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Cancer stem cell in breast cancer therapeutic resistance

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ABSTRACT

Development of therapeutic resistance and metastasis is a major challenge with current breast cancer (BC) therapy. Mounting evidence suggests that a subpopulation of cancer stem cells (CSCs) contribute to the cancer therapeutic resistance and metastasis, leading to the recurrence and death in patients. Breast cancer stem cells (BCSCs) are not only a consequence of mutations that overactivate the self-renewal ability of normal stem cells or committed progenitors but also a result of the de-differentiation of cancer cells induced by somatic mutations or microenvironmental components under treatment. Eradication of BCSCs may bring hope and relief to patients whose lives are threatened by recurrent BCs. Therefore, a better understanding of the generation, regulatory mechanisms, and identification of CSCs in BC therapeutic resistance and metastasis will be imperative for developing BCSC-targeted strategies. Here we summarize the latest studies about cell surface markers and signalling pathways that sustain the stemness of BCSC and discuss the associations of mechanisms behind these traits with phenotype and behavior changes in BCSCs. More importantly, their implications for future study are also evaluated and potential BCSC-targeted strategies are proposed to break through the limitation of current therapies.

Introduction

Breast cancer (BC) is one of the most common cancers responsible for approximately 30% of new female cancer cases and ranked as the 2nd cause of cancer-related deaths in annual statistics [1]. The treatment options for BC, including breast-conserving surgery or mastectomy, radiotherapy (RT), chemotherapy (CT), hormone therapy (HT), and other novel therapies, are decided based on the individual features of clinico-pathology. For instance, mastectomy and adjuvant RT are utilized for many early BCs with curative intent. Conventional anticancer drugs can be employed as a single agent or in combinations to minimize the recurrence risk. For women with estrogen receptor positive (ER^+) or human epidermal growth receptor positive ($HER2^+$) tumors, tamoxifen or trastuzumab respectively contribute to the substantial improvements in long-term survival rate. These therapeutic options are considered as a milestone in dealing with BC.

However, many BC patients still experienced relapse in a few years and the long-term mortality remains high. The 15-year BC mortality fluctuated between 41.3% and 49.5% regardless of post-mastectomy radiation [2], indicating current therapies blend BC treatment with high degrees of uncertainty in spite of widely applied neoadjuvant therapies. BC is normally treated based on its intrinsic subtypes, which can only partially explain the biology and response to treatment. The failure of treatment to deal with intractable cancer cells has raised a question of whether there is a special population of cells in tumor heterogeneity which exhibit resistant phenotypes that favor the micrometastasis and have the potential to cause recurrence.

For the past few years, cancer stem cell (CSC) model was proposed and has received increasing interest. Collective work has revealed that tumor regeneration could be initiated by these CSCs. They are capable of self-renewal, recapitulating the heterogeneity of original tumors, and differentiating into the whole bulk of a new tumor in immunocompromised mice. Fractional irradiation caused lower level of reactive oxygen species (ROS) in breast cancer stem cells (BCSCs) compared to highly differentiated tumor cells, suggestive of a radioresistant phenotype [3]. Treating BCSCs with a multidrug CT not only increased the expressions of markers in pre-existing BCSCs but also promoted CSC-dependent non-stem cancer cells-to-CSC conversion [4]. As a result, targeting BCSCs seems to be an efficient adjuvant way to improve disease prognosis.

In this review, we summarize the latest studies about cell surface markers and signaling pathways that sustain the stemness of BCSC and

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discuss the associations of mechanisms behind these traits with BCSC generation, regulation, and transition. More importantly, their implications for future study are evaluated and potential BCSC-targeted strategies are proposed to break through the restriction of current therapies. We believe that the further exploration in this field of research will help researchers effectively identify and target BCSCs in tumors and eventually help doctors and patients achieve an improved response to BC therapy.

Is CSC the culprit of BC therapeutic failure?

The existence of CSCs was first evidenced by Bonnet and Dick [5] in human acute myeloid leukemia. These cells were similar to normal hematopoietic stem cells and can hierarchically differentiate into leukemic clone. The hierarchy resembles the differentiation process of hematopoietic progenitor cells and puts forward the necessity of targeting CSCs in cancer treatment. Based on research findings, a consensus definition of CSC was proposed by American Association for Cancer Research in 2006, and that is 'a cell within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise a tumor' [6]. Newly presented evidence suggested that BCSCs may be not only a consequence of mutations that overactivate the self-renewal ability of normal mammary stem cells or committed progenitors but also a result of the de-differentiation of cancer cells induced by somatic mutations or microenvironmental components under treatment (Fig. 1) [7]. The most relevant mutant genes that may give rise to BCSCs are listed in Table 1. Under conventional treatments that kill rapidly proliferative cancer cells, CSCs remain self-renewal and contribute to the risk of tumor recurrence. Of note, the model of colony evolution also suggests that CSCs may be not the same as the initial tumorigenic cells. There might be some variations occurring in the stemness-related genetic features of CSCs during disease progress, leading to the phenotypic and functional switches [6]. The so-called tumor-initiating feature of CSCs can therefore be only used to refer to their ability to cause a tumor in xenografts but not to address the cell-of-origin.

BCSCs were first identified and isolated by Al-Hajj [19] from a patient-derived xenograft (PDX) model in 2003. The tumorigenic subpopulation of cells displayed the surface marker of $CD44^+CD24^{-/low}$ and lack of lineage markers. In next few years, they were sequentially detected in early disseminated or peripheral circulating BC cells from patients' bone marrow and thus considered to be associated with BC recurrence and distant metastasis [20]. The presence of undifferentiated $CD44^+CD24^{-/low}$ tumor cells after CT was unfavorable in patients with invasive ductal carcinoma, and the increased proportion of $CD44^+CD24^{-/low}$ cells in tumor mass was strongly associated with lymphatic metastasis [21]. These studies provided clear evidence for the existence of BCSCs and highlighted the critical role of CSCs in BC relapse and metastasis. However, $CD44^+CD24^{-/low}$ cells are not a universal marker. In MDA-MB-231 and MDA-MB-361 cell lines, most cells display $CD44^+CD24^{-/low}$ phenotypes, but only 5% and 12% of which have tumorigenic ability, respectively [22]. Also, the correlation between the increased proportion of BCSCs in tumor tissues and poor prognosis became more significant when aldehyde dehydrogenase 1 (ALDH1) was employed in combination [23]. Such phenomenon may be due to the distribution disparity of CSC markers among different tumor subtypes [24], and, as a result, more BCSC markers are required to be found and used in combinations for a specific and efficient identification of BCSCs from different cell lines, tumor tissues, or even progression stages. The putative CSC phenotypes identified in BCs so far and their sources are showed in Table 2. Their functional contributions to BC therapeutic resistance and progression will be further discussed in the next section.

In chemoresistant or radioresistant BC cell lines and human tissues, the proportion of BCSCs was significantly increased [3]. CSCs are the root of cancer development and characterized by the common features of mammary stem cell, including quiescence, self-renewal, and differentiation potential. The self-renewal ability gives BCSC a survival advantage by efficiently repairing the DNA damage, while the differentiation potential confers BCSC a tumorigenic ability. Microenvironmental components, including exosomes, chemokines, and extracellular matrix, also play an essential role in maintaining the phenotypes through interacting with BCSC surface markers [26,30,43]. The action closely links the changeable stem-like properties to the diverse tumor microenvironments via intracellular signaling. Compared with non-CSCs, the overactivation of several transcriptional factors and signaling pathways, such as SRY (sex determining region Y)-box 2 (SOX2), Sonic Hedgehog (Hh) pathway, Notch pathway, and Wnt/β-catenin pathway, that are related to embryonic stem cell growth and differentiation can explain the stemness of BCSCs [44–47].

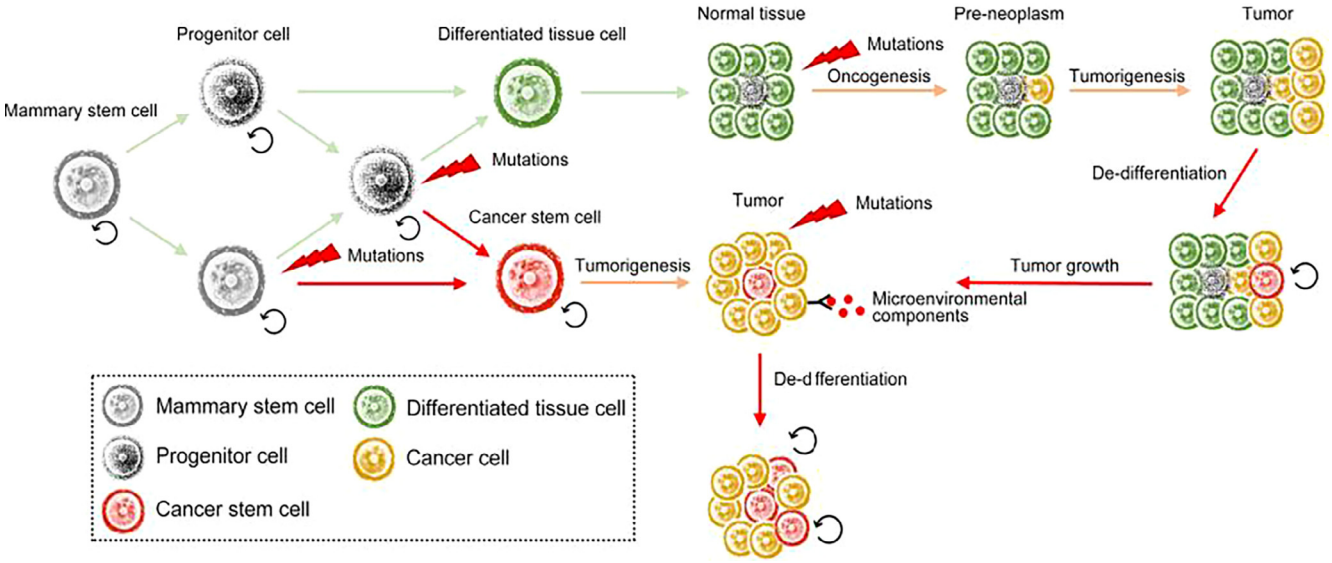

Fig. 1. Generation of BCSCs. The oncogenic mutations on mammary stem cells and progenitors can give rise to BCSCs [7]. These cells differentiate into BC cells and lead to the tumorigenesis which follows the hierarchical model. Furthermore, BC cells have the potential to de-differentiate into BCSCs due to the cellular genetic/epigenetic mutations (colony evolution) or different microenvironmental components. These two factors along with hierarchical model also collectively contribute to the breast tumor heterogeneity [8].

Table 1
The most relevant mutant genes that may give rise to BCSCs.

Mutant genes	Consequences	Specimen sources	Ref.
P53[a]	P53 loss and CD44 up-regulation	Human breast tumor	[9,10]
BRCA1[a]	Aberrant EpCAM$^+$CD49f$^+$ luminal progenitors	Human basal-like breast tumor	[11]
Wnt-1[a]	Constitutive Wnt-1 signaling which induced aberrant CD61$^+$ luminal progenitors	The pre-neoplastic tissues of MMTV-Wnt-1 transgenic mice	[12]
PTCH1[b]	PTCH1 inactivation and constitutively active Gli	Human primary breast tumor	[13]
GSK3β[a]	GSK3β inhibition and constitutively active β-catenin which induced aberrant progenitor amplification	Mouse mammary glands	[14]
KEAP1[a]	KEAP1 inactivation and NRF2 overactivation	Human breast tumor	[15]
RTK[a]	RTK activation and enhanced PI3K activity	Human breast tumor	[16]
PTEN[a]	PTEN inactivation and PI3K activation	Human breast tumor	[17]
PI3K[a]	Enhanced catalytic activity of PI3K	Human breast tumor	[18]

Abbreviations: PTCH1, patched 1; Gli, GLI family zinc finger; GSK3β, glycogen synthase kinase 3 beta; NRF2, nuclear factor E2-related factor 2; KEAP1, kelch-like ECH associated protein 1; RTK, receptor trypsin kinase; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase.
[a] Nonsynonimous somatic mutation.
[b] Synonimous somatic mutation.

Furthermore, the overactivation of antiapoptotic phosphoinositide 3-kinase (PI3K) signaling pathway and antioxidant nuclear factor E2-related factor 2 (NRF2) signaling pathway also confers BCSCs a more resistant phenotype than non-CSCs against cytotoxic drugs- or irradiation beam-induced ROS attack and apoptosis [17,48]. An example is that CSCs enjoyed hypoxic environment and highly expressed free-radical scavengers, whereby radiation caused less intracellular accumulation of ROS [49], conferring themselves a radioresistant phenotype. Additionally, some BCSCs were characterized by a dramatic overexpression of cell surface pump, such as ATP-binding cassette sub-family G member 2 (ABCG2), which impaired intracellular accumulation of anticancer drugs [25]. Along with enhanced antioxidative and antiapoptotic signaling, these BCSCs displayed stronger resistance than non-CSCs to chemotherapeutic drug-induced cytotoxicity [48]. The putative mechanisms underlying BCSC therapeutic resistance are summarized in Fig. 2.

On the other hand, some BCSCs develop the ability to escape immunosurveillance by innate immune cells or to induce immunosuppression. For example, Sultan [50] found that ALDH$^+$ 4T1 cells had decreased expressions of antigen processing and presentation genes (TAP1 and TAP2) and co-stimulatory molecule CD80 because of promoter hypermethylation. The alteration reduced the sensitivity of these BCSCs to T cell-mediated attack in immunocompetent mice [50]. In addition, CD44$^+$CD24$^-$ and ALDH$^+$ subpopulations in MCF-7, SK-BR-3, and MDA-MB-231 cells were characterized by a CD73-overexpressed phenotype [51]. Accordingly, the abnormally expressed CD73 in these stubborn tumor cells implied that CSCs might be an important part of immunosuppressive apparatus in the tumor bulk. CD73$^+$ BCSC-derived adenosine could be a previously neglected mechanism by which recurrent tumors evade the immune attack. This is because the extracellular adenosine generated by CD73 can cause the functional inhibition of T cells through direct effects on immune regulatory molecules, including regulatory T cells (Tregs), PD-ligand 1 (PDL1), indoleamine 2,3-dioxygenase (IDO), and anergy [52]. Moreover, adenosine can also activate A_{2A} receptors expressed on natural killer (NK) cells, accelerating the growth and lung metastasis of BC in mice, whereas A_{2A} receptor knockout promoted the maturation and activity of NK cells and reduced BC metastasis [53].

Table 2
The putatively effective BCSC phenotypes in principal literatures.

Phenotypes	Specimen sources	Ref.
ABCG2$^+$	Human HCC1937	[25]
ANTXR1$^+$	Mouse metastatic mammary tumor TMD231 cell line	[26]
CD29$^+$	Human MCF-7 cell line	[27]
CD61$^+$	Mouse mammary MMTV-Wnt-1 tumors	[12]
CD133$^+$	Human primary breast tumor; human MDA-MB-231, MCF-7 and ZR-75 cell lines; mouse BRCA1$^-$ mammary tumor	[28–30]
CXCR4$^+$	Human metastatic region of breast cancer and disseminating regions; human MCF-7 cell line; Mouse 4T1, 4T07, 168Farn, and 67NR cell lines	[31]
PROCR$^+$	Human MDA-MB-361 cell line; Mouse mammary fat pad-enhanced derivative of MDA-MB-231	[22,32]
CD24$^+$CD29$^+$	Mouse BRCA1-mutant primary mammary tumor; pre-neoplastic mammary tissue of virgin MMTV-Wnt1-transgenic mice	[33,34]
CD24$^+$CD49f$^+$	Mouse BRCA1-mutant primary mammary tumor	[33]
CD44$^+$CD24$^{-/low}$	Human primary breast tumor and metastatic pleural effusions; human MCF-7, BT-549, MDA-MB-231, MDA-MB-361, MDA-MB-468, T47D, ZR75, SK-BR-3, and HCC1937 cell lines; mouse BRCA1$^-$ primary mammary tumor	[19,21,22]
CD49f^{hi}CD61^{hi}	Mouse HER2/neu transgenic model	[35]
CD133$^+$ALDH1$^+$	Human invasive ductal breast tumor	[36]
CD44$^+$CD24$^{-/low}$ABCG2$^+$	Human MDA-MB-231 and MCF-7 cell lines	[37]
CD44$^+$CD24$^{-/low}$ALDH1$^+$	Human invasive ductal carcinoma; human; MDA-MB-231, MDA-MB-453, MDA-MB-468, SUM149, SUM159, SK-BR-3, ZR-75, and HCC1954 cell lines	[22,23,38]
CD44$^+$CD24$^{-/low}$EpCAM$^+$	Human MCF-7, MDA-MB-231, SUM149, and SUM159 cell lines	[19]
CD44$^+$CD24$^{-/low}$SSEA-3$^+$	Human MCF-7 and MDA-MB-231 cell lines	[39]
CD44$^+$CD49f$^+$CD133/2$^+$	Human primary ER$^-$ breast tumor and metastatic pleural effusions	[40]
CD44$^+$CD133$^+$ALDH1$^{+/hi}$	Human MDA-MB-468 cell line	[38]
CD133^{hi}CXCR4^{hi}ALDH1^{hi}	Human invasive ducal breast tumor	[41]
EpCAM$^+$CD49f$^+$	Human aberrant luminal progenitor cells from BRCA1-mutant mammary tissue	[11]
EpCAM^{hi}PROCR^{hi}SSEA-3$^+$	Human MCF-7 and MDA-MB-231 cell lines	[39]
GD2$^+$GD3$^+$GD3S^{hi}	Human MDA-MB-231 and MDA-MB-468 cell lines	[42]

Abbreviations: ABCG2, ATP-binding cassette sub-family G member 2; ANTXR, anthrax toxin receptor 1; CXCR4, CXC chemokine receptor type 4; PROCR, protein C receptor; ALDH1, aldehyde dehydrogenase 1; EpCAM, epithelial cell adhesion molecule; SSEA-3, stage-specific embryonic antigen-3; GD3S, GD3 synthase.

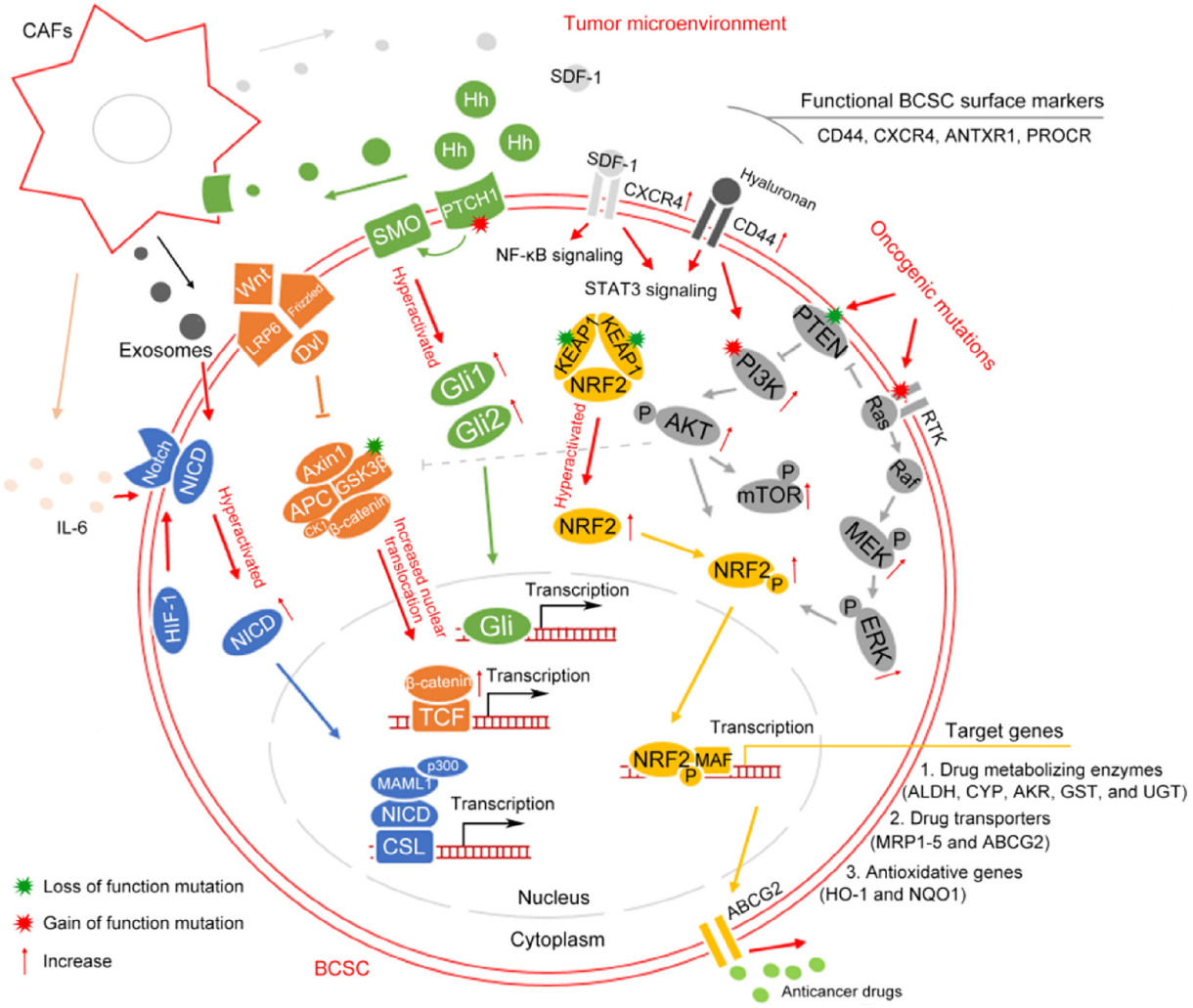

Fig. 2. Major putative mechanisms underlying the therapeutic resistance of BCSCs. These features mainly include enhanced self-renewal ability (Notch, Wnt, and Hh signaling), enhanced antiapoptotic ability (PI3K signaling), enhanced antioxidative ability (NRF2 signaling), increased efflux of anticancer drugs (ABCG2), hypoxic status (HIF-1 signaling), and tumor microenvironment-sustaining effects (exosomes or chemokines). All factors work together, contributing to a therapy-resistant phenotype of BCSC. Abbreviations: CAFs, cancer-associated fibroblasts; Hh, hedgehog; PTCH1, patched 1; SMO, smoothened, frizzled class receptor; Gli1/2, GLI family zinc finger 1/2; LRP6, LDL receptor related protein 6; Dvl, dishevelled segment polarity protein 1 pseudogene 1; APC, adenomatous polyposis coli; GSK3β, glycogen synthase kinase 3 beta; CK1, casein kinase 1; TCF, transcription factor; IL-6, interleukin 6; NICD, Notch intracellular domain; p300, E1A binding protein p300; MAML1, mastermind like transcriptional coactivator 1; CSL, CBF-1, Suppressor of Hairless, and Lag-2; CXCR4, CXC chemokine receptor type 4; SDF-1, stromal cell-derived factor 1; ANTXR, anthrax toxin receptor 1; PROCR, protein C receptor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; STAT3, signal transducer and activator of transcription 3; NRF2, nuclear factor E2-related factor 2; KEAP1, kelch-like ECH associated protein 1; p, phosphorylation; Maf, MAF bZIP transcription factor; CYP, cytochrome P450; AKR, aldo-keto reductase; GST, glutathione transferase; UGT, UDP-glycosyltransferase; MRP, multidrug resistance-associated protein; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1; ABCG2, ATP-binding cassette sub-family G member 2; RTK, receptor trypsin kinase; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; MEK, mitogen-activated protein extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase.

As conventional therapies are directed against the rapidly dividing cells rather than BCSCs, a minority population of self-renewing, long-lived, and tumorigenic BCSCs are still alive and become quiescent, setting the stage for the therapeutic resistance, recurrence, and metastasis of BC. Therefore, a more comprehensive understanding of the key markers and networks involved in the stemness of BCSCs will be necessary and helpful for finding out novel therapeutic strategies against BC.

BCSC surface markers: identification, function, and targeting

BCSC surface markers are initially used to identify and isolate CSCs from BC via flow cytometry. However, outcomes from the latest studies suggested that their activities may also determine the internal diversity among BCSCs. More importantly, as most of BCSC surface markers are membrane receptors, they are critical for the design of novel targeted drugs that would be recognized by BCSCs of interest in order to achieve a more positive response to anticancer treatment. Therefore, in this part, we first discuss the surface markers for BCSC and their potential roles in BCSC regulation.

CD44

CD44 was the first effective surface marker used to identify CSCs. As a cell receptor, CD44 mediates the communication with microenvironment through interacting with extracellular ligands, such as hyaluronan (HA). The interaction of CD44 and HA can stimulate RhoA-specific guanine nucleotide exchange factor-mediated RhoA/Grb2-associated binder-1 signaling or c-Src kinase/Twist/miR-10b/RhoA signaling that are responsible for the activation of the PI3K/AKT signaling

pathway [54]. Interestingly, Ghatak [55] reported that HA can also inhibit the PI3K/AKT pathway by activating PTEN in TA3/St cells, shedding light on the "functional switch" of CD44 that is associated with different phenotypes. Furthermore, ERM proteins (ezrin, radixin, and moesin) and Merlin may compete for the binding sites of CD44 on the cytoplasmic tail to either mediate actin cytoskeleton remodeling and invasion or inhibit ERK- and PI3K-dependent growth and migration in cancers [56]. These studies highlight the dual nature of CD44, and thus the ligand signature of CD44 in tumors will help more accurately determine the role of CD44 with distinction in the specific progress stage.

Al-Hajj [19] found that as few as 100 cells with $EpCAM^+CD44^+CD24^{-/low}$ phenotype were able to give rise to the bulk of tumors containing phenotypically diverse non-tumorigenic cells in NOD/SCID mice. The high expression of CD44 might be a result of p53 absence and is essential for the inherent resistance of BCSCs [9,10]. It was reported that HA-CD44 interaction activated Nanog/miR-21 or Nanog/signal transducer and activator of transcription protein 3 (STAT3) pathway and then enhanced antiapoptosis and increased the expression of P-glucoprotein (P-gp) that mediates the efflux of doxorubicin (DOX) and paclitaxel (PTX) in MCF-7 cells [57]. HA-decorated nanoparticles loaded with salinomycin and paclitaxel were designed to target $CD44^+$ BCSCs and demonstrated a great potential for augmenting chemotherapeutic effects *in vitro* [58]. On the other hand, $CD44^+$ BCSCs were closely associated with metastasis. The proportion of early disseminated tumor cells with $CD44^+CD24^-$ phenotype in the bone marrow of BC patients was approximately 72% [59]. These disseminating BCSCs were thought to be associated with the colonization during BC metastasis [60]. The spontaneous metastasis of $CD44^+$ BCSCs from transplanted areas to lung and lymph node in mice was observed via non-invasive imaging approaches [61]. However, an interesting finding is that distant metastatic cells were enriched for differentiated $CD44^-CD24^+$ cells [62]. To some extent, this could explain why $CD44^+CD24^-$ failed to predict poor clinical behavior in some patients, though they have higher tendency to distantly metastasize. The role of CD44 in BCSCs has sparked the clinical trial (NCT02331212) to investigate the association of HA with BCSC growth in the bone metastasis of patients. Using a 186-gene "invasiveness" expression signature in $CD44^+CD24^{-/low}$ cells, poor overall survival and metastasis-free survival can be predicted in BC patients [63].

Integrins (CD29, CD49f, and CD61)

Similar to CD44, integrins are also major cell surface receptors for extracellular ligands, such as fibronectin and laminin. They can heterodimerize with each other and mediate cell adhesion to the extracellular matrix, undertaking bidirectional communications with microenvironment. CD29, CD49f, and CD61 which encode β1, α6, and α3 subunits of heterodimer integrin, respectively, are most frequently reported in BCs and have been demonstrated to be effective BCSC markers. For example, the use of either CD29 or CD49f in combination with CD24 was able to identify CSCs from BRCA1-mutant mouse primary mammary tumor [33]. Furthermore, a subpopulation of cells separated from MCF-7 BC cells were found to carry the $CD29^+$ phenotype. Compared to parental cells, these $CD29^+$ cells showed enhanced tumorigenic ability and stronger resistance to pro-apoptosis, which may be associated with the activation of CD29 on ERK-/AKT-associated signaling pathways [27]. In BRCA1-mutant patients, basal-like BC shared the similar genetic expression signature with $EpCAM^+CD49f^+$ luminal progenitor cells in normal mammary tissues. The $EpCAM^+CD49f^+$ cells from these patients displayed aberrantly higher clonogenic activity than those from non-mutant tissues [11], suggesting luminal progenitors from BRCA1 mutation carriers may be involved in early oncogenic events. Consistently, the presence of $CD49f^+$ cells in breast tumors was associated with metastatic tendency and shorter survival time in patients [64]. Additionally, in the pre-neoplastic tissues of MMTV-Wnt-1 mice, aberrant progenitors with $CD61^+$ phenotype were found in a committed luminal progenitor population. They had strong potential to initiate mammary tumors in mice [12]. These findings provided the evidence of mutation-orientated generation of BCSCs from normal stem cells. In $Her2^+$ mouse primary mammary tumors, a subpopulation of tumor-initiating cells that showed expression signatures of TGF-β signaling and resistance to both PTX and DOX were identified using CD49f and CD61 antibodies [35]. Consistent with the role of CD61 as an epithelial-mesenchymal transition (EMT) marker, CD61 knockdown abolished the activation of TGF-β signaling by which the stemness was maintained in mouse primary mammary tumor H6O5 cells [35]. Notably, some heterodimer integrins act as a counterpart to above-mentioned integrins. For instance, the activation of α2β1 integrins can limit BC metastasis, implying that these integrins might regulate CSCs as anchors or drivers through the communication with different microenvironments, conferring them a quiescent or aggressive phenotype. Therefore, a more comprehensive and dynamic profile of integrins in BC cells may be greatly helpful for locating and targeting these CSCs.

CD133

CD133 (also known as Prominin-1) is a pentaspan and highly glycosylated transmembrane protein that defines a broad population of stem cells, including hematopoietic stem cells and endothelial progenitor cells. Although the exact role of CD133 in BC remains unclear, $CD133^+$ BC cells assuredly display CSC-like properties. These cells showed significant resistance to DNA-damaging agents and greater capability to form tumors in NOD/SCID mice [28]. Liu [29] observed that MDA-MB-231 cells with holoclone morphology exhibited $CD133^+$ phenotype and had higher colony-forming efficiency, leading to the vasculogenic mimicry [29]. Furthermore, $CD133^+$ BCSCs were found to be preferentially enriched in the tumors from patients with HT-resistant BC, promoting the self-renewal of luminal metastasis in an ER-independent way [30]. The generation of the $CD133^+$ cells was ascribed to the compensatory activation of feed-forward interleukin 6 (IL6)-Notch3 loop which restored the essential cellular oxidative phosphorylation [30]. High expression of IL6 in tumor microenvironment also favors the growth of cancer-associated fibroblasts (CAFs) *in vivo*. CAF-derived exosomes can transfer miR-221 into breast tumor cells and contribute to the up-regulation of Notch3 that is necessary for the expansion of $CD133^+$ cells [43]. Such results suggest that the activated Notch signaling pathway by IL6 might be the real target for $CD133^+$ BCSCs, though CD133 was employed for the identification. Recently, CD133-targeted polymeric nanoparticles loaded with anticancer drugs were developed. These novel agents are structurally characterized by conjugation with anti-CD133 antibody on their molecular surface that helps these tumor-killing chemicals accumulate or internalize in $CD133^+$ cells. This drug delivery system was proved to efficiently decrease the population of BCSCs and reduce their tumorigenic ability in mice [65]. Shigdar [66] have recently developed CD133-specific RNA aptamers which specifically recognize the AC133 epitope and the CD133 protein with high sensitivity in different cancer cell lines and show superior tumor penetration and retention when compared to the AC133 antibody in a 3-dimentional (3D) tumor sphere model [66]. These novel CD133 aptamers will be very helpful in future development of BCSC-targeted therapeutics. However, it is worth mentioning that CD133 is also recognized as an early marker for normal stem cells in human beings. CD133-targeted therapeutics seemingly eliminates CSCs within a tumor but might induce unpredicted myelosuppression as well, at least theoretically. Thus, more evidence should be presented to rationalize the 'targeting' of CD133-targeted CSCs eradication. Uncovering the role of CD133 in BCSCs may lead to a more accurate evaluation of $CD133^+$ tumor behavior pattern and more suitable targeted therapies.

ALDH1

ALDH1 is a NAD(P)$^+$-dependent enzyme that mediates the oxidation of intracellular aldehydes to carboxylic acids. Ginestier [67] found ALDH1 was a shared maker in normal and malignant mammary stem cells. Their study indicated that ALDH1 acted as an independent prognostic factor strongly associated with lower survival rate in BC patients [67]. *In vitro* study confirmed that CD44$^+$CD24$^-$ALDH1$^+$ MDA-MB-231 and CD44$^+$CD133$^-$ALDH1$^+$ MDA-MB-468 BCE cells exhibited stronger tumorigenic and metastatic capacity compared to ALDH1^{low}CD44^{low} cancer cells [38]. Utilizing Aldefluor® assay to identify and separate CSCs from malignant ZR-75 BC cells seemed to be more efficient than detecting CXC chemokine receptor [22].

Recently, Marcato [68] indicated that the ALDH activity of BCSCs was mostly dependent on ALDH1A3 but not ALDH1A1, further deepening the knowledge about the specific target in BCSCs. The conclusion is different from the observation found in prostate CSCs whereby ALDH1A1 is more important. The explanation for the difference is that mammary epithelium cells express a much lower basal level of ALDH1A1 than ALDH1A3. The significant association between the high expression of ALDH1A3 and BC metastasis in patients also highlighted the importance of ALDH1A3 [68]. Normally, ALDH1A3, as well as 1A1, 1A2, and 1A7, regulates early differentiation of stem cells through mediating the oxidation of retinol to retinoic acid (RA), which is vital for maintaining the differentiated potential of tissue-specific stem cells [67]. Interestingly, sphere formation assay showed ALDH blockade or inhibiting retinoid signaling increased the proportion of CSCs in several BC cell lines, including 184A1, SUM149, SUM159, and HCC1954, and gene set enrichment analysis also indicated that the self-renewal-related pathways were further activated in these cell lines [69]. However, the reason why blocking ALDH enhanced the stemness of BC is still unclear. Thus, these results suggest that although ALDH1 can be used to distinguish CSCs, more evidence needs to be displayed to prove the rationalization of ALDH1 as a potential therapeutic target for CSCs. A transgenic animal model might be useful in explaining this observation in the future study.

CXC chemokine receptor type 4 (CXCR4)

CXCR4 is a membrane chemokine receptor. Stromal cell derived factor 1 (SDF-1, also known as CXCL12) is the only ligand for the activation of CXCR4. The SDF-1/CXCR4 signaling inherits their role in facilitating the migration of CXCR4$^+$ BCSCs to the metastatic sites. Both CXCR4 neutralization by antibody and knockout inhibited the growth of orthotopically transplanted breast tumor and impaired their directional metastasis to lymph nodes and lung [70]. Mukherjee [31] found that non-migratory BCSCs facilitated the conversion of non-stem cancer cells to metastatic CXCR4$^+$ BCSCs in human primary BC tissue. Their findings not only indicated the potential of CXCR4$^+$ as a BCSC marker but also provided evidence for a CSC-mediated non-CSCs-to-CSCs conversion. The converted cells showed decreased E-cadherin and increased Vimentin, suggesting that they experienced EMT [31]. However, whether EMT causes the CXCR4 phenotype or is a consequence of CXCR4 activation is unknown.

The overactivation of CXCR4 is closely associated with tumor microenvironmental changes. The secretion of SDF-1 can be induced by a variety of insults from cytotoxic agents, irradiation, and hypoxia, implying current therapies might be the trigger of metastasis of CXCR4$^+$ cells. Furthermore, CAFs can also increase the SDF-1 level in tumor microenvironments. CAFs-induced SDF-1/CXCR4 signaling supports cancer cell stemness and metastatic phenotypes via the NF-κB signaling *in vivo* [71]. As CAFs may come from mesenchymal stem cells (MSCs) conversion induced by aberrant signaling, the contributions of MSCs to the metastatic phenotypes of CXCR4$^+$ BCSCs warrant further study. CXCR7 is another receptor for SDF-1, which are predominantly expressed on another subpopulation of tumor cells. CXCR7$^+$ cells can alleviate the desensitization of CXCR4$^+$ cells to the chronic exposure of SDF-1 by scavenging microenvironmental SDF-1 in a feedback regulation and maintain the constitutive activation of SDF-1/CXCR4 signaling, promoting the proliferation and metastasis of CXCR4$^+$ cells [72]. In addition, CXCR4 expression can be also transcriptionally regulated by FOX family, P53, TGF-β, or Hh signaling because the binding sites in CXCR4 promoter or proximal enhancer regions were detected via genomic analysis in several BC cell lines, such as Hs5787T, T47D, MDA-MB-361, MDA-MB-231, MCF-7, and MDA-MB-468 [70]. A recent study found that SDF-1/CXCR4 signaling activation can increase the phosphorylation of 60 proteins associated with migration or invasion in CD44$^+$CD24$^-$ BCSCs that might be important mediators for CXCR4-induced maintenance of BCSCs [73]. The evidence highlighted CXCR4 as a therapeutic target to block microenvironment-induced stemness and metastatic phenotypes and made it possible to abolish the signals that are actively involved in the CXCR4 network in order to eradicate CXCR4$^+$ BCSCs.

ABCG2

ABCG2 (also known as breast cancer resistance protein) is highly expressed in several chemoresistant BC cell lines. The trans-membrane pump protects BC cells from damage by reducing the cellular dynamic accumulation of cytotoxic drugs. The behavior is more significant in BCSCs. Compared with non-stem cells, the CD44$^+$CD24$^{-/low}$ cells from MCF-7, MDA-MB-231, and SK-BR-3 BC cell lines showed higher expression of ABCG2 [37]. Similarly, the overexpression of several multidrug resistance-associated proteins and P-glycoprotein in these BCSCs were also observed [74]. These findings are well supported by the pharmacodynamic studies indicating that a wide range of chemotherapeutic drugs within cells can be excreted by those transporters [15].

Because of the critical role of ABCG2 in BC chemoresistance, it was employed to separate chemoresistant BCSCs (side population cells) from tumors. The isolation can be achieved by detecting ABCG2-mediated Hoechst 33,342 dye efflux via cytometer. Leccia [25] used ABCG2 antigen to sort BCSCs from BRCA1-mutated HCC1937 BC cells and found the method was more effective for CSC identification than detecting CD44$^+$CD24$^-$ [25]. ABCG2$^+$ cells had enhanced potential for tumorigenicity and metastasis, and their presence in tumors could predict poor clinical response to CT, suggesting ABCG2 is a good independent marker for BCSC identification. However, since the intrinsic chemoresistance of CSCs also lies in the enhanced ability of DNA repair and antioxidation (the radioresistant phenotypes in BCSCs just manifested this point) while normal cells depend on ABCG2 to excrete xenobiotics, targeting ABCG2 may exert restricted effects on CSCs but increase unexpected damage to important organs. Thus, the application of ABCG2 inhibitors against BC should be re-considered and more *in vivo* evidence should be displayed.

Anthrax toxin receptor 1 (ANTXR1)

ANTXR1 is a tumor-specific endothelial marker that mediates tumor angiogenesis. The higher expression of ANTXR1 on CD44$^+$CD24$^-$ and ALDH1$^+$ TMD231 BC cell surface was uncovered by Chen [26]. Their work indicated that overexpression of ANTXR1 activated key genes in cell proliferation, DNA replication, and Wnt signaling pathway, conferring enhanced tumorigenic and metastatic potentials upon those BC cells [26]. By detecting ANTXR1, a subpopulation of malignant BCSCs could be sorted [26].

ANTXR1 partly mediates the extracellular matrix-induced stemness. It is well known that collagen VI was concentrated in breast tumors, generating a microenvironment that promotes cancer progression and metastasis. The C5 peptide of collagen VI α3 chain is the natural ligand for ANTXR1. The highly expressed ANTXR1 catered the abundant extracellular collagen VI and thus sustained the stem-like property of BCSCs *in vivo* [26]. As ANTXR1 is selectively expressed on the surface of

cancer cells that are dependent on ANTXR1 to accelerate angiogenesis or maintain stemness, ANTXR1-targeted therapeutics may suppress breast tumor growth and weaken BCSCs without excessive damage to normal tissues. This has been demonstrated in an mouse model in which the melanoma growth was disrupted by ANTXR1 knockout while other tissues remained unaffected [75]. However, the related evidence has not yet been reported in BC, with further study to warrant the potential of ANTXR1 as a therapeutic target for BCSCs.

Epithelial cell adhesion molecule (EpCAM)

EpCAM (also known as CD326 or epithelial-specific antigen, ESA) is considered as a marker for epithelial tumors and has been found to be associated with invasive BCs. Based on its role in promoting or preventing epithelial cell–cell adhesion, recent studies indicated that EpCAM plays an important role in cancer cell migration and metastasis. By detecting $EpCAM^+$ cells, a population of circulating tumor cells (CTCs) or disseminated tumor cells (DTCs) can be separated from peripheral blood of BC patients. These $EpCAM^+$ cells contained a subpopulation of metastasis-initiating BC cells that can initiate bone, lung and liver metastasis in immunocompromised mice and be used to predict unfavorable metastatic behavior in BC patients [20]. Shigdar [76] developed an EpCAM-specific-RNA aptamer. This 19-nt RNA aptamer interacts specifically with a number of live human cancer cells derived from breast, colorectal, and gastric cancers that express EpCAM, but not with those not expressing EpCAM [76]. The EpCAM aptamer-mediated survivin silencing can sensitize CSCs to doxorubicin in a BC animal model, demonstrating that this strategy for *in vivo* CSC targeting is a useful approach against BC chemoresistance [77]. However, the loss of cell adhesion is, theoretically, a prerequisite for cancer cell migration. This was evidenced by the loss of EpCAM expression of some CTCs and DTCs after EMT in patients with metastatic BCs [78]. Also, it was reported that there was a subpopulation of $EpCAM^-$ tumor cells in association with BC metastatic potential and chemoresistance in patients [64], suggesting that it might be insufficient to use single EpCAM as a surface marker for the identification and isolation of CSCs from breast tumors. Although $EpCAM^+$ cells account for the majority of epithelial tumor populations, the detecting methods primarily based on the EpCAM expression may not only underestimate the number of CTCs but also result in the off-target effect in eradicating solid BCSCs.

Protein C receptor (PROCR)

PROCR plays as a counterpart in maintaining the balance of tissue factor-mediated procoagulant effects through binding to coagulation proteases, such as protein C. It has been proved to be a specific CSC marker for triple negative BC. Hwang-Verslues [22] reported that $PROCR^+$ MDA-MB-361 and MDA-MB-231 cells showed 2-fold and 9-fold increase on colony-forming efficiency, respectively, compared with $PROCR^-$ cells [22]. PROCR was demonstrated as a marker of a unique population of mouse multipotent mammary stem cells with high capacity of regeneration and differentiation into all lineages of the mammary epithelium [79]. Interestingly, these cells exhibited EMT signatures, which proposes the possible origin of $PROCR^+$ BCSCs. PROCR inhibition by receptor blocking antibodies (αEPCR-1535) markedly reduced the tumorigenic ability of $PROCR^+$ MDA-MB-231 cells in the orthotropic model, indicating PROCR may be functionally involved in the maintenance of BCSCs [32]. However, the underlying mechanisms are poorly understood. PROCR could mediate initial BC cell migration by activating protein C but may limit cancer progression at an advanced stage through promoting tumor necrosis. Additionally, since the coagulation factor ligands for PROCR can be synthesized and regulated by tumor-associated macrophages, the stem-like properties of $PROCR^+$ cancer cells may have an unknown association with the microenvironment [32]. Thus, the correlation of PROCR-mediated coagulation protease signaling with tumor microenvironment and the role of PROCR in BCSCs is worth of further investigation. Nonetheless, the role of PROCR as a marker may shed light on a more efficient method for identifying CSCs from triple negative BC that is currently characterized by a $CD44^+CD24^{-/low}$ phenotype.

GD2

GD2 is a b-series ganglioside expressed mostly on the cell membrane. A small fraction of $GD2^+$ cells identified from MDA-MB-231 cell line were capable of forming mammospheres and initiating tumors with as few as 10 cells in immunocompromised mice [80]. Most of $GD2^+$ cells isolated from human mammary epithelial cells expressing H-Ras oncogene displayed $CD44^+CD24^-$ phenotype [80]. GD3 synthase (GD3S) is involved in the biosynthesis of GD2 and associated with EMT program in BC. The expression of GD3S was markedly increased in $GD2^+$ BCSCs [42]. GD3S knockdown significantly reduced GD2 expression and disrupted their ability to migrate and form mammospheres, suggesting that the generation of $GD2^+$ BCSCs may be associated with GD3S-mediated EMT [42]. Consistently, GD3S knockout compromised the initiation and maintenance of EMT in SUM159 and MDA-MB-231 BCE cells, while GD3S overexpression activated c-Met signaling, up-regulated GD2 and GD3, and contributed to the stem cell-like properties and metastatic competency in MDA-MB-231, MDA-MB-468, and MCF-7 cell lines [81]. Furthermore, the highly expressed GD3 activated epidermal growth factor receptor (EGFR) signaling in BC cells and may confer BCSCs a resistant phenotype to EGFR inhibitors [42]. The high expression of GD3S may be related to NF-κB activation in $GD2^+$ BCSCs. Inhibition of NF-κB signaling could reduce the expression of GD3S and the proportion of $GD2^+$ BCSCs, impairing the ability of BC cells to form new tumors or to metastasize [82]. These results indicate GD2 and GD3S are closely linked and associated with BCSC properties. Although the positive expression of GD2 can be used to identify CSCs from BC cells, GD3S-related signals that maintain the stemness are the central targets against tumor growth and metastases. Thus, future development of GD3S-related target strategies might be enlightening for BCSC therapy.

Central signaling pathways sustaining BCSCs

As mentioned above, the regulating functions of BCSC surface markers cannot be abstracted from intracellular signaling. In many cases, the aberrant activation of several important signaling pathways in BC cells, as consequences of genetic mutations, epigenetic modifications, or communications with microenvironment by surface markers, generates and sustains the stem-like nature and may be a direct cause of therapeutic resistance. Thus, a better understanding of these signaling pathways may provide a useful signature that shapes the properties of BCSCs, and then targeted therapy can be developed.

Hh signaling pathway

Hh signaling activation gives cancer cells a survival advantage with self-renewal features as it does in organizing embryonic stem cell growth and differentiation (Fig. 2). Normally, Hh signaling is upregulated in quiescent breast stem or progenitor cells but disappears when cells undergo differentiation. Although some possible activating mutations were reported by early studies, they failed to be confirmed in larger sample sets [13]. Hence, the aberrant activation of Hh signaling in BCSCs is poorly understood. It was reported that transgenic overexpression of GLI1 induced heterogeneous mammary tumors with progenitor markers in mice, whereas the tumor growth cannot be suppressed by transgenic deinduction [83], implying it was self-maintained. Consistently, BCSCs can activate the Hh signaling of CAFs within the stromal interactions *in vitro* by secreting Hh [45], which triggered cytokine release from CAFs to microenvironment and in turn promoted expansion and self-renewal of CSCs.

The Hh pathway inhibitors, such as sonidegib and vismodegib, that act as cyclopamine-competitive antagonists of smoothened (SMO) are currently used in practice for basal-cell carcinoma. However, these inhibitors are less investigated in BCs perhaps because they only showed fine efficacy towards tumors that harbor definite mutations in the Hh pathway. Thus, further study revealing the mechanisms underlying activated Hh signaling in BCs may provide some ideas for developing Hh-targeted drugs. Recently, a Phase I clinical trial reported that the combined treatment consisting of sonidegib and paclitaxel demonstrated tolerable and satisfactory effect on patients with advanced BC (NCT01954355), sheding light on the application of SMO inhibitors against BC. Additionally, targeting Hh signaling of stromal CAFs seems to be an efficient way to destroy the backbone of BCSCs. Optimistic results have been obtained using a monoclonal antibody (mAb) (5E1), though they are still undergoing pre-clinical testing [84].

Notch signaling pathway

The hyperactivation of Notch signaling cascade independent on canonical ligand induction marks a self-renewing cell population in basal BCs (Fig. 2) [46]. Notch signaling is regulated by cytokines, such as IL-6, in the tumor microenvironment. The increased IL-6 was detected in human breast tumors treated with HT. It activated cellular Notch3 signaling that can replace the ER-dependent survival mechanism and confer cancer cells a self-renewal phenotype, while blocking Notch signaling markedly decreased the self-renewal efficiency of $CD133^{high}ER^{low}$ BCSCs in HT-resistant MCF-7 cells [30]. Furthermore, hypoxia can promote the activation of the Notch pathway. The interactions between HIF-1α and Notch1 triggered the self-renewal behaviors, dictating the de-differentiation of general cancer cells to CSCs *in vitro* [85]. This may partly explain how BC cells acquire stemness in a hypoxic condition.

Notch pathway blockade may demonstrate a good efficacy in dealing with recurrent BC. This is because the blockade can not only decrease populations of BCSCs in tumors but also prevent therapy-induced Notch activation. For instance, Notch signaling is in a feed-back loop with VEGFR-1 and VEGFR-2 signaling, regulating the tumor angiogenesis in BCs [86]. Antiangiogenic VEGF inhibitors could activate Notch signaling, leading to the tumor growth. Additionally, trastuzumab or tamoxifen treatment can also unexpectedly trigger Notch activation. It suggests conventional drugs in combination with Notch inhibitors may help achieve better response to anticancer therapy and minimize the risk of recurrence. The well-investigated Notch signaling inhibitors were γ-secretase inhibitors, such as PF-03084014, which displayed anti-metastatic and anti-tumor activity by inhibiting the self-renewal ability in BC xenograft models [87]. Other promising γ-secretase inhibitors that entered ongoing Phase I/II study of BC treatment include RO4929097 (NCT01151449), LY3039478 (NCT02784795), and MK-0752 (NCT00645333). CB-103 that blocks Notch signaling by targeting the complex of transcriptional activation in the nucleus will be evaluated in Phase I/II clinical trials (NCT03422679) in the future. Additionally, several delta-like ligand 4 mAb, such as MEDI0639 and REGN421, have also entered Phase I clinical trials (NCT01577745 and NCT00871559, respectively) for patients with advanced solid malignancies. These inhibitors have demonstrated satisfactory therapeutic values with tolerated toxicity in metastatic BCs until now. The upcoming results may provide strong clinical evidence to evaluate whether targeting Notch signaling is promising in BC therapy.

Wnt/β-catenin signaling pathway

Wnt proteins normally maintain a balance between stemness and differentiation in normal stem niches and regulate cell fate. However, constitutive Wnt/β-catenin activation due to the loss of function in negative regulators underlies the tumorigenesis in mouse mammary gland [14] (Fig. 2). The appearance of aberrant progenitor cells may account for Wnt-induced tumors, and the hyperactivated Wnt signaling in these progenitors contributes to the radioresistant phenotype *in vivo* [12], suggesting a CSC-promoting role of overactivated Wnt signaling pathway. Wnt inhibitors thus may represent an effective intervention against BCs. For example, targeting Wnt pathway with sulforaphane demonstrated a significant inhibitory effect on the tumor-initiating ability of BCSCs *in vivo* without any damage to normal differentiated cells [88]. A Phase I study of OMP-54F28 that directly targets Wnt was finished in year 2017 (NCT01608867), promoting the clinical application of Wnt pathway inhibitors against BCSCs in the future.

NRF2 signaling pathway

NRF2-mediated antioxidative pathway is an emerging mechanism that, at least partly, accounts for the chemo-/radio-resistant natures of cancer cells (Fig. 2). Compared with general BC cells, a much higher expression signature of NRF2 and target genes in BCSCs was uncovered, which is a collective result of proteasome reduction and p62 increase [48]. These cells benefit from the lower ROS level under CT/RT, whereas NRF2 silence retarded the formation of mammospheres and reversed the therapy-resistant phenotypes in MCF-7 cells [48]. On the other hand, several genes involved in the pentose phosphate pathway (PPP) were also regulated by hyperactivated NRF2 in cancers [89]. It shunts glucose and glutamine into enhanced PPP that may push quiescent cancer cells into proliferative status or bypass treatment-induced metabolic blockade, termed “metabolic network adaptations”. However, its associations with the survival nature of BCSC have not yet been fully elucidated.

Since cancer cells generate more ROS than normal cells and CSCs require low level of ROS to maintain quiescence and self-renewal, combined utilizations of chemicals that dampen their antioxidant capacity were thought to make tumors vulnerable to CT/RT with normal tissues largely uninfluenced. For the purpose, a variety of natural inhibitors of NRF2, such as apigenin, all-trans retinoic acid, brusatol, chrysin, cryptotanshinone, luteolin, trigonelline, and wogonin were tested in pre-clinical cancer models [15]. Unfortunately, the exact targets through which these inhibitors exert an inhibitory effect on NRF2 are poorly investigated and their “druggability” remains unevaluated. Nonetheless, the pivotal role of NRF2 signaling provides a new train of thought for novel adjunctive therapy with potential to beat chemo-/radio-resistant natures of BCSCs.

PI3K/AKT/mTOR signaling

The sustained activation of the PI3K signaling in BCs was frequently reported in the last few years and can be basically attributed to the genetic mutations in the components of this network (Fig. 2). For example, the gain of function mutations in upstream receptor tyrosine kinases could aberrantly upregulate the activity of PI3K [16], while the loss of PTEN function was found in about 50% of BC patients [17]. Furthermore, the highly prevalent somatic mutations in *PIK3CA* can also increase the catalytic activity of PI3K in BCs. Cooperating with the heterozygous loss of *Apc,* mutant *PIK3CA* triggered centrosome amplification and increased tolerance to tetraploidization in BC cells [18]. Both can induce irreversible genomic alternations and promote tumorigenesis, indicative of an oncogenic role of the PI3K signaling. Our recent study has demonstrated the activation of the PI3K/AKT/mTOR signaling pathway was associated with enhanced CSC phenotypes and EMT in radioresistant prostate cancer cells, indicating the importance of this pathway in regulating CSCs [90]. In BCSCs, the PI3K/Akt/mTOR signaling pathway mainly mediates CD44-regulated metastasis. In addition, the sustainable stemness was also partly dependent on PI3K-regulated transactivation of several self-renewal pathways, such as Wnt/β-catenin signaling, in triple negative BCs [47].

Inhibiting PI3K signaling pathway to inhibit tumor growth is not a new idea. Rapalogs, such as everolimus and temsirolimus that showed

better pharmacodynamic parameters and pharmacokinetic properties than rapamycin, have been used for several years and demonstrated a good efficacy [91]. The failure of rapalogs to inhibit mTORC2 and negative feedback loop on the pathway spurred the development of dual inhibitors, including dactolisib (NVP-BEZ235) and NVPBGT226, that showed inhibitory effect on both PI3K and mTORC1/2 [91]. NVP-BEZ235 demonstrated potent anticancer activity in trastuzumab-resistant BT474 BC xenografts [92]. Meanwhile, novel adenosine triphosphate (ATP)-competitive mTOR kinase inhibitors, including AZD-8055 (NCT00731263), OSI-027 (NCT00698243), and INK128 (NCT03097328), were also being proposed in BC treatment. However, although these second-generation inhibitors may show ideal inhibitory effect on PI3K signaling, BCSCs seem to be neglected. It was reported that PI3K inhibition by buparlisib can stimulate Wnt pathway in a feedback way that conferred triple negative BC cells a stem-like feature [47]. Also, PI3K inhibition by rapalogs could promote GLP-1-dependent stemness features in MDA-MB-231 and MCF-7 cells, indicative of a pleiotropic effect of PI3K inhibitors [93]. Therefore, further investigation is warranted to understand the distinct mechanisms and effects of the PI3K pathway inhibitors among different cancer subtypes as synergic drugs may be required.

Therapeutic strategies and challenges for targeting BCSCs

Currently, targeting key signaling cascades that are dysregulated in cancer cells is still dependent on the high-throughput screening of compounds and structural modification of existing drugs. Although a large number of new compounds entered into clinical trials annually and have shown satisfactory efficacy, many are withdrawn due to the poor tolerance and safety concerns. Also, some of new-generation drugs failed to abolish the immortality and stemness of CSCs that underlie the most of malignant behaviors of BCs. Therefore, CSC-targeted strategy is of great significance to bring us one step closer toward overcoming safety issues and therapeutic failure. The most relevant pre-clinical inhibitors and clinical trials against BCSCs are summarized in Table 3.

The presence of BCSC surface markers provides an efficient way to identify BCSCs. Compared to normal tissue, BCSCs maintain higher expressions of these markers, which allows novel antibodies to recognize and block the signaling pathways of interest, making BCSCs vulnerable to cytotoxic agents. A good example of this is the anti-CD133 mAbs-conjugated polymeric nanoparticles. With chemotherapeutic drugs loaded, they were more efficiently internalized by CD133$^+$ cells and effectively inhibit the tumor regrowth in an MDA-MB-231 xenograft model [65]. This proposes a new idea of double-targeted drug delivery system against BCSCs. However, delivering effective inhibitors to central signaling pathways might be a more efficient approach considering the intrinsic resistance nature of CSCs to anticancer drugs (Fig. 3). For instance, the NRF2 pathway inhibitors can be used to sensitize CSCs to RT/CT, while Notch inhibitors can be used to abolish self-renewal ability. Of note, some CSCs may share the same surface markers with normal stem cells. Thus, it requires that targeting CSCs in BC therapy should be very carefully considered to avoid side effects, which in turn requires absolutely specific BCSC surface markers and signaling pathway proteins to be identified. Furthermore, as CSCs remain quiescent under treatment, tumor growth inhibition is not the convincing evidence for the potential of CSC-targeted drugs. The effect of decreasing the percentage of CSCs in tumors should be considered in future studies.

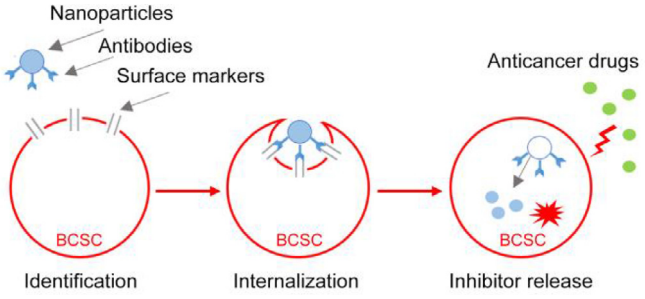

Fig. 3. A schematic picture showing a drug delivery system for targeting BCSCs. The nanoparticles are proposed to load stemness-related pathway inhibitors with antibodies targeting BCSC surface markers. The conjugates can identify BCSCs and enter the target cells via internalization. The intracellular inhibitor release can be achieved by microenvironmental regulations (potential of hydrogen) or external intervention, such as light and heat [95]. The delivery system exerts dual inhibitory effects on BCSCs via blocking both surface and intracellular tumorigenic factors. This approach may improve conventional therapies by eradicating BCSCs and avoiding the damage of normal tissues.

CSCs within a tumor may present shifting targets during cancer progression. This will be a major challenge for CSC-targeted therapeutics because the antibody used is largely subject to the identification of CSC surface markers in breast tumors. Specifically, the cells that drive tumor growth may evolve during treatment or metastasis due to the intrinsic instability of genome and epigenetics. The therapy-

Table 3
The most relevant pre-clinical inhibitors and clinical trials against BCSCs.

Targets	Pre-clinical inhibitors	Ref.	Clinical trials (identifier)
CD44$^+$ CSCs	HA-decorated nanoparticles and salinomycin	[58]	Role of HA in causing cancer stem cell growth in the bones of patients with breast cancer (NCT02331212)
Integrins	AIIB2	[94]	Unknown
CD133$^+$ CSCs	CD133-targeted polymeric nanoparticles and CD133-specific RNA aptamers	[65,66]	Unknown
EpCAM$^+$ CSCs	EpCAM-specific RNA aptamers	[76]	Unknown
PROCR	αEPCR-1535	[32]	Unknown
Hh signaling	5E1 monoclonal antibody	[84]	Phase I trial of SMO inhibitor sonidegib in combination with paclitaxel in patients with advanced solid tumors (NCT01954355)
Notch signaling	PF-03084014	[87]	Phase I/II study of γ-secretase inhibitors, including RO4929097 (NCT01151449), LY3039478 (NCT02784795), MK-0752 (NCT00645333), and the transcriptional activation complex inhibitor CB-103 (NCT03422679), for BC treatment. Phase I trials of DLL4 mAbs, including MEDI0639 (NCT01577745) and REGN421 (NCT00871559), for advanced solid malignancies
Wnt/β-catenin signaling	Sulforaphane	[88]	Phase I study of OMP-54F28 for solid tumors (NCT01608867)
NRF2 signaling	All-trans retinoic acid, alkaloid trigonelline, and brusatol	[15]	Unknown
PI3K signaling	NVPBGT226	[91]	Phase I/II study of novel ATP-competitive mTOR kinase inhibitors, including AZD-8055 (NCT00731263), OSI-027 (NCT00698243), and INK128 (NCT03097328), for advanced tumors

Abbreviations: HA, hyaluronan; EpCAM, epithelial cell adhesion molecule; PROCR, protein C receptor; Hh, hedgehog; SMO, smoothened, frizzled class receptor; DLL4, delta-like ligand 4; mAbs, monoclonal antibodies; NRF2, nuclear factor E2-related factor 2; PI3K, phosphoinositide 3-kinase; ATP, adenosine triphosphate.

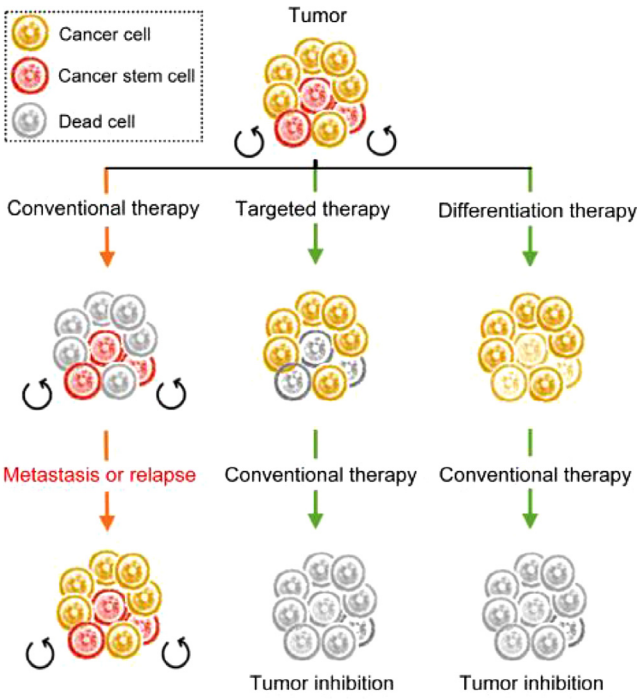

Fig. 4. A schematic diagram demonstrating targeting BCSC with different approaches. Current conventional therapies only target rapidly proliferative cancer cells but not CSCs, leading to the therapeutic escape of BCSCs and tumor recurrence and metastasis after several years. In contrast, the BCSC-targeted therapy in combination with conventional therapies has potential to decrease the risk of BC therapeutic failure. The differentiation therapy can also disrupt the stemness of BCSCs and make them more sensitive to anticancer drugs regardless of the shifting surface markers.

selective evolution or phenotype transition induced by microenvironments or other heterogeneous cells within a tumor may render CSC markers or signaling patterns changeable. This variable further complicates the requirements of high-throughput screening platforms for CSC markers. Recent studies developed and employed PDX BC animal models to investigate the regulation of CSCs in cancer progress [30]. The model demonstrated promise for pre-clinically testing the internalization of drug delivery by CSCs with the intention of improving tumor cell targeting [96]. However, concerns for using these animal models lie in that the single immunocompromised microenvironment cannot replicate the real evolution process occurring in human BC progress. Perspective studies should be considered to use human fresh BC tissue samples and culture techniques with a microenvironment-mimicking microfluidic chip [97]. Such techniques can mimic the microenvironment of biological tissues with fidelity and might provide fire-new avenues for detecting and targeting dynamic features of BCSCs.

The rapid development of nanomaterials and deep understanding of CSCs have made it possible to eradicate CSCs without marker identification. For instance, polyelectrolyte conjugated gold nanorods (AuNRs) were found to be more specifically internalized by CSCs. Xu [95] used AuNRs loaded with salinomycin that mainly kills CSCs to treat MCF-7 cells and found this conjugate could be internalized by $ALDH^+$ BCSCs much more and faster than non-stem cells with impressive efficiency in reducing the stem-like $ALDH1^+$ MCF-7 cells [95]. This is an example of developing BCSC-targeted approach with reference to the natural features of nanomaterials instead of identifying BCSC surface markers, suggesting that novel biomaterials provide CSC targeting with an extensive enlightenment. Further studies need to be performed in CSCs identified by different surface markers and in certain cancer subtypes. Furthermore, a therapeutic option by inducing CSC differentiation was also developed. Such agents enforce CSCs to differentiate into highly differentiated tumor cells that are easily killed by anticancer agents regardless of the involved CSC markers or models (Fig. 4). For instance, salinomycin can significantly decrease the proportion of CSCs by more than 100-fold relative to PTX through inducing CSC terminal epithelial differentiation and inhibit mammary tumor growth [98]. The underlying mechanism may be associated with its inhibitory effect on Wnt signaling. Similarly, BMP4 is another differentiation inducer that can inhibit β-catenin activation through inhibiting the PI3K and Wnt signaling pathways. A combination of BMP4 with chemotherapeutic drugs, such as oxaliplatin and 5-fluoracil, can effectively inhibit the *in vivo* growth of CSCs [99]. Additionally, interferon-β (IFN-β) treatment can promote the differentiation of mesenchymal/CSC to a less aggressive epithelial/non-CSC state, which shows a promising effect on depleting the population of CSCs in triple negative BC cells [100]. These findings expedite the progress of feasible CSC-targeted strategies from a new perspective.

Conclusion

Current evidence suggests CSC is a key target for clinical BC therapy to overcome resistance and recurrence, while findings related to surface markers and signaling network make it well-founded to develop BCSC-targeted modalities. The emergence of novel drug delivery systems can precisely remove residual BCSCs, underlying the curative promise of BCSC concept. However, the changeable and complex natures of BCSCs create several challenges. In these cases, extracorporeal tissue culture techniques with microenvironment-mimicking models seem to be a future-oriented way for optimizing the advance of efficient BCSC-targeted therapeutics. Furthermore, differentiation therapy represents an efficient way to eradicate BCSCs regardless of the shifting surface markers.

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Conflict of interest

There is no interest conflict in this research.

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