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REVIEW

Improving cancer therapy by targeting cancer stem cells: Directions, challenges, and clinical results

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Abstract

Cancer stem cells (CSC) are a rare cell population within a tumor characterized by the ability to form tumors following injection into an immunocompromised host. While the role of CSC has been clearly established in animal models, evidence of their clinical relevance has been harder to demonstrate. A number of markers, or combination thereof, have been used to detect and measure, although non-specifically, CSC in almost all human tumors. Several pathways have been identified as crucial for, but not necessarily unique to, CSC survival and proliferation, and novel agents have been designed to target such pathways. A number of such agents have entered early phase development. Further, drugs that have long been marketed for non-oncological indications have been redirected to oncology as they appear to affect one or more of such pathways. This article aims to review the available evidence on the clinical relevance of CSC from a drug development standpoint and the results of early phase clinical trials of agents interfering with the above pathways. It also discusses limitations of current clinical trial design and endpoints to demonstrate anti-CSC activity as well as possible strategies to overcome these limitations.

Key words: Cancer stem cells; Cancer; Time to new metastasis; Tumor heterogeneity; Drug development

Core tip: Cancer stem cells are a rare cell population with two embedded fundamental properties: self-renewal and differentiation into the heterogeneous lineages of cancer cells that comprise the tumor. While the identification and role of such cells has been clearly established *in vitro* and in animal models, uncertainty remains as to the role they play in human tumors and their relevance as therapeutic targets. The article summarizes and discusses the latest findings and challenges on cancer stem cell research, the significance of these cells beyond experimental models and suggests avenues for development of cancer stem cell targeting agents.

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INTRODUCTION

In order to explain tumor heterogeneity, the clonal theory of cancer initiation and progression was proposed[1], stating that each cell within a tumor has equal potential in acquiring genetic and/or epigenetic changes, conferring growth advantages and generating new tumors. This model presumes that all cells within a given tumor have the same tumorigenic potential, and it is called classical or stochastic.

In the cancer stem cell paradigm of tumors, heterogeneous neoplastic cells are organized in a hierarchy where only a small subset of cancer cells, the cancer stem cells (CSC), is responsible for tumor initiation and growth maintenance. According to a consensus definition[2], a CSC is a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor. CSC could be tissue stem cells or a more differentiated progeny, which acquired self-renewal capacity[3-5]. There are 2 mechanisms that could mediate the transformation of normal stem cells to CSC[6,7]: (1) Early progenitor cells can gain mutations, which gives them self-renewal capacity; and (2) Fully differentiated cells or cells in the late progenitor stage can become de-differentiated to acquire the properties of stem cells.

Activation of oncogenes or inactivation of tumor suppressor genes are the major types of mutations involved in these processes. CSC may indeed arise from normal stem cells by mutation of genes that make the stem cells cancerous, but this may not be the case in all tumors. For example, in blast crisis chronic myelogenous leukemia (CML), a committed granulocyte-macrophage progenitor may acquire self-renewal capacity and thus "reacquire" stem-like properties due to the effects of later mutations[2]. It is conceivable that more differentiated cells can, through multiple mutagenic events, acquire the self-renewal capacity and immortality that typify CSC. In these examples, a differentiated cell, not the tissue stem cell, eventually evolves to become a full-blown CSC.

The first compelling evidence on the existence of CSC was presented by Bonnet *et al*[8] through their seminal work on leukemic stem cells, where they showed that these cells possess characteristic functional properties of stem cells. They demonstrated that the cell capable of initiating human acute myeloid leukemia in non-obese diabetic mice with severe combined immunodeficiency (SCID)-termed the SCID leukemia-initiating cell-possesses the differentiative and proliferative capacities and the potential for self-renewal expected of a leukemic stem cell. The SCID leukemia-initiating cells were able to differentiate *in vivo* into leukemic blasts, indicating that the leukemic clone is organized as a hierarchy.

CSC have been identified also in solid tumors, including breast, lung, colon, prostate and pancreatic cancers, all supporting the model that cancer derives from a subpopulation of CSC capable of self-renewal to initiate and sustain tumor growth[9,10].

Functional assays were performed in all of these studies, in which cell suspensions of cancer cells were inoculated into immunodeficient mice. This assay is currently considered the gold standard for studying the degree of stemness in a subpopulation of human cancer cells that drives tumorigenicity. In fact, CSC are not necessarily derived from stem cells although they share functional similarities to normal stem cells.

Therefore, the definition of CSC remains mainly operational, *i.e.*, the ability to form tumors in an animal model, which is, although imperfect[11], regarded as the best functional assay to meet the two critical criteria of the consensus CSC definition[2]. The implementation of this approach explains the use of alternative terms in the literature, such as "tumor-initiating cell" and "tumorigenic cell" to describe putative CSC.

However, the results of this assay have been questioned, as it may measure the ability of human tumor cells to grow in immunodeficient mice and not necessarily the actual frequency of CSC in the tumor. It has been demonstrated that only rare human cancer cells (0.1%-0.0001%) form tumors when transplanted into immunodeficient mice. However, modifications to xenotransplantation assays (*i.e.*, the degree of immunodeficiency of mice) can dramatically increase the detectable frequency of tumorigenic cells, demonstrating that they are common in some human cancers[12]. Furthermore, introducing human tumor cells in Matrigel containing a cocktail of growth factors to a foreign species such as a mouse may compromise the growth of human cancer cells[13], indicating the importance of the tumor microenvironment in tumorigenesis. Last, the nature of the proteolytic enzymes used, the duration of incubation, and the temperature at which the tumor cells are dissociated into single cells in order to perform xenotransplantation might further result in the under-estimation of the percentage of CSC, even with the same type of tumor. It follows that if the cancer initiating cells are not rare, the hierarchical model of CSC may be questioned[12]. It should be stressed that the aforementioned findings do not disprove the CSC paradigm. The CSC paradigm is directly related to tumor heterogeneity and tumor's hierarchical organization, and not to the absolute number of these cells[14]. Thus, evidence of CSC in many human tumors has been described using the xenotransplantation model by highlighting that a fraction of tumor cells, and not all cancer cells, are tumorigenic and may thus represent a distinct therapeutic target[15].

From a therapeutic perspective, it is important to acknowledge that other targets exist beyond the CSC themselves to affect this cell population. In fact, the tumor microenvironment, the CSC niche, and cytokine loops play essential roles in the maintenance of CSC and in tumor growth and development[16,17].

Table 1 Commonly used Cancer Stem Cells Markers

Marker	Association with chemoresistance	Reported malignancies
CD34		Hematological malignancies (in combination with CD38)[8,36]
CD38		Hematological malignancies (in combination with CD34)[8,36]
CD24	Yes (in combination with CD44)[63]	Breast, prostate, pancreas (in combination with CD44)[15,23]
CD44	Yes (in combination with CD24)[63]	Breast, pancreas, stomach, prostate, head and neck, ovary, colon (in combination with CD24)[15,23]
CD133	Yes[24,25]	Brain, pancreas, lung, liver, prostate, stomach, colon, head and neck[15,23]
ALDH	Yes[60,61]	Breast, lung, head and neck, colon, liver, ovary[15,26]

ALDH: Aldehyde dehydrogenase.

For example, Feng *et al*[18] demonstrated that tumor microenvironment stromal cells create a growth advantage for CSC in a model of multiple myeloma. In that study, the proliferative capacity of multiple myeloma stem cells was stronger when grown in the presence of stromal cells from patients with myeloma than it was in the presence of stromal cells from normal control bone marrow.

If CSC are relatively refractory to therapies (*e.g.*, chemotherapy, radiotherapy) that have been developed to eradicate the rapidly dividing cells that constitute the majority of the non-stem cell component of tumors, then such therapies are unlikely to be curative and relapses would be expected. If correct, the cancer stem cell hypothesis would require adjustments to the way tumors are diagnosed and treated. The objective would have to be not only to eliminate the bulk of rapidly dividing but terminally differentiated components of the tumor responsible for tumor-associated symptoms, but also the minority stem cell population that fuels tumor growth and is responsible for disease relapse and metastases.

CANCER STEM CELL SURFACE MARKERS

Besides discussion about the pitfalls of the functional assay used to identify CSC, it is intuitive that such an assay is unfit for clinical development of any novel drug as it would require ready availability of immunodeficient mice to be injected with single cell suspensions of patient-derived cancer cells before and after treatment, if one is to demonstrate presence of CSC and their reduction following treatment.

Therefore, more practical assays have been developed to be implemented in clinical research. An *in vitro* tumorsphere assay was used to evidence the self-renewal properties of CSC in human brain tumors[19,20]. This assay is now widely used in clinical research inasmuch as it requires live cells, and this requirement may not be always satisfied in large, multicenter clinical trials. The easiest way to identify and measure CSC within tumors or in the bloodstream would be to use CSC-specific or - associated cell surface marker proteins. By staining cells with antibodies against these markers, populations of interest could be easily identified and measured by either flow cytometry (which requires live cells) or immunohistochemistry (which does not require live cells).

Several markers are commonly used to isolate CSC in normal and tumor tissue, but it is not clear if they are universal markers for characterizing CSC from all tumor types. Furthermore, expression of markers may not be restricted to the CSC population and may be present in normal stem cells, early progenitor cells and normal tissues.

Although many markers have been proposed to define CSC populations, here we focus on the ones most widely used in the literature (Table 1).

The pentaspan transmembrane glycoprotein CD133, also known as prominin-1, was discovered as the target of the AC133 monoclonal antibody, specific for the $CD34^+$ population of hematopoietic stem cells. Subsequently, it was shown to be expressed by a number of normal stem and progenitor cells including those of the epithelium, where it is expressed on the apical surface[21]. The biological role of CD133 has yet to be clarified.

Best known for being expressed on the tumor-initiating population of brain neoplasms[22] the $CD133^+$ phenotype has recently been used to define the CSC populations in lung, pancreatic, liver, prostate, gastric, colorectal, as well as head and neck cancers, and $CD133^+$ cells clearly generated tumors in immunocompromised mice more efficiently than their CD133-counterpart[23]. Also, $CD133^+$ CSC display resistance to traditional chemotherapies[24,25].

A valid stem cell marker among several malignant and non-malignant tissues is aldehyde dehydrogenase (ALDH). ALDH is an intracellular enzyme that oxidizes aldehydes. It holds the attractive distinction of being not only a potential marker of "stemness", but it potentially plays a role in the biology of tumor initiating cells as well[26].

Traditional methods such as immunohistochemistry and blotting are used to detect levels of ALDH in tissues and cells, but the method of choice to measure ALDH activity is the flow cytometry-based Aldefluor assay[27]. In the past few years, Aldefluor has been used to characterize CSC in breast, lung, head and neck, colon, and liver tumors and cell lines. Although CSC are enriched in $ALDH^+$ populations in several tissues,

it is important to acknowledge possible limitations of this marker, especially when used as a single marker. Enzymatic activity measured by Aldefluor is much more transient than the expression of traditional surface markers and may be altered by treatment of tumors or cells[23]. ALDH activity has been used as a marker for CSC in malignancies characterized by the presence and/or absence of cell-surface markers like the $CD44^+/CD24^{-/low}$ and $CD133^+$ populations such as breast cancer and ovarian cancer, respectively[9,28] and early reports suggest that the use of ALDH may "refine" the CSC population because the CSC populations defined above and the $ALDH^+$ populations rarely overlap completely[23].

CD44 is a transmembrane glycoprotein which mediates cell adhesion and migration by binding extracellular matrix components such as hyaluronic acid and osteopontin, or by activating receptor tyrosine kinases related to tumor progression, invasion and metastasis. CD44 is reported as a CSC marker across several tissues, including breast, pancreas, gastric, prostate, head and neck, ovarian, and colon[15]. In both preclinical models and clinical research, however, CD44 is most commonly used in combination with other markers, *e.g.*, CD24.

CD24 is a glycosylphosphatidylinositol-linked cell surface protein expressed in various solid tumors[29]. Expression of CD24 in adult non-malignant tissue is limited to B cells, granulocytes, and epithelial cells[30,31]. The normal function of CD24 outside of B cells is poorly understood. The presence or absence of CD24 on the cell surface has been used as a marker for putative CSC. Breast and prostate CSC ($CD44^+/CD24^-$) were found to have increased adhesion, invasion and migration characteristics when compared with CD24-expressing cells[32], and prostate cells that were able to migrate through Matrigel had suppressed CD24 expression along with decreased levels of E-cadherin[33].

The presence or absence of CD24 as a CSC marker seems to be tissue specific, *i.e.*, breast ($CD44^+/CD24^{-/low}$)[9], prostate ($CD44^+/CD24^-$)[34] and pancreatic ($CD44^+/CD24^+/ESA^+$)[35] cancers.

All the above markers have been used alone or in combination to define CSC populations in solid tumors. The most commonly used markers to define CSC in hematological malignancies are CD34 and CD38, which are used in combination[8,36]. Also for these markers, expression is not restricted to CSC, as they are expressed also by normal hematopoietic and non-hematopoietic cells as well as hematopoietic stem and progenitor cells (CD34).

CLINICAL EVIDENCE FOR A ROLE OF CSC IN CANCER INITIATION, RELAPSE AND METASTASES

Following the demonstration that only CSC identified in patients' tumor cells by the previously described markers can form tumors following injection into immunodeficient mice, a number of studies has been conducted to measure CSC in human tumors and to explore their association with prognosis. These studies are important as they would contribute to validate CSC as a therapeutic target and to confirm the correlation between the presence of such cells in tumor samples with unfavorable outcome, independent of any other prognostic factor. Methodologically, most of these studies are retrospective, which in itself carries limitations due to change over time in diagnostic and therapeutic procedures. Breast cancer is by far the most thoroughly investigated solid tumor. Two meta-analyses have been published. The first reported on 9 studies including a total of 2121 patients and demonstrated that the presence of $ALDH^+$ CSC and $CD44^+/CD24^{-/low}$ CSC was significantly associated with poor overall survival[37]. All of the studies included in the meta-analysis used either ALDH or CD44/CD24 as CSC marker. More recently, a second meta-analysis on 3274 patients using ALDH as the sole CSC marker (sharing 7 out of 16 studies and more than 2000 patients with the first meta-analysis) confirmed the association between the presence of $ALDH^+$ CSC and reduced patient survival irrespective of the different cutoff values used in the different studies to define ALDH positivity[38]. However, breast cancer can be divided into several subtypes, either into histopathological categories based upon expression or lack of hormone receptors and HER2 amplification, or into molecularly identifiable categories using gene expression signatures[39]. The presence and clinical significance of CSC have been investigated in most subtypes[40,41]. In this respect, it is interesting to note that CSC have been shown to be represented at an increased percentage in some breast cancer subtypes. For example, Triple Negative Breast Cancer (TNBC) has been described as the most CSC enriched breast cancer subtype[42-44] Further molecular dissection of TNBC identified mesenchymal stem cell-Like[45] and claudin-low[46] as the most CSC enriched subgroups. It has been shown *in vitro* that breast cancer cell lines of the luminal subtype do not always contain a sizeable $ALDH^+$ cell population, whereas 16/16 basal-like cell lines displayed a CSC population identified by the same marker[47]. Indeed, with a cutoff for positivity set at $\geqslant$ 5% in most studies, it would appear that a minority of tumors display a sizeable CSC population identified by a single marker or combination of markers (*i.e.*, $CD44^+/CD24^-$, $ALDH^+$)[37,38]. It remains to be demonstrated whether this is due to suboptimal markers to identify CSC and/or the limited overlap between CSC populations identified by either marker alone[41,48,49].

In other solid tumors similar results have been reported, although the number of patients studied is in general far lower. In ovarian cancer, the presence of $ALDH^+/CD133^+$ cells in debulked primary tumor specimens correlated with reduced disease-free and overall survival in 56 ovarian cancer patients[28]. These findings were confirmed by a meta-analysis of 1258

patients from 7 studies, in which ALDH was used as a single CSC marker[50]. In prostate cancer, detection of ALDH$^+$ CSC correlated with shorter median survival in hormone naïve, but not in castration resistant, tumors. The two groups consisted of 100 and 107 patients, respectively[51]. In gastric cancer, presence of ALDH$^+$ CSC was found to be an independent prognostic factor for both overall survival and recurrence-free survival[52] in a population of 216 patients. In pancreatic cancer, expression of the CSC markers CD133, CD44 and CD24 was found to correlate with poor prognosis in a sample of 65 patients[53]. In hematological malignancies, it has been found that CD34$^+$/ALDH$^+$ CSC in the bone marrow of 68 patients with acute myeloid leukemia at diagnosis correlated with reduced survival probability[54]. In conclusion, retrospective studies have found a negative prognostic role of pretreatment CSC, in keeping with their alleged resistance to chemo- and radiotherapy.

EVIDENCE OF CSC RESISTANCE TO CHEMOTHERAPY

Breast cancer is the solid tumor in which this issue has been explored most thoroughly. Breast cancer CSC have been shown to display resistance to commonly used chemotherapeutic agents (*e.g.*, paclitaxel) *in vitro*[55] and in mice[56,57]. In patients, neoadjuvant (*i.e.*, administered before surgery) chemotherapy represents the ideal setting for assessing the efficacy of chemotherapy against CSC, as the primary tumor is readily available to serial biopsies before, during and at the completion of treatment. In the majority of clinical trials of neoadjuvant chemotherapy, the proportion of CSC identified by marker expression and/or mammosphere forming efficiency was found to be increased following treatment[58-60] despite reduction of tumor size, highlighting that chemosensitivity of the bulk tumor cells is not shared by CSC.

In these studies, 56% or fewer patients had ALDH$^+$ CSC in their tumors at diagnosis, using a cutoff of $\geqslant$ 5% to $\geqslant$ 20% (reviewed in[61]). In keeping with preclinical findings[41,62], it was reported that the ALDH$^+$ phenotype[60,61], or the CD44$^+$/CD24$^-$ phenotype[63], is associated with chemoresistance However, the studies by Lee *et al*[59] and Gong *et al*[62] came to the opposite conclusion, *i.e.*, ALDH$^+$ CSC correlated with increased pathologic or clinical responses, respectively. The reasons for this discrepancy could be different chemotherapy regimens administered to patients, different patient populations, or different antibody and cutoff values used to evaluate ALDH positivity in breast cancer samples.

The study by Alamgeer *et al*[61] is the only prospective evaluation of ALDH$^+$ cells in serial biopsies of breast cancer. Biopsies were obtained before neoadjuvant chemotherapy, following 4 cycles of either one of two chemotherapy regimens, and at completion of treatment in 119 patients. Dynamic changes of ALDH were recorded, with patients switching from ALDH$^-$ to ALDH$^+$ and vice versa in 27% and 19% of cases, respectively, at the biopsy after 4 cycles of chemotherapy. There are several possible explanations for these findings. First, there may be interconversion between the stem cell like and non-stem cell like phenotype, as previously described in preclinical models[64]. However, conversion from ALDH$^+$ to ALDH$^-$ with chemotherapy alone (*i.e.*, without a CSC targeting agent) is not in keeping with the alleged chemoresistance of CSC. Second, ALDH$^+$ cells have been described to be localized at the center of the tumor mass, whereas CD44$^+$/CD24$^-$ cells are localized at the edges[49]. Thus, serial biopsy of a tumor mass may lead to increase or decrease of either cell population depending on the area sampled. Third, for biopsies negative at baseline, which convert to positive after 4 wk of treatment, there might have been ALDH positivity below the 5% cutoff value of an otherwise positive baseline sample. However, the most intriguing finding of the study is that the disease-free survival of those patients who did not achieve pathologic complete response (pCR) but displayed elimination of ALDH$^+$ cells in the primary tumor, is similar to that of patients experiencing pCR[61].

Another prospective study, the AVASTEM trial, was designed to evaluate whether the addition of bevacizumab to conventional chemotherapy alters the proportion of CSC in breast cancer patients receiving neoadjuvant treatment. It is an open label, randomized phase II trial in which tumor biopsies are required at baseline and after the 4 initial cycles of treatment to evaluate variations in the ALDH$^+$ CSC population[65]. Preclinical work demonstrated that anti-angiogenic agents (*e.g.*, bevacizumab) may increase breast cancer CSC *via* the generation of tumor hypoxia[66]. Indeed, CSC have been described to reside near hypoxic regions in solid tumors[67-70]. Results of AVASTEM will confirm or disprove these preclinical findings.

MOLECULAR SIGNALING PATHWAYS AS THERAPEUTIC TARGETS IN CSC

Signaling pathways are essential for normal stem cells with respect to self-renewal, proliferation and differentiation. Accumulating evidence suggests that maintenance of the CSC population in different human tumors employs the same signaling pathways as in normal stem cells, although an alteration of these pathways during the development of cancer has occurred, and this event has led to dysregulation of stem cell self-renewal and contributes to tumor proliferation[71,72]. Therefore, targeting these aberrant signaling pathways that are important for the formation of CSC seems to offer a new strategy for cancer therapy.

Most of these pathways have been described in dedicated reviews[15]. In this section, we will present

Table 2 Pathways and drug candidates targeting cancer stem cells

Pathway	Targets	Compounds	Clinical development
Hedgehog	Smoothened	[1]Vismodegib[80-82]	Medulloblastoma Pancreatic cancer Hematological malignancies
Notch	γ-secretase DLL4	MK-0752[94] Demcizumab[88-90]	Breast cancer Pancreatic cancer NSCLC Ovarian cancer
Wnt	β-catenin	MEDI0639 Vantictumab[101]	Solid tumors Breast cancer Pancreatic cancer NSCLC
		OMP-54F28[102]	Ovarian cancer Pancreatic cancer Hepatic cancer
CXCR1/2-CXCL8	CXCR1	Reparixin[113,114] SCH563705	Breast cancer
IL-6/JAK/STAT3	CXCR1/2 IL-6 JAK1, JAK2	[2]Tocilizumab [3]Tofacitinib [4]Chloroquine	Breast cancer[57]

[1]Erivedge™, FDA and EMA approved for metastatic or locally advanced basal cell carcinoma; [2]RoActemra™, FDA and EMA approved for rheumatoid arthritis; [3]Xeljanz™, FDA approved for rheumatoid arthritis; [4]FDA approved for malaria and extraintestinal amebiasis. DLL4: Delta-like ligand 4; FDA: Food and Drug Administration; NSCLC: Non-small-cell lung carcinoma; CXCR1/2-CXCL8: CXC chemokine receptor 1/2-interleukin 8; IL-6/JAK/STAT3: Interleukin 6/Janus kinase 2/Signal transducer and activator of transcription 3.

and discuss clinical data concerning anti-tumor activity and side effects related to targeting of each pathway.

The hedgehog signaling pathway

The hedgehog (Hh) pathway plays a crucial role in development and patterning during mammalian embryogenesis, and it is essential for the maintenance of stem cells[73]. The Hh pathway is activated when Hh protein binds to and inhibits the transmembrane protein called Patched, leading through activation of the Smoothened function to the regulation of target genes involved in many cellular functions including metastasis, proliferation, survival, and pathway auto-regulation[74,75]. Deregulation of the Hh pathway is associated with numerous human malignancies and is a critical factor affecting the outcome of treating the disease[76]. Recently, it has been suggested that the Hh pathway is essential for the maintenance of CSC in various human cancers, including colorectal, pancreatic and gastric cancer[77,78], and it is also responsible for treatment resistance of cancer cells[79]. Thus, inhibitors that block any step of the Hh signaling pathway may result in reduction of CSC and overcoming treatment resistance.

One Hh inhibitor, vismodegib, is approved for treatment of metastatic or locally advanced, unresectable basal cell carcinoma. Vismodegib 150 mg/d administered orally produced a sizeable and lasting response rate, including complete responses, in patients with basal cell carcinoma, suggesting activity also on bulk tumor cells[80,81]. In a randomized trial of vismodegib *vs* placebo in patients with basal cell nevus syndrome, an autosomal dominant disorder causing a markedly increased incidence of basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma, new basal cell carcinoma formation rate was decreased by vismodegib from 29/year to 2/year[82]. This tumor prevention capability would suggest suppression of CSC activity, however, no formal investigation of anti-CSC activity has been reported. Vismodegib administration may result in adverse reactions, so that in the latter trial, 54% of patients had to discontinue treatment due to adverse reactions[82], with 25% of patients experiencing serious adverse events in the phase Ⅱ trial regarding basal cell carcinoma[81]. Ongoing and completed clinical trials using Smoothened antagonists were recently reviewed by Amakye *et al*[83] (Table 2).

Notch signaling pathway

The Notch signaling pathway plays several crucial roles in the communication between cells[84,85]. The Notch pathway is activated through ligand-receptor interactions of four receptors (Notch-1-Notch-4) and five Notch ligands (Delta-like1, 3, 4 and Jagged1, 2)[85], resulting in the expression of multiple target genes. The Notch pathway is involved in stem cell proliferation, differentiation, and apoptosis, but its role in tumorigenesis can be either oncogenic or oncosuppressive[85]. In fact, Notch functions as an oncogenic protein in most human cancers including cervical, lung, colon, head and neck, prostate and pancreatic cancer, while it may act as a tumor suppressor in hepatocellular carcinoma, skin and small cell lung cancer[86,87].

The delta-like ligand 4 (DLL4) contributes to CSC self-renewal and vascular development and can bind to any of the 4 receptors. Two anti-DLL4 monoclonal antibodies are in development (Table 2). Demcizumab (OMP-21M18, Oncomed) is a humanized IgG2 antibody specific for DLL4 with as dual mechanism of action: targeting of cancer stem cells and inhibition of angiogenesis. It has been evaluated as a single agent in patients with advanced malignancies[88] and in two phase Ib dose escalation clinical trials for frontline treatment of metastatic pancreatic adenocarcinoma[89] and non-squamous non-small cell lung cancer[90] in combination with gemcitabine and carboplatin + pemetrexed, respectively. In pancreatic adenocarcinoma, median Progression-Free Survival observed did not differ significantly from what would be expected from single agent treatment with gemcitabine in the same patient population[89]. In order to comply with the new standard of care in this disease[91], patients are now being treated with demcizumab in combination with gemcitabine + nab-paclitaxel[89]. In non-squamous non-small cell lung

cancer, median Progression-Free Survival was around 6 mo[90]. In both studies, the objective response rate was significant (25% and 46%, respectively), but the dual mechanism of action makes it difficult to disentangle effects on CSC from anti-angiogenic activity. The most common adverse reactions included nausea, fatigue and hypertension, the latter of which was managed with anti-hypertensives[88-90]. Also, late cardiopulmonary toxicity was recorded in a few patients treated for over 5 mo, leading to a shorter course of treatment and monitoring for early signs of toxicity by serial measurements of B-type natriuretic peptide levels[89,90].

MEDI0639 (medimmune) is an IgG1 lambda (IgG1λ) antibody that selectively binds to DLL4. Clinical results are undisclosed at the time of writing (Table 2).

Notch signaling affects lineage-specific differentiation and self-renewal of normal human breast stem cells[92]. Moreover, Notch-4 activity is elevated in breast CSC, and inhibiting Notch4 activity can reduce the breast CSC population, thereby suppressing tumor initiation[93]. Cleavage of the Notch receptor by gamma-secretase is required to release the Notch intracellular domain, which then translocates to the nucleus, turning on genes involved in cell differentiation and proliferation. Thus, gamma secretase inhibition results in a loss of Notch function in cells. In breast cancer xenografts, the gamma-secretase inhibitor MK-0752 reduced CSC and enhanced the efficacy of docetaxel[94]. Subsequently, 30 patients with locally advanced or metastatic breast cancer were treated with escalating doses of oral MK-0752[95] plus docetaxel every 3 wk. All patients could be evaluated for toxicity. Overall, 12/30 patients experienced G3 adverse reactions including G3 diarrhea (2 patients) and G3 liver function tests elevation at the selected dose level. Further, 24 patients could be evaluated for efficacy. In 11/24 patients, partial remissions were recorded, and in 9/24 patients, stable diseases were recorded. Since docetaxel has an established activity against breast cancer[96], the response rate should be interpreted with caution. Tumor biopsies were optional for participants in the study, and were performed more frequently in patients with locally advanced disease for whom surgery following initial medical treatment was planned. Serial biopsies were taken from 6 patients, at baseline, after cycle 1 and 3 and at progression or completion of treatment. All patients but one were treated at the selected dose level. Four patients experienced Partial Response, and 2 patients achieved stable disease. CSC were evaluated by flow cytometry examining both $ALDH^+$ and $CD44^+/CD24^-$ cell populations and by mammosphere forming efficiency. $CD44^+/CD24^-$ cells decreased from baseline to surgery in 3/5 patients, while $ALDH^+$ cells decreased from baseline to surgery in 3/6 patients. Mammosphere forming efficiency decreased over the course of treatment[94]. Despite these results, later phase clinical trials with MK-0752 are not listed on http://clinicaltrials.gov, neither for breast cancer nor for any other tumor type.

Wnt signaling pathway

The Wnt signaling pathway is another developmental pathway involved in multiple biological processes including embryogenesis, development, cell proliferation, survival and differentiation[97]. The Wnt/β-catenin signaling pathway is the best characterized one of the Wnt pathways[74]. Wnt/β-catenin signaling is activated when secreted glycoprotein, Wnt ligand, binds to the cell membrane Frizzled receptors and LRP5/6 co-receptors, which results in triggering of target genes[97]. Alteration in Wnt/β-catenin signaling has been identified in various malignancies, such as leukemia, colon, breast and cutaneous carcinoma. Wnt signaling in many human tumors arises from mutations in pathway components, resulting in aberrantly high levels of activation. For example, defective mutations in the adenomatous polyposis coli gene, a key downstream regulator of Wnt signaling, result in the inappropriate stabilization of β-catenin, thus activating the Wnt cascade and inducing epithelial cell transformation in patients with familial adenomatous polyposis[98,99]. The Wnt signaling pathway is associated with the maintenance of CSC stem cell properties, but it also regulates important stem cell niches within the body, including normal hematopoietic stem cells and adult stem cells within various tissues.

OMP-18R5 (vantictumab) (Table 2), a fully human IgG2 monoclonal antibody targeting the Frizzled receptors 1, 2, 5, 7 and 8, displays strong anti-tumor activity in patient-derived xenografts in association with standard of care chemotherapy[100]. It also induces cancer cell differentiation. A phase Ib study of the single agent OMP-18R5 in 25 patients demonstrated pharmacodynamic activity on hair follicles and bone turnover. Prolonged stabilization of disease was recorded in 3 patients with neuroendocrine tumors. Analysis of pre- and post-treatment tumor biopsies showed inhibition of Wnt and CSC genes, as well as upregulation of differentiation genes[101].

OMP-54F28 is a fusion protein based on a truncated form of the Frizzled8 receptor, or Fzd8, and the Fc fragment of human immunoglobulin IgG1. It is designed to bind Wnt ligands thus blocking Wnt signaling. In patient-derived xenograft models, OMP-54F28 acts synergetically with chemotherapy in a broad spectrum of malignancies. This fusion protein was tested as a single agent in a phase Ⅰ study in patients with advanced solid tumors[102]. Intravenous injections every 3 wk did not result in any grade ≥ 3 adverse reactions at doses up to 20 mg/kg, double the target efficacious dose established by animal data and pharmacokinetic modeling. Most common (> 20% of patients) grade 1-2 adverse reactions were dysgeusia, decreased appetite, fatigue, muscle spasms, nausea and vomiting. No objective responses were recorded in 25 patients treated in 7 dose escalation cohorts, whereas several patients experienced prolonged stabilization of the disease. Pharmacodynamic modulation of Wnt pathway genes was shown in hair follicles. Five patients experienced doubling of the bone turnover marker

β-C-terminal telopeptide, consistent with Wnt pathway inhibition in bone. This adverse reaction was easily managed with a single dose of zoledronic acid. Phase Ⅰb studies of OMP-54F28 in combination with gemcitabine and nab-paclitaxel (pancreatic cancer), carboplatin and paclitaxel (ovarian cancer) and sorafenib (hepatocellular carcinoma) are currently in progress (Table 2).

CXCR1/2-CXCL8 signaling pathway

CXCL8 [formerly interleukin (IL-8)] is a small soluble protein belonging to the chemokine family[103]. It was originally identified as a potent neutrophil activator and chemotactic factor mainly secreted by activated monocytes and macrophages[104,105]. The biological effects of CXCL8 are mediated by two class A, rhodopsin-like guanine-protein-coupled receptors: CXC chemokine receptor 1 (CXCR1) (IL-8RA) and CXCR2 (IL-8RB)[106,107]. CXCL8 is upregulated in a wide variety of solid cancers, such as prostate, gastric, bladder, ovarian, lung cancer and melanoma. It has also been reported to contribute to multiple hallmarks of cancer, such as increased proliferation, angiogenesis, invasion, and metastases[108,109]. CXCL8 is overexpressed in breast cancer, compared with normal breast tissue, and although there is substantial evidence that CXCL8 may promote breast cancer initiation and progression, more recent evidence indicates that this cytokine is a key regulator of CSC activity[110]. A search for actionable molecules on $ALDH^+$ breast cancer CSC identified CXCR1 as a target almost exclusively expressed by CSC as compared with bulk tumor cells[47]. It was shown *in vitro* that breast cancer CSC proliferate in response to exogenous CXCL8 and that addition of a small molecular weight antagonist of CXCR1/2 (reparixin, formerly repertaxin)[111] or a blocking anti-CXCR1 (but not anti-CXCR2) monoclonal antibody depleted CSC *in vitro*[56]. Interestingly, a FAS-FASL mediated bystander effect killed the vast majority of bulk tumor cells *in vitro*, suggesting the possibility of synergistic effects with chemotherapy[56]. In breast cancer patient-derived xenografts or human breast cancer cell lines, the combination of docetaxel and reparixin was more effective than chemotherapy alone in reducing tumor size[56]. As expected, administration of the single agent reparixin did not result in tumor shrinkage over a 4 wk treatment period. However, tumors recovered from mice that had been treated with reparixin alone or in combination with chemotherapy displayed a far lower abundance of CSC than tumors recovered from mice receiving chemotherapy alone[56]. In addition, reparixin reduced metastasis formation in mice following injection of luciferase-transfected human breast cancer cells into the bloodstream[56]. These results were integrated into a model in which following administration of chemotherapy, dying bulk tumor cells release CXCL8 and FASL. However, $CXCR1^+$ CSC are sheltered from apoptotic signals from FASL unless CXCR1 is blocked by reparixin. Reparixin is a clinical grade molecule already used in patients with non-oncological conditions[112]. Therefore, clinical trials of oral reparixin were started in combination with weekly paclitaxel in HER2-negative pretreated metastatic breast cancer (NCT02001974)[113], and as a single agent in a window of opportunity trial in HER2-negative operable breast cancer (NCT01861054)[114]. Indirect support for this model comes from two lines of evidence. First, Bolha *et al*[55] reported release of CXCL8 from tumor cells exposed to taxane *in vitro*. Also, they observed a marked and dose-dependent increase in mammosphere forming efficiency in TNBC tumor cells recovered from immunocompromised mice treated with 2 doses of paclitaxel[55]. Second, Singh and colleagues[115] demonstrated measurable IL-8 levels in pleural effusions and ascites from breast cancer patients When tumor cells from pleural effusions and ascites were cultured *in vitro*, a direct correlation between IL-8 levels and CSC activity could be measured by mammosphere formation[115]. Surface CXCR1 was detected on the majority of mammosphere cells, and a CXCR1/2 inhibitor, SCH563705, blocked the effects of exogenous CXCL8 on mammosphere formation[115]. In partial contrast to findings by Ginestier pointing to a role for CXCR1 but not CXCR2 in anti-CSC activity[56], CXCR2 inhibition was shown to lead to a significant growth inhibition of basal like breast cancer cell lines *in vitro*[116], further highlighting the potential of CXCR1/2 inhibition. The activity was far less pronounced on non-basal like breast cancer cell lines. Also, in the report by Ginestier, most of the cell lines and patient-derived xenografts were triple negative breast cancers[56]. Although basal like and triple negative breast cancers do not fully overlap[44], these findings suggest that basal like and/or triple negative breast cancer could be most sensitive to CXCR1/2 inhibition, possibly due to an enrichment in CSC in these breast cancer types[42-44].

A possible role for CXCL8 on CSC has been recently highlighted also in pancreatic cancer[53]. The authors found a positive correlation between CXCR1 and both CD44 and CD133 expression in human pancreatic cancer samples. Furthermore, addition of exogenous CXCL8 *in vitro* increased sphere formation, CSC population, and cell invasion of pancreatic cancer cells, all these effects being reversible upon addition of a CXCR1-blocking monoclonal antibody[53].

CXCR1/2 blockade may lead to anti-CSC effects also by an indirect mechanism. In fact, mesenchymal stem cells, a stromal cell type secreting factors that sustain CSC, are often recruited to tumors by tumor cell-derived CXCL8[117]. Thus, inhibition of CXCL8 activity and its homing signal may prevent localization of mesenchymal stem cells to the tumor stroma, hindering the development of a supportive CSC niche[15].

Finally, considering the multiple roles played by CXCL8 in cancer (reviewed in[118]), it should be borne in mind that inhibition of CXCL8 biological activity may lead to anti-tumor activity also by other mechanisms not directly related to CSC, such as inhibition of angiogenesis and modification of the leukocyte infiltration into tumors.

IL-6/Janus kinase 2/Signal transducer and activator of transcription 3

The IL-6/Janus kinase 2 (JAK2)/Signal transducer and activator of transcription 3 (Stat3) pathway was found to be preferentially activated in $CD44^+/CD24^-$ breast CSC[116]. This opens the possibility of targeting CSC by compounds interfering with any of the 3 pathway components. Approved anti-IL-6 compounds (tocilizumab, anti-IL-6R mAb) and JAK inhibitors (*i.e.*, tofacitinib) are available for the treatment of rheumatoid arthritis, thus making this pathway a readily available clinical target. Furthermore, it has been shown in preclinical studies that breast cancer cell resistance to trastuzumab, an approved monoclonal antibody for treatment of $HER2^+$ breast cancer, is mediated by an IL-6 feedback loop, leading to an expansion of the CSC pool[119]. Indirect support for a role played by JAK2 comes also from preclinical studies using chloroquine (another approved drug for treating malaria), which suppressed $CD44^+/CD24^-$ breast CSC *via* inhibition of the JAK2-Stat3 pathway[57].

HER2/AKT

HER2 has been reported to be an intrinsic regulator of breast cancer CSC[120,121]. This regulation occurs through activation of the Wnt/β-catenin pathway *via* Akt mediated phosphorylation of GSK3B and nuclear translocation of β-catenin[122]. In patients with HER2-positive breast cancer undergoing neoadjuvant treatment, lapatinib administration led to a decrease, although not a statistically significant one, in both the proportion of $CD44^+/CD24^-$ CSC and mammosphere forming efficiency[58]. However, HER2 regulation of CSC possibly extends to HER2-negative breast cancer (reviewed in[123], as suggested by the clinical benefit observed in patients with HER2-negative breast cancer receiving adjuvant anti-HER2 treatment[124]. It has been shown that in HER2-negative breast cancer, CSC selectively express, and are regulated by, HER2[125]. This is in line with the observation that *in vitro*, lapatinib inhibits CXCL8-stimulated mammosphere formation from both HER2-positive and HER2-negative patient breast cancer samples, highlighting a key role of HER2 in regulating CXCR1/2 mediated activation of breast cancer CSC[115]. This data was put into context in a model in which CSC activity is increased following CXCL8 binding to CXCR1/2 *via* both HER2-independent and HER2-dependent (through CXCR1/2 mediated transactivation) mechanisms[115]. From a clinical standpoint, this data suggests that combination therapy with anti-HER2 and anti-CXCR1/2 compounds may affect breast cancer CSC to a greater extent than either compound alone[115,126].

REPURPOSED DRUGS

A number of approved drugs that have been used for many years in non-oncological indications have been shown to have anti-tumor activity *in vitro* and/or *in vivo*. The obvious advantage of such drugs is the extensive knowledge of their safety profile (although not in association with standard of care chemotherapy), which may open the possibility for single agent window of opportunity trials in a preoperative setting for selected cancers, or possibly neoadjuvant trials if overlapping toxicities are not to be expected[127]. In other instances, high throughput screening programs have identified known compounds as potential CSC targeting agents[128,129]. Several such drugs are being tested as anti-CSC.

Chloroquine is an antimalarial drug. It is capable of inhibiting autophagy, which is a mechanism exploited by cancer cells to cope with stress[130]. In particular, autophagy has emerged as a mechanism by which CSC maintain their capability of self-renewal[131]. *In silico* gene expression analysis of the $CD44^+/CD24^-$ treatment resistant breast cancer cell population identified chloroquine as a candidate anti-CSC compound[57]. *In vitro* chloroquine reduced, in a dose-dependent manner, mammosphere forming efficiency in TNBC cell lines as well as $CD44^+/CD24^-$ cells when used alone or in combination with paclitaxel[57]. *In vivo*, growth of orthotopically implanted tumors as well as lung metastases were reduced, and so were CSC when chloroquine was administered in combination with paclitaxel[57]. A clinical trial (NCT01446016) is currently in progress, in which chloroquine is administered in combination with a taxane or taxane-like drug (*i.e.*, nab-paclitaxel and ixabepilone) to women with advanced or metastatic breast cancer, who have not responded to anthracycline therapy. An interim report showed that in two out of three patients, from whom pre- and post-treatment biopsies were taken, a clear reduction in $CD44^+/CD24^-$ cells was recorded[57]. Chloroquine has been investigated as a CSC targeting agent not only in breast cancer but also in CML[132].

A significant number of studies has been performed with the anti-diabetic drug metformin[133]. Its activity against CSC has been described in a number of preclinical reports[134,135], whereas the issue of its clinical activity against CSC has remained unexplored despite a number of window of opportunity studies in patients with operable breast cancer that established safety of the drug in this setting[136].

Itraconazole and arsenic trioxide, two drugs approved for fungal infections and acute promyelocytic leukemia, respectively, have been shown to be Hedgehog pathway inhibitors[137,138]. In an open label phase 2 clinical trial in patients with basal cell carcinoma, a 24% decrease in tumor area, a 65% reduction in Hedgehog pathway activity and a 45% reduction in the proliferative marker Ki-67 were observed following treatment with itraconazole in comparison with untreated patients[139]. The potential for all-trans retinoic acid as an anti-CSC agent has been recently and thoroughly reviewed[140].

Salinomycin, which is used in chicken fodder as a coccidiostat, has been shown to reduce the proportion of CSC by > 100-fold relative to paclitaxel, a commonly

used breast cancer chemotherapeutic drug[128]. Treatment of mice with salinomycin inhibits mammary tumor growth *in vivo* and induces increased epithelial differentiation of tumor cells[128]. In addition, global gene expression analyses show that salinomycin treatment results in loss of expression of breast CSC genes previously identified by analyses of breast tissues isolated from patients[128]. However, no clinical trials testing salinomycin are registered on http://clinicaltrials.gov.

Thioridazine, an antipsychotic drug, selectively targets leukemic stem cells while sparing normal hematopoietic stem cells[129]. The drug antagonizes dopamine receptors that are expressed on CSC[129]. It is currently being tested in a phase I trial in combination with cytarabine in patients with acute myeloid leukemia (NCT02096289).

ISSUES IN DESIGNING CLINICAL TRIALS FOR CSC-TARGETING AGENTS

Early drug development in cancer usually requires signs of anti-tumor activity as measured by shrinkage of metastatic lesions. Once this is demonstrated, a novel agent is then moved to the adjuvant setting, *i.e.*, after removal of the primary tumor, to reduce recurrence risk. Based on the CSC model, and considering that CSC represent only a tiny percentage of the total tumor cells (*i.e.*, < 5%) of any given tumor, single agent CSC-specific agents would not be expected to display antitumor activity measurable by RECIST criteria[141]. As a consequence, in metastatic patients such agents would not be expected to affect tumor growth and tumor-related symptoms in the short term. Thus, in this setting, CSC-specific agents likely need be tested in combination with standard of care chemotherapy that will address the proliferating, non-CSC portion of the tumor. When a CSC-targeting agent is used in combination with standard of care chemotherapy, the objective response rate is still an obvious endpoint, but its relevance is mostly to ensure that the CSC-targeting agent does not hinder chemotherapy activity. In fact, it is unlikely that a specific CSC-targeting agent augments chemotherapy activity as measured by the objective response rate unless indirectly mediated through a bystander effect on bulk tumor cells[56].

The same consideration applies to neoadjuvant chemotherapy, the purpose of which is to downsize the primary tumor and to eliminate micrometastases. Also, in this setting, it is not intuitive that a CSC-specific agent can significantly increase the rate of pCR as compared to chemotherapy alone, unless a bystander effect is postulated. In this specific setting, one may hypothesize that in a randomized clinical trial, the rate of pCR as well as complete and partial clinical responses between the two treatment groups could be very similar, but disease-free survival may differ, favoring the regimen including a CSC-targeting agent. Partial support for this hypothesis comes from the study by Alamgeer *et al*[61], in which patients who experienced less than pCR but had eliminated $ALDH^+$ CSC in their primary tumor following neoadjuvant chemotherapy had the same disease-free survival as patients achieving a pCR.

Therefore, the objective response rate *per se* is not necessarily indicative of anti-CSC activity, whereas the duration of response would be more informative. These considerations are relevant for the identification of effective compounds for adjuvant treatment, in which activity on CSC is expected to be most relevant, according to the CSC paradigm[123]. Evaluation of pre- and post-treatment biopsies is conceivably the most compelling evidence for anti-CSC activity. However, post-treatment biopsy can be difficult to obtain from metastatic patients[94]. Other challenges also may hinder the value of repeated biopsies even in the neoadjuvant setting, in which tumor tissue is readily available. In breast cancer, the most widely used CSC markers are $ALDH^+$ and $CD44^+/CD24^-$. However, it has been shown that cells displaying either phenotype are largely not overlapping, with each marker(s) identifying a distinct CSC population[41,48,49]. Furthermore, breast cancer CSC can transition from one phenotype to the other. Little is known about the role, and the therapeutic relevance, of each CSC population in primary breast cancer. However, recent evidence shows that $ALDH^+$ cells are proliferative, epithelial cell-like CSC, whereas $CD44^+/CD24^-$ CSC are quiescent, mesenchymal-like cells poised to invade and metastasize[49]. In keeping with their different putative role in cancer, $ALDH^+$ and $CD44^+/CD24^-$ cells tend to be localized in different areas of the tumor, *i.e.*, center and edges, respectively[49]. The location of these CSC populations in anatomically distinct areas of a tumor, coupled with their plasticity, poses theoretical challenges to comparative measurement of CSC in serial biopsies taken before, during and after treatment.

In drug development, in order to treat patients with potentially curable diseases with a novel agent, a robust safety database from the metastatic setting or from other indications for repurposed drugs must be available. Thus, most novel agents designed to target CSC will be tested first in patients with metastatic disease due to safety reasons. One may argue that the metastatic setting is less than ideal to test CSC-targeting agents, since they are conceivably best suited to prevent or reduce metastatic spread and thus should be applied to the (neo)adjuvant setting. In order to circumvent these limitations, surrogate clinical endpoints have been devised to evaluate the potential of a novel agent to reduce the metastatic spread of tumors[142]. This concept fits into the CSC model and can be applied to the development of a CSC-targeting agent which, as a novel chemical entity or biologic,

cannot be tested in settings of earlier disease stages due to safety concerns. Time to new metastasis (TTM) is an exploratory endpoint that measures the time elapsing from study entry or randomization to detection of metastatic lesions in a new organ system as compared to baseline[143]. Examples of application of this endpoint can be found in lung cancer[144,145]. In a less stringent fashion, this endpoint can be measured also until appearance of new lesion(s) in an organ already metastatic at baseline. In breast cancer, considering the proportion of patients developing either new metastatic sites or new lesions at their first tumor progression[146], this endpoint can certainly be read with the possibility of demonstrating the potential for a novel agent to reduce metastatic spread. It should be noted that TTM has also immediate clinical relevance since the development of new metastases in the brain, bones or viscera can lead to significant morbidity. The data with denosumab in the prevention of bone metastases in patients with castration-resistant, non-metastatic prostate cancer[147], as well as the reduction of skeletal-related events in patients with metastatic breast cancer[148], is reminiscent of this endpoint. However, TTM is a challenging endpoint to measure, as in several metastatic tumors, patients with progression will be put on another treatment so that TTM can be read only until RECIST 1.1 progression[143]. The most thorough way to assess TTM would be to design trials allowing treatment beyond progression[143]. One possible way to measure anti-CSC activity circumventing the need for serial biopsies would be to analyze cancer cells and/or tumor DNA in patients' bloodstream. The prognostic role of circulating tumor cells (CTCs) has been established since 2004[149,150]. However, the relationship between the number of CTC and tumor-localized or circulating CSC has not been established yet. The potential, but also the current pitfalls, of CTC and circulating DNA have been recently reviewed elsewhere[151,152].

Targeted anti-cancer therapies are best developed in conjunction with biomarkers (*e.g.*, crizotinib, vemurafenib) that can measure treatment efficacy and/or can identify patients with a higher chance to benefit from the treatment. As discussed earlier, not all tumors display a sizeable CSC population, although this finding may be related to the sensitivity of the assay used and the use of a single marker. The ideal biomarker for pharmacodynamic evaluation of cancer stem cell therapies would be able to accurately measure the proportion of stem cells within the tumor, so as to select patients with a high proportion of stem cells for treatment, to identify presence of the target on CSC for targeted therapies, and to determine on pre- and post-therapy specimens (*i.e.*, tumor or blood) whether the proportion of stem cells within the tumor decreased under the therapy. Unfortunately, the tiny percentage of CSC in a tumor mass makes it difficult to develop a companion diagnostic for any CSC targeting agent with a molecular target.

CONCLUSION

The existence of multiple subpopulations of cells within a tumor with distinct tumor initiating capacity has been clearly demonstrated[14,15]. The clinical relevance of CSC is being highlighted by both retrospective analyses and prospective studies, challenging clinical paradigms[61]. There is still controversy about the most suitable markers to identify CSC in order to understand their role and therapeutic relevance in different stages of human cancers, as well as about monitoring effects of CSC targeting agents. There is a requirement to identify cell surface marker and gene activity profiles that can be readily and reliably used to identify CSC in human tumors. However, as these genes frequently operate in other cell types, they cannot be called "stemness" genes. In order to identify true signatures and pure populations, microarray and genome-wide techniques can be applied to detect trends in genetic and epigenetic models for cancer stem cells. This is especially true for cells expected to be rare, such as cancer stem cells, the expression signature of which would be saturated by the majority of non-stem cells in a whole tumor sample. Even after a cancer stem cell signature from a particular type of tumor is identified, one cannot assume that a given signature is useful for identifying cancer stem cells in a different tumor type, unless it is validated by a functional assay. The use of gene inactivation to eliminate "stemness" or gene activation to engender "stemness" would be required to functionally link any marker to stem cell identity. This can be applied to clinical trials as exemplified by the study that identified RPL39 and MFL2 as potential targets in CSC[153].

A number of novel or repurposed agents interfering with pathways considered relevant for CSC are being evaluated in patients with advanced tumors following demonstration of activity in patient-derived xenografts. A recommended phase 2 dose has been identified for most of such agents either alone or in combination with chemotherapy. For some agents, pharmacodynamic demonstration of target pathway modulation of non-cancer targets (*e.g.*, hair follicles) has been obtained, and some long term responders or at least non-progressors have been described. Formal demonstration of an anti-CSC effect is still lacking in most cases, due to a number of factors including the challenge to obtain serial biopsies in metastatic patients, and the interference of several anti-CSC agents with multiple pathways makes it difficult to disentangle the relative contribution of the anti-CSC *vs* non-anti-CSC activity. Clinical surrogate endpoints may prove useful in suggesting an anti-CSC activity, such as reduction of metastatic spread measured as time to new metastasis[143]. However, in order to seek marketing authorization, a CSC-targeting agent should demonstrate an advantage at least in terms of Progression-Free Survival and Disease-Free Survival as compared with standard of care in the metastatic and

(neo)adjuvant setting, respectively. In this respect, the goals recently put forward by the American Society of Clinical Oncology Cancer Research Committee[154] may be within reach if the CSC model holds true for a given malignancy. Achievement of these goals raises the issue of patient selection, a rather unexplored issue thus far, since trials to date have been conducted on unselected patient populations. First, it is not known whether the CSC paradigm is universal to all human cancers[14]. Second, it has been clearly established that administration of chemotherapy in the adjuvant setting (*i.e.*, following surgery of the primary tumor) increases 30-year disease-free survival as compared with surgery alone in node-positive breast cancer patients[155]. This suggests that a fraction of patients does not seem to require a CSC targeting agent, whereas the opposite is entirely possible in another set of patients or a different breast cancer subtype. Third, aberrant activation of any of the pathways discussed here due to mutations, as seen in a proportion of tumors, may represent one selection step. Lastly, it is possible that CSC may eventually show resistance to CSC targeting agents. At least two drug resistance pathways have been identified for the Hedgehog inhibitor vismodegib in patients[156,157].

In conclusion, given the extraordinary complexity of CSC clinical targeting, a bench to bedside to bench circle[94] is the most sensible approach to develop anti-CSC agents.

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