Adam10 (176). Colon cancers are usually caused by mutations activating Wnt signaling (177) yet exhibit high Wnt activity preferentially in tumor cells located close to stromal myofibroblasts, indicating that Wnt activity is regulated by extrinsic cues. Myofibroblasts secrete hepatocyte growth factor (HGF), which signals to cancer cells and activates β-catenin-mediated transcription and tumor stemness (178). Fgf4 is another growth factor secreted by CAFs that stimulates ovarian CSCs via Fgfr2, leading to upregulation of Sox2 that enhances tumor-propagating potential (179). Several other paracrine factors have been reported to contribute to the crosstalk between CAFs and CSCs, such as chemokines (Ccl2) (180), Annexin a1 (181), Igf2 (182), and prostaglandins (Pge2) (183).

Interestingly, CAFs are activated in response to anticancer therapy and may support tumor relapse. Chemotherapy-treated human CAFs promote colorectal CSC self-renewal and tumor-initiating potential via paracrine secretion of cytokines such as Il-17a (184). These data suggest that chemotherapy induces changes in tumor microenvironment that support CSC survival and tumor relapse.

CANCER STEM CELLS AND METASTASIS

Metastasis is a complex, multistep process requiring tumor cells to leave the primary tumor, migrate to a distant site, and colonize the new site to initiate their growth. Increasing evidence suggests that metastasis is initiated by specialized tumor cells that present CSC properties (185).

Sequencing of metastatic human cancers has revealed that the metastatic clones emerged late during tumor genetic evolution; the vast majority of the mutations are shared between the primary tumor and the metastases (186, 187), suggesting that the metastatic potential is acquired by nongenomic factors. In addition, in human colon cancer, as well as in other types of tumors, only a subset of long-term self-renewing CSCs can drive metastasis following their transplantation into immunodeficient mice, providing evidence that only a subpopulation of cancer cells are at the origin of metastasis (188). Further, both metastatic dissemination and the acquisition of CSC properties are promoted by EMT (97). In renal carcinoma, metastatic potential is regulated by epigenetic factors; loss of repressive epigenetic markers (PRC2-dependent H3K27me3 and DNA methylation) activates the expression of chemokine receptor Cxcr4, which promotes migration, and cytohesin 1–interacting protein (CYTIP), which supports cell survival (189). The metastatic SCs are characterized by additional features such as dormancy and plasticity. Metastases can occur years after a successful treatment of the initial tumor, indicating the long-term latency of the metastatic seeds. In accordance, cancer cells detected in the bone marrow of patients are quiescent, and this quiescence protects the cells against cytotoxic agents. Exiting the dormant state to proliferate and form a metastatic tumor involves several pathways, including MAPK and BMP (185). Interestingly, some studies have shown that distinct populations of CSCs are responsible for tumor growth and metastatic seeding (36, 190).

EMT confers metastatic potential on cancer cells, and circulating tumor cells show features of EMT (191). Upon arrival at the destination, cancer cells shut down EMT transcription factors (Twist1 and Prrx1), undergo MET, and regain epithelial traits in order to colonize the distant organs (102, 192).

Interactions of metastatic SCs with the microenvironment govern metastatic seeding, survival, dormancy, colonization, and growth (185). In a mouse model of mammary tumors, a small population of $CD24^{+}CD90^{+}$ CSCs induces the expression of stromal Periostin at the metastatic site, allowing their initial expansion by increasing Wnt signaling. Blocking the function of Periostin prevents metastasis, suggesting that preventing de novo niche formation could be a valuable strategy for preventing and treating metastatic diseases (193).

A key challenge in the treatment of cancer is the late occurrence of distant metastases, sometimes years or decades after the diagnosis and treatment of the primary tumors. Dormant tumor cells reside in close proximity to the vascular niche of lung, bone marrow, and brain, and endothelial-derived thrombospondin-1 induces sustained tumor quiescence; however, during neoangiogenesis these quiescent tumor cells reenter the cell cycle through the secretion of active Tgf-β1 and Periostin from endothelial tip cells, suggesting that a stable vascular niche promotes tumor metastasis dormancy, whereas sprouting vasculature during active neoangiogenesis stimulates metastatic outgrowth (194).

CANCER STEM CELLS AND RESISTANCE TO THERAPY

A fundamental property of CSCs is their capacity to maintain tumor propagation. CSCs may also be inherently resistant to medical therapy and contribute to tumor relapse, although the CSCs that propagate the tumor and the cancer cells that are resistant to medical therapy can be different. These cells can be either intrinsically resistant to therapy, and thus persist after treatment and

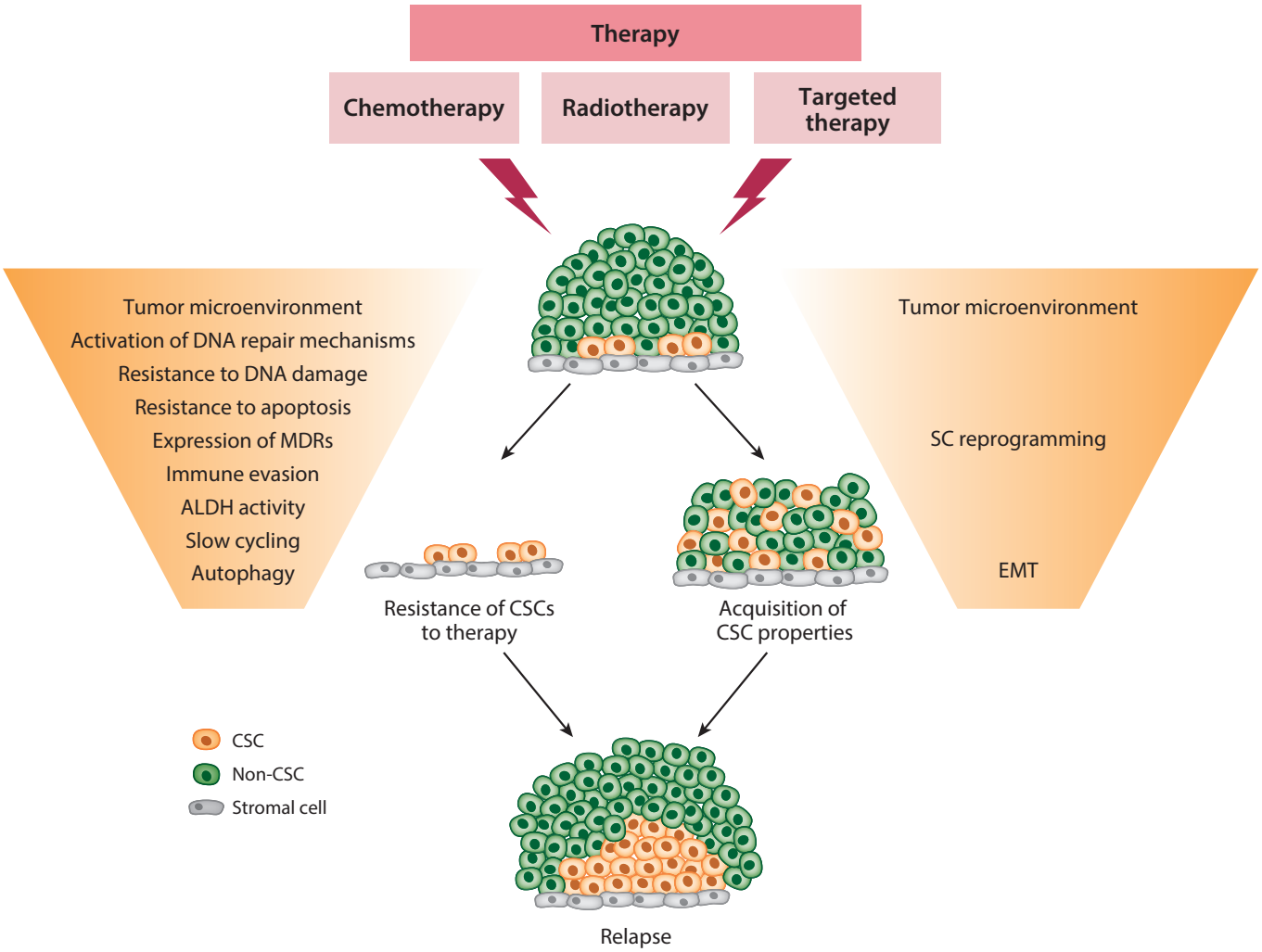


Figure 3

Mechanisms controlling the resistance of CSCs to medical therapy. Multiple intrinsic and extrinsic mechanisms induce CSC resistance to therapy. During or after therapy, non-CSCs may reacquire CSC properties by EMT, as well as by transcriptional and epigenetic reprogramming, and also contribute to tumor relapse. Abbreviations: ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition; MDRs, multidrug resistance proteins; SC, stem cell.

cause a relapse, or extrinsically instructed by the tumor microenvironment to become resistant under the selective pressure of therapy (**Figure 3**).

Several studies show that CSCs become enriched following chemo- or radiotherapy, suggesting that therapy induces or selects cancer cells with CSC properties. Ionizing radiation induces upregulation of $CD133^+$ CSCs in GBM xenografts (195). Breast $Thy1^+CD24^+$ CSCs are enriched in tumors after radiation therapy (196). Enrichment of preleukemic HSCs in AML patients during remission indicates that resistance to therapy is an early feature of CSCs (197). Lung cancer cell lines that acquire resistance to the Egfr tyrosine kinase inhibitor gefitinib (198) and GBM cells that acquire resistance to the anti-Vegfa bevacizumab show EMT and CSC properties (144). In GBM, lineage-tracing experiments suggest that temozolomide resistance is accompanied by an expansion of the CSC pool, possibly through dedifferentiation of non-CSCs (199).

Like their normal tissue counterparts (200), CSCs seem to be resistant to DNA damage–induced cell death (88). When exposed to radiation therapy, breast CSCs contain lower levels of reactive oxygen species (ROS) than nonstem cancer cells due to the expression of higher levels of ROS scavengers and are thus protected against ROS-induced DNA damage (196). Further, brain CSCs show more efficient DNA damage response than non-CSCs. $CD133^+$ GBM CSCs resist radiotherapy by activating DNA damage checkpoints and repairing radiation-induced DNA damage more efficiently than $CD133^-$ cells. Pharmacological inhibition of the DNA damage checkpoints Chk1 and Chk2 sensitizes CSCs to radiotherapy (195).

Other mechanisms may account for CSC resistance to therapy (201). Cancer cells that have undergone EMT are usually more resistant to therapy. EMT cells are usually slow cycling and have lower levels of ROS, and EMT TFs may induce other important functional properties directly controlling resistance to therapy by regulating apoptosis, autophagy, or other mechanisms (201). Zeb1-induced EMT and tumor stemness confer resistance to therapy in different tumor types (185, 202–204). Ionizing radiation induces activation of Atm, which phosphorylates and stabilizes Zeb1 in response to DNA damage. Zeb1, through interaction with Ups7, activates Chk1, promoting DNA repair and resistance to radiation (205). As Zeb1 is regulated by miR-203, which itself is regulated by epigenetic mechanisms, treatment by the histone deacetylase inhibitor mocetinostat restores miR-203 expression, represses Zeb1, and restores drug sensitivity (203). Loss of miR-205 is associated with tumor relapse in breast cancer patients. Therapeutic delivery of miR-205 sensitizes tumors to radiation-induced cell death by targeting Zeb1 and the ubiquitin-conjugating enzyme Ubc13 (204), further suggesting that targeting EMT and miRNAs constitutes a promising approach to stimulate drug sensitivity in CSCs.

Some CSCs, like their normal SC counterparts (206), express high levels of multidrug resistance (MDR) or detoxification proteins, which are transporters facilitating drug efflux. Consistent with this observation, in gliomas, side populations identified by their capacity to rapidly efflux Hoechst 33342 dye are highly tumorigenic and express the ABCG2 transporter in response to PTEN/PI3K/Akt signaling, leading to their resistance to temozolomide (207). ALDH, which catalyzes the oxidation of aldehyde, marks a population of CSCs in various tumors that has been suggested to contribute to therapy resistance (30, 201, 208). ALDH-mediated resistance to therapy involves complex mechanisms, including metabolism and detoxification of chemotherapeutic agents and the activation of the prosurvival PI3K/Akt and MAPK/ERK pathways (201).

By combining lentiviral lineage tracking with DNA profiling of human colorectal cancers through serial xenograft passages in mice, researchers have shown that different clones contribute differently to the clonal dynamics of tumor cells over time (71). Some clones contributed to tumor growth at each passage, whereas other clones contributed initially and were later exhausted. Still other clones were present at a lower level in the initial passage but became more frequent at latter passage, suggesting their slow-cycling or fluctuating cycling properties. Interestingly, oxaliplatin administration selected for slow-cycling or dormant clones that became dominants. This study demonstrates that, besides genetic diversity, tumor cells display important functional diversity during tumor progression, which contributes to cancer growth and resistance to therapy (71). Likewise, in a GBM mouse model, slow-cycling $Nestin^+$ CSCs resisted temozolomide and were responsible for tumor regrowth after therapy (76). In bladder cancer, resistance of tumor cells to chemotherapy was caused by slow-cycling CSCs that were stimulated to proliferate in between cycles of chemotherapy, similar to the recruitment of normal SCs during tissue repair (209). The proliferative response of CSCs was promoted by PGE2 release by cancer cells that were killed by the chemotherapy. Targeting PGE2 by monoclonal blocking antibody or by the administration of cyclooxygenase-2 inhibitor attenuated chemoresistance and suggested that targeting this

pathway in between cycles of chemotherapy may enhance the therapeutic response in bladder cancer.

The tumor microenvironment also contributes to CSC resistance to therapy. In prostate cancer, DNA damage induces Wnt16b expression in B lymphocytes, which subsequently signal in a paracrine manner to activate the Wnt pathway in tumor cells, which in turn attenuates the effects of cytotoxic chemotherapy in vivo and promotes tumor cell survival and disease progression (210). In colorectal cancer, chemotherapy activates CAFs to secrete Il-17A, which stimulates CSC self-renewal and tumor growth (184). CAFs also secrete Hgf, which acts through Met receptors and inhibits CSC response to anti-Egfr antibodies (211). Chemoresistant CSCs produce proinflammatory cytokines to polarize $CD14^+$ monocytes toward M2-like macrophages that in return increase the tumorigenic properties of CSCs (212). Hypoxia increases resistance of non-small-cell lung cancer cell lines to gefitinib therapy by generating CSCs through activation of Igf1 receptors (213). Hypoxia induces resistance of CML SCs to Abl1 tyrosine kinase inhibitor imatinib through Hif1a signaling (156). In pancreatic cancer, survival of CSCs in hypoxic conditions was mediated by autophagy (214).

Targeting CSCs necessitates a better understanding of the mechanisms that lead to their resistance to therapy. Integrin β3 marks a population of TPCs in breast, lung, and pancreatic cancer that are resistant to treatment with tyrosine kinase inhibitor erlotinib (215). Integrin β3 forms a functional complex with Kras and RaiB that activates the NF-κB pathway and promotes resistance to therapy. Inhibitors of the Alk and Egf receptor tyrosine kinases are used to treat lung cancers harboring *eml4-alk* translocations or activating mutations of *egfr*; however, rapid resistance to therapy develops in these tumors. RNAi screening identifies Med12 as a determinant of response to Alk and Egfr inhibitors. Cytoplasmic Med12 interacts with Tgf-βr2 and inhibits Tgf-β signaling. Loss of Med12 results in activation of Tgf-βr signaling, which induces drug resistance. Inhibition of Tgf-β signaling restores drug responsiveness, suggesting that inhibition of Tgf-β signaling could be a therapeutic strategy in resistant tumors that have lost Med12 (216). Accordingly, in SCC, Tgf-β-responding cells are a subset of basal $CD34^+CD44^+Itga6^{high}$ cells that are enriched in TPCs. Tgf-β-responding cells are slow cycling and located at the invasion front in the vicinity of the tumor vasculature and display an EMT phenotype. Interestingly, Tgf-β signaling in CSCs increases their resistance to cisplatin via the Nrf2/p21 pathway, by enhancing glutathione metabolism of the drug, which diminishes its efficacy (172).

Given the important roles of CSCs during tumor growth and relapse after therapy, the goal of cancer therapy should be their elimination or their terminal differentiation. Lineage ablation experiments in mouse models provide encouraging evidence of how the elimination of CSCs induces the regression of tumors and their delayed relapse after chemotherapy (53, 76, 77). Additionally, targeting the microenvironment during therapy should be important to avoid the dedifferentiation of non-CSCs into a CSC-like state and, consequently, tumor relapse.

PERSPECTIVES

Several recent studies have shown that in their native microenvironment in vivo, tumor growth is sustained by long-lived CSCs, whereas more committed tumor cells contribute only transiently to tumor outgrowth (63, 65–68, 71); however, it is still unclear whether all tumor types contain such hierarchical organization of tumor growth in vivo and whether, during tumor progression, all tumor cells present long-term self-renewal capacity—behaving as CSCs.

Although EMT is often associated with CSC features such as enhanced tumor-propagating potential, future studies are required to determine whether EMT is a generalized feature of CSCs or whether EMT cells represent a second pool of CSCs with more slow-cycling and invasive

properties, leading to metastasis, whereas the bulk of tumors are maintained by more primitive multipotent CSCs that do not present EMT features. New transplantation assays that will better recapitulate the tumor stroma found in the primary tumors will allow researchers to study the influence of each of the different components of the tumor microenvironment—including immune cells, inflammatory cells, ECs, and CAFs—on the tumor phenotype, and further specify the role of the microenvironment in the regulation of tumor heterogeneity.

The link between genetic tumor heterogeneity and functional tumor heterogeneity is still poorly understood. Do the new mutations that confer selective clonal advantage occur in a CSC, or do those mutations confer increased fitness and tumor stemness in committed cells? Are CSC units (i.e., their corresponding progeny) the pillar of genetic evolution in cancer? New studies are needed to evaluate the number of CSCs and the size of their respective units at different stages of tumor progression and metastasis, and to define how they compete or collaborate during tumor growth.

Finally, a much better understanding of the mechanisms regulating CSC resistance to therapy is required to prevent tumor relapse. New treatment strategies should combine conventional therapy and molecules that specifically target CSCs or their extrinsic and intrinsic regulators.

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