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REVIEW

Breast cancer stem cell: the roles and therapeutic implications

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Abstract Breast cancers have been increasingly recognized as malignancies displaying frequent inter- and intratumor heterogeneity. This heterogeneity is represented by diverse subtypes and complexity within tumors, and impinges on response to therapy, metastasis, and prognosis. Cancer stem cells (CSCs), a subpopulation of cancer cells endowed with self-renewal and differentiation capacity, have been suggested to contribute to tumor heterogeneity. The CSC concept posits a hierarchical organization of tumors, at the apex of which are stem cells that drive tumor initiation, progression, and recurrence. In breast cancer, CSCs have been proposed to contribute to malignant progression, suggesting that targeting breast cancer stem cells (BCSCs) may improve treatment efficacy. Currently, several markers have been reported to identify BCSCs. However, there is objective variability with respect to the frequency and phenotype of BCSCs among different breast cancer cell lines and patients, and the regulatory mechanisms of BCSCs remain unclear. In this review, we summarize current literature about the diversity of BCSC markers, the roles of BCSCs in tumor development, and the regulatory mechanisms of BCSCs. We also highlight the most recent advances in BCSC targeting therapies and the challenges in translating the knowledge into clinical practice.

Keywords Biomarker · Epithelial–mesenchymal transition · Signal pathway · Microenvironment · Therapy

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Introduction

The cancer stem cell (CSC) hypothesis has challenged the traditional view of cancer development. This theory implies a hierarchical organization with a rare population of cells residing at the apex that enable tumor formation and progression. This small subset of tumorigenic cells, referred to as CSCs, are defined as a distinct population of cancer-initiating cells that possess the properties including self-renewal and the ability to generate both further stem cells and more differentiated cells forming the bulk primary tumor [1]. Although CSCs account for only a tiny part of the bulk cells, they have been thought to be responsible for therapeutic resistance [2]. Thus, combining traditional chemoradiotherapy with CSC-based therapies should probably provide a high-efficient and low-toxic treatment for cancer therapy. In addition to CSC, there are some other terminologies describing this subpopulation of cells, e.g., stem-like cancer cell, tumor-initiating cell, tumorigenic cell, side population cell, and clonogenic stem-like cell. In most cases, CSC has now been used interchangeably with these terminologies. There are two theories that describe the origin of CSC: either from adult tissue stem cell via malignant transformation or the dedifferentiation of a lineage committed cell that has acquired stem cell characteristics through mutation [3]. Severe nonobese diabetic severe combined immunodeficient (NOD/SCID) mouse was used as a xenograft model to study the proliferation and self-renewal potential of CSCs. In the case of breast cancer, as few as 100 $CD44^{+}/CD24^{-}$ tumor cells, which were subsequently proven to be breast cancer stem cells (BCSCs), were capable of initiating tumors when injected into mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors [4].

Recent works have identified some distinct types of BCSCs with various markers [5]. Some signaling pathways as well as tumor microenvironment have been shown to mediate the generation of multiple bulk cell progeny from BCSCs [6, 7]. Given the capacity of BCSCs to generate bulk primary tumor, the combination of BCSC targeting agents and individualized therapies of breast cancer may improve the clinical outcome. Indeed, a number of drugs targeting BCSCs are undergoing early stage clinical trials. New insights into the current knowledge of the properties and regulatory mechanisms of BCSCs are important for the development of more effective therapeutic strategies in breast cancer.

Evolutions of CSC model

Tumors are composed of different subtypes with distinct morphologies and behaviors, and the source of tumor heterogeneity remains unclear. The CSC hypothesis was proposed to describe a subpopulation of cancer cells, with fundamental properties of self-renewal and differentiation that can give rise to tumors displaying genomic and phenotypic heterogeneity [8]. The concept implies that a small fraction of cancer cells with stem-like properties, rather than the vast majority of cancer cells, contribute to the occurrence of tumor heterogeneity and malignant progression. The first evidence of stem-like cells was reported in breast cancer [4]. These stem-like breast cancer cells exhibit expression markers similar to those found in multipotent progenitor cells, indicating that these BCSCs can originate from mammary stem cells (MaSCs). Theoretically, BCSCs can arise from any stage throughout the differentiation process [9].

There are passionate debates favoring or opposing the CSC theory. The clonal evolution model states a quite different view in explaining how tumor heterogeneity comes about. The clonal evolution model highlights the occurrence of random mutations and clonal selection in tumor diversity and carcinogenesis. In the clonal evolution model, the cancer cells are stochastically organized, and any cancer cell can self-renew and give rise to a large number of offspring once it acquires mutations and selective advantages [10]. In breast cancer, a recent report has unraveled that the common mutation *PIK3CA*^{H1047R} is capable of inducing multipotency and multilineage potential during tumorigenesis [11].

In fact, neither of these two theories is sufficient to explain tumor heterogeneity, because the CSC hypothesis ignores intra-tumor genetic heterogeneity, whereas the clonal evolution model cannot explain the functional diversity of cell states. Notably, increasing evidence has proved that the CSC hypothesis and clonal evolution model are not mutually exclusive. A novel notion has been generally accepted in which CSCs may evolve and acquire driver mutations over time during tumor progression [8]. It describes that the increasing mutational burden causes impairment of maturation programs, enhances self-renewal capacity, and increases the properties of CSCs. The tumor becomes functionally homogeneous without steep hierarchy when the frequency of CSCs accumulates to a high enough level, as the majority of cells can self-renew and few non-CSCs progenies are generated [8]. It provides a new framework for studying the underlying mechanisms of cancer heterogeneity; however, it remains unclear whether such a novel model can be applied to breast cancer.

BCSC markers

A panel of markers has been strongly recommended to define the BCSC subpopulation. It was validated that the BCSCs isolated from cell lines and primary tumors by these specific markers were able to reconstitute the parent tumors in xenografts. The $CD44^+/CD24^{-/low}$ phenotype is by far the most commonly used marker to characterize BCSCs since it was first documented in 2003 [4] (Table 1). The stem property of $CD44^+/CD24^{-/low}$ cells was further demonstrated by more tumorigenicity studies, colony formation, migration, and invasion assays [12]. CD44 is a cell surface adhesion molecule that mediates cell–cell and cell–extracellular matrix (ECM) interactions through binding to hyaluronic acid (HA), whereas CD24 is a small glycoprotein involved in negatively regulating the activity of chemokine receptor CXCR4, which can mediate breast cancer metastasis. Thus, the $CD44^+/CD24^{-/low}$ breast cancer cells should possess an effective ability to induce the malignant progression due to the combination of increased CD44 expression and decreased CD24 expression. However, there is a debate over the relationship between $CD44^+/CD24^{-/low}$ phenotype and tumorigenicity. The percentage of $CD44^+/CD24^-$ cells did not always correlate with tumorigenicity, but a few $CD44^+/CD24^-/ESA^+$ cells could form tumors instead [13]. In another study, $CD44^-/CD24^+$ status, rather than $CD44^+/CD24^-$, identified patients with worse prognosis in breast cancer [14].

The controversy of $CD44^+/CD24^-$ cells calls for better BCSC markers. ALDH1 has come to the forefront as an additional indicator of the BCSC population (Table 1). ALDH1 is a detoxifying cytosolic enzyme that oxidizes intracellular aldehydes, and the ALDH1 activity can be assessed by the ADELFLUOR assay. ALDEFLUOR-positive subpopulation isolated from human breast tumors was highly

Table 1 BCSC markers and expressions

Markers	Descriptions[c]	Species	Study methods	Expressions in different cell lines or clinical subtypes
The most commonly used				
$CD44^+/CD24^{-/low}$, $CD44^+/CD24^{-/low}/Lineage^-$, $ESA^+/CD44^+/CD24^{-/low}$, $ESA^+/CD44^+/CD24^{-/low}/Lineage^-$ [4]	CD44: a cell surface glycoprotein involved in cell–cell interactions, cell adhesion and migration CD24: a small glycoprotein modulating growth and differentiation signals	H	Colony formation, proliferation, migration, invasion and tumorigenicity studies	MDA-MB-468: −, MDA-MB-231: +++, HCC1937: ++, T47D: −, MCF7: −, ZR75: −, SKBR3: −, MDA-MB-361: +++ [27][a]; positively associated with BLBC ($P = 0.028$) and luminal B ($P = 0.050$), but negatively with luminal A ($P = 0.025$) [28]
$ALDH1^+$, $ALDH1^+/CD44^+/CD24^-$ [15]	ALDH1: a protein of the aldehyde dehydrogenase family involved in retinol metabolism and the regulation of the metabolic responses	H/M	Tumorigenicity study	MDA-MB-468: +, MDA-MB-231: +, HCC1937: +, T47D: −, MCF7: +, ZR75: +, SKBR3: ++, MDA-MB-361: − [27][a]; T47D: +, BT-20: ++, MDA-MB-157: +, MDA-MB-231: + [29][b]; positively associated with HER2-OE ($P < 0.001$) and BLBC ($P = 0.027$), but negative with luminal A ($P < 0.001$) [28]
$CD133^+$ [16]	CD133: a transmembrane glycoprotein thought to function in maintaining stem cell properties by suppressing differentiation	M	Tumorigenicity study	MDA-MB-468: +++, MDA-MB-231: −, HCC1937: −, T47D: −, MCF7: −, ZR75: −, SKBR3: −, MDA-MB-361: − [27][a]; T47D: 0, BT-20: 0, MDA-MB-157: 0, MDA-MB-231: 0 [29][b]
Other markers and combinations thereof				
$CD29^{hi}/CD24^+$ [19]	CD29: a membrane receptor of integrin family involved in cell adhesion and recognition	M	Tumorigenicity study	NA
$CD29^{low}/CD24^+/CD61^+$ [19]	CD61: a cell surface proteins of integrin family involved in cell adhesion and cell surface-mediated signaling	M	Tumorigenicity study	NA
$CD24^+/CD29^+/CD49f^+$ [20]	CD49f: a membrane protein of integrin family involved in cell surface adhesion and signaling	H/M	Migration and metastasis studies	NA
$CD24^{high}/CD49f^{hig}h/DNER^{high}$ [17]	NA	H	Sphere-forming study	NA
$CD24^{high}/CD49f^{high}/DLL1^{high}$ [17]	DLL1: a member of the delta/serrate/jagged family involved in cell-to-cell communication	H	Sphere-forming study	NA
$CD49f^+/DLL1^{high}/DNER^{high}$ [17]	NA	H	Sphere-forming study	NA
$PKH26^+$ [17]	NA	H	Sphere-forming study	NA
$Proteosome^{low}$ [18]	NA	H	Tumorigenicity study	NA
$PROCR^+$, $PROCR^+/ESA^+$ [21]	PROCR: a receptor for activated protein C involved in the blood coagulation pathway	H/M	Tumorigenicity study	MDA-MB-468: −, MDA-MB-231: ++, HCC1937: −, T47D: −/+, MCF7: −, ZR75: −, SKBR3: −, MDA-MB-361: ++ [27][a]; T47D: ++, BT-20: ++, MDA-MB-157: ++, MDA-MB-231: ++ [29][b]
$Sca\text{-}1^+$ [22]	NA	H/M	Tumorigenicity study	NA
$MUC1^+/CD24^+$ [23]	MUC1: a membrane-bound protein of the mucin family involved in cell adhesion and cell signaling	H	Tumorigenicity study	Positive expression rates: luminal A: 40 %, luminal B: 44 %, HER2: 33 %, basal-like: 13 % (P = NS) [25]

Table 1 continued

Markers	Descriptions[c]	Species	Study methods	Expressions in different cell lines or clinical subtypes
$Thy1^{+}/CD24^{+}$ [24]	Thy1: a cell surface glycoprotein of the immunoglobulin superfamily involved in cell adhesion and cell communication	H	Tumorigenicity study	NA
$CD44^{+}/Vimentin^{+}$ [25]	Vimentin: a member of the intermediate filament family responsible for maintaining cell shape, integrity of the cytoplasm, and stabilizing cytoskeletal interactions	H	Expression study	Positive expression rates: luminal A: 12 %, luminal B: 6 %, HER2: 11 %, basal-like: 81 % ($P < 0.0001$) [25]
$CD44^{+}/Osteonectin^{+}$ [25]	Osteonectin: a cysteine-rich acidic matrix-associated protein involved in extracellular matrix synthesis and promotion of changes to cell shape	H	Expression study	Positive expression rates: luminal A: 7 %, luminal B: 0 %, HER2: 0 %, basal-like: 25 % (P = NS) [25]
$CD24^{+}/CK18^{+}$ [25]	NA	H	Expression study	Positive expression rates: luminal A: 58 %, luminal B: 38 %, HER2: 50 %, basal-like: 6 % ($P = 0.0008$) [25]
$CD24^{+}/GATA3^{+}$ [25]	GATA3: a protein of GATA family involved in endothelial cell biology	H	Expression study	Positive expression rates: luminal A: 92 %, luminal B: 69 %, HER2: 33 %, basal-like: 6 % ($P < 0.0001$) [25]

BCSC breast cancer stem cell, *H* human, *M* mouse, *BLBC* basal-like breast cancer, *HER2-OE* human epidermal growth factor 2-overexpressed, *NS* not significant, *NA* not applicable

[a] − 0 %, + 0–5 %, ++ 5–70 %, +++ 70–100 %

[b] 0 0 %, − 0.05–0.5 %, + 0.5–10 %, ++ 10–90 %, +++ 90–100 %

[c] The descriptions were cited from NCBI (http://www.ncbi.nlm.nih.gov/gene/)

enriched in tumorigenic capacity [15]. CD133, also known as Prominin 1, is a transmembrane glycoprotein and was found to have characteristics similar to $CD44^{+}/CD24^{-/low}$ cells from *BRCA1*-knockout mice [16] (Table 1). Additionally, enhanced PKH26 dye-retaining capacity [17], low proteasome activity [18], expression of CD29, CD61 [19], CD49f [20], PROCR [21], Sca-1 [22], MUC1 [23], Thy1 [24], vimentin, osteonectin, CK18, GATA3 [25], and various combinations thereof including $CD24^{high}/CD49f^{high}/DNER^{high}$, $CD24^{high}/CD49f^{high}/DLL1^{high}$, and $CD49f^{+}/DLL1^{high}/DNER^{high}$ [17], are correlated with stem cell activity in breast cancer (Table 1). Furthermore, the expression patterns of these BCSC markers varied in the human and mouse [26], and also varied greatly among different breast cancer cell lines and primary tumors [27–29].

These studies imply that BCSCs themselves are heterogeneous. The variability and complexity of evolutionary processes, factors in tumor microenvironment, and genetic mutations might contribute to the diversity of CSCs [5]. It is therefore crucial to identify the most clinically relevant BCSC markers for therapeutic targets.

Properties of BCSCs

There is now substantial evidence that only a small fraction of cancer cells are able to initiate tumor growth and drive metastasis. This small population of cancer cells is termed CSCs, or cancer cells with stem-like properties, which were first proposed in human leukemia [30] and later in solid tumors including the breast [4]. CSCs in distinct mesenchymal-like and epithelial-like states were mainly localized either at the tumor invasive front or in interior hypoxic zones, with remarkably different capacity for tissue invasion, dissemination, and growth at metastatic sites [31]. Moreover, CSCs were capable of forming functional tumor blood vessels by transdifferentiating into endothelial progenitor cells, endothelial cells (ECs), or vascular smooth muscle-like cells [32]. Hence, it has been demonstrated that CSCs can drive tumor initiation, mediate metastasis, and result in therapy resistance [33] (Fig. 1). Considering this, breast cancer with a high proportion of CSCs is correlated with a poor outcome [34].

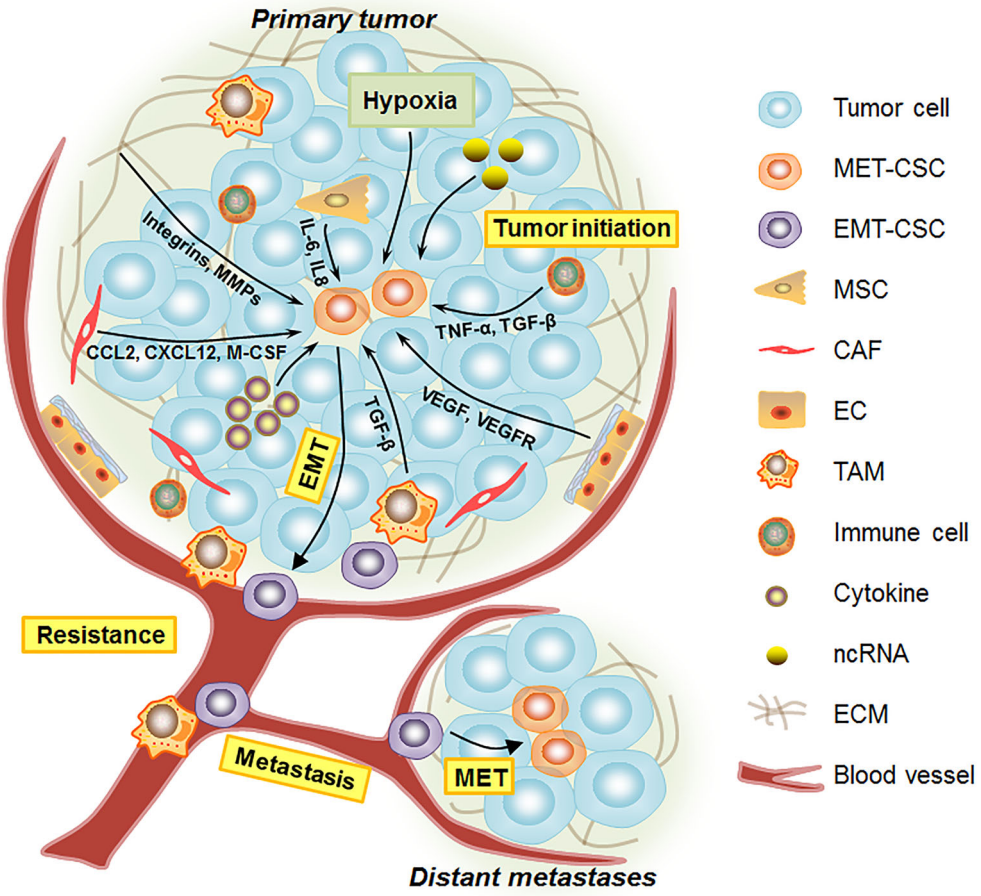

Fig. 1 Properties and regulation of BCSCs. This schematic diagram represents the interactions between CSCs and the surrounding tumor microenvironment, which have a direct effect on breast cancer cell malignancy and lead to tumor initiation, EMT, MET, metastasis, and therapeutic resistance

Tumor initiation

The CSC hypothesis has fundamental implications for carcinogenesis in the breast, as BCSCs were demonstrated to be able to recapitulate the parent tumors in previous xenotransplantation studies. In this respect, Al-Hajj et al. first reported that a small subset of human breast cancer cells having a $CD44^{+}/CD24^{-/low}$ phenotype could efficiently form tumors in NOD/SCID mice, whereas tens of thousands of $CD44^{-}/CD24^{+}$ cancer cells could not [4]. Further studies also found that BCSCs could form xenograft outgrowths with limiting cells compared to the other tumor cells [15, 16]. These data support that BCSCs play a crucial role in breast cancer initiation and growth.

Additionally, transit-amplifying cells (TACs), an early intermediate in transition between stem cells and differentiated cells, have also shown contributions in tumor initiation. TACs are short-lived but can expand rapidly, providing progeny that differentiate into mature cells of varying lineages [35]; hence, this population is greater in number and more frequent in proliferation than stem cells. TACs of tumors suffer from replication stress and thus accumulate mutations, but the mutations would be quickly lost and hence would be rendered harmless. However, if a TAC sustains a mutation that confers indefinite self-renewal, or if this cell already has additional mutations required for malignancy by virtue of descent from a tissue stem cell, then malignancy can arise from a TAC [36]. The EGFR–HER2 module represents an important functional marker for clonal expansion of TACs and their interference with stem cells. According to this model, active EGFR–HER2 of TACs expands these progenitors and promotes their dedifferentiation to stem cells [37]. Given that the EGFR–HER2 module controls dedifferentiation as well as proliferation of TACs, it is predictable that agents blocking EGFR or HER2 might inhibit some tumors because of their effects on the TAC-to-CSC transition.

Epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET)

EMT is a phenotypic process by which the epithelial cells convert to mesenchymal ones, and it has been documented to promote cancer progression. Several studies have reported that the EMT process is correlated with generating cells with stem-like properties [38]. It was demonstrated

that AXL, which regulates a number of signal transduction pathways including NF-κB, STAT, Akt and MAPK, was constitutively activated in BCSCs and induced EMT by regulating the expression of EMT markers such as E-Cadherin, N-Cadherin, Snail and Slug [39]. The up-regulated transcription factor *FOXC2* induced EMT in human mammary epithelial cells and its suppression contributed to reduced CSCs in aggressive breast cancer cell lines [40]. Some other EMT-associated transcription factors such as *TWIST* [41] have also been shown to induce stemness in breast cancer. It has been proposed that BCSCs can acquire metastatic capacity via EMT. *Vimentin* is one of the key genes involved in regulating EMT, and triple negative breast cancer (TNBC) showed the highest rate of $CD44^+$/vimentin$^+$ cells compared to other breast cancer subtypes [25]. The $CD44^+/CD24^-$ breast cancer cells were considered to have increased EMT potential [42]. It indicates a possible link between tumor aggressiveness and the EMT capacity of BCSCs.

Actually, recent work has identified two types of BCSCs with distinct properties: a more quiescent mesenchymal-like state labeled as EMT-CSCs and a more proliferative epithelial-like state labeled as MET-CSCs. EMT-CSCs was a population identified as $CD44^+/CD24^-$ with signatures of EMT such as low expression of E-Cadherin and high expression of vimentin. MET-CSCs, on the other hand, were always characterized by ALDH + phenotype with signatures of MET such as high level of E-Cadherin and low level of vimentin [31]. More importantly, the transition between these two states was thought to be critical for tumor metastasis and it was likely to be regulated by the tumor microenvironment through cytokine and chemokine signaling [33]. The EMT-CSCs are localized at the invasive edge of the tumor where they are capable of entering the circulation and forming micro metastases at distant sites, while the MET-CSCs locate more centrally and allow the transition back to the proliferative epithelial state to generate a tumor at the secondary site. The two BCSC states can switch when the invasive edge becomes the interior of the tumor and further researches are needed to more conclusively understand the clinical implications of the plasticity in BCSCs.

Metastasis

Metastasis remains one of the major causes of mortality in breast cancer and currently no standardized therapy is available for metastatic breast cancer. Not all breast cancer cells in primary tumors possess metastatic potential, and only a small subpopulation of cells can home to distant tissues or organs [38]. An increasing body of evidence has identified that BCSCs are such a subset of metastatic cells. A gene profiling study revealed that $CD44^+/CD24^-$ cells from primary breast tumors displayed a gene expression profile related to increased metastasis and poor clinical outcome [43]. Further studies observed that early disseminated cancer cells in the bone marrow of breast cancer patients displayed the $CD44^+/CD24^-$ phenotype [44]. Likewise, cells from lung metastases highly expressed CD44 in a xenograft model of human TNBC [45]. Moreover, ALDH-positive cancer cells showed significantly greater metastatic capacity compared to ALDH-negative cells in xenografts [46]. These studies strongly suggest a metastatic role of BCSCs.

Resistance

Several lines of evidence have suggested that BCSCs display relative resistance to conventional therapies, both in pre-clinical models and clinical trials. In vitro, breast cancer cells with BCSC phenotype did not respond to chemotherapy, while paclitaxel treatment enriched the cells expressing the BCSC phenotype [47]. Clinically based studies demonstrated that there was an increase of cells expressing the $CD44^+/CD24^-$ phenotype in primary tumors following chemotherapy [48]. BCSCs were also found to be resistant to radiotherapy in different breast cancer cell line models [49]. Additionally pre-clinical studies observed that resistance to endocrine therapy was always accompanied by an increase in the proportion of BCSCs [50]. This is supported by clinical studies in which neoadjuvant hormone therapy led to an increased BCSC subset [51]. This evidence suggests an intrinsic resistance of BCSC subpopulations to anticancer therapies. The quiescence of CSCs makes them insensitive to DNA-damaging agents and radiation. These studies suggest that targeting of BCSCs might be an effective therapeutic strategy for breast cancer.

Regulation of BCSC characteristics

BCSCs are by no means a fixed population. It has been shown that breast cancer cells are capable of shifting between stem-like and non-stem-like states [52]. This plasticity indicates that the regulatory mechanisms of BCSCs are extensive and complex. Several pathways have been identified to be involved in the induction and maintenance of stemness. In addition, interactions between BCSCs and the tumor microenvironment, non-coding RNAs (ncRNAs), have also been demonstrated to regulate BCSCs biology (Fig. 1).

Stemness pathways

Notch signaling pathway plays a critical role in cell fate determination by maintaining a balance of proliferation, differentiation and apoptosis. In mammals, the Notch pathway comprises four transmembrane receptors (Notch1–4) interacting with five ligands (DLL1, 3, 4, Jagged1, 2). Upon binding to the ligand on the neighboring cell, the Notch receptor is activated and cleaved by γ-secretase, releasing the Notch intracellular domain (NCID). Then the NICD translocates to the nucleus where it interacts with other co-factors (e.g., CBF1) and induces target gene transcription [53] (Fig. 2). Breast cancer cells with increased Notch activity exhibited BCSC features including increased sphere formation, expression of BCSC markers, and tumor initiation capacity [54]. Notch activity was increased in BCSCs, whereas its inhibition decreased BCSC numbers and inhibited tumor initiation [55]. Knocking-down Notch or treating with Notch inhibitor reduced the $CD44^{+}/CD24^{-}$ BCSC population and decreased the formation of brain metastases from breast cancer [56]. These findings suggest that the Notch pathway may be a potential therapeutic target in breast cancer. Given the diversity of receptors and ligands in the Notch pathway, γ-secretase has been one of the main targets of Notch signaling in preclinical and clinical trials, and these compounds have shown efficacy in reducing BCSCs [57, 58] (Table 2).

The Wnt signaling pathway modulates a balance between stemness and differentiation in some types of cancer cells. Wnt proteins are a family of secreted mediators. Binding of the Wnt ligand to the heterodimer receptor (Frizzled and LRP) activates the complex composed of AXIN, APC, and GSK3β. This leads to the nuclear translocation of β-catenin that binds to TCF/LEF, which regulates downstream targets transcriptionally [59] (Fig. 2). BCSCs displayed relatively increased Wnt pathway activity and higher level of therapeutic resistance compared to non-BCSCs in bulk tumor cells [60]. Furthermore, the ligand Wnt3a increased, while the inhibitor

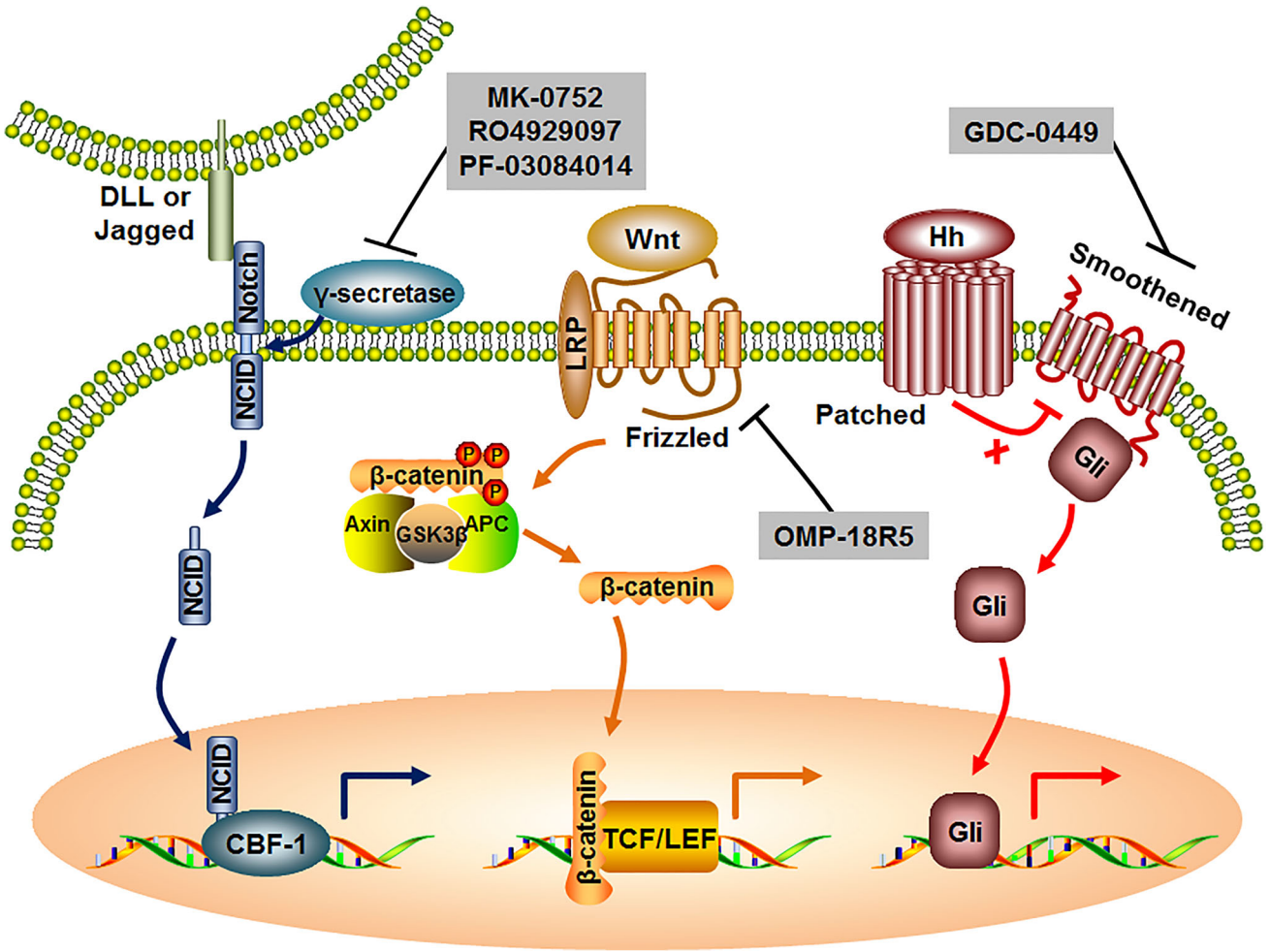

Fig. 2 The major signaling pathways and targeting drugs of BCSCs. This figure illustrates three signaling pathways, the Notch pathway (*blue arrows*), Wnt pathway (*orange arrows*), and Hedgehog pathway (*red arrows*)

Table 2 Clinical trials of BCSC-targeting therapies

Agent	Target	Trial phase (trial number, status)	Patients (number)	Combined therapy	Outcome measures	Results
Notch pathway-targeting						
MK-0752	γ-secretase	Phase I (NCT00756717, active)	Early stage, ER-positive breast cancer (22)	Tamoxifen or AI	Safety and tolerability	NA
		Phase I/II (NCT00645333, completed)	Advanced or metastatic breast cancer (30)	Docetaxel	DLT, MTD	Manageable toxicity and reduced BCSCs [57]
		Phase I (NCT00106145, completed)	Metastatic or locally advanced breast cancer (24) and other solid tumors (79)	NA	Safety and tolerability	Good tolerability and Notch pathway inhibition [58]
RO4929097	γ-secretase	Phase I (NCT01238133, terminated)	TNBC (14)	Paclitaxel and carboplatin	DLT, MTD	NA
		Phase I (NCT01071564, terminated)	Advanced or unresectable breast cancer (13)	Vismodegib	AEs, DLT, MTD	NA
		Phase I (NCT01149356, terminated)	Advanced or metastatic breast cancer (15)	Exemestane	MTD, safety and tolerability	NA
		Phase I (NCT01208441, terminated)	Post-menopausal hormone receptor-positive stage II/III breast cancer (28)	Letrozole	DLT, MTD	NA
		Phase II (NCT01151449, active)	Advanced, metastatic, or recurrent TNBC (3)	NA	ORR, 6-month PFS	NA
PF-03084014	γ-secretase	Phase II (NCT02299635, terminated)	Advanced TNBC (19)	NA	OR	NA
		Phase I (NCT01876251, terminated)	Advanced breast cancer (30)	Docetaxel	DLT, PFS	NA
Wnt pathway-targeting						
OMP-18R5 (vantictumab)	Frizzled7	Phase I (NCT01973309, recruiting)	Locally recurrent or metastatic breast cancer (34)	Paclitaxel	Safety and tolerability	NA
Hedgehog pathway-targeting						
GDC-0449 (vismodegib)	Smoothened	Phase I (NCT01071564, terminated)	Metastatic or unresectable breast cancer (13)	RO4929097	AEs, DLT, MTD	NA
		Phase II (NCT02694224, recruiting)	TNBC (40)	Paclitaxel, epirubicin, and cyclophosphamide	pCR, cCR, molecular changes	NA
BCSC-targeting						
Bivatuzumab mertansine	CD44v6	Phase I (NCT02254005, completed)	CD44v6-positive metastatic breast cancer (24)	NA	MTD	Fatal toxic epidermal necrolysis [100]
		Phase I (NCT02254031, terminated)	CD44v6-positive recurrent or metastatic breast cancer (8)	NA	MTD	NA
CSC vaccine	BCSC	Phase I/II (NCT02063893, completed)	Metastatic breast cancer (40)	NA	Safety and immune responses	NA

Table 2 continued

Agent	Target	Trial phase (trial number, status)	Patients (number)	Combined therapy	Outcome measures	Results
Multiplasmid vaccine	CD105/Yb-1/SOX2/CDH3/MDM2	Phase I (NCT02157051, recruiting)	HER2-negative stage III/IV breast cancer (30)	NA	Immunologic efficacy, toxicity	NA
Microenvironment-targeting						
Reparixin	CXCR1/2	Phase I (NCT02001974, completed)	HER2-negative metastatic breast cancer (33)	Paclitaxel	Safety and effect	NA
		Phase II (NCT02370238, recruiting)	Metastatic TNBC (190)	Paclitaxel	PFS, mPFS, OS, ORR	NA
		Phase II (NCT01861054, terminated)	Early breast cancer (20)	NA	Markers and pathways	NA

CSC cancer stem cell, *BCSC* breast cancer stem cell, *TNBC* triple-negative breast cancer, *ER* estrogen receptor, *HER2* human epidermal growth factor 2, *AI* aromatase inhibitor, *DLT* dose-limiting toxicity, *MTD* maximum tolerated dose, *AE* adverse events, *ORR* overall response rate, *PFS* progression-free survival, *mPFS* median PFS, *OR* objective response, *pCR* pathologic complete response, *cCR* clinical complete response, *OS* overall survival, *NA* not applicable

DKK1 decreased mammosphere formation in breast cancer cell lines [61]. For TNBC, β-catenin silencing significantly reduced the ALDH + BCSCs, as well as the expression of stem cell-related target genes including *BMI-1* and *c-MYC* in vitro, and led to markedly smaller and slower formation of tumors in vivo [62]. A recent study shows that the Wnt pathway inhibitor pyrvinium pamoate reduces both $CD44^{+}/CD24^{-/low}$ and ALDH + BCSCs and inhibits the self-renewal and metastasis of these BCSCs [63]. Moreover, resveratrol, a natural polyphenolic compound, inhibited BCSCs and induced autophagy via suppressing the Wnt/β-catenin signaling pathway [64]. However, there are currently few clinical researches testing the efficacy of the Wnt pathway inhibitors in breast cancer (Table 2). The effects of these agents on BCSCs warrant further investigation in clinical trials.

Hedgehog (Hh) pathway plays a role in normal mammary development and carcinogenesis. The Hh ligands are secreted proteins including Sonic (Shh), Indian (Ihh), and Desert (Dhh). The Hh ligands exert their activity via binding to another transmembrane receptor, Patched 1 or Patched 2, which constitutively inhibits the Hh pathway activity by interacting with Smoothened, another transmembrane protein downstream in the pathway. When the Hh ligand binds to the Patched, Smoothened is activated and releases a GLI family of transcription factors (Gli1–3), thereby regulating downstream target genes [65] (Fig. 2). A wide range of evidence has shown the important role of Hh pathway in maintaining BCSCs. The mRNA and protein expressions of Smoothened in $CD44^{+}/CD24^{-}$ cells were markedly higher than those in non-$CD44^{+}/CD24^{-}$ cells, while the ablation of Smoothened by transfecting siRNA led to decreased Shh activity and expression of Shh downstream genes, including *STAT3*, *BCL2*, and *CCND1* [66]. Increases in $CD44^{+}/CD24^{-}$ BCSCs and mammosphere formation were observed after docetaxel treatment, whereas these increases were eliminated by co-treatment with Hh inhibitors [67]. Despite the promising preclinical findings about the role of the Hh pathway in BCSCs, there are currently few clinical studies validating these results (Table 2). Therefore, further clinical investigations are required to identify the efficacy of the Hh pathway inhibitors in BCSCs.

In addition, TGF-β, TNF-α/NF-κB, and receptor tyrosine kinase (RTK) signaling pathways have also shown contributions to the development of BCSCs [68]. These pathways may provide potential targets for breast cancer therapy.

Microenvironment

It is now recognized that the tumor microenvironment, which is also known as "niche", plays a crucial role in supporting and maintaining CSCs [69]. The CSCs are regulated by complex interactions with the components of the tumor microenvironment, such as mesenchymal stem cells (MSCs), cancer-associated fibroblasts (CAFs), ECs, tumor-associated macrophages (TAMs), other immune cells, and ECM, through networks of cytokines and growth factors [70]. Tumor cells within the CSC niche produce factors that stimulate CSC self-renewal, induce angiogenesis, and recruit immune and other stromal cells that secrete additional factors to promote tumor cell invasion and migration [71] (Fig. 1).

Hypoxia is able to enrich the CSC population and induce the CSC phenotype in many cancers. The proportion of $ESA^+/CD44^+/CD24^-$ BCSCs and colony formation rate were markedly increased after hypoxia treatment in MDA-MB-231 cells [72]. Moreover, hypoxia increased the proportion of $CD44^+/CD24^{-/low}$ and ALDH + BCSCs, both in primary tumors and cell lines [73]. It has shown that hypoxia, through hypoxia-inducible factor (HIF), causes expansion of BCSC subpopulation by up-regulation of embryonic stem cell markers including *NANOG*, *OCT4*, *SOX2*, *KLF4*, *c-MYC*, and *microRNA-302* [74]. Further studies showed that HIF-dependent *ALKBH5* expression mediated the up-regulation of *NANOG* and enrichment of BCSCs in the hypoxic tumor microenvironment [75]. Thus, HIF or other hypoxia-related molecules might be putative therapeutic targets for breast cancer treatment.

Elevated levels of cytokines and growth factors produced by tumor cells enhance the proliferation and survival of CSCs and recruit immune and other stromal cells, which secrete additional growth factors, forming a positive feedback loop that promotes tumor cell invasion and metastasis [70]. Paracrine or autocrine signals between BCSCs and surrounding stroma are also involved in regulating the BCSC phenotypes. In a recent study, interleukin (IL)-8 and -6 secreted by human umbilical cord-derived MSCs can activate the autocrine IL-8 and IL-6 signaling in MCF-7 cells and induce $CD44^+/CD24^-$ BCSCs, which subsequently promote migration in vitro and metastasis in vivo [76]. Secreted IL-6 was sufficient to convert non-BCSCs to BCSCs by up-regulating *OCT4* gene expression through the IL-6–JAK1–STAT3 signal transduction pathway [77]. CAFs-derived chemokine (C–C motif) ligand 2 (CCL2) stimulated the stem cell-specific, sphere-forming phenotype and BCSC self-renewal in breast via inducing Notch1 expression [78]. The interaction between ECs and $CD44^+/CD24^-$ cells was also involved in regulating BCSCs. It described a feedback loop that tumor cells first secreted endothelial stimulatory signals, such as VEGF, FGF12, PTN and NF1, and thereby stimulated ECs to secret PDGFB, which in turn promoted cancer cell proliferation [79].

TAMs constitute a major cell population in the breast tumor microenvironment. It has been suggested that TAMs come from polarized macrophages, which lead to their pro-tumor phenotypes that facilitate tumor growth and stimulate angiogenesis [80, 81]. Yang et al. showed that TAMs could promote CSC-like phenotypes in murine breast cancer cells through the paracrine EGFR/Stat3/Sox-2 signaling pathway [82]. Co-injection of TAMs with CSCs was found to significantly augment the tumor growth compared to injection of $CD44^+/CD24^-$ cells alone, strongly supporting that TAMs may play a crucial role in BCSC maintenance [83]. It has shown that TAMs are often found in the surroundings of blood vessels of breast cancer [84], and the number of TAMs in vessel areas is positively correlated with blood vessel density in breast cancer [85]. Meanwhile, TAMs, CAFs, newly generated blood vessels, and other stromal cells accumulated at the invasive front where CAFs secreted macrophage colony stimulating factor (M-CSF) to turn on TAMs' pro-angiogenic switch [71].

Also, T cells can participate in breast cancer promotion when recruited to the tumor microenvironment. CD4+ helper T cells and TAMs could secrete TNF-α, which up-regulated NF-κB signaling pathways to induce Slug, Snail, and Twist and increase the cross-talk with the TGF-β signaling pathway which stimulated self-renewal [86, 87]. Increased infiltration of CD8+ cytotoxic T cells and FOXP3+ regulatory T cells was associated with unfavorable histologic features, including high histological grade and highly aggressive steroid receptor-negative status [88]. The infiltration of CD4+ and CD8+ T lymphocytes was closely correlated with the BCSC phenotype and EMT [89].

The ECM is an essential component of both normal and cancer stem cell niche and plays multiple roles in maintaining stem cell properties [90]. ECM anchorage restricts stem cells in the niche and thereby allows them to be exposed to paracrine and cell–cell contact signals that are important for maintaining stem cell properties. The ECM also maintains stem cell properties via some other features such as ECM stiffness that affects cell fate determination. Abnormal changes of ECM would block the cellular differentiation process, resulting in a decrease of differentiation and an increase of stem cells [90]. Increased ECM stiffness in breast cancer promoted transcriptional coactivator with PDZ-binding motif (TAZ) activity, resulting in an increase of BCSCs [91, 92], which suggests the potential role of ECM in breast cancer progression by promoting the self-renewal ability of BCSCs.

ncRNAs

The plasticity of tumor cells can also be regulated by ncRNAs, mainly including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) [93]. A set of ncRNAs have been found to be associated with BCSCs, either inhibiting or promoting BCSC properties. The miRNAs differentially expressed between BCSCs and non-BCSCs, such as *miR-200 cluster*, *miR-183 cluster*, *miR-221–222 cluster*, *let-7*, *miR-142* and *miR-214*, could target the genes and pathways responsible for stem cell maintenance [94]. Also, the deregulations of various miRNAs, such as *let-7*, *miR-7*, *miR-10* and *miR-15a*, have been indicated to contribute to drug resistance in breast cancer [95]. It has been illustrated that overexpression of *let-7a* decreases cell proliferation and mammosphere formation of BCSCs in a

KRas-dependent manner, both in vitro and in vivo [96]. Stable overexpression of *miR-10b* in MCF-7 cells increased self-renewal capacity and expression of stemness and EMT markers, whereas inhibiting *miR-10b* resulted in a decrease in BCSCs self-renewal [97]. *Linc00617*, the human ortholog of evolutionarily conserved lncRNA *TUNA*, was demonstrated to up-regulate the expression of stemness factor SOX2 in breast cancer cells, which was accompanied by induction of stem cell properties [98]. The Shh–Gli1 pathway-associated lncRNA-Hh stimulated the activation of Hh signaling, thereby increasing Gli1, SOX2, and OCT4 expressions for the maintenance of BCSCs [99]. Thus, BCSCs can probably be suppressed at the transcriptional level by targeting these deregulated ncRNAs. However, the clinical relevance of these ncRNAs and their potential as therapeutic targets still need to be verified by further studies.

Therapeutic implications of BCSCs

The CSC hypothesis has crucial implications for the development of cancer therapy. As mentioned before, BCSCs are relatively resistant to conventional therapies targeting the tumor bulk. Novel approaches targeting BCSCs [100] and pathways or factors regulating BCSCs [57, 58], have shown promising results in tumor inhibition (Table 2). These findings have led to the establishment of novel therapeutic strategies that combine BCSC and bulk cell targeting agents.

Based on molecular profiling, breast cancers that express estrogen receptor (ER) and/or progesterone receptor (PR) are subdivided into luminal types. In luminal breast cancers, the expansion of BCSCs was triggered by estrogen-induced paracrine FGF/FGFR/Tbx3 signaling pathway [101], or progesterone-induced receptor activator of the NF-κB ligand (RANKL) [102]. It indicates that the addition of BCSC targeting agents to hormonal therapies might increase the clinical benefit. Recently, some novel targeted agents, such as mTOR inhibitor everolimus and cyclin-dependent kinase 4/6 (CDK4/6) inhibitor palbociclib, have shown efficacy in combination with endocrine therapy in ER-positive breast cancer. Interestingly, the mTOR pathway [103] and cyclin D-CDK4/6 complex [104] have been reported to play roles in the regulation of stem-like cell activity. These findings imply that the clinical benefit of mTOR or CDK4/6 inhibitors might be attributed to their ability in inhibiting BCSCs, supporting the combination of BCSC targeting agents and endocrine therapies in luminal breast cancers.

Human epidermal growth factor 2 (HER2)-positive tumor constitutes another molecular subtype of breast cancer with an aggressive biologic behavior. The development of anti-HER2 agents, including trastuzumab, pertuzumab, lapatinib, and trastuzumab emtansine (T-DM1), has greatly improved the clinical outcomes of HER2-positive breast cancer patients. It has been demonstrated that HER2 cooperates with c-MYC to markedly increase self-renewal and drive a stem-like phenotype in breast cancer [105], whereas HER-2 blocking can reduce BCSCs [106]. Even though HER2-targeting agents display high efficacy at first, most patients eventually develop resistance. It has been shown that IL-8 regulates BCSC activity via binding to its cognate receptors, CXCR1 and CXCR2, and targeting CXCR1/2 significantly reduces BCSC activity and increases the efficacy of inhibiting HER2 [107]. This raises the intriguing possibility of combining BCSC targeting and HER2 targeting agents in HER2-positive breast cancers.

TNBC, which is characterized by lack of expression of ER, PR, and HER2, has the highest level of cells expressing BCSC markers compared to the other subtypes [108]. There are currently no effective targeted therapies available for TNBC patients because of the absence of hormone receptors and HER2 expressions. Cytotoxic chemotherapy is the only established treatment in TNBC, and this therapy initially shows clear benefit; however, patients invariably develop resistance. Studies with TNBC cells suggest that BCSCs with self-renewing and tumor-initiating capacities are responsible for the resistance and relapse. Thus, the addition of BCSC targeting agents to traditional chemotherapy can probably improve the treatment efficacy in TNBC.

The BCSC state plasticity has been suggested as an important issue in cancer therapy. It has been proven that BCSC is not a fixed population, because bulk tumor cells are capable of dedifferentiating into BCSCs. A transition between the differentiated breast cancer cells and stem-like cells was observed in vitro though combined expression of the transcription factors *SLUG* and *SOX9* [109]. A further in vivo study undertook exome sequencing of CSCs from 12 breast cancer patients along with paired primary tumor samples and found that the majority of somatic mutations were shared between BCSCs and bulk primary tumor, which implies a dynamic switch between BCSCs and differentiated cell states [110]. Furthermore, a mathematical model has found that dedifferentiation and plasticity substantially reduce the effectiveness of CSC-targeted therapies and increase the rates of resistance [111]. However, the actual contribution of dedifferentiation in tumor progression and resistance remains unclear, and thereby further studies should be designed to analyze how this plasticity can be applied to facilitate better therapeutic treatments.

Although these studies exhibit promising results in targeting BCSCs, there are some critical limitations. One

question is that all the approaches used to characterize BCSCs, including mammosphere formation assay and xenotransplantation experiments, are processed in an artificial microenvironment. Tumorigenicity studies are conducted in immunodeficient mice, which is quite different from the real immune system of humans. The second question is that there are numerous biomarkers of BCSC, as well as the dynamic switch between stem-like and non-stem-like states during tumor progression. How to deal with the spatial and temporal heterogeneity remains to be answered. The third question is specificity. How BCSC targeting agents can specifically target BCSCs remains an important issue, since some markers are also expressed in normal MaSCs.

Conclusion

The CSC hypothesis provides an important model for cancer research. The crucial roles of BCSCs in breast cancer initiation, metastasis, and resistance highlight the pressing need for developing novel therapies to eradicate these cells. A growing number of cell surface markers are being discovered to identify BCSCs. Several stemness pathways, tumor microenvironment, as well as ncRNAs have been demonstrated to be involved in regulating BCSCs, suggesting that these regulators may represent future therapeutic targets of breast cancer. Accumulating evidence has shown the efficacy of targeting BCSCs in inhibiting stem-like properties, as well as reversing drug resistance in vitro and in vivo. It has also been proposed that combination therapies targeting BCSC and bulk cell may be more effective than single therapy. However, the majority of studies are still in the early stages and it remains difficult for clinical practice. Thus, continuing effort in establishing clinically relevant biomarkers of BCSC is urgently needed for translating the knowledge from laboratory to clinical practice.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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