Review

Breast Cancer Stem Cell Heterogeneity, Plasticity and Treatment Strategies

Mengting Chen [1], Suling Liu [1,2,3,*]

1. Fudan University Shanghai Cancer Center & Institutes of Biomedical Sciences; State Key Laboratory of Genetic Engineering; Cancer Institutes; Department of Oncology; Key Laboratory of Breast Cancer in Shanghai; The Shanghai Key Laboratory of Medical Epigenetics; Shanghai Key Laboratory of Radiation Oncology; The International Co-laboratory of Medical Epigenetics and Metabolism, Ministry of Science and Technology; Shanghai Medical College; Fudan University, Shanghai 200032, China; Email: mtchen1107@outlook.com;
2. Jinfeng Laboratory, Chongqing 401329, China
3. Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center for Cancer Medicine, Nanjing Medical University, Nanjing 211166, China

* **Correspondence:** Suling Liu; Email: suling@fudan.edu.cn

Abstract

Breast cancer stem cells (BCSCs) are a small proportion of stem-like breast cancer cells with more tumorigenic and metastatic capacity in comparison to the bulk tumor cells, which are capable of self-renewal and generating differentiated cells. BCSCs are resistant to chemotherapy and radiotherapy. More and more research indicate that BCSCs are heterogeneous and have plasticity, which refer to their ability to switch between different subtypes of stem-like cells and differentiated cells. There is some evidence showing that BCSC heterogeneity and plasticity plays a role on therapeutic resistance and metastasis of breast cancer. In this review, we discussed the roles of extrinsic (rewired tumor microenvironment, including hypoxia, microbiota) and intrinsic (pro-tumor signaling) factors on regulating BCSC heterogeneity and plasticity, leading to various malignant behaviors of BCSCs, including epithelial-mesenchymal transition (EMT)/mesenchymal-epithelial transition (MET), immune evasion, vasculogenesis and so on. Moreover, we also discussed the potential therapeutic strategies to target BCSCs.

Keywords: breast cancer stem cell; tumor microenvironment; plasticity; heterogeneity; targeting therapy

1. Introduction

Breast cancer is the most common cancer in women worldwide, and is a leading cause of cancer mortality in women [1]. Treatment of breast cancer is largely based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2/ERBB2). The hormone receptors ER/PR-positive patients account for around 70% of breast cancer patients, who are treated with hormonal therapy [2]. HER2 expression, which is observed in approximately 15–25% of breast cancers, is mainly relevant in the choice of anti-HER2 targeted therapy. Triple-negative breast cancer (TNBC), defined by the absence of all three markers, is the most aggressive subtype of breast cancer due to the lack of targeted therapeutic options. About 15% of breast cancers are TNBC [2]. Chemotherapy and immunotherapy are currently the most commonly used systematic therapies in TNBC patients. However, tumor recurrence, metastasis and treatment resistance remain the leading causes of mortality, particularly in TNBC patients.

Substantial advances have been made in understanding the heterogeneity of breast cancer. Cancer stem cells (CSCs) are a subpopulation of cancer cells that can self-renew and differentiate into different cell lineages, thereby contributing to tumor relapse and treatment resistance. Self-renewal refers to a process that occurs during cell division, whereby at least one daughter cell maintains stem cell-like phenotypes, including long-term proliferative potential [3]. The CSC self-renewal capacity can be assessed by serial passaging of cancer cells *in vivo* [4]. CSCs have been discovered in many types of cancer, including breast cancer, glioblastoma, colon cancer and prostate cancer [5]. One of the most profound implications of CSCs is their contribution to tumor heterogeneity. Due to their clinical significance, several biomarkers have been identified to characterize CSCs, correlating with diagnosis, therapy sensitivity and prognosis. Dormant CSCs are thought to be more resistant to current therapies [6]. Importantly, CSCs exhibit high plasticity, modifying their phenotypic and functional appearance under different conditions [7]. By understanding the specific markers and signaling pathways associated with CSCs, novel treatments could potentially inhibit their self-renewal or induce differentiation, thus eradicating the seeds of the tumor. Here, we discussed the heterogeneity and plasticity of CSCs in breast cancer and novel treatment strategies to target CSCs.

2. Breast Cancer Stem Cells (BCSCs)

BCSCs are a small proportion of breast cancer cells that are capable of self-renewal and generate differentiated cancer cells [8]. The origin of BCSCs is still unclear. One of the theories is that they derive from mammary stem cells (MaSC), as they share similar markers and a MaSC-enriched population has been observed in preneoplastic tissue of mammary tumor-prone mice [6]. MaSCs possess the ability to differentiate into various mammary cell lineages, contributing to the gland's functional architecture during puberty, pregnancy, and lactation. MaSCs lacks the expression of hormone receptors in mice and they might become BCSCs after gaining the mutation [9]. Further defining the regulation mechanisms of MaSC might help us understanding the origin of BCSCs.

BCSCs have been extensively studied due to their malignant behavior such as tumorigenesis, therapeutic resistance and high metastatic potential. The classic method to identify CSCs involves cell sorting by flow cytometry to isolate cell populations based on specific surface markers, followed by inoculation of cells at limited dilutions in serial xenotransplantation assay to assess the tumor-initiating cell frequency [3]. A number of alternative approaches include three-dimensional (3D) culture systems and single-cell RNA sequencing, *etc* [3]. The main characteristics of BCSCs are their high heterogeneity and plasticity, which contribute remarkably to their malignancy and pose a challenge for efficient cancer therapeutics. Heterogeneity refers to their subpopulations that express different cellular markers. Plasticity refers to their ability to switch between different states regulated by the tumor microenvironment and pro-tumor signaling pathways.

The mammary gland is a highly regenerative organ that goes through significant changes during the menstrual cycle, pregnancy, lactation, and involution, and stem cells play important roles during these process. How these changes affect BCSCs and breast oncogenesis is still controversial. A previous study showed that pregnancy in the adult mouse does not alter the proportion of mammary epithelial stem/progenitor cells [10]. Epidemiological studies have demonstrated that an early, full-term pregnancy at young age may reduce the lifetime risk of breast cancer [11]. However, pregnancy generally offers protection against the development of ER/PR$^+$ tumors, while the breast cancers that emerge shortly after pregnancy typically do not express these hormone receptors [12]. During pregnancy, the breast undergoes significant morphological and functional changes, primarily driven by a surge in hormones such as estrogen and progesterone. These hormonal shifts stimulate mammary gland development and differentiation, leading to the proliferation of epithelial cells and increased stromal support. The microenvironment with growth factors and cytokines promotes the survival and expansion of BCSCs [13]. Furthermore, another study revealed that parity reduced mammary repopulating activity but did not affect mammary stem cells defined as CD24$^+$ CD29/CD49f^{hi} in mice [14].

3. BCSC Heterogeneity and Plasticity

A number of subpopulations within breast cancer have been identified to possess high tumor-initiating potential. The most commonly used markers to identify BCSCs include CD24$^-$ CD44$^+$, ALDH, and CD49f [15,16].

The initial BCSC population to be identified was that of CD24$^{-/low}$ CD44$^+$ cells [4]. These cancer cells demonstrate a substantial capacity for tumor initiation. As few as 100 breast cancer cells with EpCAM$^+$, high levels of CD44, and low levels of CD24 expression were able to efficiently form tumors when inoculated in immune-deficient mice [4]. The tumorigenic subpopulation could be serially passaged. Each time, these cells generated new tumors containing additional CD44$^+$CD24$^{-/low}$ Lineage$^-$ tumorigenic cells as well as the phenotypically diverse mixed populations of non-tumorigenic cells present in the initial tumor [4]. CD44 is a cell-surface glycoprotein that binds hyaluronic acid (HA) and many other extracellular matrix components [17]. CD44 is involved in cell-cell interactions, cell adhesion and migration. Alternative splicing of CD44 could determine breast cancer stem cell state [18]. CD44 standard splice isoform

(CD44s) is the predominant isoform expressed in BCSCs and positively associates with the CSC gene signatures. In contrast, the CD44 variant splice isoforms (CD44v) exhibit an inverse association with the CSC signatures [18]. Mounting evidence have suggested that CD44 functions not only as CSC markers but also as critical regulators of cancer stemness, including self-renewal, tumor initiation, and metastasis [17].

Another frequently used BCSCs marker is aldehyde dehydrogenase 1 (ALDH1). ALDH1 is a member of the aldehyde dehydrogenase protein family, which catalyzes the oxidation of intracellular aldehydes. The measurement of ALDH1 activity via the Aldefluor assay is a frequently employed method for the analysis of BCSCs in both cell lines and animal models. ALDH1 plays a role in the early differentiation of BCSCs through oxidizing retinol to retinoic acid [16]. The activity of ALDH1A1 in BCSCs has been demonstrated to remodel myeloid-derived suppressor cells, thereby promoting breast cancer progression [19].

CD49f, also known as integrin-α6, is a further BCSC marker, as well as a common CSC marker [20]. It forms homodimers with β4-integrin, thereby facilitating binding to laminin and enhancing epithelial cell adhesion to the extracellular matrix [21]. CSCs, which are enriched in the $CD24^+ CD29^+/CD49f^+$ cell population from a Brca1-mutant mouse model, exhibited a markedly elevated migration capacity relative to $CD24^- CD29^-/CD49f^-$ cells [22]. The dynamics of the $CD49f^+$ tumor-initiating population is associated with taxane resistance in TNBC [23].

Notably, $CD24^{-/low} CD44^+$ mesenchymal-like BCSCs are primarily quiescent and localized at the tumor invasive front, whereas $ALDH^+$ epithelial-like BCSCs, are proliferative, and are located more centrally [24]. Although breast cancer cells with both $CD24^{-/low} CD44^+$ and $ALDH^+$ expression represent a rare population, they have been observed to exhibit the greatest tumorigenic and invasive capacity [24]. Importantly, a recent study has demonstrated that ALDH1A3 is the key regulator that determines the balance of $ALDH^+$ and $CD24^- CD44^+$ breast cancer stem cells [25]. While ALDH1A3 increases the number of $ALDH^+$ breast cancer cells, it inversely suppresses the $CD24^- CD44^+$ population by retinoic acid signaling. It can therefore be concluded that BCSCs constitute a heterogeneous population, which is regulated by a number of different factors.

The abundance of BCSCs in different breast cancer subtypes has also been explored. The tumors enriched with $CD44^+ CD24^-$ or $ALDH^+$ subpopulation were more correlated with basal-like and HER2-positive breast cancers [26,27]. In line with these results, BCSCs were rare in $ER^+ PR^+$ luminal subtype breast cancers [28]. Moreover, within a tumor, $CD44^+ CD24^-$ cells showed lower ER and PR expression compared to $CD44^- CD24^+$ cells [28]. These results may also explain the poor prognosis of TNBC and HER2-positive patients.

The causes of BCSC heterogeneity is complicated and not yet fully elucidated. One theory is the occurrence of series of mutations in differentiated somatic cells. A key aspect of this theory is the concept of de-differentiation of differentiated cell, allowing them to be reverted to a more stem-like state under certain conditions [29]. This plasticity may underlie the heterogeneity observed in BCSCs, as different differentiated cells may respond uniquely to mutagenic stress, leading to diverse tumor phenotypes and behaviors. In contrast, the second theory proposes the occurrence of mutations in MaSC or progenitors [30]. This theory suggests that BCSCs arise from mutations occurring in the

normal mammary stem cell compartment or its immediate progenitors. These mutations can lead to the dysregulation of key pathways involved in stem cell maintenance and differentiation, resulting in the heterogeneity and plasticity of BCSCs. However, sequencing analysis revealed that the majority of somatic mutations are shared between CSCs and bulk tumors [31]. Therefore, future efforts are still needed to deeply understand the causes of BCSC heterogeneity and plasticity.

4. Extrinsic Factors Regulating BCSCs Heterogeneity and Plasticity

The heterogeneity of BCSCs is typically attributed to their inherent plasticity. This plasticity refers to their capacity to transition between stem-like and differentiated states [32]. Multiple mechanisms have been identified as regulators of BCSCs plasticity and heterogeneity. Here we discuss how the tumor microenvironment components, hypoxia, tumor-resident microbiota, and tumor cell intrinsic signaling pathways influence the heterogeneity and plasticity of BCSCs.

4.1 Tumor ecosystem

Breast cancer consists of multiple cell types, including a large variety of immune and other host cells, which create the tumor microenvironment (TME). The crosstalk between all these cellular and structural components is essential for breast cancer growth and progression by accumulating phenotypic and genetic heterogeneity. The tumor microenvironment has been identified as a driving force behind BCSC heterogeneity and plasticity, with interactions between immune cells, stromal cells, and the extracellular matrix influencing BCSC expansion. The immune cells in the TME regulate BCSC plasticity through the establishment of positive feedback loops [33]. Here, we summarize ways in which the various cell types that comprise the TME, including macrophages, myeloid-derived suppressor cells, T cells and cancer-associated fibroblasts, contribute to the BCSC stemness and plasticity. The mechanisms are summarized in **Figure 1** (created with BioGDP.com [34]).

4.1.1 Immune cells

Macrophage

Tumor-associated macrophages (TAMs) constitute the most abundant immune population in the breast tumor microenvironment. TAMs have been demonstrated to facilitate cancer cell proliferation and metastasis, while also exerting immunosuppressive effects on the adaptive immune cells that populate the TME. Macrophages that have been activated are typically classified into two categories: M1-like macrophages, which are primarily involved in pro-inflammatory responses, and M2-like macrophages, which are primarily involved in anti-inflammatory responses.

Recent studies have demonstrated that TAMs promote epithelial-mesenchymal transition (EMT) and the cancer stem cell properties in breast cancer via the CCL2/AKT/β-catenin signaling [35,36]. In addition, Wang *et al*. recently reported that Rab13, a small GTPase, is highly expressed in BCSCs. Rab13 was found to control the membrane translocation of C-X-C chemokine receptor type 1/2

(CXCR1/2), allowing tumor cells to interact with TAMs and cancer-associated fibroblasts, and establish a supportive BCSC niche [37]. Furthermore, macrophage-induced ERK-TGF-β1 signaling in MCF7 breast cancer cells result in cancer stem cell plasticity and EMT which are completely reversible [38]. Additionally, M2-like TAMs contribute to increase cancer cell stemness in TNBC cells via secreting VEGFA, which were evaluated with stem cell markers, including CD24, CD44, OCT-4, Nanog and SOX-2. Reciprocally, elevated VEGFA expression by TAM-educated TNBC cells acts as a regulator of macrophage polarization [39]. In light of these findings, it can be concluded that TAMs play a pivotal role in regulating BCSC stemness and plasticity.
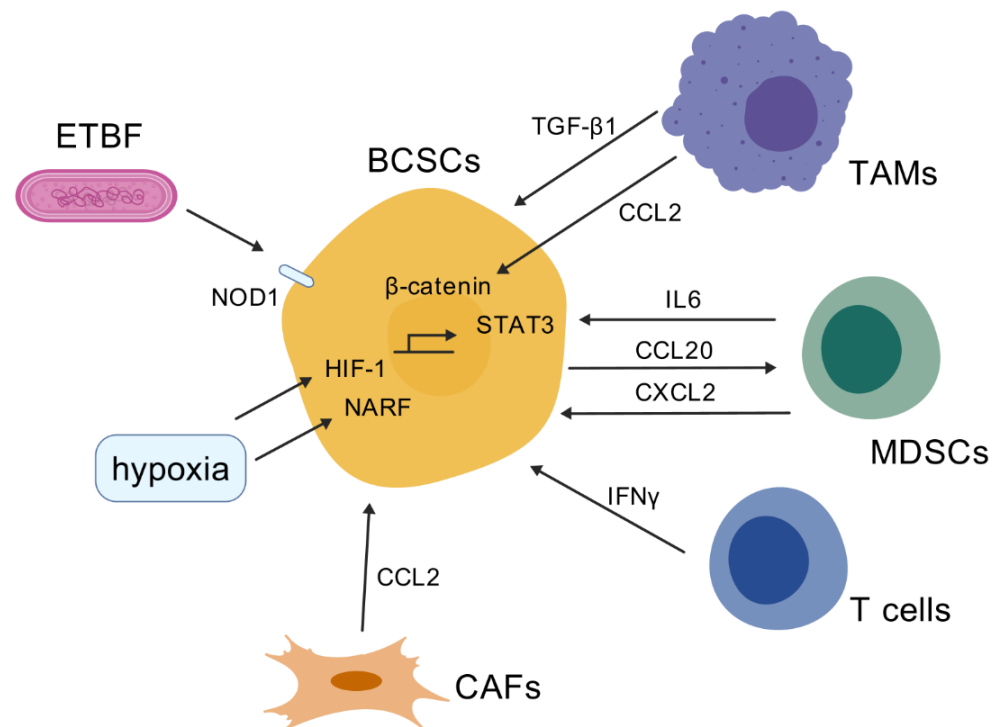

Figure 1. Mechanisms of BCSC plasticity regulated by the tumor ecosystem. The plasticity and heterogeneity of BCSCs are influenced by the immune cells, fibroblasts, hypoxia, and microbiota present in the tumor niche. This contributes to treatment resistance and metastasis. TAMs: Tumor-associated macrophages; MDSC: myeloid-derived suppressor cells; CAFs: cancer-associated fibroblasts; ETBF: enterotoxigenic bacteroides fragilis. Created with BioGDP.com.

Myeloid-derived suppressor cell (MDSC)

Myeloid-derived suppressor cells (MDSCs) are activated neutrophils and monocytes with potent immunosuppressive activity. Two distinct subtypes of MDSCs have been identified: monocytic myeloid-derived suppressor cells (M-MDSCs) and granulocytic polymorphonuclear MDSCs (PMN-MDSCs). M-MDSCs show a stronger immunosuppressive effect [33]. Nonetheless, PMN-MDSCs play an important role in regulating tumor featured immune responses [40].

MDSC contribute to the development of stem-like phenotypes in breast cancer cells, which in turn facilitate immune suppression and tumor recurrence. For example, MDSCs promoted tumor growth by enhancing sphere formation and increasing the percentage of ALDH+ BCSCs in breast cancer as well as by suppressing T-cell activation. These effects were mediated by the cross-talk

between the IL6-STAT3 and NOTCH pathways in cancer cells with MDSC [41]. MDSC was found to be correlated with the presence of BCSCs in breast cancer samples and was also identified as an independent predictor of poor survival outcomes [41]. Additionally, it was demonstrated that PMN-MDSCs, which are modulated by CCL20 from cancer cells, can increase ALDH+ BCSCs and stemness genes (eg. KLF4, NANOG, SOX9, *etc*.) via the CXCL2-CXCR2 pathway in breast cancer [42].

T cell

Although T cells are known for their tumor-killing ability, recent studies reveal that ineffective immune responses not only fail to clear a tumor, but also promote stemness and tumor-forming capacity in cancer cells. Interferon gamma (IFNy) produced by activated T cells following immunotherapy directly converts non-CSCs to CSCs [43]. IFNy enhances several CSC phenotypes, including resistance to chemo- and radiotherapy, as well as metastasis formation [43]. Cognate interaction with nonlytic CD8+T cells increased the proportion of CD44+CD24− BCSCs in a cell-to-cell contact- or proximity-dependent manner [44].

4.1.2 Cancer-Associated Fibroblast (CAF)

CAFs play a pivotal role in the reactive stroma within the TME, as they not only interact with cancer cells extensively via secreted cytokines or cell−cell adhesion but also indirectly influence cancer cells via remodeling of the extracellular matrix and immune cell infiltration [45]. For instance, primary CAFs activated by cocultured breast cancer cells produce higher levels of chemokine CCL2, which induces the CSC phenotype in breast cancer [46]. Increased CCL2 expression in activated fibroblasts required STAT3 activation by diverse breast cancer-secreted cytokines, and in turn, induced the mammosphere formation and self-renewal of expansion in breast cancer cells via NOTCH1, constituting a cancer-stroma-cancer signaling circuit [46]. In addition, Su *et al*. have found that CD10+GPR77+ CAFs provide a survival niche for CSCs, thereby promoting tumor formation and chemoresistance [47]. Mechanistically, CD10+GPR77+ CAFs are driven by persistent NF-κB activation via p65 phosphorylation and acetylation, which is maintained by complement signaling via GPR77. In line with this notion, targeting these CAFs with a neutralizing anti-GPR77 antibody abolishes tumor formation and restores chemosensitivity [47]. On the other hand, BCSCs reciprocally promote the formation of CAFs. Breast cancer stem cells are found to upregulate IRF6 in stromal fibroblasts to induce stromagenesis, in order to sustain the BCSC tumor niche [48].

4.1.3 Hypoxia

Hypoxia is a key feature of the breast cancer TME that promotes expression of the transcription factor hypoxia-inducible factor 1 (HIF-1) and is correlated with unfavorable prognosis and chemoresistance. HIF-1 induces the expression or activity of stem cell pluripotency factors, including NANOG, KLF4, OCT4 and SOX2 [49,50]. It is also noteworthy that HIF-1 facilitates the evasion of phagocytosis and the maintenance of BCSCs by activating the transcription of CD47 [51]. CD47 functions as a mediator of immune evasion by delivering a 'don't eat me' signal to macrophages [52]. Hypoxia-induced secretion

regulates the dissemination of CSCs, mediated by JAK-STAT signaling [53]. A recent study found that nuclear prelamin A recognition factor (NARF) acts as a hypoxia-induced coactivator for OCT4-mediated BCSC specification [49]. It was also illustrated that HIF-2α, but not HIF-1α, induces breast cancer cell stemness under hypoxia [54]. Mechanistically, hypoxia inducible factor-2α (HIF-2α) activates superoxide dismutase 2 (SOD2) under hypoxic conditions, thereby decreasing mitochondrial reactive oxygen species (mtROS) levels. The reduced mtROS transported to the endoplasmic reticulum subsequently activate unfolded protein response (UPR) via GRP78, which eventually confer chemoresistance of cancer cells, increase of OCT4 expression, and both CD44$^+$CD24$^-$BCSC and ALDH1$^+$ BCSC populations [54]. Accordingly, depletion or inhibition of HIF-2α was observed to abolish the hypoxia-induced sphere-forming ability and the percentage of CD44$^+$CD24$^-$ cell subpopulation [54]. Of note, glycolysis was found to reprogram BCSC under hypoxic conditions. Pyruvate dehydrogenase kinase 1 (PDK1), a key glycolysis gene, was found to be overexpressed in BCSCs and accumulated in hypoxic regions. The inhibition of PDK1 led to a remarkable reduction in the percentage of ALDH$^+$ BCSC subpopulation, expression of stemness-related transcriptional factor, and sphere-forming ability and tumor growth in breast cancer [55]. Taken together, hypoxia has been identified as one of the critical factors in the TME that sustain the BCSC stemness.

4.1.4 Tumor-resident microbiota

The roles of microbiota for cancer cell stemness, metastasis and response to treatment remain poorly understood, having only recently emerged as a topic of investigation. Tumor-resident intracellular microbiota, albeit at low biomass, were shown to promote metastatic colonization in breast cancer [56]. It is noteworthy that microbiota *enterotoxigenic Bacteroides fragilis* (ETBF) was found to be highly enriched in tumors of breast cancer patients who did not respond to taxane-based neoadjuvant chemotherapy [57]. Mechanistically, the toxic protein BFT-1 secreted by ETBF, directly bound to the cytosolic pattern recognition receptor NOD1. NOD1 increased BCSCs by activating the NOTCH1-HEY1 signaling pathway. In line with this notion, NOD1 inhibition and ETBF clearance was shown to increase the chemosensitivity of breast cancer by impairing BCSCs [57]. However, evidence on how the tumor-resident microbiota regulate breast cancer stemness and plasticity is still rare, which warrants further investigation.

4.2 Intrinsic signaling in tumor cells

4.2.1 Notch signaling

It is well established that Notch signaling plays a pivotal role in sustaining the self-renewal and tumor-initiation capacities of BCSCs. The Notch pathway involves four Notch receptors (Notch1-4) and five ligands (Jagged1, Jagged2, Delta-like1 (Dll1), Dll3, and Dll4). The association between NOTCH3 expression and tumor metastasis in ERα$^+$ and TNBC models has been identified [58]. The abrogation of NOTCH3 expression was found to significantly reduce the self-renewal and invasive capacity of *ex vivo* breast cancer cells, restoring a luminal CD44^{low}/CD24^{high}/ERα^{high} phenotype [58]. Additionally, NOTCH4 has been shown to enhance tumorigenesis in TNBC

cells via upregulating Nanog expression [59]. NOTCH4 also maintains quiescent mesenchymal-like BCSCs in TNBC cells via activating SLUG and GAS1 [60]. The Notch ligand Dll1 is also a key factor in tumor growth and metastasis and promotes stemness of breast cancer cells [61]. Activation of the NF-κB pathway occurs downstream of Dll1 and is associated with chemoresistance. In addition, a recent study has demonstrated that the tumor suppressor FERM domain-containing protein 3 (FRMD3) functions as an endogenous activator of the Notch signaling pathway, facilitating the basal-to-luminal transformation in mammary epithelial cells [62].

4.2.2 Wnt/β-catenin signaling

The Wnt/β-catenin signaling plays critical roles in the proliferation, migration, and treatment resistance of BCSCs. β-catenin is a key downstream effector of the Wnt signaling pathway. Following the binding of Wnt family proteins to the Frizzled receptor, β-catenin translocates to the nucleus where it interacts with LEF1 and TCF1 to activate canonical targets including CD44, cyclin D1, c-Jun, c-Myc, Met, and MMP-7 [63]. Notably, a recently identified unique subcluster of Socs3^{high}CD11b$^-$CD27$^-$ immature NK cells in TNBC samples expressed a reduced cytotoxic granzyme signature and, in mice, were responsible for activating cancer stem cells through Wnt signaling. The activation of BCSCs by NK cells subsequently enhanced tumor progression in mice, whereas depletion of NK cells or Wnt ligand secretion from NK cells resulted in a decrease in tumor progression [64]. In addition, FBXW7 blocks the activation of the Wnt/β-catenin signaling pathway to inhibit the percentage of ALDH$^+$ and CD44$^+$CD24$^-$ BCSCs in TNBC by degrading CHD4 protein through ubiquitination [65]. Furthermore, the secretome of BCSCs has been shown to contain MIF, which plays a crucial role in potentiating glycolysis and the subsequent evasion of immune responses. MIF was shown to increase c-MYC-mediated transcriptional upregulation of the glycolytic enzyme aldolase C by activating WNT/β-catenin signaling [66]. Therefore, the Wnt/β-catenin signaling serves as a promising target to inhibit BCSCs.

4.2.3 Hedgehog (Hh) signaling

The Hedgehog (Hh) signaling pathway plays critical roles in regulating cell fate and cell growth during the embryonic development. It is recognized as a key player in tumor growth, stemness and metastasis. Three canonical Hh ligands have been identified: Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH) [67]. Notably, TSPAN8 has been demonstrated to promote BCSC stemness through activating Sonic Hedgehog signaling [68]. Moreover, the Hedgehog signaling mediates the interaction between BCSCs and cancer-associated fibroblasts (CAF). CSCs secrete the Hedgehog ligand SHH, which regulates CAFs via paracrine activation of Hedgehog signaling. Conversely, CAFs secrete factors that facilitate the expansion and self-renewal of CSCs [69]. The administration of the Hedgehog inhibitor vismodegib decreases CAF and CSC expansion, consequently leading to a reduction in tumor formation. Similarly, in mouse models of TNBC, the Hedgehog ligand produced by neoplastic cells reprograms CAFs to provide a supportive niche for the acquisition of a chemo-resistant, CSC phenotype via FGF5 expression and production of fibrillar collagen [70]. In line with this notion, stromal treatment of patient-derived xenografts with smoothened inhibitors (SMOi)

downregulates CSC marker expression and sensitizes tumors to docetaxel. In addition, the Hh signaling also regulates aerobic glycolysis, which is critical for sustaining cancer cell stemness. For instance, ETV4, a key transcription factor in regulating glycolytic gene expression, was shown to promote BCSC stemness by activating CXCR4-mediated sonic Hedgehog signaling [71].

5. Impact of BCSC Heterogeneity and Plasticity

The heterogeneity and plasticity of BCSCs contribute remarkably to their high malignancy. BCSCs are able to switch between different states in response to corresponding signals and environmental stimuli. The consequences of such plasticity include increased invasive capacity via epithelial-mesenchymal transition, immune evasion, vasculogenic mimicry and resistance to chemotherapy and radiotherapy (**Figure 2**).
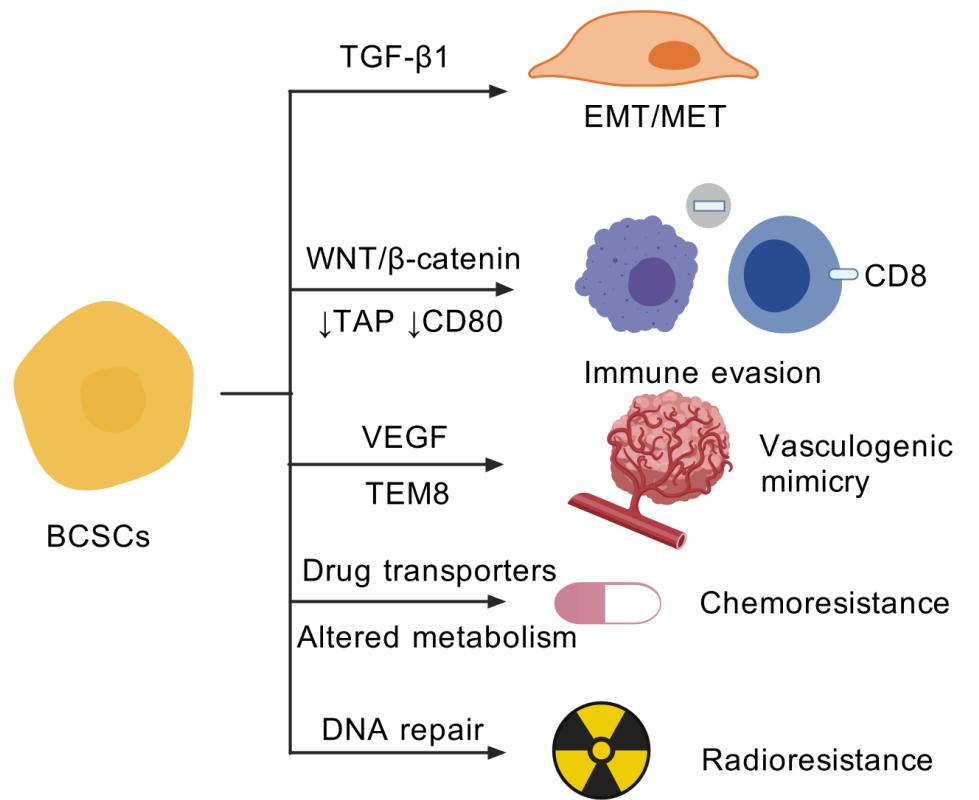

Figure 2. Impact of BCSC plasticity and heterogeneity in breast cancer malignancy. BCSCs can transit between mesenchymal and epithelial states in response to stimulation by TGF-β1. They are capable of evading immune cell-mediated killing through the activation of WNT/β-catenin or the reduction of antigen processing (TAP) genes and co-stimulatory molecule CD80. VEGF and TEM8 promotes vasculogenic mimicry in BCSCs. EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition (MET). Created with BioGDP.com.

5.1 Epithelial-mesenchymal transition (EMT) and Mesenchymal-epithelial transition (MET)

BCSCs display plasticity that enables them to flexibly transit between EMT and MET states. The EMT and MET processes play crucial roles in the metastasis of cancer. EMT is a cell process in which epithelial cells undergo a reversible

phenotype change marked by the loss of adhesion ability and acquisition of mesenchymal features. The process is regulated by multiple transcription factors, including ZEB1, Snail and Twist [72]. Tumor cells that have undergone an EMT-like process are more likely to enter the circulation through intravasation and disseminate to distant organs. Conversely, the MET state allows tumor cells to reside in metastatic sites. These phenotypic changes are induced by a wide variety of extracellular signals. Notably, mesenchymal-like BCSCs are characterized as $CD24^-CD44^+$, while epithelial-like BCSCs are $ALDH^+$. The gene-expression profiles of mesenchymal-like and epithelial-like BCSCs resemble those of distinct normal basal and luminal stem cells and are remarkably similar across different molecular subtypes of breast cancer [24]. In addition, the BCSCs isolated from primary TNBC specimens express epithelial and mesenchymal markers concomitantly [73]. BCSCs are susceptible to the EMT inducer TGF-β1, leading to the increase of mesenchymal genes and enhanced cell migration [73].

5.2 Immune evasion

BCSCs contribute to the immune evasion phenotype, which may influence the efficacy of immunotherapy. The mechanisms include a reduction in neo-antigens, antigen presentation and crosstalk with immune cells. For instance, radioresistant BCSCs have been observed to enhance DNA double-strand break repair and replication fork protection ability to immunogenic cytosolic DNA, thereby inhibiting the intracellular immune response [74]. Of note, $ALDH^+$ cells in breast cancer, but not $CD44^+CD24^-$ cells, have reduced expression of transporter associated with antigen processing (TAP) genes and co-stimulatory molecule CD80, which would decrease susceptibility to T cells [75]. Moreover, the upregulated glycolysis in BCSCs cells, which is mediated by WNT/β-catenin signaling suppresses intratumoral cytolytic $CD8^+$ T cells and proinflammatory macrophages while increasing regulatory T cells and tumor-associated neutrophils in the TME [66].

5.3 Vasculogenic mimicry (VM)

The rapid growth of cancer requires an efficient blood vessel network to provide enough oxygen and nutrition. VM elucidates the formation of fluid-conducting channels by highly invasive and genetically unstable tumor cells, thereby contributing to tumor metastasis and treatment resistance [76,77]. The primary pathway involved in tumor angiogenesis is the VEGF pathway. In addition, alternative neovasculogenic pathways play critical roles in tumor progression, as they facilitate the formation of a perfusable vasculogenic-like network, known as vasculogenic mimicry (VM) [78]. Recent studies revealed the associated between $CD133^+$ and $USP44^+$ cancer stem cells and vasculogenic mimicry in breast cancer [79,80]. Of note, tumor endothelial marker 8 (TEM8) has been identified as a marker for VM-forming breast tumor-initiating cells [78]. TEM8 increased active RhoC level and induced phosphorylation of SMAD5, in a cascade essential for promoting stemness and VM capacity of breast cancer cells [78]. Moreover, evidence indicates that endoplasmic reticulum stress was shown to inhibit Matrigel-induced vasculogenic mimicry of breast cancer cells [81]. In light of these findings, it seems reasonable to suggest that targeting the vasculogenic capacity of BCSCs may prove an effective strategy for the treatment of breast cancer.

5.4 Chemoresistance and radioresistance

More and more research shows that BCSCs are that they are resistant to chemotherapies and radiotherapy. Besides the mechanisms mentioned above, other factors also contribute to the treatment resistance. In terms of chemoresistance, CSCs often exhibit upregulation of ATP-binding cassette (ABC) transporters, such as ABCG2 and MDR1 [82,83]. These efflux pumps actively extrude chemotherapeutic agents from the cells, reducing drug accumulation and efficacy. Moreover, BCSCs can enter a quiescent state, remaining in a non-dividing phase of the cell cycle [84]. The dormancy provides a protective mechanism against agents such as chemotherapies that target rapidly dividing cells, rendering conventional chemotherapies ineffective. Furthermore, BCSCs can utilize distinct metabolic pathways to survive under low nutrient conditions and evade the cytotoxic effects of chemotherapies. For instance, fatty acid β-oxidation is found to be critical for BCSC self-renewal and chemoresistance [85]. Regarding to radioresistance, BCSCs possess enhanced DNA repair mechanisms, including upregulated expression of repair proteins, enabling them to efficiently repair the DNA damage induced by radiation, thereby surviving from the treatment [86].

6. Preclinical/Clinical Therapeutic Strategies Targeting BCSCs

Given the pivotal roles that BCSCs play in tumorigenesis and treatment resistance, many therapeutic approaches have been tested in preclinical settings alone or in combination with chemotherapy. A summary of recent novel preclinical therapeutics, along with their respective targets and effects on BCSCs, is presented in **Table 1**.

Table 1. Preclinical therapeutics targeting BCSCs

Name	Target	Effects	Reference
Small molecule inhibitor			
Sonidegib	SMO inhibitor	Sensitizes tumors to docetaxel, improve survival and reduce metastatic burden	[70]
Alpelisib	PI3 kinase p110a	Inhibits BCSCs stemness and EMT	[87]
Quercetin/Luteolin	CD73	Inhibits cancer stem cells and increases lymphocyte infiltration	[88]
Ropivacaine	AKT	Suppresses stem cells-like properties of breast cancer cells	[89]
FTY720	SphK1	Inhibit BCSC proliferation	[90]
HDAC3 inhibitor	HDAC3	Suppresses the CSC subpopulation of TNBC	[91]
Antibody-based therapy			
Tocilizumab	IL6	Potentiates cisplatin cytotoxicity in TNBC	[92]
Dinutuximab	GD2	Inhibits TNBC growth by targeting GD2+ breast cancer stem-like cells	[93]
CAR-T therapy			
DLL4-targeted CAR-T cells	DLL4	Eliminates CSCs and reshape immune microenvironment in HER2+ breast cancer	[94]
TEM8-targeted CAR-T cells	TEM8	Kills tumor endothelial cells and TEM8+ TNBC cells; offsetts the formation of mammospheres	[95]

The first strategy involves the development of small molecule inhibitors targeting BCSC signalings or the TME. For instance, targeting the Hh signaling pathway may prove an effective strategy to overcome BCSC plasticity. In the phase I clinical trial EDALINE, three of the 12 patients with metastatic TNBC derived clinical benefit from combination therapy with the SMO inhibitor Sonidegib and docetaxel chemotherapy, with one patient experiencing a complete response [70]. In accordance with this hypothesis, novel combinations of JAK2 inhibitors (ruxolitinib and pacritinib) with SMO inhibitors (vismodegib and sonidegib) synergistically suppressed breast cancer stem cells, tumor growth and metastasis [96]. Additionally, CD73 expression is required for the maintenance of CD44$^+$CD24$^-$ BCSCs. Quercetin and luteolin, which are commonly used as over-the-counter supplements, combined with paclitaxel could effectively downregulate paclitaxel-enhanced CD73 and CSC-promoting pathways YAP and Wnt. The triple-drug combination could inhibit paclitaxel-enriched CSCs, suppress the growth of TNBC cell lines and patient-derived xenograft organotypic cultures [88]. The FDA-approved PI3 kinase p110a inhibitor, alpelisib (BYL-719), has the potential to inhibit the stemness and EMT phenotype in BCSCs in 3D cultures. A comparison of eribulin-resistant breast cancer cell lines revealed that BYL-719 effectively overcame drug resistance [87].

In addition to small molecule inhibitors, the use of currently approved antibody also showed promising effects. Notably, pharmacological inhibition of IL-6 by tocilizumab potentiates cisplatin cytotoxicity and targets cancer stem cells in TNBC [92]. New antibodies are also in development. The antibody targeting GD2 (Dinutuximab), which is expressed in around 60% of primary TNBC, is able to inhibit TNBC growth by targeting GD2$^+$ breast cancer stem-like cells [93].

The implementation of immune cytotherapy also showed promising results. For instance, DLL4-targeted CAR-T therapy has been shown to eliminate cancer stem cells and reshape immune microenvironment in HER2$^+$ breast cancer, thereby sensitizing neoadjuvant chemotherapy [94]. Likewise, the TEM8 CAR T cells targeted breast cancer stem-like cells, offsetting the formation of mammospheres [95].

Besides preclinical studies, multiple therapeutic agents targeting BCSC in combination with chemotherapy have been tested in clinical trials. Relevant studies have been summarized in **Table 2**. The metabolism of CSCs in hypoxia suggests an altered balance between mitochondrial oxidative phosphorylation and glycolysis [97]. In line with this notion, specific drugs targeting mitochondrial metabolism, leading to increased ROS levels, may inhibit CSCs. Many classes of FDA-approved antibiotics, including doxycycline, actually target mitochondria. A recent clinical trial (NCT06452394) aims to check for the clinical efficacy of doxycycline to target the BCSCs and improve the response to neoadjuvant chemotherapy in ER+/HER2− breast cancers.

Moreover, cirmtuzumab is a receptor-tyrosine-kinase like orphan receptor 1 (ROR1) antibody [98]. A phase 1b study has evaluated the safety and efficacy of cirmtuzumab combined with paclitaxel for metastatic or locally advanced, unresectable breast cancer (NCT02776917). The stem cell makers, ALDH and CD133, from primary pre-treatment and post-treatment tumor specimens were measured to assess the effects on BCSCs. Furthermore, CXCR1 has been identified as a druggable target on BCSCs. The CXCR1 inhibitor, reparixin, in

combination with paclitaxel has been tested in TNBC patients. Unfortunately, the primary endpoint of prolonged PFS was not met [99].

Table 2. Clinical trials targeting BCSCs.

Trial NCT Number	Type	Name of Intervention Agent	Drug Target or Biomarker	Breast Cancer Subtypes	Number of Patients Enrolled/ to be Enrolled
NCT01190345	Phase II	Bevacizumab	VEGF	Not specified	75
NCT06452394	Phase II	Doxycycline	A broad-spectrum of bacteria	ER+/HER2-	50
NCT02776917	Phase Ib	Cirmtuzumab	ROR1	HER2-	22
NCT02254005	Phase I	Bivatuzumab Mertansine	CD44v6	Metastatic Breast Cancer	24
NCT06331169	Phase Ib	Anlotinib	VEGFR/ FGFR/ PDGFR/ c-kit	HER2-Low Advanced and/or Metastatic Breast Cancer	42
NCT05550415	Phase II	Simvastatin	Vimentin/ HMGCR	TNBC	26
NCT00645333	Phase I/II	MK-0752	Notch	Advanced or Metastatic Breast Cancer	30

7. Concluding Remarks and Future Perspectives

Overall, the studies on the heterogeneity and plasticity of BCSCs underscores the complexity of these cells and their roles in tumorigenesis, tumor progression and treatment resistance. Accumulating evidence have proved that a number of factors contribute to the plasticity of BCSCs. These include pro-tumor signaling, immune cells and stromal cells in the TME, hypoxia, non-coding RNAs (e.g., miRNAs, and lncRNAs) and the recently discovered tumor-resident microbiota. A comprehensive understanding of the molecular mechanisms and environmental factors that drive BCSC plasticity is essential for the development of targeted therapies and the improvement of patient outcomes. A disruption of the tumor-TME network may prove an effective strategy for the treatment of breast cancer.

Despite fundamental achievements, several questions still remain elusive. First of all, the eradication of BCSCs may prove challenging due to their inherent heterogeneity. The manner in which distinct subtypes of BCSCs compete or collaborate at various stages of tumor progression remains to be elucidated. Recent advances in spatial transcriptomics and single-cell sequencing may facilitate the resolution of these questions. Secondly, it is crucial to choose the optimal treatment regimens of combining BCSC-targeted therapy with systemic therapy, while minimizing the incidence of adverse effects. Thirdly, the majority of BCSC-targeted therapies have been evaluated in preclinical models. Further clinical trials are required to validate the efficacy and safety of these treatments in patients with breast cancer.

Declarations

Ethics Statement

Not applicable.

Consent for Publication

Not applicable.

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Competing Interests

The authors have declared that no competing interests exist.

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