

Cancer Stem Cells: A Moving Target

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Abstract Even though the number of anti-cancer drugs entering clinical trials and approved by the FDA has increased in recent years, many cancer patients still experience poor survival outcome. The main explanation for such a dismal prognosis is that current therapies might leave behind a population of cancer cells with the capacity for long-term self-renewal, so-called cancer stem cells (CSCs), from which most tumors are believed to be derived and fueled. The CSCs might favor local and distant recurrence even many years after initial treatment, thus representing a potential target for therapies aimed at improving clinical outcome. In this review, we will address the CSC hypothesis with a particular emphasis on its current paradigms and debates, and discuss several mechanisms of CSC resistance to conventional therapies.

Keywords Cancer · Cancer stem cells · CSCs · Drug resistance · DNA repair · Genomic instability · ABC transporters · Pathobiology

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Introduction

The discovery of human cancer stem cells (CSCs) has important diagnostic and prognostic implications, and holds significant promise for the development of novel therapeutic strategies. Nevertheless, until recently, no drugs that effectively target CSCs have been in clinical use. It remains challenging to understand what makes a CSC different from a normal stem cell (SC), and to target malignant cells while sparing their normal counterparts. Normal SCs and CSCs not only share pathways that regulate self-renewal and multipotentiality [1], but also share other mechanisms regulating response to environmental stress [2, 3], including DNA repair machinery [4, 5], as well as adenosine triphosphate-binding cassette (ABC) transporters [6, 7]. These mechanisms can afford protection to CSCs, shielding them from the adverse effects of chemotherapeutic insult.

To explore the possibility of selectively targeting and destroying CSCs as a successful approach for increasing the survival of cancer patients, we conducted an extensive review of published CSC papers. Here, we describe their discovery and the mechanisms conferring their drug resistance that should be considered for therapeutic protocol design and application.

The Cancer Stem Cell Hypothesis: History and Open Questions

Cancer was suggested to derive from SCs more than 150 years ago. In the late 1800s, Cohnheim, a German pathologist, introduced a theory that tumors may arise from an “embryonal rest” that lies dormant until induced to transform [8]. However, not until 1994 were CSCs actually identified, thanks to the advent of flow cytometry. Dick and

colleagues reported that human acute myeloid leukemia (AML) is organized as a hierarchy that originates from a primitive hematopoietic cell possessing a $CD34^+/CD38^-$ phenotype [9, 10]. Later, a hierarchical organization was also reported in breast cancer [11]. Similar to leukemia, only a minority of breast cancer cells identified with a $CD44^+/CD24^{-/low}$ phenotype were tumorigenic in xenograft assays. Additional evidence for the existence of CSCs rapidly followed these studies. The CSCs were identified in brain and colon cancers [12–14], and since then in most human cancers. However, despite the first wave of enthusiasm surrounding the CSC field, no consensus has emerged about cell surface marker profiles that define CSCs, hindering the development of effective SC-based approaches to diagnose and treat cancer. Not only do CSC marker phenotypes vary among different cancer types, but they might also vary among patients with the same disease. For example, although most AMLs follow the CSC model, the leukemogenic activity is not restricted to the $CD34^+/CD38^-$ fraction in all patients [15]. Also, concerning solid cancers, there have been huge controversies over the definition of the cell surface signature that characterizes the CSC fraction. Initially described as a unique marker for immature cells in the lower part of the intestinal crypt [16], CD133 was later found to be ubiquitously expressed on differentiated colonic epithelium in both adult mice and humans [17]. Surprisingly, both $CD133^+$ and $CD133^-$ metastatic colon cancer cells initiated tumors, raising the question of whether CD133 is a bona fide marker for colon CSCs [17]. Additional investigations revealed that CD133 remains expressed on the membrane during differentiation, but is not recognized by common antibodies due to a reduced glycosylation that likely affects CD133 folding [18]. Nevertheless, concerning this particular form of cancer, a list of other markers was reported. In the study of Dalerba et al. [19] expression of CD133 displayed a variable pattern, with some tumors scoring as homogeneously negative, some as predominantly positive, and others as a mixture of positive and negative cells. Since not all tumors expressed CD133, the combined expression of $EpCAM^{high}/CD44^+/CD166^+$ was suggested as being more robust for the isolation of colon CSCs [19]. In our study, putative colon CSCs were analyzed for 24 different CD markers [20]. Heterogeneous expression of CD26, CD44, CD227, CD56, CD64 and CD133 was found. Of note, we described an efficient long-term culture system for the expansion of colon CSCs, based on a feeder layer derived from rat mammary adenocarcinoma. The CSCs cultivated using this system exhibited self-renewal and multipotentiality, as well as tumor-initiating ability in vivo.

Amongst all the CSC markers proposed, the glycoprotein CD44 gained much interest due to the observation that its variant CD44v6 promotes oncogenic signals required

for tumor cell invasion and migration [21]. Despite this, contradictory opinions regarding the role of CD44 in metastasis have also been reported [22–28]. Controversies on CD44 function may depend on lack of distinction of its isoforms, which might possess divergent functional activities. Indeed, the way CD44 radically modifies cell behavior seems to be dependent on the unique pattern of glycosylation, itself determined by alternative splicing [29]. Nevertheless, two distinct papers have recently provided a way to target CD44 in CSCs from two different tissues, but neither has focused its attention on a specific CD44 variant. Cheng et al. [30] provided evidence that decreasing CD44 levels by forcing the expression of mature microRNA (miR)-199a may prevent growth, invasion, migration and multidrug resistance of $CD44^+/CD117^+$ ovarian CSCs. Anti-CD44 antibody-mediated liposomal nanoparticle delivery was then proposed to selectively attack CSCs of hepatocellular carcinoma [31].

Characterization of CSC-specific cell surface markers can be the first step towards the identification of pathways activated in CSCs. In colon cancer, a Wnt signaling gradient with the highest levels of activity in CSCs and the lowest in their differentiated progeny, was reported [32]. Thus Wnt is an attractive target for new therapeutics. Similar to the Wnt pathway, Hedgehog (HH) and Notch signaling may play a critical role in cancer by dictating CSC fate decisions [33, 34]. Strategies targeting these developmental pathways are currently being explored.

Despite intensive research, many reports call for caution in the acceptance of the CSC hypothesis. Hierarchical organization in a cancer does not imply that it originated from normal SCs, since cancer can also arise through mal-differentiation or arrested differentiation of committed progenitors, as well as dedifferentiation of fully differentiated cells [35]. Leukemias offer a good example. Committed myeloid cells can be reprogrammed by oncogenic factors (i.e., BCR/ABL and PML-RAR α fusion protein) to become progenitor-like cells that then develop the disease [36, 37]. Similarly, most fully differentiated cells in the central nervous system (CNS), upon defined genetic alteration, can acquire the capacity to dedifferentiate to a more progenitor-like (SC) state [38•]. Therefore, tumor progression likely requires a permissive microenvironment composed of cell types and molecular pathways that can sustain both differentiation of tumor cells and the maintenance of tumor stem-like cells. Activation of epithelial-mesenchymal transition (EMT) has also been reported to induce somatic cells to acquire stem-like properties. Indeed, triggering of an EMT program in immortalized human mammary epithelial cells (HMLEs) results in the acquisition of mesenchymal traits and in the expression of stem-cell markers [39]. Additionally, although no direct experimental evidence is currently available, cell fusion

has been suggested to generate CSCs [40]. Spatial and temporal changes in the cell-of-origin likely influence CSC properties [41]. Subtypes of medulloblastoma and of ependymoma arise from neural SCs isolated from regionally and developmentally distinct regions of the CNS that are susceptible to transformation by different gene mutations [42, 43]. Temporal changes in the regulation of the phosphoinositide 3-kinase (PI3K) pathway lead to temporal changes in the mechanisms that regulate hematopoietic stem cell (HSC) function and leukemogenesis [44••]. Particularly, *Pten* deletion increases PI3K pathway activation in adult, but not neonatal, HSCs. Since neonatal HSCs and other hematopoietic cells can harbor mutations in *Pten* and *p53* without transforming into leukemia until adulthood, Morrison's group suggests that mutated cells may persist for years in children before a change in developmental context renders these mutations competent to induce leukemia. This can clearly explain why the spectrum of disease and treatment successes differ markedly between pediatric and adult patients [45].

Treatment resistance and disease recurrence have been largely attributed to a CSC quiescent state [46]. However, it is not clear whether or not CSCs are dormant. Not only does the CSC proliferative state have fundamental implications for therapy, but the abundance of CSCs also influences the design of new therapies. Tumorigenic cells in cancers which follow a suggested CSC model are usually rare. Nevertheless, whether or not CSCs are truly rare remains an open question. Xenotransplantation assays may underestimate CSC frequency. Using the highly immunocompromised NOD/SCID Il2rg^{-/-} mice, Quintana et al. [47] reported a high percentage of melanoma cells with the potential to proliferate extensively and form new tumours. Thus, melanoma may not follow a CSC model. As a consequence, if tumors possess a small fraction of tumorigenic cells, anti-cancer therapies may be identified based on their ability to selectively kill these cells rather than the bulk population of non-tumorigenic cancer cells. Alternatively, if cells with tumorigenic potential are common, it will not be possible to treat cancer successfully by only focusing on a small fraction of cells.

Importantly, while some cancers have been hypothesized to initiate as a SC disease, disease progression may occur by clonal evolution of their CSCs [48]. As a consequence, therapy resistance cannot be exclusively linked to the SC origin of cancer, but is most probably the result of the progressive accumulation of genetic and epigenetic changes (Fig. 1).

Taken together, the CSC hypothesis is not a universal model that applies to all cancers and not even to all patients with the same disease. As cancer's Achilles' heel, CSCs have been intensively studied to develop more effective therapies. However, many unanswered questions still exist

about the CSC hypothesis. Identifying the cell(s)-of-origin for each cancer type is an important prerequisite for the development of personalized treatment strategies.

DNA Repair and Genomic Integrity

There are many mechanisms by which CSCs can acquire resistance to anti-cancer therapies. Cells encounter stressors from the environment (ionizing radiation) and from intracellular by-products (reactive oxygen species) that cause DNA damage. This damage must be repaired or the cell is destined for death by checkpoint mechanisms. There is little consequence if this occurs in differentiated cells of an organ, however, if this occurs in a SC, the entire lineage can be compromised [3]. Therefore, SCs have been equipped with better repair mechanisms than their more differentiated progeny in an attempt to maintain genomic integrity and persistence through an organism's life [4]. Four major repair pathways can be distinguished: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and recombination repair. The faster the repair is made, the less p53 is activated and apoptosis is prevented, thus conserving the SC pool [5•]. If CSCs arise from normal SCs, it is likely they retain this proficient DNA repair, allowing them to survive and propagate.

Disruption of the mechanisms that regulate cell-cycle checkpoints, DNA repair and apoptosis results in genomic instability, whose role in cancer continues to be debated. Indeed, genomic instability, including genetic and/or epigenetic alterations, may lead to cellular death or provide a fertile ground for additional mutations. From this point of view, genomic instability seems to be a mere consequence of tumor progression, but we believe it is responsible for the stepwise accumulation of molecular alterations which drive not only tumor progression and metastatic spread, but also tumor initiation. Genomic instability could start in the long-lived, self-renewing SC, allowing for their transformation into CSCs. Indeed, evidence from several experiments indicates that SC transformation might be an early event in carcinogenesis. Colon cancer provides a clear example. In many cases of this disease, the first mutation occurs in the Adenomatous Polyposis Coli (APC) gene. Transformation of normal SCs through loss of APC is an extremely efficient route towards initiating intestinal adenomas [49]. Interestingly, APC mutations not only deregulate the Wnt pathway, but also increase the assembly of BER proteins and as a consequence, DNA repair activity [50]. Similarly, p53 mutations, which occur late in the adenoma-carcinoma sequence of colorectal cancer (CRC), facilitate DNA repair by increasing the levels of apurinic/apyrimidinic endonuclease (APE1), a BER pathway effector [51]. Elevated levels of APE1 also confer enhanced survival to MCF-7

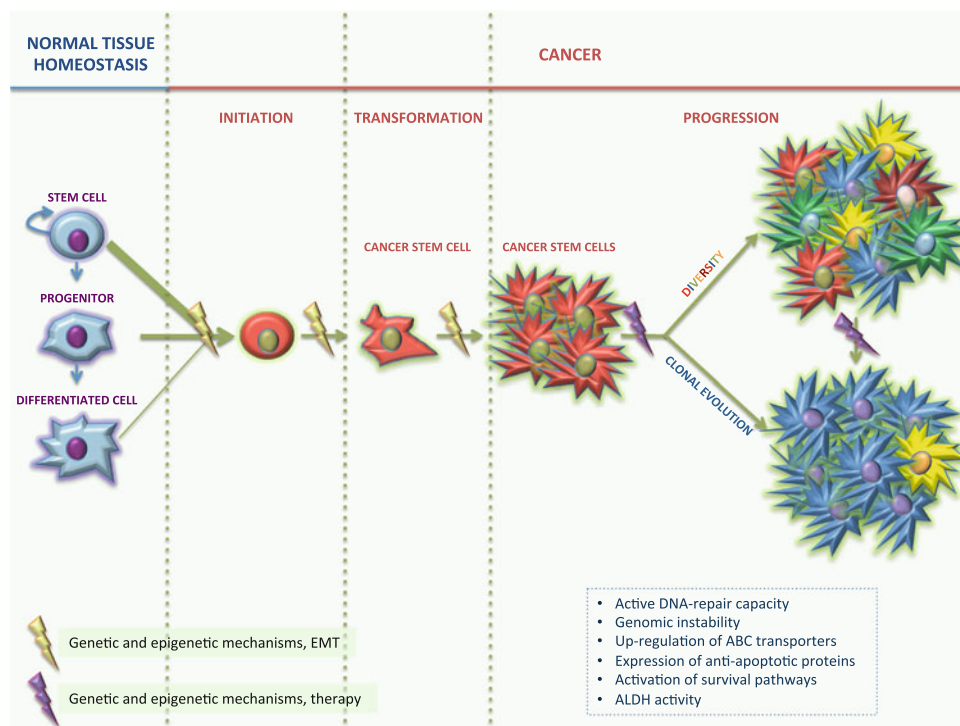


Fig. 1 Cancer develops through definable stages: initiation, transformation and progression. Cancers that follow the CSC model can derive from the accumulation of genetic and epigenetic changes in a normal SC, a committed progenitor cell or a fully differentiated somatic cell. Activation of EMT can also induce mature cells to acquire stem-like properties. Cancer can then progress as a SC disease, giving rise to a hierarchically organized heterogeneous population of cancer cells, or strictly adhere to the clonal evolution/stochastic model, generating further aggressive subtypes undergoing a positive Darwinian selection. In another scenario, hierarchically

organized cancers progress by clonal evolution of their CSCs. The choice may be dictated by a number of molecular mechanisms altering cell behavior, and may, therefore, change from patient to patient. Therapeutic treatment can also enhance the existing genomic instability, further promoting CSC expansion. Mechanisms regulating responses to environmental stress, including DNA-repair, genomic instability, ABC transporters, anti-apoptotic proteins, survival pathways and ALDH can afford protection to CSCs, shielding them from the adverse effects of chemotherapeutic insult

cancer-initiating cells following exposure to ionizing radiation [52]. Of note, small-molecule inhibitors of proteins involved in BER and inhibitors of cell-cycle checkpoints have been developed with encouraging results in colon and glioblastoma studies [53, 54].

Our lab was the first to indicate a correlation between CSCs and genomic instability in colon cancer [20]. All tumor cells analyzed expressed human karyotypes with clonal and non-clonal numerical and structural aberrations. The presence of genomic instability in CSCs has many therapeutic implications. Over time, genomic instability produces diverse tumor cell clones, which compete for resources and evolve by natural selection. Multiple cycles of genome rearrangement and clonal selection eventually lead to the emergence of aggressive tumor cell phenotypes. Indeed, high genomic instability indices often correlate with poor prognosis in some types of cancer. Interestingly, ionizing radiation and/or chemotherapy could enhance the existing genomic instability, further promoting tumor selection and CSC expansion. This concept has been well demonstrated for chronic myeloid leukemia (CML), where

following radiation or chemotherapy exposure, CSCs are induced to further alter their behavior and resist death stimuli [55]. Moreover, reversion of the anticancer drug-induced polyploidization can lead to the emergence of new CSCs [56].

Taken together, while existing therapies could have the harmful effect of increasing the selective pressure on CSCs or of generating new CSCs, therapies aimed at reducing DNA repair activity and/or the exacerbation of the cancer cell unstable phenotype may prove clinically relevant in the treatment of a variety of cancers. How DNA repair activity and genomic instability affect resistance to existing chemotherapeutic agents is therefore an urgent task to address.

ABC Transporters

The active DNA-repair capacity and genomic instability are not the only characteristic to affect CSC resistance. Initially, CSCs seemed resistant to chemotherapeutics that

target rapidly dividing cells due to their innate slow-cycling characteristic [46]. More recent in-depth studies indicate that further resistance comes from the expression of ABC transporters [6]. Using ATP hydrolysis, these transporters translocate substrates across biological membranes against a concentration gradient [57•]. It is the ABC transporter that gives the SC “side-population” (SP) its ability to efflux fluorescent dyes [7]. Mouse knockouts for several ABC transporters have suggested their protective role against drug-induced cytotoxicity [58]. Amongst the most extensively studied ABC transporters in cancer are ABCB1, ABCG2 and ABCC1 [57•]. The co-presence of more than one transporter in cancer cells has pushed for development of treatments targeting more than one, including polyphenols and curcumin [59]. In vitro studies showed that curcumin could effectively target FOLFOX (Oxaliplatin, 5-Fluorouracil, Leucovorin)-surviving colon cancer cells, inferring that curcumin could enhance chemotherapeutic responses by preventing the emergence of chemoresistant cells [60]. Interestingly, ABCG2 transporter activation in FOLFOX-resistant HCT-116 and HT29 cells could be reduced by Schlafen-3 (Slfn-3), a protein with a role in cellular differentiation in intestinal mucosa [61].

Additional studies indicate that ABC transporters in CSCs play a critical role conferring higher drug resistance than in more differentiated cancer cells. Indeed, ABCG2-expressing CD133⁺ colon cancer-derived tumor spheres were found to be more resistant to standard chemotherapy than their differentiated progeny [62]. The SP cells in neurospheres derived from primary glioblastoma tumors had more ABCG2 activity with respect to their differentiated progeny and were eliminated through ABCG2 inhibition [63]. Studies on lung [64], brain [65] and pancreatic [66] cancer correlated resistance in SP cells expressing ABCG2 with the CSC phenotype, identified through CD133 glycoprotein expression.

Combination therapy with modulators of ABC transporters may allow anti-cancer drugs to more effectively attack and kill cancer cells. Initially developed based on its ability to inhibit epidermal growth factor receptor (EGFR), Erlotinib was later found to increase intracellular accumulation of anti-cancer drugs that otherwise would have been pumped out through ABC transporters. In AML, Erlotinib activity enhanced chemotherapy-induced cytotoxicity by multiple mechanisms, including the downregulation of surface-exposed pumps and the modulation of their ATPase [67]. Conversely, other compounds such as low molecular weight heparin (LMWH) decrease cancer resistance by increasing proteasome-mediated ABC transporter degradation [68].

Studies on ABC transporters have often been correlated with patient response to therapy. Interestingly, chemotherapy was found to increase resistance via ABC

transporter up-regulation. Indeed, breast cancer patients resistant to 5-fluorouracil (5-FU) treatment showed an increased expression of ABCG2 [69]. Similarly, in another study, all hepatocellular cancer specimens exhibited increased ABCG2 expression after chemotherapy [70, 71]. Lower miR-451 levels, a negative regulator of ABCB1, were also found in patients who had received an Irinotecan-based chemotherapy for metastatic CRC and did not respond favorably [72]. These studies clearly show the clinical significance of resistance via ABC transporters characteristic of the CSC population.

Other Methods of Resistance

There are yet other factors that can confer resistance to CSCs. Brain CSCs show increased resistance in response to treatment with chemotherapy due in part to expression of anti-apoptotic proteins [73]. Breast progenitor cells maintain their resistance by differential regulation of survival pathways such as the aforementioned Wnt pathway [74]. In breast, colon, liver and pancreatic cancers, resistance has been linked to increased expression and activity of aldehyde dehydrogenases (ALDHs) in CSCs, a family of enzymes involved in several detoxifying pathways, against which specific inhibitors have been designed [75]. Mechanisms conferring resistance to CSCs are indicated in Fig. 1.

Normal Versus Cancer Stem Cells: Implications for Therapy

As already discussed in this review, normal and cancer SCs share many characteristics such as cell surface markers, survival pathways, DNA repair mechanisms, and ABC transporters. They both have the ability to self-renew and differentiate into the multiple cell lineages of their parent organ while having a higher clonogenicity than other, more differentiated, cells [1]. While these observations have been made in breast [76] and in the hematopoietic systems [10], the intestine offers a good model to compare normal and cancer SC subsets. Lineage tracing studies allowed the identification of Wnt target gene LGR5 as a marker of mouse intestinal SCs. Indeed, LGR5⁺ cells were able to generate all the epithelial lineages of the adult organ [16] as well as form crypt-villus structures in vitro, recapitulating the in vivo architecture and differentiation status [77]. Lineage tracing studies also revealed LGR5⁺ SC activity in mouse intestinal adenomas [78•]. Importantly, specific deletion of APC in LGR5⁺ intestinal SCs produced rapidly growing adenomas, suggesting LGR5 as a bona fide intestinal CSC marker [49]. While LGR5 expression is a

shared characteristic between normal and cancer SCs, its expression is higher in cancer than in normal tissue and positively correlates with Ki67 staining, histological grade, and lymph node involvement and distant metastases [79]. Recent evidences indicate that LGR5 does not simply mark normal and cancer SCs of the intestine, but is also a regulator of Wnt responses, cell motility and cell–cell adhesion [80].

Besides Wnt, another shared pathway between normal and cancer SCs of the intestine is the Notch pathway, which normally regulates cell lineage fate decisions [81, 82]. Indeed, emergence and self-renewal of colon CSCs were shown to be dependent on Notch [83]. Notch signaling functions through γ -secretase, an enzyme which acts to release the intracellular domain of the Notch receptor, allowing translocation to the nucleus and activation of transcription [84]. Treatment with a γ -secretase inhibitor (GSI) converts both normal intestinal and intestinal adenoma cells to post-mitotic goblet cells, reduces proliferation and induces apoptosis, indicating Notch has a critical role in proliferation [83, 85–87]. Likewise, GSIs have been found to inhibit the self-renewal and proliferation of breast CSCs [88]. If GSIs are to be used in combination with chemotherapy, a delicate balance will be required to target CSCs while sparing normal SCs [89].

Regardless of how they come to be, the fact that CSCs share so many fundamental characteristics with normal SCs is a difficult obstacle to overcome. The possibility of adverse effects is easy to ignore in in vitro settings that promise therapies effectively targeting the CSC population. Homeostasis requires a delicate balance of DNA repair, transport, proliferation and self-renewal to sustain tissue maintenance without allowing the formation of CSCs. Systemic treatments targeting these shared pathways may harm normal cells in a way that tips the balance to a different disease later in life. Therefore, it is necessary to identify distinct differences that can be exploited between normal and cancer SCs from the originating organ such as specific markers or opposing molecular mechanisms. Alternatively, treatments that offer protection and/or recovery for normal cells may prove profitable.

Conclusion

Cancer response to environmental stressors, including treatment, is regulated by DNA repair, genomic instability and expression of ABC transporters in CSCs. Stochastic alterations in response to these stressors may cause the identity of the CSC to change over time allowing only the fittest cell to prevail. Any therapy targeting CSCs may also destroy healthy tissues. A priority for researchers will be to identify important differences between normal and cancer

SCs so that therapies can be designed specifically to target CSCs while sparing normal SCs.

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- Of importance
- Of major importance

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