Cancer stem cells: Concepts, challenges and opportunities for cancer therapy

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Abstract

Cancer stem cells (CSCs) are a subpopulation of cancer cells with self-renewal capacity, fuels tumor growth and contributes to the heterogeneous nature of tumors. First identified in hematological malignancies, CSC populations have to date been proposed in solid tumors in various organs. In vitro and in vivo assays, mouse genetic models, and more recently the rise of single-cell sequencing technologies and other "-omics" methodologies have not only facilitated the identification of novel CSC populations but revealed and clarified novel properties of CSCs. Increasingly, both cell autonomous and CSC niche factors are recognised as important contributors of CSC properties. The deepened understanding of CSC properties and characteristics would enable and facilitate the rationale design of CSC-specific therapeutics that would, ideally, have high selectivity for cancer cells, eliminate tumor bulk and prevent tumor recurrence. Addressing these issues would form some of the key challenges of the CSC research field in the coming years.

1. Introduction

Over the past decades, a subpopulation of cells within cancers termed cancer stem cells (CSCs) have become an obsession for many researchers. In this chapter we give a background to this subject of intense research drawing from historical perspectives, models, key features, techniques of study of CSCs and finally offer some future research directions for the field that would be key to understand CSCs and their implications for cancer therapy.

2. Historical background, features, and controversies of CSCs

Tumors are made up of a myriad of cells that have functional and phenotypic heterogeneity. One of the factors to account for the functional and phenotypic diversity in tumors is a distinct population of self-renewing malignant cell population termed cancer stem cells (CSCs). CSCs are distinguished by the ability to self-renew and having the developmental potential to recapitulate a variety of cell types found in a tumor.

The first ideas of CSCs came from the hematopoietic system. Normal hematopoeitic stem cells can give rise to leukemic stem cells which are cancer initiating cells of acute myeloid leukemia (AML) [1, 2]. From these

studies, a cellular hierarchy in tumorigenesis was first proposed where CSCs at the apex can give rise to more-differentiated, short-lived progeny. Of note, while there is clear evidence of the presence of CSCs in tumors arising in blood cell lineages that mirror normal developmental cues, there is considerable debate on the presence of CSCs in solid tumors. This is, in part, due to the technical challenges to isolate and characterise functional CSCs (discussed below) in these tumors. Nonetheless, at present, many studies have identified putative CSCs in a variety of solid tumors such as the breast, colon, brain, skin and the intestine (Table 1) Notably, even within a single tumor, several distinct CSC populations may exist [3, 4].

Two main models (and their variations) (Figure 1) have been proposed to describe tumor growth and the acquisition of heterogeneity. The hierarchical model (mediated by single or multiple CSCs) assumes a fixed or rigid cellular hierarchy akin to normal development. At the top of the hierarchy is the CSC. Below that, at each level of the hierarchy, cells can gain or lose a mutation that will result in the formation of differentiated, heterogeneous clones. A stable CSC phenotype suggested in this model precludes the stochastic interconversion of stem-like cells and more differentiated cell types.

A more fluid or dynamic model termed the stochastic or clonal evolution model suggests that cells can enter and exit the stem cell state depending on intrinsic factors like genetic evolution, cell state changes, and extrinsic microenvironmental stimulus. This model suggests that all cells would have the same tumor initiating capacity, yet tumor initiating capacity is stochastically restricted to a subset of cells within the tumor population. With appropriate stimuli (e.g. gain of an advantageous mutation), any cell can potentially give rise to a dominant clone within the tumor.

It is pertinent to note that both models are not mutually exclusive and can be viewed as integrated processes. Stochastic events could allow cells to dedifferentiate to become CSCs and which would then give rise to hierarchically organised cell populations. These processes could be reiterated during the course of tumor evolution.

Several features of CSCs deserve clarification here. To the uninitiated, the term CSC may evoke some assumptions that are not necessarily warranted. Indeed, the term suggests that the cell-of-origin of CSCs is *necessarily* a normal stem cell as in the case of AML [1, 2], intestinal

cancer [5] and basal cell carcinoma [6]. This notion, however, is being steadily disproved in various liquid and solid tumor models which show that non-stem cells or differentiated progenitor cells may adopt a CSCs phenotype and contribute to tumor growth and bulk, as suggested by the stochastic model [7-9]. These observations highlight the plasticity of cellular phenotypes during tumorigenesis.

In relation to normal stem cells characteristics, CSCs have been thought to be quiescent and divide asymmetrically. The experimental evidence supporting these traits are few, and warrant further clarification due to their implications on therapy (see section below).

Another frequently debated issue is the frequency of CSC in a given tumor which is usually assumed to be small akin to the frequency of stem cells in normal tissues. While many studies show a low frequency of CSCs in tumors, at least a few observations counter this claim. When lymphomas and leukemias of mouse origin are transplanted into histocompatible mice, a very high frequency (at least 1 in 10) of the tumor cells can seed tumor growth [10]. In melanoma, it has been proposed that CSC frequency can vary between 2.5 to 41% [11].

3. Methods to study CSCs

The CSC field has traditionally relied on a few methods to study CSCs, oftentimes drawing inspiration from the hematopoietic stem cell field which has pioneered most of them. The purification and isolation of CSCs by specific cell surface marker expression in AML has shown that a particular subset of cells in the hematopoietic niche characterised by CD34+CD38- was able to give rise to AML [1, 2]. Attempts to isolate CSCs from solid tumors such as the breast, colon, brain, intestine and skin by cell surface marker expression or immunophenotyping have proven to be more challenging. Part of the challenge and difficulty is the observation that there is no one universal CSC marker for all tumors from various organs. Moreover, CSCs marker expression could change with tumor stage and evolution due to cellular and phenotypic plasticity [12].

However, several more common markers may be helpful in initial assessments of CSC activity such as CD44, CD133 and EpCAM (see table 1 for a list of CSC markers). Another intriguing marker of CSCs which is usually used to assess CSC activity is ALDH, originally identified in hematopoietic stem cells [13]. ALDH activity is linked to drug resistance. In solid tumors, several studies have pointed to ALDH high activity as a marker for CSCs in malignant human breast stem cells [14] and many other solid tumors. Like other molecular markers, ALDH expression may change during tumor evolution. [15].

Besides the immunophenotyping techniques described above, the self-renewal capacity of CSCs is typically assessed by the ability of cells to form spheres in vitro [16]. Many efforts have been devoted to the derivation of 3D tumor sphere models from a variety of organs to study CSCs. Our own work show that non-small cell lung carcinoma (NSCLC) tumor spheres have enhanced CSC or tumor initiating characteristics compared to their isogenic adherent cell lines counterparts (Wang et al, manuscript in review).

CSCs are defined by clonal long-term repopulation capacity. As such, candidate CSCs can also be assessed by limiting-dilution transplantations, oftentimes with supportive material such as Matrigel, in immuno-compromised mouse hosts. The assay tests for the ability of transplanted cells, at low or clonal densities, to form a tumor in vivo and recapitulate the phenotypic and heterogeneity of the parental tumor. By definition, CSCs should be able to initiate tumors in multiple rounds of in vivo serial passaging. In the absence of other corroborating evidence drawn from other assays, this particular feature has led some in the field to adhere to the more puristic term “tumor- initiating cells” or “tumor propogating cells” over the more contentious CSCs. Moreover, it has been proposed that the term CSC should be restricted to cells that can be

prospectively isolated from tumors. If this is not the case, the functional term TIC is perhaps more appropriate.

The limiting-dilution transplantation method, when performed with a range of cell doses, can also be used to estimate the frequency of CSCs, and compare the “stemness” of various CSC populations within or across tumors. However, this approach suffers from fact that the transplantation site, typically subcutaneous, may not fully recapitulate the native tumor microenvironment essential for tumor growth and produce artificial selection biases. For example, whereas primary serous ovarian cancer contain a large proportion of CD133+ CSCs, the majority of xenografted tumors contain a large proportion of CD133- TICs [17]. It is imperative that researchers test a variety of transplantation sites and methods when assaying CSCs.

Techniques that rely on isolating and studying CSCs in vitro, though informative, may mask or lead to biases in true CSC characteristics in vivo. Studies of normal mammary stem cells have shown that whilst a single basal cell can give rise to the entire epithelial network of the mammary gland ex vivo [18, 19], using lineage tracing methods, basal cells are shown to be unipotent in vivo [20, 21]. Van Keymeulen and Lee et al [22] show by in vivo lineage tracing that basal and luminal cells only become multipotent upon overexpression of a potent oncogene like

PIK3CA-H1047R or in combination with p53 loss-of-function. This and other observations highlight the importance of niche and microenvironment-specific signals that govern cellular fates (see section below on intrinsic and extrinsic factors).

The caveats of studying CSCs in vitro have intensified efforts in developing new animal models and in vivo lineage tracing methods which are touted as the gold standard for studying CSCs. In lineage tracing, a cell or populations of cells are marked by a promoter-specific transgenic reporter. Upon genetic recombination, the transgenic reporter, typically a fluorescent marker or beta-galactosidase, is transmitted to all of its progeny. This would allow the establishment of cellular hierarchy in vivo. Using this method, Boumahdi et al [23] show that basal skin cells expressing Sox2 represent a CSC population in squamous cell carcinoma. Importantly, upon lineage ablation of the Sox2 population, tumors were eradicated, demonstrating that Sox2+ cells make up an important cell lineage that sustains tumor growth. Similarly using lineage retracing methods, the Clevers group show the important contribution of Lgr5+ cells in intestinal and colorectal tumors in vivo [5]. Likewise, Nestin+ cells make up a substantial part of glioblastoma [24].

In the absence of markers or when markers are unstable, unbiased lineage tracing methods can be particularly informative to track CSCs and observe clonal cell growth over a long time frame. Zomer A (2013) et al [25] performed unbiased lineage tracing in mouse model of breast tumors to characterize the nature of the tumor growth and identified the presence of CSCs. Using Rosa-Cre confetti reporter that effectively labels any cell in the tumor independent of biased cell marker expression, researchers detected the presence of large unicolor cell progenies in vivo, suggesting the contribution of a single cell population. An improved intravital imaging further confirmed the presence of these large clones in the same mice that had multicolor smaller clones at the start of the recombination. These results suggest that, while multiple cell types may initially contribute to tumor propagation at varying levels, a single population ultimately dominates. This mode of tumor growth is reminiscent of the hierarchical CSC model (Figure 1).

Using CA-30 somatic based mutation tracing, the presence of CSC-mediated tumor propagation was also observed in vivo in mouse adenomas [26]. Depending on the number of CSCs present and the size of the adenomas analyzed, small or large clones emerging from single cells were observed and remained stable for more than 1 year suggesting the long term contribution of CSC that fuel the growth of the adenomas in

vivo. Such somatic-based lineage tracing techniques could be useful to study CSCs in human tumors. Indeed, a combination of nuclear and mitochondrial DNA lesions and methylation patterns elucidated the clonal dynamics of human colorectal adenomas [27]. In addition, CRISPR/Cas9-based lineage tracing methods that are widely applicable in various model systems could be powerful tools for future research.

4. Single cell analysis of CSCs

To date, our understanding of CSCs have been mostly derived from studying cells in groups, obscuring observations that may be gathered from analyses at the single cell level. With the advances in single cell analyses, transcriptomic, epigenetic and protein expression profiles differences between two cells are being captured at the highest resolution and we are now in a better position to clarify the concepts of ‘cell states’, ‘cell fate’ and ‘cell potential’ (Figure 2). In order to define the presence of distinct CSCs and differentiate it from non-CSCs, it is critical to understand if CSCs and non-CSCs that may present themselves as distinct cell types (based on the expression of a surface markers) have (1) dynamic and oscillating genetic programs suggesting a high chance of inter-conversions and plasticity (cell states), (2) are separated by refined and

stable genetic programs suggesting a non-convertible and compartmentalized cell types (cell fate) or (3) if CSCs and non-CSCs respond to activating or inactivating stimuli enabling them to contextually produce cells of all lineages in the tumor (cell potential). While current bulk cell analyses can display differences between CSCs and non-CSCs, minor oscillating gene expression levels, openness of the chromatic regions and the additional regulatory measures during mRNA translation steps must be characterized at the single cell level to understand if the tumor is governed by stable or unstable CSC population(s). This would allow the identification of irreversible or permanent phenotypic markers, if any, for the prospective isolation and characterization of CSCs. Prior to single cell assays, a landmark study published in 2008 that analysed differential levels of Sca-1 expression in cells by flow cytometry suggested that what was previously described as distinct progenitor populations based on Sca-1 low or Sca-1 high expressions were not two distinct cell types but were the same cells at different stages of the cell cycle that exhibited an unsynchronized transcriptional machineries leading to varying levels of Sca-1 gene and protein levels [28].

In human glioblastomas single-cell analysis revealed new transcriptional programs that were different from the dominant transcriptional program obtained using bulk analyses and proposed the presence of the hybrid cell

states and their impact on patient prognosis [29]. In addition, single cell profiling of colorectal tumors identified the presence of new FAP^{-ve} stromal fibroblast that may not respond to potential FAP-directed therapies and aid in paracrine TGF-b signaling for tumor cell survival and propagation. Rather provocatively, this report showed that EMT-signatures were enriched in fibroblasts compared to epithelial tumor cells [30], an observation that would have been masked by bulk tumor analyses.

Put together, these findings and many other studies in normal homeostasis and cancer (reviewed in [31]), continue to stir debates on how to view phenotypic and functional cell types in normal and neoplastic samples and the subsequent definitions for 'cell states', 'cell fate' and 'cell potential'. Thus, in addition to bulk cell analyses, single cell assays are and will be enormously useful to understand the biology of tumor heterogeneity, identify and characterize novel cell types and identify potential therapeutic targets.

5. EMT and CSCs

EMT and CSCs have been inextricably linked in many instances. During cancer evolution, cells may, from a polarised epithelial organisation, adopt

an undifferentiated, migratory, and invasive mesenchymal cell state. This process mirrors the morphogenetic events that occurs during embryonic development. Intriguingly, tumor cells have been shown to exhibit features of embryonic cells [32, 33]. In cancer, EMT occurs upon over-expresssion of classical transcription factors Zeb1, Twist1, Snail and Slug, leading to the gain of stem-cell like properties, tumorigenicity [34-36] and the formation of metastasis [37]. Other co-activators may be involved in this process. For example, together with Slug, Sox9 has been shown to be an important co-activator to enhance EMT and promote metastasis in breast cancer cell line xenografts [38]. In addition, the activation of signalling pathways (TGF-β, FGF, EGF, HGF, Wnt/beta-catenin and Notch) and hypoxia may induce EMT.

The potential to undergo EMT may differ between cell types, leading to differential CSC features. While skin squamous cell carcinomas (SCC) derived from interfollicular epidermis are generally well-differentiated, hair follicle stem cell-derived SCCs frequently undergo EMT, efficiently form secondary tumors, and possess increased metastatic potential. These differences are due, in part, to distinct chromatin landscapes and gene regulatory networks that cooperate to prime EMT gene expression particularly in the hair follicle lineages [39].

It is increasingly recognised that EMT is not a binary process but a dynamic one which produces a variety of intermediate cell states. Indeed, subtypes of ovarian cancer with varying E and M characteristics and corresponding tumorigenic properties have been proposed [40]. A recent study elegantly demonstrated a variety of EMT phenotypes in vivo. By profiling tumor cells by cell surface marker expression and single cell RNA-seq, Pastushenko et al [41] uncovered subpopulation of cells with E, M and hybrid EM states. Intriguingly, all subpopulations of cells exhibited similar tumor initiating frequency as assessed by transplantation into immunocompromised mice. However, hybrid epithelial and mesenchymal cell populations show the greatest lung metastasis potential while mesenchymal cell populations in general showed higher levels of cellular plasticity and invasiveness. This report and others have also unveiled specific spatial localisation of EMT cell populations in tumors. In breast cancer, specific CSC populations have been proposed for epithelial and mesenchymal regions within the same tumors. While ALDH marked epithelial CSC, CD24-CD44+ marked the mesenchymal CSCs that were specifically located at the invasive front and less proliferative compared to the epithelial CSCs [3]. Similarly, single cell analyses of head-and-neck SCC have uncovered cells with partial EMT features that spatially localize to the leading edge of primary tumors and facilitate invasion [42].

EMT may form part of the mechanistic basis of the stochastic model where cells along the hierarchy may stochastically adopt a more mesenchymal cell state which will contribute to increasing stemness. Indeed, populations of non-CSC have been shown to spontaneously undergo EMT under appropriate conditions, acquiring CSC-like cell-surface markers and an enhanced capacity to seed tumors in mice [43, 44].

6. CSC metabolism

The identification of deregulated cell metabolism as a hallmark of cancer has opened new avenues for cancer research. Increasingly, CSCs have been shown to harbour unique metabolic phenotypes that are unique from non-CSCs.

Several reports suggest that CSCs are more glycolytic (Warburg effect) than other differentiated cancer cells in vitro and in vivo in osteosarcoma, glioblastoma, breast cancer, lung cancer, ovarian cancer and colon cancer. Glycolysis was also found to be the preferred metabolic programme in radioresistant sphere-forming cells in nasopharyngeal carcinoma [45] and CD133^{+}CD49f^{+} TICS in hepatocellular carcinoma [46].

Apart from glycolysis, CSCs may exhibit increasing reliance on oxidative phosphorylation (OXPHOS) for metabolism. For example, patient-derived

glioblastoma relied more on OXPHOS than their differentiated progeny. The same is true for sphere-forming and CD133^{+} cells for both glioblastoma [47] and pancreatic ductal adenocarcinoma (PDAC) [48]. Importantly, these studies also suggest that OXPHOS is intricately linked to self-renewal and in vivo tumorigenenic capacity of CSCs. Metabolic vulnerabilities associated with amino acid metabolism have also been described in CSCs. CD166+ CSCs in NSCLC were found to exhibit high expression of the enzyme glycine decarboxylase (GLDC) which promotes tumorigenesis via its metabolic activity [49]. Following this study, using isogenic tumorsphere and adherent NSCLC models, Wang et al (manuscript in review) identified MAT2A and methionine pathway as a vulnerability in the same CD166+ NSCLC CSCs. Small molecule chemical inhibition of MAT2A by FIDAS was effective in hampering its tumor initiating capacity.

Altered lipid metabolism may be another hallmark of CSCs. Self-renewal in both hematopoietic stem cells and leukaemia-initiating cells appears to be dependent on FAO [50, 51] while Inhibition of FAO with JAK/STAT3 inhibitors preferentially eliminates CD44+ breast CSCs compared to non-CSCs [52]. A recent study show that CD44 high metastasis-initiating cells of oral carcinoma express high levels of the CD36 fatty acid receptor and lipid metabolic genes [53].

Altogether, such metabolic vulnerabilities and presumably many more that are going to be uncovered with more sophisticated metabolite profiling techniques and flux analyses may be used as a basis for CSC targeted therapy (reviewed in [54])

7. Cell-autonomous, niche or both? Intrinsic vs extrinsic factors that govern CSC behavior

Genetic and epigenetic changes that occur within the cell undoubtedly contribute to clonal expansion and tumor growth. In addition to these changes, the tumor microenvironment plays a critical role in determining CSC function and properties. The tumor microenvironment consists of immune cells, stromal cells, blood vessels and their secreted factors. Intriguingly, through paracrine interactions, the tumor microenvironment has been shown to initiate stem cell like programs in cancer cells [55, 56]. The inflammatory microenvironment of the tumor may also influence CSC properties. During intestinal tumorigenesis, a bidirectional conversion between CSCs and non-CSCs can be triggered by an inflammatory stroma, which is characterized by elevated NF-κB and Wnt signaling, leading to dedifferentiation of non-CSCs that acquire tumor-initiating capacity [57]

Tumor and microenvironment interactions are often bidirectional. There is evidence pointing to factors produced by CSCs and endothelial cells in the tumor microenvironment that can transform normal fibroblasts into cancer-associated fibroblasts (CAFs) (reviewed in [58]). In turn, CAFs, far from being a passive player in the tumor ecosystem, can promote tumor progression and the induction of stemness. One such example is in pancreatic cancer where CAFs can enable tumor cells to undergo EMT through the secretion of cytokines IL-6 and TCF21 [59]. As well, CSCs in glioblastomas have been shown to secrete VEGF to promote the development of vasculature [60].

It is pertinent to note that the contribution of extrinsic factors could be very different in liquid and solid tumors. While there is some evidence that show that the bone marrow niche [61] and various cytokines and growth factors can govern the fate of LSCs [62], unlike blood, cells in epithelial tissues are in direct contact with different cell types and complex extracellular matrix throughout tumorigenesis, and it is reasonable to propose that, cells originating in epithelial tumors respond more readily to these external factors. Such factors cause CSCs in epithelial tumors to be easily reprogrammed, resulting in remarkable plasticity, expression of varying cell surface markers, and different responses to different assays.

As a result, there are oftentimes ambiguous interpretation of the existence and the nature of CSCs in these tumors.

8. CSC and implications for cancer therapy

The presence of CSCs has important implications for cancer therapy. Cancers that have a CSC-associated molecular signatures often correlate with poor patient prognosis [63]. Interestingly, even in patients with diverse driver mutations, gene expression signatures that are specific to CSCs and normal stem cells are good prognostic markers for patient outcome [63, 64]. This shows that signaling pathways that drive stemness may be advantageous to the cell.

Tumors are known to evolve resistance mechanisms against commonly used therapeutic compounds and this resistance is observed in both the therapies that use specific molecular inhibitors or broad spectrum compounds targeting proliferative cells. Despite using a highly sophisticated chemotherapeutic strategy, or ionizing radiation, a subset of tumor cells remain or develop resistance that later contribute directly to the development of new chemo-resistant tumors. It is not clear if resistant cells pre-exist or develop resistance at the time of therapy but it has been widely speculated that, CSCs are inherently chemoresistant or highly quiescent to evade therapies specific to proliferative cells. Expression of

known CSC markers such as ABC transporters that often efflux drugs such as doxorubicin and paclitaxel [65, 66] out of the cell may provide the link between CSC and chemoresistance. Conjugating nano particles to the drugs can decrease the efflux activity of ABC transporters but there is limited evidence in support of this and a number of clinical studies focused on ABC transporters are underway [67]. ALDH, a known CSC marker in many tumors is also known to be involved in chemoresistance but its exact mechanisms and the effect of its enzymatic activity remains to be characterized. B-catenin mediated Wnt signaling, Notch signaling and BCL-2 pathways that are often associated with CSCs may also aid in chemo and other drug resistance [68-70]. Notably, combined inhibition of Wnt signaling and Hedgehog signaling was sufficient in eliminating a Lgr5+ cell population that emerged after vismogenib treatment of basal cell carcinoma [71]. It is important to note that, the mechanisms of drug resistance by ABC transporters, ALDH or other signaling pathways are different in different tumors and the efficacy of each CSC marker as a potential drug resistant target must be studied in the context of a specific tumor type.In this section we will review a few strategies that are currently used or considered to specifically target CSCs in cancer therapy.

In the following section we will review a few strategies that are currently used or considered to specifically target CSCs in cancer therapy (Figure 3).

8.1 Reversing EMT

The reversal of cell states from mesenchymal to epithelial or the induction of mesenchymal to epithelial transition (MET) is an attractive strategy to reduce cancer cell stemness, perturb invasiveness and migratory properties and the development of metastasis. One such proof-of-concept is the activation of PKA in mammary mesenchymal cells which was shown to be effective in inducing a more epithelial phenotype and increase sensitivity of cells to chemotherapy [72]. Strategies to reverse the epigenetic reprogramming induce by Zeb1 in pancreatic and breast cancer cells models have also been shown repress stemness and overcome drug resistance [73]. Besides overcoming drug resistance, it could be envisioned that the induction of MET could limit the tumor initiating capacity of mesenchymal cells.

A major challenge in this therapeutic strategy is to define a therapeutic window that would promote epithelialisation in primary tumors, while

excluding metastatic cells that have already disseminated from primary tumors. The latter scenario may conceivably promote epithelial colonisation leading to the undesirable formation of potentially detrimental secondary tumors.

8.2 Targeting ALDH

ALDH activity is known to be involved in chemoresistance but its exact mechanisms and the effect of its enzymatic activity remains to be characterized. Targeting ALDH could be a viable therapeutic strategy - simultaneous knockdown of ALDH and gemcitabine in pancreatic adenocarcinoma cells is shown to induce apoptosis and decrease proliferation in vitro [74]. Many small molecule inhibitors for ALDHs have been successfully developed. Of these, inhibiting ALDH1 with diethylaminobenzaldehyde (DEAB) has been shown to sensitize ALDH+ breast cells to paclitaxel and epirubicin [75]. ALDHs can also be targeted with vitamin A-related compounds, known as retinoids which have been shown to increase effectiveness of standard chemotherapy (Loo et al, manuscript in preparation) [76-78]. These drugs activate retinoid acid signaling which decreases the expression of stemness markers promoting cellular differentiation, promotes cell cycle arrest and decreases cellular proliferation and reduces tumor growth in mice (reviewed in [79]).

8.3 Immunotherapy for CSCs

Immune cells could be harnessed to eradicate CSCs. In AML, targeting of the receptor CD47 by monoclonal antibodies in CD34+ LSC is sufficient to promote phagocytosis by macrophages while sparing normal cells [80]. Moreover, in combination with Rituximab, anti-CD47 therapy show an increased efficacy in eradicating non-Hodgkin lymphoma [81]. Another approach that is clinical trials involves the administration of autologous dendritic cell vaccines to glioblastoma patients [82]. With our increased understanding of unique molecular regulators that are unique in CSCs, CAR-T cell based immunotherapies against CSCs may dominate therapeutic options in the near future (reviewed in [83]).

Several challenges with this therapeutic strategy remain nonetheless. CSCs seem to have unique immune evasion features including overexpression of PD-1/PD-L1 molecules. In melanoma, the ABCB5+ CSC cellular subset selectively express the B7.2 (a CTLA4 ligand) and PD-1 (PD-L1 receptor) as compared to bulk and negative populations [84]. Similarly, in lung SCC, SCA1+NGFR1+ cells which display increased tumor-propagating activity compared with bulk cells also show enrichment for PD-L1 expression [85]. One mechanism to account for the increase in PD-L1 expression in CSCs have been proposed recently. In breast

cancer, EMT may enrich PD-L1 expression in CSCs by the EMT/β-catenin/STT3/PD-L1 signaling axis. Consequently, the induction of MET downregulates PD-L1 and promotes anti-tumor immunity [86]

8.4 Targeting self-renewal and reprogramming pathways of CSCs

Targeting the molecular regulators of reprogramming that limits the ability of cells to gain stem-like state, inter-convert to other cell types or direct towards differentiation are potential therapeutic strategies for CSCs. Pharmacologic inhibition of reprogramming or self-renewal pathways in CSCs may have therapeutic value (reviewed in [87]). Several pre-clinical models lend support to this approach - inhibition of the Hedgehog pathway in leukemias inhibited the expansion of imatinib-resistant CML [88, 89]. Notch pathway inhibition in brain cancer promoted its sensitivity to radiation [90]. However, it is increasingly clear that combination approaches to overcome the crosstalk among Notch, Hedgehog and Wnt pathways, as well as other signalling pathways would be more effective than single agents or combined single agents-chemotherapy regiments [87]. An ongoing clinical trial explores the effect of cirmtuzumab, a ROR1-based humanized monoclonal antibody drug, on patients with relapsed or

refractory chronic lymphocytic leukemia (CLL) [91]. Aberrant expression of ROR1 is seen in many malignancies and has been linked to RhoGTPase activation and cancer stem cell self-renewal. However, toxicity may be a concern given that such self-renewal pathways are also activated in normal stem cells,

8.5 Targeting the CSC niche

Some attempts to target the malicious CSC niche have already shown promise. Targeting hypoxia could be useful to eradicate quiescent, drug-resistant cells. HIF-1α and HIF-2α, which promote cell cycle via c-Myc, represent a promising target for therapy for glioma patients [92, 93]. Anti-angiogenic therapies may be helpful to limit CSC function in vivo. VEGF inhibition can deplete the tumor vasculature and ablate self-renewing CSCs and inhibit tumor growth. Similarly, blocking DLL4-mediated signaling in tumor and vascular cells is effective to inhibit growth of colon tumor xenografts [94]. Lastly, depletion of TAMs by inhibiting either CCR2 or M-CSF receptor resulted in decreased CSCs in pancreatic tumors, improved chemotherapeutic efficacy, inhibited metastasis, and increased antitumor T-cell responses [95].

One interesting way to decrease CSC function is to disrupt the interactions of CSC and the niche. This could involve disrupting chemokine receptors that are expressed on CSCs such as CXCR4 [96, 97].

9. **Summary and Perspectives**

Until recently, cancer has always been perceived as a group of cells with uncontrolled proliferation and initial cancer therapies have been focused on halting proliferation or promoting apoptosis. The CSC concept has provided researchers with a new way to look at tumors and re-think strategies for cancer therapy. Since the discovery of CSCs, we are now viewing and studying tumors as highly regulated models with extensive heterogeneity, clonal cell cooperations which evolve mechanisms to undergo metastasis and resist therapy.

The presence and the biology of CSCs remains to be clarified with systematic characterization of tumor-type specific CSCs in patients with reliable and reproducible phenotypic markers and molecular targets that are distinguishable from non-CSCs. Studies that characterize CSC content and catalog the heterogeneous cell types in patient tumors with long-term prognosis implications are necessary to translate the laboratory findings of CSCs to a potential diagnostic tools or therapeutic applications in clinics. With the advent of single cell techniques, researchers are now

beginning to characterize the heterogeneity and the presence of CSCs in patient tumors and a refined CSC catalog in multiple tumor types is expected to be revealed in the next few years. These efforts, together with the deepening knowledge pool from various approaches will allow the design of specific therapies that target CSCs which, hopefully, may lead to the complete eradication of cancer.

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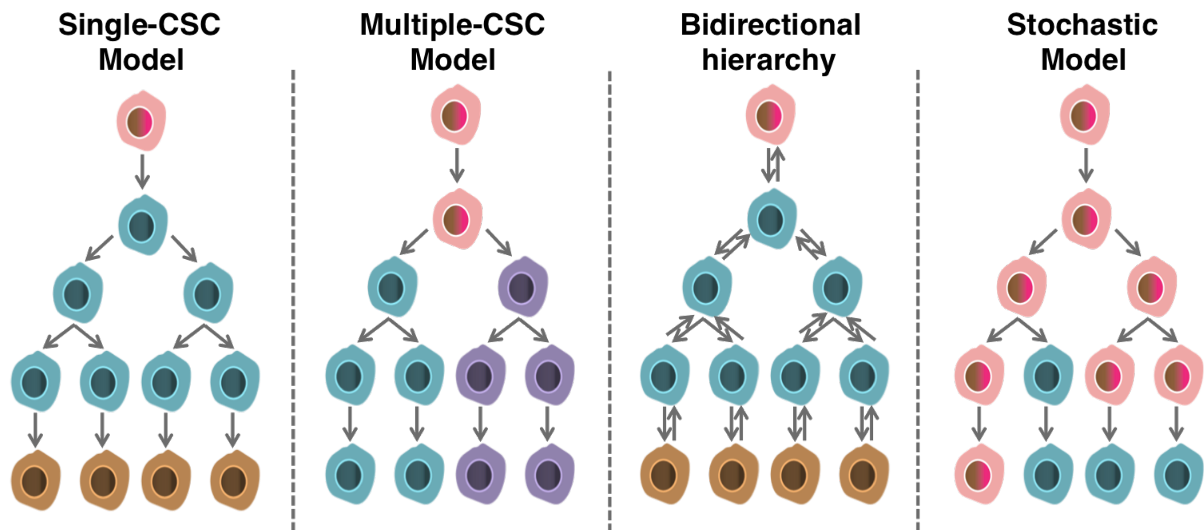

Figure 1: CSC models: Tumors can be propagated by a single or multiple CSCs (pink cell) that produces single or multiple progenitors/transient amplifying cells (blue and purple cells) and differentiated cells (brown cells) in a hierarchical manner. In some tumors, all cells display plasticity and are able to produce all cell types in the tumor (stochastic model). Lastly, CSCs subjected to intrinsic and extrinsic changes generate CSC or progenitors/transient amplifying cells (blue and purple cells). This implies that the overall CSC content may change at all times.

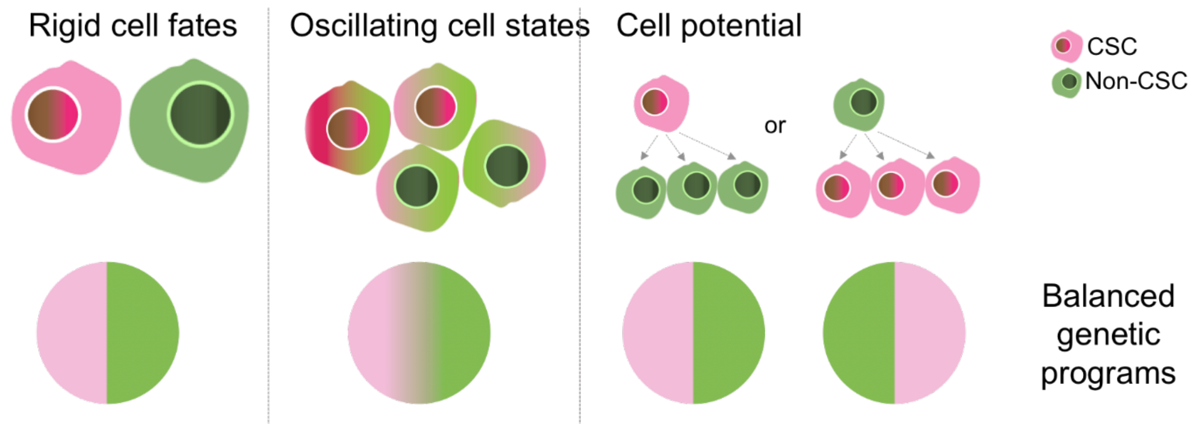

Figure 2: Identification of CSCs or non-CSCs: Differences between two cells could occur as different cell fates (with green and red cells displaying distinct genetic programs), cell states (where most cells share genetic programs common to green and red cells but a balanced expression favors cells biased either towards green or red identities) or cell potential (where the distinct red or green cells can generate other lineages of each other depending on the external stimuli or the assay used to test the potentials)

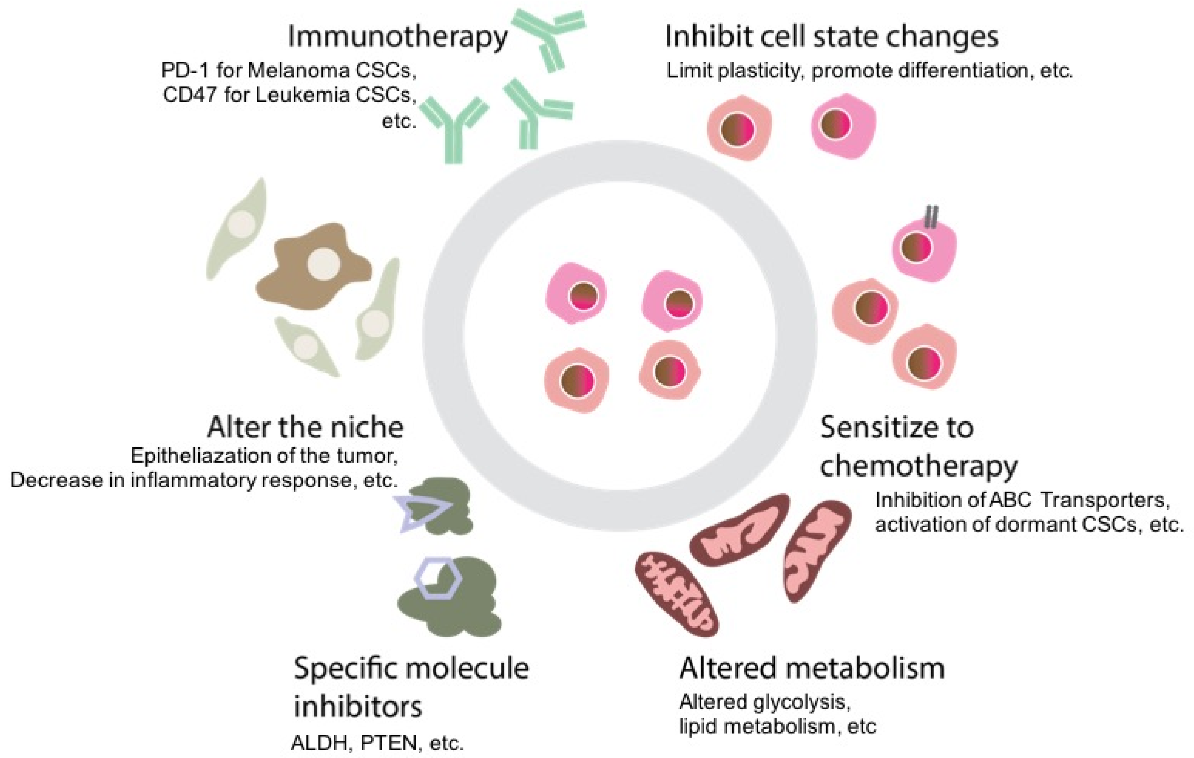

Figure 3: Therapeutic strategies for CSCs. The unique characteristics of CSCs present new options for cancer therapy which could be complemented with existing systemic therapies such as chemotherapy (see text for more details).

Table 1: CSC markers in specific tumor-types

Tumor type	CSC marker	Reference
Acute myeloid leukemia	$CD34^+CD38^−$,$CD47^+$, $CCL\text{-}1^+$,$CD96^+$, $TIM3^+$, $CD32^+$, $CD25^+$, ALDH	[1, 2, 80, 98-101]
Non-Hodgkin's lymphoma	$CD47^+$	[81]
Bladder	$EMA^−CD44v6^+$	[102]
Bone sarcoma	$Stro\text{-}I^+CD105^+CD44^+$	[103]
Breast	$CD44^+CD24^{−/low}$, EPCAM, ALDH	[104]
Brain	$CD133^+$	[105]
Colorectal	$CD133^+$, EpCAM, Lgr5 $CD133^+$, $CD166^+$, $CD44^+CD24^+$	[5, 106-108]
Gallbladder	$CD44^+CD133^+$	[109]
Gastric	$CD44^+CD24^+$	[110, 111]

Head and neck	CD44+, CD24+	[112, 113]
Liver	CD133+, CD44+CD90+ ESA^{+}CD133^{+}CD90^{+}CD44^{+}CD24^{+} EpCAM	[114-116]
Lung	Scal+CD45−Pecam−CD34+	[117]
Melanoma	CD20+	[118]
Ovarian	CD44+CD117+	[119-121]
Pancreas	CD24+CD44+ESA+ ESA^{+}CD44^{+}CD24^{+} EPCAM	[122]
Prostate	CD44+α2β1+CD133+ , EpCAM, CD44, Sca1+	[123]
Renal	CD105+	[124, 125]
Skin SCC	Sox2	[23, 126]
Skin BCC	Sox9	[127]
Intestinal	Lgr5	[5]
Esophagus	CD90	[128]

Table 2: Methods to identify, assess, and isolate CSCs

Techniques	**CSC characteristic assessed**	**Advantages**	**Disadvantages**	**Model systems**
Immunophenotyping / cell surface marker expression	Stemness	Easy, fast Efficient way to isolate putative CSCs	Limited applicability	Cell lines, Tumors
Hoechst dye exclusion, side population	Quiescence	Easy, fast	Limited applicability	Cell lines, Tumors
ALDH activity	Drug resistance	Easy, fast	Limited applicability	Cell lines, Tumors

Limiting dilution transplantation and serial passaging	Tumor initiation	Ability to assess tumor heterogeneity Quantitative measure/estimate of CSC frequency	Expensive Reliance on immuno-compromised models	Cell lines, Tumors
Sphere formation	Self-renewal	Easy Ability to derive patient-models	Limited applicability	Cell lines, Tumors
Lineage tracing	Self-renewal, clonogenicity	In vivo context Ability to study long-term repopulation	Expensive, Heavy reliance on mouse genetic models	Mouse, Zebrafish
Single-cell profiling	Cellular hierarchy	Unbiased	Expensive Heavy reliance on computation	Tumors

		Ability to assess tumor heterogeneity	methods and power	