Cancer Stem and Progenitor-Like Cells as Pharmacological Targets in Breast Cancer Treatment

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Supplementary Issue: Targeted Therapies in Breast Cancer Treatment

Valéria B. de Souza[1,2] and André A. Schenka[1,2]

[1]Department of Pharmacology, School of Medical Sciences, State University of Campinas (UNICAMP), São Paulo, Brazil.
[2]Department of Anatomic Pathology, School of Medical Sciences, State University of Campinas (UNICAMP), São Paulo, Brazil.

ABSTRACT: The present review is focused on the current role of neoplastic stem and progenitor-like cells as primary targets in the pharmacotherapy of cancer as well as in the development of new anticancer drugs. We begin by summarizing the main characteristics of these tumor-initiating cells and key concepts that support their participation in therapeutic failure. In particular, we discuss the differences between the major carcinogenesis models (ie, clonal evolution vs cancer stem cell (CSC) model) with emphasis on breast cancer (given its importance to the study of CSCs) and their implications for the development of new treatment strategies. In addition, we describe the main ways to target these cells, including the main signaling pathways that are more activated or altered in CSCs. Finally, we provide a comprehensive compilation of the most recently tested drugs.

KEYWORDS: breast cancer, stem cell, cancer stem cell, anticancer drugs

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CORRESPONDENCE: schenka@fcm.unicamp.br; schenka@hotmail.com

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Introduction

Cancer has a major impact on humans worldwide, both in terms of incidence and mortality.[1] Despite the significant decrease in incidence and death rates seen over the last couple of decades in developed countries as a direct result of improvements in diagnostic and treatment strategies, inverse epidemiological trends have been detected in developing countries.[1–3] Moreover, the survival of patients with end-stage solid tumors is still low everywhere, irrespective of the socioeconomic status of the country.[4] In this context, breast cancer is particularly relevant, since it is the main cause of death among cancers in women worldwide and a perfect prototype of the epidemiological patterns stated above.[5]

For many years, the answer to the question of why malignant neoplasms resist the main therapeutic modalities (even when combined), both primarily (treatment failure) and secondarily (tumor recurrences), has involved many theories, the most recent of them being the cancer stem cell (CSC) concept.

Normal and cancer stem cells. Stem cells participate actively in different physiological processes and developmental stages of pluricellular organisms. Accordingly, they have been classified into many subtypes, two of which stand out: the embryonic and the adult stem cells. Embryonic stem cells derive from the first division of a fertilized egg and give rise to virtually all cell types during intrauterine life, while adult stem cells are present in different somatic tissues and give rise to only the specific cell types of these adult tissues, hence bearing a lesser multilineage potential when compared to embryonic stem cells.

Normal stem cells (NSCs), regardless of subtype, have two main defining properties. First, they can renew themselves, which allows self-perpetuation and maintenance of a pool of totipotent stem cells.[6–8] Self-renewal can occur by means of symmetric mitosis in which a stem cell produces two daughter stem cells, or asymmetric division in which a stem cell produces a daughter stem cell and another cell that is committed to a certain line of differentiation.[6–9] Second, NSCs can differentiate into multiple lineages (such as epithelial and mesenchymal), thus replacing and maintaining the major functional elements that characterize the surrounding tissue. In the mammary gland, for example, these differentiating cells generate two main cell types: 1) luminal epithelial cells, which line internally ductal and lobular structures, and 2) myoepithelial cells, which are contractile cells enclosing the former.[10]

Besides these two fundamental characteristics, NSCs have other features that increase significantly their chance of survival when challenged by xenobiotics. NSCs are naturally protected against xenobiotics, especially those able to modify nucleic acids, because they are quiescent (ie, in G_0 phase) most of the time and express a number of efflux pumps, such as the ATP-binding cassette (ABC) superfamily of transporters.[10]

CSCs are a subpopulation of cells found within any type of malignant neoplasm (ie, hematological or solid neoplasms), usually comprising <2% (especially in breast cancer cases) or more, depending on cancer type and detection assay.[11,12] Currently, CSCs are related to several and confusing synonyms in the literature, which include terms like tumor stem cells, neoplastic stem cells, tumor initiating cells, tumorigenic cells, and cancer progenitor (or progenitor-like) cells.

Currently, there is no consensus on the definition of the terms "cancer stem cell", "cancer progenitor cell", and "tumor-initiating cell". In some studies, these terms are used loosely and interchangeably as synonyms. In others, the use of "cancer stem cell" is limited to a more immature, totipotent (ie, full multilineage potential) stem cell, while "cancer progenitor cells" is generally applied to designate CSC daughter cells with more restricted capacity of differentiation (ie, stem cells with less multilineage potential). "Tumor initiating cells", on the other hand, can be applied to neoplastic cells that account for the successful occurrence of xenotransplants and metastasis, even if they do not bear other stem-cell-defining features (eg, the expression of stem cell phenotypic markers) and regardless of their status/post in the maturation hierarchy. Therefore, "tumor initiating cells" can be used as a broad synonym for CSCs or cancer progenitor cells. Furthermore, it may also be used by those who are not convinced of the existence of CSCs, when referring to the first cells that reach and successfully colonize a given tissue, in xenotransplant assays or in metastatic spread processes.

The most employed term, namely "cancer stem cell", derives from the observation that they bear most of the fundamental features of NSCs as pointed out above.[6–8] They are capable of self-renewal by means of symmetric or asymmetric mitosis, thereby controlling tumor maintenance and growth. They can give rise to all cell types seen within a certain tumor, which explains its morphologic heterogeneity and similarities between primary and metastatic neoplasm.[7] It is to be noted that their tumorigenic activity is not limited to the metastatic phenomenon (ie, giving rise to a new tumor mass within the same organism), but also enables them to form tumors when transplanted into immunodeficient animals.[7] Finally, they usually display low proliferation rates and are frequently found to express a variety of cytoplasmic membrane-bound efflux transporters.[13]

Efflux transporters, also known as efflux pumps or ABC transporters, are ATP-dependent pumps that can promote the translocation of substrates across biological membranes against a concentration gradient.[13] By doing so, these transporters help in protecting different cell types against the potential toxic effects of many xenobiotics (including several chemotherapeuticals). ABC transporters have been found to be highly expressed on normal and CSCs, and contribute to multidrug-resistance phenomena in the latter case. Forty-eight ABC transporter encoding genes have been identified in the human genome, and they are categorized into seven subfamilies A–G.[13] The most studied and relevant efflux pumps for CSCs so far, from the pathophysiologic point of view, are ABCB1 and ABCG2. ABCB1 or P-glycoprotein (P-gp) is the product of the *MDR1* gene and provides resistance against a multitude of structurally unrelated hydrophobic compounds (including chemotherapeutic agents such as etoposide, doxorubicin, and vinblastine).[13] ABCG2, also known as BCRP (breast cancer resistance protein) or ABCP (ABC transporter in placenta), is a 72-kDa protein capable of transporting doxorubicin, mitoxantrone, topotecan, methotrexate, and tyrosine kinase inhibitors, among other substances.[13]

Despite these similarities with NSC, they differ in that the mechanisms that normally regulate these processes are absent or anomalous, such that in response to variable selection pressures they may continuously originate more adapted/resistant clones.[14]

Historical aspects: the evolution of the CSC concept. It is generally accepted that the CSC hypothesis started with Cohnheim, who postulated in 1875 that NSCs, which had been misplaced during embryonic development, could later be implicated in tumorigenesis.[8,15,16]

This hypothesis was based on the many biologic similarities that can be traced between embryonic and neoplastic tissue. Indeed, both tissues are composed of cells that can self-renew, originate distinct cell types, migrate, resist toxic substances, and live for longer periods.[17,18] In addition, ovarian and testicular teratomas contain a variety of cell types that are not normally found in these primary sites, suggesting that such tumors could originate from cells with multilineage potential, just like embryonic stem cells.

Subsequently, in 1974, Pierce further developed Cohnheim's concept by suggesting that malignant neoplasms could initiate from NSCs that had accumulated carcinogenic mutations that impair normal regulatory mechanisms of proliferation and differentiation.[16,19] Carcinogenic mutations take time to occur and accumulate in a single cell, but NSCs are long-lived, so it makes sense that these cells should be the preferred origin of malignant neoplasms.[8,18,20,21] Moreover, extra mutations would be necessary for a differentiated cell to acquire the self-renewal capacity, while this is an innate feature of NSCs.[22]

Despite the theoretical background summarized above, the first solid evidence for the stem cell origin of cancer came in 1997 with the demonstration by Bonnet and Dick[23] that only very immature CD34+/CD38– cells, derived from acute myeloid leukemia patients, could successfully reconstitute the referred malignancy in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Since then, the existence of neoplastic cells with stem cell-like features has been demonstrated in most if not all malignant neoplasms, including solid tumors such as breast cancer, prostate adenocarcinomas, brain gliomas, lung cancer, colorectal carcinomas, and melanoma.[17,24–27] In these studies, such cells are often denominated CSCs. It seems that the CSC concept has received

greater acceptance and development among leukemia and breast cancer studies; however, a growing number of studies show that the model can be generalized to other solid tumors as well (in particular, gliomas and colorectal cancers).[24,25] It is important to emphasize that, regardless of the type of neoplasia, a better understanding on the biology of these cells, particularly on the signaling pathways that control their growth, is needed. It is clear that the current lack of reliable CSC markers hampers significantly the development of new CSC-specific drugs.

The first report on the presence of CSCs on solid tumors was made by Al-Hajj et al[17] and involved breast cancer. Using flourescence-activated cell sorting (FACS), they isolated a tumorigenic population of cells with the phenotype $CD44_{+}$/$CD24_{-/low}$. Less than 200 of these cells were sufficient to generate tumors when xenotransplanted into NOD/SCID mice, although an average of 50,000 were needed in the unsorted population to produce the same results. Enhanced tumor-forming capacity of $CD44_{+}/CD24_{-/low}$ cells was later confirmed by many others.[14,28] Recently, by contrast, some critics have suggested that the CSC hypothesis could be simplistic and artificial, since the gold standard for defining stemness is the tumorigenicity in immunodeficient mouse models. They argue that the mammary fat pads of immunodeficient mice may not necessarily be a realistic surrogate for the microenvironment/niche where CSCs thrive in the human body. Therefore, some have proposed a more complex model of cancer development, merging the classic "clonal evolution" model (often referred to as the stochastic model) and the concept of CSCs.[29]

Cancer stem cells and carcinogenesis models. In the course of history, several models of carcinogenesis have been proposed.[30] Lately, at least two main models have survived criticisms to become the most commonly reported theories in the literature on cancer: (1) the clonal evolution and (2) the stem cell models (Table 1).

The classic or stochastic clonal evolution model postulates that any normal cell (regardless of its maturation status or hierarchical post in a given tissue) may originate a malignant neoplasm and that all cells within a tumor may contribute in varying degrees to its maintenance and further development.[30–32] According to this theory, cancers originate and evolve as a consequence of the cumulative/multistep acquisition of genetic and epigenetic alterations, which depend on random phenomena as well as on certain driving forces (or selection pressures) such as the exposure to carcinogenic and therapeutic agents.[33] Compelling evidence from clinical studies on B-cell lymphoblastic leukemias supports this model.[32]

The other model is represented by the CSC hypothesis, which states that cancers arise not from any cell type of a given somatic or germinal tissue but exclusively (or at least most frequently) from stem cells.[31,32] Again, upon the progressive accumulation of genetic/epigenetic aberrations, this transformed stem cell (from now on called CSC) would then be responsible for the maintenance, repopulation, progression, and local/systemic dissemination of the malignant process.[31,34–37] The CSC model is supported mainly by studies on germ line and breast cancers.[32] In breast cancer, on the basis of a growing body of evidence, it has been hypothesized that tumor initiation would take place preferentially in normal mammary stem or progenitor cells expressing the CSC marker CD44.[38] Furthermore, it has been assumed that the relative frequency of these cells would also determine tumor progression by increasing the chances of metastasis and of a worse clinical outcome.[39,40]

Breast cancer is not a single disease with a single tumorigenesis pathway but a highly heterogenous group of diseases from both clinicopathologic and molecular points of view. Currently, based on gene expression profiling (or alternatively, on immunohistochemistry phenotyping), breast cancer can be classified into five molecular subtypes: luminal A, luminal B, HER2/neu-positive, and triple-negative/basal-like. These subtypes reflect differences not only in the expression of estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor type 2 (HER2/neu) but also in metastasis rates and post-treatment recurrence.[41–43] Furthermore, a growing number of studies now suggest that

Table 1. Brief summary of the main carcinogenesis models reported in the literature of cancer: clonal evolution (stochastic) versus the stem cell models.

	STOCHASTIC	CANCER STEM CELL
Origin of the neoplastic process	Any cell type (including a stem cell)	The cancer stem cell (a mutated stem cell)
Maintenance of the neoplasia	Any cell type that proves to be resistant to the presenting selection pressures	The cancer stem cell
The existence of neoplastic cells with stem cell features	It is just another phenotypic subtype of cancer cell (frequently associated with heterogeneous tumors), and possibly bearing a greater potential to promote resistance	The cancer stem cell (a "stable" subtype of cell)
Supporting evidence	The existence of cancer stem cells has not been demonstrated in all malignancies	It is "easier" to obtain a neoplasia from a mutated stem cell than from a normal well-differentiated cell. Most neoplasms have cells with stem cell phenotypic features

Notes: Refs: Shackleton et al,[32] Kakarala and Wicha,[10] Al-Hajj and Clarke,[9] Dick,[60] Polyak and Hahn.[42]

the presence of CSCs in breast tumors is highly associated with specific subtypes[44,45]

In support of this theory, Honeth et al[46] recently demonstrated a significant association between basal-like phenotype—a poor prognosis molecular subtype of breast cancer—and the number of CD44+/CD24− cells. Additional experimental studies have also confirmed the relationship between CD44+/CD24− breast cancer cells and increased in vitro expression of other stem cell biomarkers (such as the capacity for mammosphere formation), not to mention enhanced invasion, resistance to radiation, and metastatic potential.[47–49] Also, consistent evidence derived from clinical studies demonstrates that CD44+/CD24− breast cancer cells express an invasive gene signature that is associated with an increase in the risk of distant metastases.[38,49,50]

Most importantly, CD44+/CD24− should not be regarded as the only CSC profile to predict increased aggressiveness and worse prognosis. Honeth et al,[46] in the study mentioned above, states that not all basal-like tumors contain CD44+/CD24− cells, suggesting the CSC phenotype may not be limited to this expression profile and that the quest for alternative breast CSC markers should proceed. As a result, other markers and specific expression profiles have been associated with CSC features, including adverse outcomes. Stingl et al,[51] for instance, reported a significant association between the fundamental stem cell characteristics of self-renewal and multilineage potential and the expression of the stem cell markers CD24, CD29, and CD49F. In agreement with these findings, Shackleton et al[32] demonstrated enhanced tumorigenic capacity among CD29high/CD24+ and CD49Fhigh/CD24+ cells.

Some studies have provided the description of full organ reconstitution from a single normal epithelial stem cell, and this fact bears significant implications for the isolation/detection of stem cells from other tissues. It is not yet certain whether there is a stable hierarchy of stem/progenitor cells in breast tissue, such as the one described in bone marrow hematopoietic tissue. Some evidences suggest that one single stem cell would be sufficient to reconstitute a complete mammary gland, although distinct progenitor cells (ie, first-generation daughter cells of a single stem cell) would be necessary for the development of different histologic components, such as ductal and lobular structures.[32] It is likely that β1-integrin (CD29) and α6-integrin (CD49f) participate in the interactions between stem cells and mammary stroma. The identification of the genes that are differentially expressed within stem and progenitor cells could contribute to the discovery of new stem cell and CSC markers.

As stated previously, many critics of this hypothesis claim that the current gold standard for assessing CSCs (ie, heterotransplantation of human neoplastic cells into immunocompromised mice) may be biased by the selection of cells that are more adapted to surviving and proliferating in the mouse microenvironment with foreign growth factors and cytokines.[52,53] In the light of these criticisms, intermediate models combining elements of both models[1] have been created, adding considerable complexity to the current understanding of tumorigenesis. These merged models predict that the frequency of CSCs in each patient should vary considerably and be dependent on the type of cancer, dominant mutations, as well as gene amplifications and deletions. Furthermore, these mixed models propose that dominant CSC clones could emerge during tumor progression, as resistant CSCs are preferentially selected by ongoing therapies.[7,54]

The distinction between the classic clonal evolution model and the CSC hypothesis is not just an academic one, because these models have different therapeutic implications. In the clonal evolution model, cure can be achieved only if treatment resulted in the death of all potentially resistant clonal subpopulations, whereas in the CSC model, resolution is possible only by the eradication of CSCs. Even in mixed models, the doubt persists because the origin and nature of CSCs remain unclear. Are they dedifferentiated cells that have acquired a more stem cell-like phenotype, or are they NSCs that through longevity have accumulated a sufficient number of mutational hits required for carcinogenic transformation? Evidence suggests that conventional chemotherapy targets the bulk of the tumor cells, allowing slow-cycling cells such as CSCs to persist after treatment and promote further metastatic disease.[8]

Despite the current theoretical controversies, it is important to note that regardless of the true origin of cancer, it is possible to detect neoplastic cells with stem cell features in most malignant neoplasms (from leukemias to solid tumors) and to consistently confirm their relationship with local agressiveness, systemic dissemination, therapeutic resistance, and worse prognosis.[55,56] So, at least for treatment purposes, perhaps we should put aside the concept of CSCs as the primary origin of cancer (as emphasized by the CSC hypothesis), and focus on the more practical concept of CSCs as (1) potential drivers of therapeutic failure in most established neoplasms and, consequently, (2) major targets in pharmacological and pathophysiological studies of cancer.

Limitations to the study of CSCs. The study of CSCs has two major constraints. First, CSCs account for a very small subset of the neoplastic cells (usually <2%) and the isolation techniques can be laborious.[23,57–60] Second, even now the identification and characterization of CSCs is limited by the lack of specific markers and biomarkers.[61]

Currently, there are four main approaches to the detection and quantitation of CSCs, and they are all based on their fundamental properties, such as (1) the capacity to originate solid tumors in immune-deficient mice (the tumorigenicity assays), (2) the ability to form spheres in cultures (such as the mammosphere and neurosphere assays),[18] (3) the presence and activity of antixenobiotic defense mechanisms (eg, membrane efflux pumps and aldehyde dehydrogenase 1 expression and functional assays),[62,63] and (4) the expression of specific cell markers (most of which are constitutively displayed on the

surface of the cells) and whose detection depends mostly on immuphenotyping techniques, such as immunocytochemistry and flow cytometry.[17] Although a detailed description of these methods is beyond the scope of this review, it is worth mentioning that the first approach is the closest to the definition of the "gold standard" (though seriously limited by ethical and biological criticism, as already established). In addition, sphere-forming tests and those assays designed to assess anti-xenobiotic mechanisms are limited by "logistics" and technical difficulties because they require considerable amounts of fresh CSC-rich specimens. Because of these relevant problems, the last approach has become the most widely recommended and reported in the literature.

Ways of Targeting Cancer Stem Cells and Successful Pharmacological Agents

Targeting CSCs can, theoretically, be achieved by exploring two of their fundamental properties, namely (1) the deregulated pathways implicated in self-renewal, and (2) typical surface or intracellular stem cell markers. Here, we summarize the current knowledge about these specific targets and the studies describing the most promising agents (see Table 3), with emphasis on breast cancer literature.

Signaling pathways. The signaling pathways that are most frequently deranged in CSCs are Notch, Hedgehog, Wnt, p53, and HER-2. The aberrant activation of Notch-1 favors chemoresistance and radioresistance[47] of CSCs, whereas Hedgehog, Wnt, and HER2 expressions seem to correlate with stem renewal and increased CSC numbers.[64–66] Because of this, Notch, Wnt, Hedgehog, and HER-2 have been studied as critical signaling pathways for the self-renewal process, proliferation, metastasis, and tumor development.[67–69]

Recent studies have shown that the inhibition of the Notch pathway by gamma-secretase inhibitors (GSI) (eg, dual antiplatelet therapy, DAPT) results in the reduction of CSC marker expression and parallel decrease in tumor growth in vivo. In glioblastoma studies, Notch pathway blockade by GSIs reduced the immunoexpression of CSC markers (such as CD133 and nestin) in neuroespheres. In addition, by blocking the Notch pathway, the cells lose their colony-forming efficiency both in vitro and in vivo.[70] In preclinical studies, Schott et al[71] have shown that the inhibition of the Notch pathway could reduce the number of CSCs in xenograft models of breast cancer. The same authors have also demonstrated in clinical trials the viability of combining GSI and a chemotherapeutic agent (docetaxel) for advanced breast cancer, while encouraging further studies to define better drug combinations. These findings have been confirmed for several other malignancies using preclinical models.[72,73] As a result, these compounds have entered clinical trials.[71,74]

In breast cancer, it is important to mention that any novel strategy to target Notch must take into account potential crosstalks with other prominent signaling pathways, such as those involving ERs and the product of the HE2 oncogene.[75] For instance, in ER+ cells, estrogens inhibit Notch activity, while anti-estrogens and estrogen withdrawal can activate Notch.[76] Notch signaling, in turn, may stimulate ER-dependent transcription, suggesting the existence of feedback mechanisms controlling Notch–estrogen crosstalk.[77] These data indicate that the combined inhibition of estrogen and Notch pathways may prove to be effective in treating luminal-type breast cancers.[76] Similarly, the combined inhibition of Hedgehog and Notch signaling by Genetech's GDC-0049 and Roche's RO4929097, respectively, has resulted in a more efficacious anti-neoplastic effect, thus highlighting their role in CSC pathology and possible Hedgehog–Notch interactions.[55,78,79]

The Hedgehog pathway by itself has been shown to play a prominent role in chronic myeloid leukemia (CML) pathogenesis by regulating the process of self-renewal of CSCs.[80] Using the Hedgehog antagonist cyclopamine, Zhao et al[81] improved the efficacy of tyrosine kinase inhibitors by depleting CSCs and subsequently improving survival of CML-bearing mice.

Concerning the Wnt/β-catenin canonical pathway, which is one of the most studied molecular pathways in oncogenesis, a number of inhibitors have been tested. These include non-steroidal anti-inflammatory drugs, molecularly targeted agents (such as the CREB-binding protein/β-catenin antagonist ICG-001), and biologic inhibitors (antibodies, RNA interference agents, and recombinant proteins).[82] These attempts to inhibit this pathway followed the evidence provided by Heidel et al[83] and Hu et al,[84] who first showed that the Wnt/β-catenin pathway is involved in CSC renewal (particularly, in CML), and that deletion of the β-catenin results in a significant loss of remaining CSCs in the bone marrow of mice bearing CML, previously subjected to imatinib therapy.[83,84]

Another promising way to inhibit CSCs may be achieved by targeting tumor supressor genes such as *p53*, which has been implicated in the self-renewal of these cells. Korkaya and Wicha[11] suggest that a deregulation in *p53* and in *PTEN* genes could lead to an altered self-renewal, which could lead to resistant tumors. Although fundamental in many aspects of carcinogenesis, *p53* has not been addressed as a specific target in the context of CSC inhibition.

Finally, targeting these signaling pathways remains a challenge, since they are held as crucial in the homeostasis of NSCs. Therefore, inhibiting these signaling pathways may be detrimental to the maintenance of normal tissues.[85] Moreover, one should consider the possibility of a CSC subclone developing resistance to the inhibition of any one of these signaling pathways, thus preventing future combination therapies targeted to CSC-associated signaling pathways.[86]

Phenotypic stem cell markers. In this case, the therapeutic strategy is to target surface or intracellular antigens that are known to be preferentially expressed by CSCs. Several of these markers have been investigated with the use of diagnostic antibodies, which allows the identification, isolation/separation,

and monitoring of leukemic and solid tumor CSCs, in both preclinical and clinical settings.[6] In spite of the dispute concerning the specificity of these molecules as true markers of the CSC phenotype, they have been consistently associated with resistance to conventional therapy, including chemo- and radiotherapy, by different sources.[55] CD34, CD44, CD133, and EpCAM are the most commonly used proteins to identify CSCs in various cancers (Table 2).[87] For that matter, they have become major targets in the development of new therapeutical monoclonal antibodies (MoAbs) against several types of cancer.[55] Successful examples in preclinical studies include the P245 anti-CD44 and the MT110 anti-EpCAM MoAbs, both of which exhibited activity against breast cancer stem cells in xenograft mice models.[55] It is important to remember, however, that what is generally considered as "typical" CSC markers may vary considerably among cancer types. For instance, the profiles CD44+/CD24– and ALDH1+/CD44+/CD24–/lin– are more frequently used as CSC markers in breast and prostate cancers, while CD133 is the preferred CSC marker for brain and colorectal tumors.[12,17,24,70]

The expression of CSC marker proteins can be heterogenous both intra- and inter-tumors. Such heterogeneity may not only undermine the primary response of the tumor to MoAbs but also favor the development of secondary resistance. Therefore, future studies should concentrate on the variability of CSC marker expression across different types neosplams and stages of tumor progression, in order to facilitate the personalization of CSC-targeted medicine. Other equally illustrative examples of recent experiences with anti-CSC agents, not mentioned in the text, are summarized in Table 3.

Concluding Remarks

- Despite the growing number of publications dedicated to the study of CSCs as major therapeutic modality, there are still many unsolved questions, particularly regarding their existence as phenotypically stable cell types/subpopulations and the best methods to detect them. In our opinion, as long as there is no consensus on the true nature of CSCs and on the most reliable methods to identify them (specially, in different sample contexts), preclinical studies seeking to demonstrate an anti-CSC effect should be done with more than one detection method. When using immunophenotyping-based methods, at least two CSC markers/profiles (optimized for tumor type/site) should used.
- In the past decade, approximately 40 different substances have been tested as possible anti-CSC agents in the context of breast cancer, half of which are represented by repurposed drugs.
- Unfortunately, in most instances, the molecular mechanisms that account for the alleged anti-CSC effect were not clearly demonstrated. In addition, only a minority of studies provided in vivo supporting evidence for the in vitro findings, not to mention that only very few studies investigated the risk of adverse effects concerning NSCs. Local or systemic inhibition of NSCs and progenitor cells should be a major concern in preclinical studies

Table 2. Main cancer stem cell immunophenotypic markers across different neoplasms.

STEM CELL MARKER	SYNONYM	MOST COMMONLY FOUND ON	PUTATIVE ROLE OF THE MOLECULE
CD24	Heat stable antigen	Breast CSCs	Adhesion molecule expressed in the majority of lymphocytes and differentiating neuroblasts
CD44	–	Breast and prostate CSCs	Surface glycoprotein cell–cell interaction, cell adhesion, and migration
ALDH1	–	Normal and cancer stem cells in a wide range of tissues	ALDH isoform involved in the metabolism of aldehydes and retinol
EpCAM	Epithelial-specific antigen (ESA)	Breast and pancreatic CSCs	Transmembrane glycoprotein involved in Ca^{2+} dependent cell–cell interactions associated to cell signaling, migration, proliferation, and differentiation
CD133	Prominin-1	Gliomas and colorectal carcinoma CSCs	Glicoprotein coded by *PROM1* gene in human genome. Highly expressed in plasma membrane protrusions of several epithelial cell types. Important for the topological organization of plasma membranes
Oct-4	POU5F1	Cancer stem cells in a wide range of tissues	Protein coded by *POU5F1* gene in human genome. Commonly expressed on undifferentiated tumor cells
CD34	–	Intestinal, hepatic, and pancreatic CSCs	Cell adhesion glycoptrotein
c-Kit	CD117	Intestinal, hepatic, and pancreatic CSCs	Tyrosin kinase receptor coded by the *KIT* gene. Expressed in hematopoietic stem cells and in granulocyte precursors
CD10	CALLA	Head and neck squamous cell carcinoma CSCs	Surface metalopeptidase, expressed in lymphoid progenitor cells, and in immature B cells in the bone marrow

Note: Adapted from Klonisch et al[5] and Oliveira et al.[41]

Table 3. Preclinical drug development of CSC-specific pharmacological agents for breast cancer treatment.

CLASS	COMPOUND	MAIN EFFECT (CONCERNING CSCs)	SPECIFICITY (CSC VS NORMAL SC)	MODEL	PROPOSED MECHANISM	REFERENCES
Repurposed drugs	5-Azacytidine	↓ Tumorsphere and migration	Not established	In vitro	Not established	Chang et al[88]
	Acetaminophen	↑ Differentiation ↓ Migration and expression of efflux pumps	Not established	In vitro	Not established	Takehara et al[89]
	Benzylisothiocyanate (extracted from cruciferous plants)	↓ Expression of CSC markers	Not established	In vitro and in vivo	↓ Tyrosine kinase RONas	Rao[90]
	BMPs (bone morphogenetic proteins) 2/7 heterodimer	↓ Expression of CSC markers	Not established	In vitro and in vivo	↓ TGFβ-driven Smad signaling	Buijs et al[91]
	CDK4 inhibitor (Millipore, Billerica, MA, Cat. # 219476)	↑ Differentiation and ↓ Expression of CSC markers	Not established	In vitro	Cell cycle arrest	Han et al[92]
	Cisplatin	↑ Differentiation and ↓ Expression of CSC markers	Not established	In vitro	Not established	Prabhakaran et al[93]
	Curcumin	↓ Expression of CSC markers	Not established	In vitro	Downregulation of Wnt signaling	Charpentier et al[94]
	Curcumin + Epigallocatechin	↓ CSC marker expression	Not established	In vitro	Downregulation of STAT3–NFκB signaling	Chung and Vadgama[95]
	Disulfiram	↑ CSC apoptosis and ↓ Expression of CSC markers	Not established	In vitro	↑ MAPK pathways and EDG1/S1P1 pathways	Liu et al,[96] Robinson et al,[97] Yip et al[98]
	Fenretinide (a derivative of vitamin A)	↓ Tumorsphere	Low cytotoxicity to normal cells	In vitro and in vivo	Inhibition of cell-cycle-related genes	Wang et al[99]
	Flubendazole	↑ Differentiation ↓ Migration and expression of CSC markers	Not established	In vitro and in vivo	Arrested cell cycle at G2/M phase and induced monopolar spindle formation through inhibiting tubulin polymerization	Hou et al[100]
	Huaier aqueous extract	↓ CSC marker expression	Not established	In vitro	Inactivation of Hedgehog pathway	Wang et al[101]
	Metformin	↓ CSC proliferation	Not established	In vitro and in vivo	Not established	Barbieri et al,[102] Hirsch et al,[103] Jung et al,[104] Cufí et al[105]
	3-*O*-Methylfunicone (isolated from Penicillium pinophilum)	↑ CSC apoptosis	Not established	In vitro	↓ Survivin, hTERT, and Nanog-1 gene expressions	Buommino et al[106]
	Salinomycin	↓ Expression of CSC markers	Not established	In vitro	Not established	Lu et al[107]
	Simvastatin	↓ Expression of CSC markers	CSC-specific	In vitro and in vivo	Not established	Rennó et al[108]
	Thioridazine	↓ Expression of CSC markers	CSC-specific	In vitro	Antagonism of dopamine receptors on CSCs	Sachlos et al[109]
	Tranilast	↓ Tumorsphere and expression of CSC markers	Not established	In vitro and in vivo	Activation of aryl hydrocarbon receptor	Prud'homme et al[110]
	Trastuzumab	↓ Expression of CSC markers	Not established	In vitro and in vivo	Not established (but probably independent of HER2 status)	Ithimakin et al[111]
	Vitamin D compounds: BXL0124 and 1a25(OH)2D3	↓ Expression of CSC markers	Not established	In vitro and in vivo	Not established	So et al,[112] Wahler et al[113]
	Cisplatin + TRIAL	↓ Tumorsphere	Not established	In vitro	Inhibition of Wnt-1 signaling	Yin et al[114]
	CRLX101 (nanoparticle-drug) conjugated with camptothecin	↓ Expression of CSC markers	Not established	In vitro and in vivo	Inhibition of TOPO-1 and HIF-1α	Conley et al[115]

(*continued*)

Table 3. (*Continued*)

CLASS	COMPOUND	MAIN EFFECT (CONCERNING CSCs)	SPECIFICITY (CSC VS NORMAL SC)	MODEL	PROPOSED MECHANISM	REFERENCES
Classic and novel anticancer agents	Mitochondrial targeting liposomes incorporating daunorubicin and quinacrine	↑ CSC apoptosis	Not established	In vitro and in vivo	Activation of pro-apoptotic Bax protein	Zhang et al[116]
	Nanoparticles combining decitabine or doxorubicin	↓ Tumorsphere and ↓ Expression of CSC markers	Not established	In vitro and in vivo	Not established	Li et al[117]
	D-Gluco-, D-galacto-, and D-manno-configured 2-amino-2-deoxy-glycerolipids	↓ Tumorsphere and ↑ CSC apoptosis	Not established	In vitro	Not established	Samadder et al[118]
	Pegylated liposomal doxorubicin	↓ Expression of CSC markers	Affects normal mammary gland stem cell function	In vivo	Not established	Chun et al[119]
	Doxorubicin and all-trans-retinoic acid (ATRA)	↓ Expression of CSC markers	Not established	In vitro and in vivo	Not established	Sun et al[120]
	Doxorubicin conjugated to gold nanoparticles via hydrazone bonds	↓ Tumorsphere, tumorigenesis, and CSC marker expression	Not established	In vitro and in vivo	Not established	Sun et al[121]
	Epigallocatechin gallate analogs (synthetic analogs of the green tea polyphenol)	↓ CSC marker expression	Not established	In vitro	Activation of AMPK	Chen et al[122]
	Everolimus	↑ CSC apoptosis	Not established	In vitro and in vivo	Not established	Liu et al[123]
	Ganetespib	↓ CSC marker expression	Not established	In vitro In vivo	Decreased HIF-1α levels and decreased expression of multiple mRNA products of known HIF-1 target genes	Xiang et al[124]
	Gd-metallofullerenol nanomaterial	↓ CSC marker expression	Not toxic to normal mammary epithelial cells	In vitro and in vivo	Not established	Liu et al[125]
	IMD-0354 (inhibitor of NF-κB with anti-inflammatory activity)	↓ CSC marker expression	Cytotoxic effect on non CSCs	In vitro and in vivo	Inhibition of NF-κB pathway	Gomez-Cabrero et al[126]
	Lapatinib	↓ Expression of CSC markers ↓ Tumorsphere	Not established	In vitro	Not established	Farnie et al[127]
	Notch1 blocking short hairpin RNA (+ paclitaxel)	↓ Tumorsphere and expression of CSC markers	Not established	In vitro	Reversion of paclitaxel-induced resistance by downregulation of Notch-1	Mao et al[128]
	PCIAC133–saporin (photochemical internalization for the endosomal escape of the CD133-targeting immunotoxin AC133–saporin)	↓ Expression of CSC markers	Not established	In vitro	Not established	Bostad et al[129]
	RNA aptamers against CD44	↓ Expression of CSC markers	Not established	In vitro	Not established	Ababneh et al[130]
	Sorafenib (+ radiation)	↓ Tumorsphere and expression of CSC markers	Not established	In vitro	↓ HIF-1α expression	Lee et al[131]
	Triterpenoid CDDO-Imidazolide	↓ Tumorsphere and expression of CSC markers	Not established	In vitro	↓ Protein levels of Notch receptors, TGF-b/Smad (pSmad2/3), and Hedgehog downstream effectors (GLI1)	So et al[132]

like these, given the biological similarities between NSCs and CSCs. Furthermore, a better understanding on the underlying mechanisms of action of these drugs could foster the discovery of molecular targets that would be specific to CSCs and safer for NSCs.

Author Contributions

Conceived and designed the experiments: VBS and AAS. Analyzed the data: VBS and AAS. Wrote the first draft of the manuscript: VBS and AAS. Contributed to the writing of the manuscript: VBS and AAS. Agree with manuscript results and conclusions: VBS and AAS. Jointly developed the structure and arguments for the paper: VBS and AAS. Made critical revisions and approved final version: VBS and AAS. Both authors reviewed and approved of the final manuscript.

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