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Breast Cancer Stem Cells: A Review of Their Characteristics and The Agents That Affect Them

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Abstract

The evolving concept that cancer stem cells (CSCs) are the driving element in cancer development, evolution and heterogeneity, has overridden the previous model of a tumor consisting of cells all with similar sequentially acquired mutations and similar potential for renewal, invasion and metastasis. This paradigm shift has focused attention on therapeutically targeting CSCs directly as means of eradicating the disease. In breast cancers, CSCs can be identified by cell surface markers and are characterized by their ability to self-renew and differentiate, resist chemotherapy and radiation, and initiate new tumors upon serial transplantation in xenografted mice. These functional properties of CSCs are regulated by both intracellular and extracellular factors including pluripotency-related transcription factors, intracellular signaling pathways and external stimuli. Several classes of natural products and synthesized compounds have been studied to target these regulatory elements and force CSCs to lose stemness and/or terminally differentiate and thereby achieve a therapeutic effect. However, realization of an effective treatment for breast cancers, focused on the biological effects of these agents on breast CSCs, their functions and signaling, has not yet been achieved. In this review, we delineate the intrinsic and extrinsic factors identified to date that control or promote stemness in breast CSCs and provide a comprehensive compilation of potential agents that have been studied to target breast CSCs, transcription factors and stemness-related signaling. Our aim is to stimulate further study of these agents that could become the basis for their use as stand-alone treatments or components of combination therapies effective against breast cancers.

Keywords

Breast cancer; cancer stem cells; pluripotency; differentiation

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1.1 Introduction

Breast cancer is a phenotypically diverse cancer with a large degree of inter- and intra-tumoral genetic and epigenetic heterogeneity. Breast tumors are divided into subtypes based on hormonal receptor status—specifically based on their expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). ER and PR are expressed either alone (ER+/PR− or ER−/PR+) or together (ER+/PR+) in a majority of breast carcinomas, and are used as biomarkers and prognostic factors to guide clinical management[1]. ER+ breast cancers are well differentiated and less aggressive relative to ER- breast cancers. Co-expression of both ER and PR receptors carries better prognosis when compared to ER+/PR− or ER−/PR+ cases[2]. HER2+ carcinomas, cancers comprising about 25% of all breast cancer cases, feature the most aggressive phenotype among invasive breast cancer[3]. However, a pathologically complete response often can be achieved from HER2-targeted therapy along with conventional chemotherapy[4]. Breast carcinomas that do not express ER, PR, or HER2 are referred to as triple negative breast cancers (TNBC) and constitute about 15–20% of breast cancer cases. These are a group of genetically and phenotypically heterogenous tumors with poor prognosis and limited responsiveness to treatment[5]. Additional functional biomarkers have been investigated for potential implications in diagnosis, treatment, and predictions of drug resistance and prognosis; these include antigen Ki-67 (KI-67; cell proliferation), programmed death-ligand 1 (PD-L1; immune response), HER2Δ16 (drug resistance), and matrix metalloproteinase 9 (MMP-9; invasion and metastasis)[6].

Compelling evidence indicates that within a cancer, there is a subpopulation of cells known as tumor-initiating cells (TICs) or cancer stem-like cells (CSCs) that are responsible for the tumor initiation, chemo-/radio-resistance and relapse[7, 8]. This population is characterized by a stem-cell gene expression signature, drug-resistant phenotype and self-renewal capacity *in vitro* and *in vivo*[9]. CSCs can self-renew through division and give rise to the bulk of tumor cells in the mass through replication and differentiation from the stem cell compartment[10, 11]. Thus, targeting CSCs can be a promising therapeutic strategy for eradicating breast cancer.

Two models have been proposed to explain the evolution of CSCs[12]. According to the clonal evolution model, genetic mechanisms are the culprits underlying clonal expansions, with the stepwise acquisition of mutations in single clones culminating in tumor progression. This is followed by selection of more aggressive dominant subclones having a survival advantage and tumorigenic potential[13]. Meanwhile, the CSC model hypothesizes a role for nongenetic mechanisms as the source of intra-tumoral heterogeneity. In this model, cancers originate from a small subpopulation of tumor cells that can initiate tumorigenesis. CSCs were first identified in acute myeloid leukemia, when a $CD34^{+}/CD38^{-}$ subpopulation of human leukemia cells transplanted into immunocompromised (NOD/SCID) mice, perpetuated the disease and underwent leukemic transformation and differentiation *in vivo* to form the bulk of the cells phenotypically identifiable as leukemic[14]. CSCs have now been identified in a variety of cancer types, including breast cancer, colon cancer, melanoma, prostate cancer, lung cancer, and glioblastoma[15].

In this review, we examine the known markers identifying and characterizing breast CSCs (BCSCs), the signaling pathways and transcription factors that appear to regulate stemness properties and agents that target them and that might be exploited in treatment of the disease.

1.2 Cancer stem cells

1.2.1 Identification of BCSCs

Stem cell surface markers that are used to isolate BCSCs provide key insights into BCSC biology along with opportunities to develop therapeutics that target them. To date, CSCs in various human cancers have been identified by using one or multiple cell surface markers in fluorescence-activated cell sorting (FACS); measuring functional markers such as aldehyde dehydrogenase 1 (ALDH1) enzyme activity and ATP-binding cassette (ABC) transporter expression; single-cell DNA sequencing; and screening side population cells with the Hoechst-33342 dye exclusion technique[15]. Identifying, isolating, and characterizing the BCSC populations has so far primarily utilized cell surface markers. In particular, the CD44, CD24, and ALDH1$^+$ markers have become increasingly used to isolate BCSCs, characterize them, and use them as prognostic markers for patients[16].

CD44, a non-kinase single-span transmembrane glycoprotein that binds hyaluronan, is involved in controlling cell proliferation, survival, and differentiation; it thus regulates CSC properties including self-renewal, tumor initiation, metastasis, and radio- and chemo-resistance. Alternatively-spliced variants of CD44 play roles in tumor development and progression. CD44 expression is high in BCSCs; its downregulation induces differentiation and sensitizes the cells to chemotherapy[17, 18]. CD24 is a glycosylphosphatidylinositol-linked cell surface glycoprotein that has been implicated in immunological functions, tumorigenesis, chemoresistance, and metastasis. CD24 expression is low or absent in BCSCs, and its upregulation is associated with poor prognosis in the luminal A and TNBC subtypes[19]. ALDH1 is a member of group of enzymes that oxidize intracellular aldehydes to carboxylic acids. Its activity is measured by the ALDEFLUOR assay, which assesses nine active isoforms of ALDH; in breast cancer, high ALDH1 activity is associated with stem-like features and chemoresistance. ALDH1$^+$ breast cancers are also characterized by being ER-, EGFRII$^+$ and Ki-67^{hi} [20]. Suppression of ALDH1 decreases tumorigenicity and cell migration[21].

BCSCs were first isolated from xenografts using a combination of cell surface markers: CD44$^+$/CD24$^{-/low}$ Lin$^-$. The cells with this phenotype are tumorigenic in numbers as low as 100 cells; in contrast, those with different phenotypes failed to form tumors even with tens of thousands of cells[7]. A high CD44/CD24 ratio is directly correlated with cell proliferation and tumorigenesis, as indicated by increased formation of mammospheres *in vitro* and xenograft tumors[21]. In addition, CD44$^+$/CD24$^-$ breast cancer cells are enriched for EMT-associated traits, including expression of matrix metalloproteinase 1 (MMP-1), vimentin, and zinc finger E-box binding homeobox 1 (ZEB1); this is suggestive of interplay between EMT and CSC status[22]. These cells also demonstrate increased expression of the molecular chaperones glucose-regulated protein 78 (GRP78) and 94 (GRP94), which regulate endoplasmic reticulum homeostasis in stem cell development and in invasion of cancer[23]. Furthermore, the cells exhibit dysregulation of major signaling pathways otherwise involved

in the regulation of normal mammary stem cells, such as the Notch, Hedgehog, and Wnt/β-catenin pathways; blockage of these pathways by chemotherapeutic agents inhibits the CSC-like phenotype and tumorigenesis[24]. In mice, breast cancer cells derived from BRCA1-deficient mammary tumors show increased numbers of $CD44^+/CD24^-$ and $CD133^+$ cells and increased expression of stem cell-associated genes including Oct4, Notch1, Aldh1, Fgfr1, and Sox1[25]. In the clinical context, the $CD44^+/CD24^-$ phenotype is associated with resistance to cytostatic agents, grade of malignancy, and patient survival[26]. Furthermore, $CD44^+/CD24^-$ BCSCs are resistant to radiation treatment and demonstrate increased expression of Jagged-1, Notch-1, and p-S6K1 (a major downstream regulator of the mTOR pathway)[27]. The radioresistance of these cells is mediated through upregulation of the checkpoint kinase pathway (CHK); application of the CHK inhibitor, debromohymenialdisine, effectively overcoming the resistance[28].

Regarding ALDH as a CSC population marker, Ginestier *et al.* found that ALDH1 enzymatic activity is high in a subpopulation of breast carcinomas having tumorigenic and self-renewal abilities both *in vivo* and *in vitro*[29]. $ALDH^{hi}CD44^+$ subpopulations of BCSCs are resistant to chemotherapy and radiotherapy and feature increased expression of glutathione-S-transferase pi, p-glycoprotein, and checkpoint kinase 1 (CHK1). Pretreatment of these cell populations with all-trans retinoic acid or the ALDH inhibitor diethylaminobenzaldehyde (DEAB) significantly sensitizes the stem-like breast cancer cells and reduces resistance[30]. In MCF-7 xenograft tumors, ALDH1A1 (an isoform of ALDH1) promotes tumor angiogenesis by upregulating the retinoic acid/HIF-1α/VEGF signaling pathway, thereby affecting breast cancer progression[31]. In $ALDH1^+$ BCSCs, the Wnt/β-catenin signaling pathway, known to regulate stem cell niche during development, is dysregulated; downregulation of Wnt expression inhibits the CSC phenotype and suppresses breast cancer metastasis[32]. In ductal carcinoma in situ (DCIS), expression of ALDH1 along with enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), a marker implicated in stem cell maintenance and renewal, is associated with tumor recurrence and progression to invasive breast cancer[33].

Studies of invasive breast carcinomas and breast cancer cell lines have shown basal-like tumors to be enriched with $CD44^+/CD24^-$ and $ALDH1^+$ phenotypes[34]. Quiescent mesenchymal-like BCSCs are $CD44^+/CD24^-$ and localize to the tumor periphery, whereas proliferative epithelial-like BCSCs are $ALDH1^+$ and localize in the center[35]. Table 1 summarizes the BCSC markers, their functions, target genes and relation to tumorigenesis.

In addition to cell surface markers, various functional assays are employed in the study of BCSCs; these include the mammary organoid 3D culture model, mammosphere forming assay in serum free medium, and the *in vivo* injection of FACS-sorted cells in limiting dilutions into immunocompromised mice, with consequent initiation of tumor growth[36]. Despite the multiplicity of BCSC markers and assays available, universal putative markers have yet to be resolved that can identify specific subpopulations having the most tumorigenic potential in each breast cancer case. Identification of those subpopulations is essential for the development of CSC-targeted therapy and overcoming resistance to chemo- and radio-therapeutic treatments.

1.2.2 Characteristics of BCSCs

CSCs are similar to normal stem or progenitor cells in their ability to self-renew and recapitulate heterogeneity[13]. Self-renewal is a hallmark of stem cells, in which a stem cell produces two daughter cells with stem cell properties (symmetric division) or one daughter cell with stem cell properties and a second that undergoes differentiation (asymmetric division)[37]. CSCs express transcription factors (OCT4, NANOG homeobox [NANOG], and SRY-box transcription factor 2 [SOX2]) that are found in early embryonic stem cells. The core stem cell factors regulate pluripotency and self-renewal, and their overexpression is associated with signaling pathways related to malignant transformation, tumorigenicity, tumor progression, relapse, and inhibition of apoptosis[38]. OCT4, NANOG and SOX2 markers are induced in many cancer types, including breast, prostate, lung, colorectal, and gastrointestinal cancers[39]. Likewise, normal stem cells and CSCs share common self-renewal signaling pathways including the Notch, Hedgehog, STAT3, and Wnt/β-catenin pathways; all of these are documented as being important signaling cascades in embryonic development and have been shown to contribute to tumorigenesis in multiple types of tumors[40]. The plausibility of the CSC theory in breast cancer, which hypothesizes that BCSCs are derived from normal progenitor/stem cells, is supported by phenotypic features similar to their lineage-specific normal stem cell counterparts[41].

CSCs arise from deregulation of the self-renewal program in stem cells, giving rise to their malignant transformation, or from the dedifferentiation of committed mature cells to acquire CSC-like properties[42]. In addition to self-renewal, CSCs also display quiescence in response to environmental cues. Thus, while anti-mitotic chemotherapeutic agents have been developed to target proliferating tumor cells, the resident, generally quiescent CSCs remain resistant to chemo- and radio-therapies even at high doses and so are the major cause of relapse—the living evidence of CSC plasticity and the supreme challenge faced by current therapies[43].

Ultimately, numerous intrinsic and extrinsic factors regulate CSC traits, including developmental pathways, epigenetics, stem cell transcription factors, epithelial mesenchymal transition (EMT) factors, cell cycle regulation mechanisms, apoptosis pathways, and the tumor microenvironment. All of these factors interact constantly and dynamically regulate CSC survival, proliferation, and metastasis[44]. As a consequence, CSCs exhibit a spectrum of functional and phenotypic heterogeneity, confirmed by *in vitro* clonogenic and anchorage-independent growth assays (tumor sphere assays) as well as *in vivo* limiting dilution xenotransplantation assays[45]. CSCs constitute only a small proportion (0.01–2%) of the tumor cells in a tumor mass, and isolating and identifying a pure CSC population remains challenging[46].

1.3 Major self-renewal pathways in BCSCs

CSC populations are maintained by their self-renewal capacity. The current notion of CSCs states that the self-renewal signaling and transcription factors which regulate growth and maintenance in normal stem cells are dysregulated in BCSCs[47]. The following section will discuss the major self-renewal pathways in BCSCs.

1.3.1 Notch signaling pathway

Notch is a family of four transmembrane receptors (NOTCH 1–4) that interacts with five ligands: the jagged proteins (JAG1 and JAG2) and the delta-like ligands (DLL1, DLL3, and DLL4)[48]. While canonical Notch signaling is involved in multiple cellular processes, including embryonic development, stem cell fate determination, apoptosis, cell cycle progression, self-renewal and lineage specific differentiation, non-canonical Notch signaling is associated with immune activation and breast tumorigenesis[49]. Oncogenic RAS activates NOTCH1 and upregulates the Notch ligand DLL1 along with presenilin-1 through a p38-mediated pathway. There is a correlation between Ras overexpression and upregulation of NOTCH1 in breast carcinomas[50]. In clinical breast cancer samples, Notch signaling is found to promote BCSCs by inducing expression of sirtuin 2 (SIRT2), leading to deacetylation and activation of ALDH1A1[51]. Notch1 and Notch4 signaling are higher in $ESA^{+}/CD44^{+}/CD24^{low}$ enriched BCSCs. NOTCH1 overexpression in MCF-7 and MCF10A breast cancer cells increased the abundance of the BCSC $CD44^{+}/CD24^{low}$ subpopulation, along with increasing tumor cell invasion and migration. Increased NOTCH1 expression also promotes the EMT phenotype and tumor growth *in vivo* through crosstalk with STAT3 signaling[52].

Notch signaling and expression of its target genes are also elevated in mammosphere-derived stem-like cells. Inhibition of Notch signaling by a γ-secretase inhibitor significantly reduces sphere formation, proliferation and colony formation, and also induces apoptosis[53]. Likewise, pharmacologic and genetic inhibition reduce stem cell activity in *in vitro* and tumor formation *in vivo*[54]. In $CD44^{+}/CD24^{-}$ mammospheres, the breast tumor suppressor signal peptide, CUB domain and EGF like domain containing 2 (SCUBE2) is overexpressed, with concomitant overexpression of SOX2, OCT4, and NANOG in TNBC. Ectopic expression of SCUBE2 in adherent cells promotes EMT and metastasis by activating Notch signaling and its components[55].

Notch4 expression is high in TNBC and is negatively correlated with overall survival[56]. $Notch4^{+}$ BCSCs are characterized by increased expression of stemness factors (OCT4, SOX2, NANOG), mammosphere formation *in vitro*, and tumorigenicity in a serial dilution tumor transplantation xenograft model[57]. Treating TNBC cells with mTOR inhibitors leads to increased stemness features and greater *in vivo* tumor initiating capacity. The intrinsic resistance of these cells from TORC1/2 inhibition is driven by their activated Notch1 and FGF1 pathways in association with increased mitochondrial metabolism and FGFR1 signaling. Notably, abrogation of the FGFR-mitochondrial metabolism-Notch1 axis overcomes resistance to TORC1/2 inhibitors by eliminating drug-resistant CSCs[58]. Meanwhile, JAG1-NOTCH4 receptor activation increases BCSC activity and induces tamoxifen resistance in both patient-derived tumors and xenograft models. Targeting Notch4 reverses the increase in Notch, reducing BCSC activity and improving the tamoxifen resistance[59]. Thus, in combination with other modalities, targeting the Notch pathway could be a promising strategy for enhancing the effectiveness and sensitivity of breast cancer treatment while simultaneously eradicating BCSCs.

1.3.2 Wnt signaling in BCSCs

The Wnt/Frizzled/β-catenin pathway is an evolutionarily conserved signaling pathway that plays significant roles in embryonic development and tissue homeostasis[60]. There are 19 Wnt glycoproteins that serve as ligands for the receptors Frizzled (FZD) and LDL receptor related protein 5/6 (LRP5/6)[61]. Aberrant Wnt signaling is implicated in breast cancers[62]. Wnt signaling is constitutively activated in basal breast cancer cells, affecting their self-renewal and differentiation[63]. Regulators of the Wnt signaling pathway, such as lymphoid enhancer-binding factor 1 (LEF1), cyclin D1, β-catenin, and TCF-4 are upregulated in $ALDH^+$ BCSCs. Treating 4T1 BCSCs with Wnt3a ligand induced Wnt/β-catenin signaling and transcriptional activity, while Wnt1 silencing decreased tumor sphere formation and the $CD44^+/CD24^-$ population *in vitro*, along with decreasing tumorigenesis and metastasis in xenografts[32]. Thyroid hormone receptor interactor 6 (TRIP6), an adapter protein involved in regulating the functions of CSCs, enhances stemness in breast cancer cells through activation of the Wnt/β-catenin pathway[64]. On the converse side, β-catenin silencing has been shown to reduce tumorigenesis *in vivo* and to suppress cancer stemness *in vitro* by decreasing the abundance of $ALDH^+$ breast cancer cells and the expression of stemness-related genes, including B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) and MYC proto-oncogene, bHLH transcription factor (c-Myc). In TNBC cells, such silencing also impaired formation of anchorage-independent colonies in soft agar assay and improved chemoresistance[65]. Treatment of TNBC cells with WNT-targeting pharmacological agents modulates the expression of PD-L1, a ligand for the inhibitory immune checkpoint receptor PD-1, which is highly expressed in the stem cell compartment ($ALDH^+$ or CD44v6-positive) alongside WNT signaling-related genes. This indicates a role of Wnt signaling in TNBC-related immune escape[66]. The pleiotropic effects of Wnt signaling and its components in breast cancer initiation, progression, and the maintenance of different cancer subtypes remain to be elucidated, and deeper understanding of them is essential for developing BCSC-targeted therapies.

1.3.3 Hedgehog signaling

The Hedgehog (Hh) signaling pathway is involved in animal development and tissue homeostasis and is associated with many solid tumors including pancreatic cancer, lung cancer, breast cancer, basal cell carcinoma, and hematological malignancies. Hh family members include Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH)[67]. In cancer, this pathway plays roles in malignant transformation, proliferation, drug resistance, metastasis, and the expansion of cancer stem cells[68]. Hh signaling is known to drive oncogenesis, specifically resulting from mutations in components of Hh pathway, over-expression of ligands of the Hh pathway, and maintenance of CSC phenotype through regulation of stemness-related genes[69]. The pathway is significantly upregulated in luminal B and TNBC breast cancer subtypes[70]. An earlier study in mice showed that overexpression of Gli1 under the MMTV promoter is sufficient to promote development of breast tumors expressing progenitor cell markers[71].

In mammospheres, PTCH, SMO, GLI1 and GLI2 are highly expressed, becoming down-regulated upon differentiation. Activation of Hh signaling increases mammosphere forming efficiency (MFE) and size, effects mediated by the polycomb gene BMI-1. Hh signaling is

also hyperactivated in the $CD44^+/CD24^-/Lin^-$ BCSC population[72]. In mammospheres of estrogen receptor-positive MCF-7 breast cancer cells, components of the Hh pathway (PTCH, SMO, GLI1 and GLI2) are highly expressed relative to monolayer cells; treatment with salinomycin, which targets CSCs, induced apoptosis and downregulated target genes of the Hh pathway (c-Myc, Bcl-2, and Snail) *in vitro* and reduced the tumor growth and expression of PTCH, SMO, GLI1 and GLI2 in xenograft tumors[73]. In basal-like breast cancer, increased expression of forkhead box C1 (FOXC1), an EMT-associated transcription factor, acts via activation of SMO-independent Hh signaling mediated by GLI2 to enrich CSC properties of the cancer, including $ALDH^+$ cell populations and mammosphere growth. Furthermore, expression of FOXC1 in TNBC cells confers resistance to anti-Hh drugs[74]. LncRNAs were demonstrated to regulate EMT-associated BCSC stemness through the growth arrest specific 1 (GAS1)-activated lncRNA-Hh pathway. The upregulated Hh signaling increased GLI1, SOX2, and OCT4 expression and MFE *in vitro* and tumorigenicity *in vivo*. Silencing lncRNA-Hh reversed these findings[75]. Hh signaling is also associated with chemoresistance in TNBC. Chemotherapy-induced drug resistance is mediated by GLI1 via upregulation of multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP)[76]. Ultimately, activation of the Hh signaling pathway is well-documented as a poor prognostic indicator in both hormone receptor-positive breast cancer and TNBC. However, there are limited Hh-targeted therapies available. Selective inhibition of GLI and other targets might represent an effective strategy for impeding breast cancer development and the activity of cancer stem cells.

1.3.4 TGF-β signaling

The transforming growth factor β (TGF-β) superfamily consists of 42 ligands including TGF-β, activins, Nodal, inhibins, bone morphogenic proteins (BMPs), and growth differentiation factors (GDFs)[77]. In cancer, TGF-β displays context-dependent dichotomous behaviors, being a tumor suppressor that inhibits cell cycle progression and promotes apoptosis or a tumor promoter that induces EMT and invasion[77]. Consistent with its tumor suppressor role, constitutive expression of TGF-β1 in mammary epithelial cells of xenografts increased latency of tumor growth and decreased mammary cancer risk[78]. Similarly, TGF-β reduces the BCSC population and induces luminal differentiation[79]. Loss of TGF-β-mediated tumor suppression in breast cancer is associated with downregulation of luminal markers and upregulation of basal markers[79]. In another example, transgenic expression of MMTV-driven dominant-negative TβR2 (DNIIR) in female mice decreased tumor latency and induced spontaneous tumor formation and invasion[80]. In contrast, mammary epithelial cell-specific expression of TGF-β ligands or TβRs in xenograft tumors promotes lung metastasis, while attenuation of TGF-β signaling decreases metastasis[81]. These findings suggest a paradoxical role of TGF-β signaling in inhibiting tumor initiation while promoting metastasis.

In immortalized human mammary epithelial cells (HMLE cells), TGF-β1-induced EMT generates stem cell-like cells that express EMT markers and have increased ability to form mammospheres, colonies in soft agar, and xenograft tumors[82]. Meanwhile, $CD44^+/CD24^-$ BCSCs generated by TGF-β1-induced EMT are more resistant to radiation compared to

their parental cells, mediated by upregulating antioxidant-related genes and reducing activation of death receptor pathways[83].

Accumulating evidence has implicated the epigenetic regulation of TGF-β signaling in breast cancer progression[84]. In TNBC, TGF-β1 inhibits miR-196a-3p and activates its downstream target gene neuropilin-2 to promote metastasis[85]. Meanwhile, miR-133b and miR-190 have been shown to inhibit TGF-β-induced EMT and metastasis by targeting SMAD2, indicating their roles as tumor suppressors and potential diagnostic biomarkers of breast cancer[86]. In mouse epithelial NMuMG cells, lncRNA-HIT mediates TGF-β-induced EMT and invasion by targeting E-cadherin; this long noncoding RNA is conserved in humans and elevated in invasive breast cancer. Attenuation of lncRNA-HIT resulted in decreased invasion, migration, and tumor growth[87]. Overall, due to the complexity of functional switches in TGF-β signaling, specific drugs targeting downstream signaling would be preferable as therapeutics, as they can be utilized without compromising other physiological functions of TGF-β.

1.3.5 STAT3 signaling

The transcription factor signal transducer and activator of transcription (STAT) family consists of seven highly conserved members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6; all share structural and functional similarities[88]. STAT3 is known to contribute to tumor cell proliferation, progression, metastasis, immune suppression, and stem cell self-renewal and maintenance[89]. STAT3 overexpression is found in more than 40% of breast cancers, mainly in the TNBC subtype. Aberrant activation of STAT3 promotes breast cancer development by deregulating genes implicated in proliferation, angiogenesis, and EMT[90]. In TNBC, hypoxia induces an increase in the $CD44^{high}/CD24^{low}$ BCSC population and in chemoresistance by activating STAT3 signaling. Genetic knockdown of STAT3 reverses the acquisition of stem-like features, which suggests a significant role of STAT3 in promoting the induction of cancer stemness by hypoxia[91].

Cytokines are known risk factors that induce inflammation and promote breast cancer progression. Oncostatin M (OSM), a member of the gp130 family of cytokines, has been implicated in inflammatory functions driving tumor aggressiveness and in increased STAT3 phosphorylation and STAT3-dependent IL-6 production, which promotes breast cancer progression. High expression of OSM correlates with poor breast cancer patient survival[92]. High levels of another cytokine, IL-35, are associated with poor prognosis in patients. Breast cancer cell-derived IL-35 inhibits conventional T (Tconv) cell proliferation and induces the cells to transform into IL-35-producing induced regulatory T (iTr35) cells by activating STAT1/STAT3, thereby promoting breast cancer progression[93].

MiR-124, a tumor suppressor that modulates breast cancer cell proliferation and invasion, is downregulated in breast cancer cells. Overexpression of miR-124 in TNBC decreased STAT3 and suppressed cell proliferation and invasion. Restoration of STAT3 expression reversed miR-124-mediated tumor cell invasion[94]. Similarly, miR-7 was demonstrated to act as a tumor suppressor by inhibiting breast cancer cell invasion and metastasis, decreasing BCSC populations, and reversing EMT in MCF-7 and MDA-MB-231 cell lines. These miR-7-mediated effects occurred through targeting the oncogene SETDB1, which led to

suppression of the downstream target STAT3 as SETDB1 binds to its promoter and regulates its expression[95]. All told, STAT3 signaling is not simply limited to a role in tumorigenesis but is also important in invoking the immune cell response. STAT3 will be a promising target for breast cancer prevention and therapy.

1.3.6 Other signaling in the regulation of BCSCs

Breast tumorigenesis is driven by aberrant regulation of cell signal transduction pathways owing to the accumulation of genetic and epigenetic changes over time. Apart from the aforementioned pathways, other significant signaling involved in BCSC enrichment and maintenance includes the Hippo, PI3K/Akt/mTOR and BMI-1 pathways[16]. Dysregulation of any of these individual pathways or of the interplay between them poses a risk of developing breast cancer. In addition, the receptor tyrosine kinase (RTK) class of specialized cell surface receptors respond to environmental cues by relaying appropriate signals in the tumor cell; these include epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and AXL receptor tyrosine kinase (AXL). RTKs play a multifaceted role in breast cancer development, sharing common downstream pathways such as MAPK, NF-κB, PI3K/Akt, and JAK/STAT signaling; the crosstalk with other key signaling pathways relevant to the regulation of angiogenesis, metastasis, and maintenance of BCSCs. Mutation in or overexpression of RTKs has been observed in different stages of breast cancer to lead to constitutive activation of various signal transductions that promote BCSCs and chemoresistance[96].

1.4 Signature of cancer stem cell transcription factors in breast cancer

Pluripotency in embryonic stem cells (ESCs) is regulated by a well-characterized core transcriptional network. The circuitry of this network constitutes major transcription factors of pluripotency, signal transduction machinery, and epigenetic regulators. In human embryonic stem cells, OCT4, NANOG, and SOX2 function as master regulators of pluripotency and self-renewal properties while inhibiting differentiation to control cell fate[97]. Pluripotency can be induced in adult somatic cells, as evidenced by reprogramming of adult fibroblast cells into pluripotent stem cells with characteristic features of ESCs using the OSKM transcription factors (OCT3/4, SOX2, c-Myc, and Kruppel-like factor 4 [KLF4])[98]. Astrocytes transduced with the H-ras oncogene or with OSKM factors undergo reprogramming into progenitor cells, resulting in tumorsphere formation. When these tumorspheres are transplanted as xenografts, they form heterogeneous tumors, suggesting an interplay between tumorigenicity and pluripotency[42]. It can be assumed that CSCs share characteristics with ESCs. The pluripotency transcription factors OCT4, NANOG, and SOX2 are upregulated in human cancers, including breast cancer, glioma, melanoma, and prostate cancer, and their overexpression in tumors is associated with poor differentiation, stem-like phenotype, and inhibition of apoptosis[38].

1.4.1 OCT4

OCT4, a homeodomain transcription factor of the Pit-Oct-Unc family, is one of the most important transcription factors governing pluripotency[99]. The human OCT4 gene has three transcript variants (OCT4A, OCT4B, and OCT4B1) and four protein isoforms (OCT4,

OCT4B-190, OCT4B-265, and OCT4B-164). Each alternative transcript variant and isoform demonstrates diverse expression patterns and functions[100]. Distinctive expression patterns of *OCT4* variants have been identified in different types of breast cancer: OCT4A and OCT4B are highly expressed in low-grade ductal tumors, whereas OCT4B is overexpressed in lobular type breast cancer. Expression of OCT4 variants is also associated with the expression of ER, PR, HER2 and p53[101]. Among them, OCT4A is responsible for maintenance of stemness in pluripotent embryonic stem cells[100]. Ectopic expression of Oct4 in 4T1 mouse breast cancer cells increased tumorsphere formation, expression of stem cell markers such as CD133, CD34, Sca-1, and ALDH1 *in vitro*, and tumorigenic potential *in vivo*[102]. OCT4 controls the expression of target genes by recognizing and binding to DNA regulatory regions through an octamer motif (AGTCAAAT) or by recruiting other transcription factors to regulate a specific set of genes[103].

Phenotypically, resistance to chemo- or radiotherapy is among the hallmarks of CSCs. The function of OCT4 in the stemness-mediated resistance of BCSCs to chemotherapy and irradiation is of particular interest in breast cancer. In hormone receptor-positive breast cancer, OCT4 can be used a prognosis indicator for poor clinical outcome and tamoxifen resistance[104]. Doxorubicin resistant-TNBCs showed increased CSC phenotype along with high expression of signal transducer and activator of transcription 3 (STAT3), OCT4, and c-Myc. Treatment with the STAT3 inhibitor WP1066 decreased phosphorylation of STAT3 and the expression of OCT4 and c-MYC, leading to a reduction in $CD44^+$ BCSC population and restoration of doxorubicin sensitivity[105]. OCT4 also confers resistance to irradiation by increasing clonogenic survival following irradiation and upregulating interleukin 24 (IL-24) production through STAT3 and NF-κB signaling[106].

PD-L1, a T-cell inhibitory molecule with immunomodulatory function, regulates breast cancer stemness by modulating OCT4 and NANOG. In breast cancer, its expression is associated with EMT, chemoresistance, and maintenance of stemness. PD-L1 knockdown inhibits AKT phosphorylation and mTOR activity, with downstream reduction of OCT4 phosphorylation at T235 and therefore of OCT4 activity[107]. Another regulator of OCT4 is the E3 ubiquitin ligase carboxy terminus of HSP70-interacting protein (CHIP), which was demonstrated to mediate its proteasomal ubiquitination at lysine 284 through microarray analysis of mammospheres derived from MDA-MB-231 and MCF-7 cells. CHIP overexpression decreased OCT4 stability and BCSC populations, while CHIP depletion promoted breast tumor and lung metastasis in xenografts. This finding suggests that CHIP-induced post-translational modification of OCT4 is important in maintenance of BCSCs[108].

Although OCT4 is well studied in the context of stemness maintenance, its role in metastasis remains controversial. Overexpression of OCT4 in MDA-MB-231 and 4T1 breast cancer cell lines induced E-cadherin while suppressing cell migration and invasion *in vitro* and lung metastasis *in vivo*[109]. The inhibitory effect of OCT4 on metastasis is mediated through downregulation of Rho family GTPase 1 (RND1) by binding to its promoter region[109]. In contrast, a previous study from the same group showed downregulation of OCT4 in MCF-7 cells to promote cell migration and invasion by inducing EMT (decreased E-cadherin expression and increased alpha-smooth muscle actin expression)[110]. Given the multiple regulatory effects of OCT4 on stemness, resistance and metastasis in breast cancer, a better

understanding of OCT4 for its interaction and interconnection with other markers and effectors of CSC function is essential.

1.4.2 SOX2

SOX2 is a member of the Sox (SRY-related HMG box) family member of transcription factors with a single high-mobility group DNA-binding domain. It is recognized as a key player in the regulation of early embryonic development, maintenance of undifferentiated ESCs, and cell fate determination, and its expression is dysregulated in several cancer types, including breast, prostate, brain, and lung cancers. SOX2 is additionally involved in tumorigenesis, drug resistance, poor prognosis, and metastasis, indicating a major role in cancer and positioning it as an attractive therapeutic target[111]. Overexpression of SOX2 in breast cancer cells increased mammosphere formation, while its knockdown suppressed mammosphere formation and delayed tumor formation in xenograft tumor initiation models. Mechanistically, SOX2 overexpression was induced through the activation of a distal enhancer of SOX2 promoter, the same element that natively regulates SOX2 transcription in pluripotent stem cells[112]. In ER-positive breast cancer patients, SOX2 expression is associated with poor prognosis and endocrine treatment failure, and SOX2 promotes tamoxifen resistance via activation of Wnt signaling[113]. It also targets SOX9 to regulate luminal progenitor cells and Wnt signaling activity[114]. In TNBC cases, SOX2 is implicated in BCSC chemoresistance through modulation of TWIST1. Silencing SOX2 increased paclitaxel sensitivity and diminished stemness and TWIST1 expression. This illustrates the significance of SOX2 as a connector between pluripotency, chemoresistance, and the EMT axis[115]. Likewise, SOX2 knockdown in MCF-7 cells decreased mammosphere formation, $CD44^+/CD24^-$ subpopulation, $ALDH^+$ population, viability *in vitro*, and tumorigenicity *in vivo*[113].

1.4.3 NANOG

NANOG is a homeodomain protein found in undifferentiated mammalian ESCs and pluripotent cells. Endogenous Nanog drives ESC self-renewal by maintaining the level of OCT4, which is integral to ESC function. Although Nanog is absent in differentiated cells, its abnormal expression is reported in human cancers including prostate cancer, hepatocellular carcinoma, glioblastoma, colon cancer, and breast cancer. Expression of Nanog is associated with stemness, self-renewal, and tumorigenesis[116]. When coexpressed with Wnt-1 in the mouse mammary gland, Nanog promotes mammary tumorigenesis and metastasis. Ectopic expression of Nanog in MCF-7 cells enhances colony formation, migration, and invasion *in vitro* and tumor growth *in vivo*[117]. Meanwhile, silencing Nanog reduces colony formation, cell proliferation, and invasion; it furthermore downregulates the cell cycle regulators cyclin D1 and c-Myc, leading to cell cycle arrest at G0/G1[118]. In BCSCs, Nanog and OCT4 modulate TGF-β-mediated EMT; their induction promoted invasion while knockdown of both inhibited CSC migration *in vitro*[119]. In addition, Nanog confers drug resistance in MCF-7 breast cancer cells through STAT3-mediated activation of MDR1[120], and in breast ductal carcinoma, its expression has statistically significant relationship with tumor grade, lymph node metastasis, and disease staging[121]. Tissue microarray analysis revealed that breast cancer patients with strong Nanog expression have

significantly lower disease-free survival and overall survival rates than those with weak expression[122].

1.4.4 KLF4

KLF4 is a member of the highly conserved Kruppel-like zinc finger transcription factor family, and is one of the four major transcription factors of pluripotency. It plays diverse roles in physiology and disease, with functions in cell cycle regulation, proliferation, apoptosis, differentiation, somatic cell reprogramming, and pluripotency[123]. KLF4 is differentially expressed in human cancers, and furthermore is bifunctional; it can act as either tumor suppressor or oncogene depending on the tissue, tumor type, and staging[123]. In breast cancer tissues, its protein expression is correlated with pathological type, histological grade, and lymph node involvement; low-level expression is found in normal breast epithelium, while increased expression is detected in neoplastic cells and prior to invasion[124]. In estrogen-dependent breast cancer, KLF4 acts as a tumor suppressor by regulating the transcriptional activity of ERα specifically binding to its DNA-binding region and preventing it from binding to estrogen response elements in promoter regions[125]. It is also self-regulating, in that the isoform KLF4α antagonizes the function of KLF4 and stimulates breast cancer cell proliferation by binding and retaining KLF4 in the cytoplasm, opposing its regulatory activities in the nucleus[126]. KLF4 is highly expressed in BCSCs from primary mammary tumor and breast cancer cell lines. In the MCF-7 and MDA-MB-231 cell lines, KLF4 knockdown decreased the population of $ALDH1^+$ progenitor cells; it furthermore suppressed cell migration, invasion, and mammosphere formation *in vitro* and tumorigenesis *in vivo*[127]. In BCSCs, KLF4 and the androgen receptor have been demonstrated to mediate stem cell phenotype; this effect is negatively regulated by dual specificity tyrosine phosphorylation regulated kinase 2 (DYRK2), a protein kinase that controls EMT via Snail degradation. Downregulation of DYRK2 promotes KLF4 expression and cancer stem-like properties[128].

1.4.5 MYC

MYC is a dimeric transcription factor of the basic helix-loop-helix (bHLH) DNA-binding protein superfamily that regulates a broad range of biological processes such as cell proliferation, differentiation, growth, and apoptosis; it is also implicated in embryonic stem cell self-renewal and pluripotency[129]. The MYC promoter is a downstream effector target of self-renewal pathways such as the Notch, Wnt, NF-κB and TGF-β signaling pathways[130]. Of the three MYC family members l-MYC, c-MYC, and n-MYC, the latter two play crucial roles in the maintenance of pluripotency. Co-deletion of both transcription factors in ESCs and in induced pluripotent stem cells (iPSCs) led to destabilization of pluripotency and spontaneous differentiation into primitive endoderm[131].

As an important transcription regulator in ESCs, MYC also displays similar regulatory role in CSCs[132]. In fact, MYC was first recognized as one of the most potent oncogenes, inducing neoplastic transformation of target cells and a wide variety of tumors[133]. Transient overexpression of MYC in Rat1A cells evoked genomic instability and increased tumorigenicity[134]. In breast cancer, MYC amplification is associated with disease progression; additionally, its expression is higher in TNBC than in other subtypes. MYC

overexpression in the BRCA1-deficient TNBC subtype is associated with poor prognosis[135]. Meanwhile, targeting MYC in TNBC with triptolide (C1572), a small-molecule natural product, depletes cancer-stem like cells via a proteasome-dependent mechanism[136]. In combination with MCL1 apoptosis regulator, BCL2 family member (MCL1), MYC promotes chemoresistance of CSCs in TNBC by increasing mitochondrial oxidative phosphorylation and the generation of reactive oxygen species[137]. Additionally, c-MYC is the effector target of the tumor suppressor gene p53 in mammary stem cells; loss of p53 function is implicated in the development of cancers. In breast tumors, p53 mutation activates c-MYC, leading to maintenance of cancer stemness features and expression of a mitotic gene signature, which correlates with breast cancer aggressiveness and poor prognosis[138]. Transducing MYC in HMLE cells induces luminal epithelial morphology changes, spheroid formation, and dedifferentiation into progenitor-like states. MYC-driven epigenetic changes are mediated through suppression of lineage-specific transcription factors and activation of de novo enhancers, determined by hyperactivation of the Wnt pathway, which further drives transcriptional activation of oncogenic pathways[139].

1.5 BCSCs and therapeutic resistance

Tumor relapse in breast cancer has been attributed to drug-resistant CSCs, and the persistence of CSCs after chemotherapy pinpoints this population as an ‘ultimate target’ that must be eliminated to eradicate cancer. BCSCs share many features of normal stem cells and modulate a multitude of drug resistance mechanisms, including overexpression of drug efflux pumps (e.g. ATP-binding cassette family members ABCG2, P-gp, ABCC1, ABCB5, etc.)[140], enhanced DNA repair activity[141], increased scavenging of reactive oxygen species[142], activation of anti-apoptotic proteins[143], and induction of dormancy[144, 145]. BCSCs exhibit DNA damage repair mechanisms that render them chemo- and radiation-resistant, thus targeting DNA repair pathways is a plausible approach for BCSC-directed therapy[141]. BCSCs trigger increased expression of free radical scavenging systems at lower ROS levels than do other cells, protecting them from anti-cancer agents. Doxorubicin-dependent $CD44^{+}/CD24^{-}$ BCSCs in MCF-7 cells demonstrate upregulated levels of nuclear factor, erythroid 2 like 2 (NRF2), a key transcription factor that regulates cellular responses to oxidative damage. Specifically, CD44 regulates NRF2 level through p62 expression, and NRF2 activation endows the BCSCs with aggressive phenotype and chemoresistance[142].

CSCs activate anti-apoptotic proteins that can withstand cytotoxic agents. Inhibiting these anti-apoptotic proteins (such as Bcl-2) can be a potential therapeutic avenue against chemo-resistance in BCSCs[143]. Recently, evidence has accumulated for a role of the pro-survival autophagic pathway in BCSC survival and maintenance. Autophagy flux is high in the $ALDH^{+}$ BCSC population and is essential for tumorigenicity[146]. This population of BCSCs shows chemoresistance that is enhanced by hypoxia, but the inhibition of autophagy in TNBC can overcome chemoresistance[147]. Dormant cancer cells can survive an unfavorable microenvironment and undergo reversible growth arrest; furthermore, while in a dormant state, committed tumor cells de-differentiate to become stem-like cells[148]. Tumor dormancy is characterized by upregulation of autophagic signaling (which maintains the metabolic homeostasis of dormant cancer cells), epigenetic features, stress-lenient signaling, and microenvironmental cues[149]. In BCSCs, autophagy maintains low-level expression of the

glycolysis mediator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) to sustain cellular dormancy. Inactivation of autophagy signaling components re-establishes normal-level PFKFB3 expression, culminating in the reactivation of BCSC self-renewal, tumor aggressiveness, and metastatic outgrowth[145]. Despite their indirect role in tumor growth, eradicating dormant tumor cells as the source of BCSCs and chemoresistance will offer promising therapeutic implications.

1.6 Potential compounds regulating cancer stem cells and differentiation

Loss of differentiation coupled with uncontrolled proliferation is a hallmark of malignant neoplasms. Differentiation therapy is a therapeutic strategy that re-instates endogenous differentiation programs to induce maturation in tumor cells. Upon differentiation, tumor cells revert back to a non-malignant phenotype, culminating in reduction of proliferation and metastatic potential and upregulation of differentiating markers[150]. Since chemotherapies target only rapidly-proliferating tumor cells and spare the slowly-dividing population of CSCs, relapse is common. The presence of dedifferentiated CSCs in solid tumors gives rise to their heterogeneous nature with regard to proliferation, metastasis, and relapse after radio- or chemotherapy. A prospective alternative CSC-targeted therapy is to use differentiation-inducing agents to target CSCs and self-renewal signaling, influence the functional hierarchy between tumor cells, and thereby reduce their chemo- and radio-resistance[151]. A literature search on PubMed in December 2020 yielded 4436 articles on “breast cancer stem cells”, 7758 articles on “breast cancer and differentiation” and 3450 articles on “differentiation inducing agents and cancer” for the past 5 years. From this literature search, Table 2 summarizes differentiation-promoting natural products and synthetic chemicals that have been indicated to target breast cancer stemness signaling. Some potential differentiation-inducing agents for breast cancer including all-*trans* retinoic acid (ATRA), vitamin D, and histone deacetylase inhibitors (HDACi) are discussed here.

An early success story of differentiation therapy was the use of ATRA as a clinical therapeutic agent. ATRA, an active metabolite of vitamin A, has anti-proliferative, cyto-differentiating and secondary apoptosis-inducing properties and is increasingly used in various tumors such as acute promyelocytic leukemia (APML), breast cancer, bladder cancer and ovarian carcinoma[152]. In a TNBC xenograft model, combined treatment of ATRA with the epigenetic and chemotherapeutic agents, entinostat and doxorubicin, targets CSCs and induces differentiation by activating ETS transcription factor 1[153]. ATRA induced differentiation in BCSCs by decreasing the populations of $CD44^{+}/CD24^{-}$, NANOG-positive and OCT3/4-positive MCF-7 breast cancer cells. ATRA treatment inhibited cell invasion and enhanced the sensitivity of MCF cells to radiation treatment[154]. In HER2-positive SK-BR-3 and UACC812 cancer cells with co-amplification of ERBB2 and RARA genes, ATRA induces RARα-dependent epithelial differentiation by reorganizing cytoskeletal elements and exerts anti-migratory action by down-regulating EMT-modulator NOTCH1[155]. ATRA directs the recruitment of RARβ-TET2 complex to epigenetically activate miR-200c that further inhibits PKCζ, a cell polarity protein that dictates asymmetric division of mammalian stem cells, resulting in symmetric division and downregulation of stem cell pool in breast CSCs. ChIP-sequencing analysis showed ATRA enhanced RARβ-TET2 complex

co-occupancy in promoters of genes implicated in cell differentiation such as RUNX1, BMP6, IKZF1 and CAV1[156].

Vitamin D belongs to a group of fat-soluble secosteroids produced as a result of skin exposure to UV light or obtained from dietary sources such as plants and fish[157]. Prior studies have demonstrated that the active vitamin D metabolite (1,25D3) and its analogs inhibit breast tumorigenesis *in vivo* and trigger apoptotic and autophagic cell death *in vitro*[158–160]. In addition to its effect on primary breast tumors, vitamin D compounds has demonstrated inhibitory effects on metastasis, achieved through inhibiting EMT[161]. In MDA-MB-231 breast tumor cells, 1,25D3 induced epithelial marker E-cadherin by CDH1-promoter demethylation, culminating in epithelial differentiation and reduction in tumor progression[162]. In MCF10DCIS.com xenograft tumors, the Gemini vitamin D analog BXL0124 has been shown to inhibit ductal carcinoma in situ (DCIS) progression to invasive ductal carcinoma (IDC) by maintaining the myoepithelial cell layer and basement membrane[163]. BXL0124 repressed the expression of CSC marker CD44 at both mRNA and protein levels in MCF10DCIS.com cells via vitamin D receptor (VDR)-dependent mechanism and suppressed the mammary tumor growth in xenografts.[164]. 1,25D3 and BXL0124 inhibit BCSCs by reducing the $CD44^{+}/CD24^{-/low}$ subpopulation and mammosphere forming efficiency. Treatment of mammospheres with vitamin D compounds targets stem cell phenotype markers (including CD44, CD49f, pNFκB, and c-Notch1) and pluripotency markers (such as OCT4 and KLF4)[165]. In SUM159 breast cancer cells, 1,25D3 and BXL0124 reduced the self-renewal of mammospheres and suppressed the genes related to pluripotency and Notch signaling. Vitamin D also upregulated myoepithelial differentiating markers including cytokeratin 14 and smooth muscle actin and down-regulated luminal marker, cytokeratin 5[166].

When it comes to development and stem cell differentiation, it is well-established that epigenetic regulation plays a significant role. Aberrant epigenetic modifications (including microRNAs and histone modifications) have been implicated in differentiation programs in cancer[167]; of these, microRNAs provide an appealing target for differentiation therapy. Petrelli *et al.* showed that miR-100 promotes differentiation in basal-like BCSCs, transforming the basal-like phenotype to luminal type. In basal-like breast cancer, miR-100 inhibits maintenance of BCSCs by targeting the Wnt signaling pathway and polo like kinase 1 (PLK1); conversely, its inhibition induces a stem-like phenotype[168]. Also of interest in breast cancer is the potential role of HDACi as avenues for differentiation therapy[169]. Histone acetylation is tightly controlled by histone acetyltransferases and histone deacetylases (HDAC). HDACs are implicated in multiple stages of cancer development, including the regulation of cell cycle regulation, autophagy, apoptosis and angiogenesis[170]. Aberrant expression of HDACs is associated with solid and hematological malignancies. HDACi can restore the abnormal acetylation status and reactivate the expression of tumor suppressors in cancer cells, inducing differentiation and inhibit tumor progression[171]. In TNBC cells, a pan-HDACi, Panobinostat, induced E-cadherin and repressed EMT and metastasis by inhibiting ZEB expression[172]. A low dose of the HDACi abexinostat induces BCSC differentiation in sensitive breast cancer cells, with treated cells exhibiting high expression of luminal and epithelial markers and low expression of mesenchymal markers. Furthermore, abexinostat reduces the BCSC population in patient-derived xenografts

expressing low levels of the lncRNA Xist[173]. HDACi have been evaluated in clinical trials together with other antitumor agents such as primary chemotherapeutic agents, epigenetic-targeted drugs and proteasome inhibitors to improve their efficacy and toxicity.

Other potential compounds of interest that can induce differentiation and target BCSC are acetaminophen, efatutazone and flubendazole. Acetaminophen, an anti-inflammatory drug, was evaluated for its effect on differentiation and tumorigenicity in breast cancer. Treatment of MDA-MB-231 cells with acetaminophen induced morphological changes, decreased $CD44^+/CD24^-$ and $ALDH^+$ subpopulations, altered markers for differentiation and stemness, and inhibited tumorigenicity. It also increased susceptibility to anti-tumor drugs through suppressing the expression of multidrug efflux pumps. The differentiation-inducing effect of acetaminophen is mediated through the Wnt/β-catenin signaling pathway[174]. Acetaminophen modulates the expression of EMT-related genes including CK19, TIMP1, MMP2 and TWIST, microRNAs including miR-143 and miR-146a and NOTCH signaling. It reduces the protein levels of Twist and Vimentin, and increases the level of E-cadherin in favor of differentiation. Breast cancer cells treated with acetaminophen showed a significant decrease in *in vitro* cell migration and an increase in chemo-sensitization[175].

PPARγ agonists are agents that activate endogenous PPARγ, a member of the nuclear receptor family of ligand-activated transcription factors, with profound effects on cellular differentiation, proliferation and inflammatory response in cancer tissues[176]. Efatutazone, a high-affinity PPARγ agonist, inhibited MCF10DCIS mammosphere formation and down-regulated Akt phosphorylation. Efatutazone-treated DCIS lesions in xenografts showed less invasive feature with fewer CD44+/p63+ basal progenitor cells and exhibited fat deposition along with mammary epithelial cell differentiation, suggesting that PPARγ agonists can be useful as potential differentiation inducing agent to delay invasive progression in breast cancer[177].

Flubendazole, a FDA-approved anthelmintic, is shown to inhibit breast cancer cell proliferation. It exhibits BCSC-targeted effects by inhibiting mammosphere formation and reducing the $CD44^+/CD24^-$ subpopulation in MDA-MB-231 cells. Flubendazole suppressed the expression of self-renewal genes (OCT4, SOX2, NANOG, CYCLIN D1 and C-MYC) and induced cell differentiation (increasing Oil Red O stain + cells, upregulating epithelial marker Keratin-18 and down-regulating mesenchymal markers – N-cadherin, Vimentin and β-catenin). It also enhanced the chemosensitivity of the breast cancer cells[178]. These findings demonstrate the novel use of flubendazole as a BCSC-targeted agent with differentiation inducing property.

In addition, knockdown of CD44, a BCSC marker involved in the differentiation, adhesion, and metastasis of cancer cells, sensitized breast cancer cells to doxorubicin or radiation. Its depletion induces BCSCs to differentiate into non-stem-like cells, targeting drug resistance, metastasis, and stem cell-related genes, indicating BCSC marker targeted therapy can modulate differentiation and inhibit breast tumorigenicity at the same time[179]. Collectively, the above studies illustrate the promise of differentiation agents either as a stand-alone therapy or as part of a combinatorial regimen targeting BCSCs (Fig. 1).

1.7 Conclusion

While there are a fair number of cell surface markers, receptors and ligands, intracellular signaling molecules and transcription factors that identify breast cancer stem cell subpopulations and appear responsible for their stem-like behavior, therapeutic agents that target BCSCs through these elements remain elusive. Likewise, while much *in vitro* and *in vivo* evidence indicates that induction of differentiation (or redifferentiation) of CSCs can exert clinically beneficial effects in certain malignancies, this has not yet been achieved effectively in breast cancers. Reducing the properties of stemness that make the CSC compartment resistant to conventional therapy and providing the seeds for recurrence, and inducing the return of those stem cells to their differentiated, somatic origins, could offer improved efficacy in long-term control of the disease.

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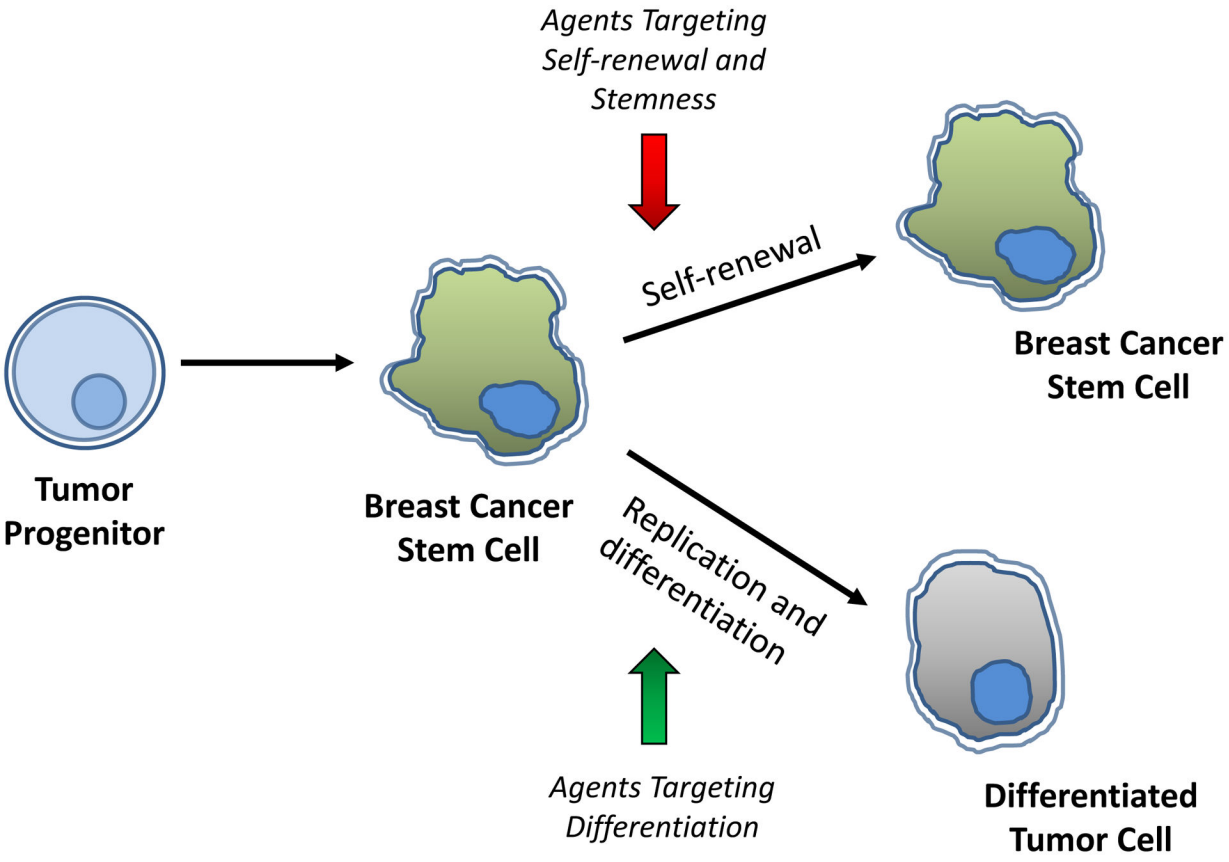

Figure 1. Breast cancer stem cell signaling, transcription factors and agents that target them.

Agents targeting the self-renewal program controlled by transcription factor mediators, OCT4, SOX2, NANOG or KLF4, agents reducing stemness by targeting aberrantly activated signaling pathways involving Notch, Wnt, Hh, STAT3 or TGF-β, and agents inducing differentiation of breast cancer stem cells by reprogramming cells into more differentiated tumor cells are to be exploited in treatment and prevention of breast cancer.

Table 1.

Breast cancer stem cell markers, their functions, target genes and tumorigenesis

Cancer cell surface marker(s)/ Transcription factor	Function in stem cell	Experimental system	Target genes/markers studied	TS/CFA	In vivo study	Ref.
ABCG2^{+} (CD338)	Tumor initiation	HCC1937	CD326/EpCAM, CD49f/α6-integrin, CD24, CD10, CD133	+	Injection of unsorted cells (4×10^5 and 2×10^6 cells)	Leccia et al[180]
ALDH$^{+/hi}$ CD44^{+}	Chemo-radio resistance	MDA-MB-231 and MDA-MB-468	GSTP1, P-gp, CHK1, CK8/18/19	+	NA	Croker et al[181]
ALDH1^{+}	Self-renewal; tumor initiation	Cells developed from patient tumors	NA	+	Injection of sorted cells (500 ~ 50,000 cells)	Ginestier et al[29]
C/EBPδ	Tumor initiation; EMT; metastasis	SUM159, MDA-MB-231, MDA-MB-468, MCF-7, T47D	IL-6, HIF-1α, CD44, N-cadherin, Vimentin, E-cadherin, Twist and STAT3, Myc, Nanog, and KLF4, OCT4, SOX2, CDH1, FBXW7, NOTCH and NICD	+	Injection of unsorted cells (3×10^6 or 0.3×10^6 cells)	Balamurugan et al[182]
CCR5	Self-renewal; DNA repair	SUM159, SUM149 and FC-IBC-02	DNA damage repair genes: FANCB, LIG3, POLE, CRY1; PI3K/Akt signaling; cell survival signaling	+	Injection of sorted cells (4000 cells)	Jiao et al[183]
CD133^{+}	Tumor initiation; self-renewal	Cell lines developed from *Brca1*Δ^{exon11}/*p53*$^{+/-}$ mouse mammary tumors	Expression of stem cell associated genes: OCT4, NOTCH1, ALDH1, FGFR1 and SOX1	+	Injection of sorted cells (50 ~ 5000 cells)	Wright et al[25]
CD24^{+}CD29^{+}/CD49f^{+}	Metastasis; EMT	BRCA-1 mutant mouse derived CSCs	E-cadherin	+	Injection of sorted cells (2×10^5 cells)	Vassilopoulos et al[184]
CD24^{+}Thy1^{+}	Tumor initiation	MMTV-Wnt-1 mouse	CK5, CK14, CK17, NOTCH4, BCL6B	+	Injection of sorted cells (50 ~ 2,000 cells)	Cho et al[185]
CD29^{lo}CD24^{+}CD61^{+}	Tumor initiation	MMTV-Wnt-1, MMTV-neu and p53 mutant mice	CK14, CK8	+	NA	Vaillant et al[186]
CD44^{+}/CD24$^{-/low}$Lineage^{-}	Tumor initiation	Human breast tumor-derived tumor cells	NA	−	Injection of sorted cells; Limiting dilution (100 ~ 5×10^5 cells)	Al-Hajj et al[7]
CD44^{+}/CD24$^{-/low}$/EpCAM^{+}	Tumor initiation; self-renewal	HMLE cells, MDA-MB-231, MCF-7, MCF10A, SUM149, SUM159, SUM1315 and SUM225	NA	+	Injection of sorted cells; Limiting dilution (100 ~ 1×10^6 cells)	Fillmore et al[187]
CD44^{+}/CD49f^{hi}/CD133/2^{hi}	Tumor initiation, self-renewal	Human breast cancer tissues	SOX2, BMI-1, NANOG	+	Injection of sorted cells; Limiting dilution (50 ~ 2500 cells)	Meyer et al[188]
CD44^{+}CD24$^{+/lo}$SSEA-3^{+} or ESA^{hi}PROCR^{hi}SSEA-3^{+}	Tumor initiation	MCF-7 and MDA-MB-231	SSEA-3, Caspase-3, capase-8, caspase-9, caspase-12 SSEA-4, globo-H, β3GalT5	+	Injection of sorted cells (10 ~ 2500 cells)	Cheung et al[189]

Cancer cell surface marker(s)/ Transcription factor	Function in stem cell	Experimental system	Target genes/markers studied	TS/CFA	In vivo study	Ref.
$CD49F^{+}/DLL1^{hi}/DNER^{hi}$	Tumor initiation; self-renewal	Human normal mammary stem cells and breast tumors	SERPINB5, TOP2A, CK5, TP63, SOX4, CD24, ADRM1, DNER, DLL1, and JAG1	+	Injection of sorted cells (500 ~ 1000 cells)	Pece e al[190]
$CD49f^{+}CD61^{+}$	Tumor initiation; self-renewal	MMTV-Her2/neu-induced primary mammary gland tumor	CSC markers: Abcg2, Aldh1, CD133, Gli1 and Tp63; differentiation marker genes: CK5, CK6, CK14, CK18; TGFβ signaling: Pai, Il6, Igfbp3, Foxc2; EMT genes: CDH2, SMA, SNAIL, TWIST1 and ZEB1	+	Injection of sorted cells (5000 cells)	Lo et al[191]
CD61(Integrin αvβ3)	Tumor initiation; self-renewal	BT-20, MDA-MB-231and MDA-MB-468	SLUG	+	Injection of unsorted cells; Limiting dilution (100 ~ 1×10^{5} cells)	Desgrosellier et al[192]
$CD70^{+}$	Self-renewal; EMT; lung-specific metastasis; differentiation	MCF-7, MDA-MB-231 and CN34	E-cadherin and Vimentin	+	Injection of sorted cells; limiting dilution	Liu et al[193]
Cx26	Self-renewal; tumor initiation	Triple negative breast cancer samples; MDA-MB-231 and HCC70	NANOG, FAK, OCT4, SOX2	+	Serial dilution injections (8,000 ~ 800,000 cells)	Thiagarajan et al[194]
CXCR2	Chemo-radio resistance; tumor initiation	Human breast cancer tissues; 4T1	ALDH, ABCG2, NOTCH1, SOX2, and NANOG	+	Injection of sorted cells (200 ~ 20,000 cells)	Wang et al[195]
$GD2^{+}$	Self-renewal; tumor initiation; EMT	Transformed HMLE cells and MDA-MB-231	GD3S; MMPs: MMP2, MMP7 and MMP19; EMT markers: N-cadherin, Vimentin, E-cadherin; stemness markers: CD44, CD24	+	Injection of sorted cells (1 ~ 10,000 cells)	Battula et al[196]
Glyoxalase 1	Self-renewal	MDA-MB-157 and MDA-MB-468	ALDH1	+	NA	Tamori et al[197]
HIF-2α	Self-renewal; chemoresistance	MCF-7 and MDA-MB-231	Stem cell markers: C-MYC, OCT4, NANOG; Notch pathway related proteins: $NOTCH^{NICD}$ and HEY2; Wnt-pathway related proteins: β-catenin, Axin2 and Survivin	+	Injection of unsorted cells (3×10^{6} or 1×10^{6} cells)	Yan et al[198]
$Lgr5^{hi}$	Self-renewal; tumor initiation; EMT	MCF-7, MDA-MB-231	EMT markers: E-cadherin, β-catenin, Vimentin, Fibronectin, Snail, slug; Cyclin D1, C-myc, CK14, and CK18, CD44, CD24	+	Injection of sorted cells (200 ~ 20,000 cells)	Yang et al[199]
miR-1	Negative regulator of breast cancer stem cells and EMT	Human breast cancer tissues; MDA-MB-231	EVI-1; EMT markers: E-cadherin, N-cadherin	−	Injection of sorted cells (5×10^{6} cells)	Wu et al[200]
miR-221	Self-renewal	Human breast cancer tissues; T47D and MCF-7	Stemness genes: NANOG, OCT3/4; β-Catenin, DNMT3b, CD44, CD24, Numb, p53	+	NA	Roscigno et al[201]

Cancer cell surface marker(s)/ Transcription factor	Function in stem cell	Experimental system	Target genes/markers studied	TS/CFA	In vivo study	Ref.
MUC1^{+}	Tumor initiation; self-renewal	MCF-7	ABCG2, CK18, CK19, EpCAM, CD49f	+	NA	Engelmann et al[202]
Nectin-4^{+}	EMT; metastasis; self-renewal	MDA-MB-231	CD44, CD133, PI3K, Akt, β-catenin, E-cadherin, Vimentin	+	Injection of unsorted cells (1×10^{7} cells)	Siddharth et al[203]
PROCR^{+}/ESA^{+}	Self-renewal; tumor initiation; EMT	MDA-MB 231	EMT markers: Vimentin, E-cadherin, SLUG, FOXC2; stem cell markers: ALDH, CD44, CD24, ESA, CD133, CXCR4, ABCG2	+	Injection of sorted cells; Limiting dilution (100 ~ 2500 cells)	Hwang-Verslues et al[204]
RUNX1	Negative regulator of self-renewal and CSC	MCF10AT1, MCF10A, MCF10CA1 and MCF-7	E-cadherin, Vimentin, FN1, VEGF, MMP13, MMP9, CXCR4, CXCL12, CSC markers: Zeb1, Twist1, CD44, CD24	+	Injection of unsorted cells (1×10^{6} cells)	Hong et al[205]
Sca1^{+}	Self-renewal; tumor initiation; chemoresistance	BALB-neuT mouse	Stem cell markers: Oct-4, CD44, CD29, CD24; differentiation markers: CK14, CK18, CK19, α-SMA	+	Injection of unsorted cells (100 ~ 10,000: cells)	Grange et al[206]
Syndecan-1	Self-renewal	SUM149 and SK-BR-3	CSC markers: CD44, CD24, ALDH; Notch signaling: NOTCH-1, −3, −4, HEY-1; Gli-1, IL-6, IL-8, gp130, STAT3, NFκB, CCL20, EGFR	+	NA	Ibrahim et al[207]
tDR-000620	Predictor of TNBC recurrence	Human patients- derived TNBC samples; MCF-7 and MDA-MB-231	SOX2, OCT4, ALDH1, CD44, CD24	+	NA	Feng et al[208]

TS/CFA: Tumor Spheroid (TS) or Colony formation assay (CFA) (+ indicates TS/CFA was used, - indicates TS/CFA was not used); NA: Not available

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Table 2.

Potential compounds regulating cancer stem cell markers and differentiation in breast cancer

Agent	Classification	Experimental Model	Significance	Ref
Acetaminophen	Synthetic chemical	*In vitro*, MDA-MB-231	mRNA level: CK19, AKT2, CD24, and TIMP1↑; MMP2, ALDH1, MMP9, TWIST, NOTCH1, and AKT1↓ Protein level: Vimentin↓/E-cadherin↑, Twist↓ Cell surface marker: $CD44^{hi}/CD24^{low}$↓, $CD44^{+}/CD24^{+}$↑ Differentiation induction of CSC: Twist↓, Vimentin↓/E-cadherin↑	Afshar et al[175]
AF38469	Synthetic chemical	*In vitro*, MDA-MB-231, MCF-7; *In vivo*, MDA-MB-231, MCF-7 xenograft (5–10 μg/day/mouse given in drinking water for 3 weeks)	Sortilin inhibition, Decreased mammosphere formation, Reduced EMT; *In vivo* efficacy: breast cancer metastasis and local infiltrative growth↓	Rhost et al[209]
Active Hexose Correlated Compound (AHCC)	Natural product	*In vitro*, MCF-7	Decreased mammosphere formation (AHCC alone and in combination with Wasabi) Observation of monocyte-to-macrophage differentiation	Corradetti et al[210]
All-*trans*- retinoic acid (ATRA)	Natural product	*In vitro*, MDA-MB-231, T47D, ZR75–1, BT549, MCF-7	RARβ-TET2 complex recruitment, leading to activation RARβ-TET2-miR-200c-PKCζ signaling - increases symmetric commitment and represses asymmetric division in CSC ChIP-seq with RARβ-TET2: co-occupancy in promoters of genes involved in differentiation - RUNX1, BMP6, IKZF1, CAV1 Restoration of normal epithelial like, well-differentiated acinar structure in 3D matrigel ↑ Tamoxifen sensitization	Wu et al[156]
		In vitro, MCF-7	Cell markers: $CD44^{+}/CD24^{-}$, NANOG, Oct3/4↓ Differentiation induction of CSC: involucrin and syndecan↑ Reduced invasiveness and migration Epirubicin sensitization	Yan et al[154]
Arsenite	Synthetic chemical	*In vitro*, MDA-MB-231	Cell surface marker: ICAM-1 expression induction (in combination with Tetrandine)	Yu et al[211]
Atorvastatin	Synthetic chemical	*In vitro*, MDA-MB-468, MDA-MB-231	Gene expression: Hippo, Notch, Wnt↓ Protein level: Yap/Taz protein↓, vimentin↓, E-cadherin↑ Differentiation induction of CSC: $CD24^{+}$↑ Reduced EMT	Koohestani mobarhan et al[212]
β-lapachone	Natural product	*In vitro*, MDA-MB-231	NQO1 induction Gene expression: CD44, ALDH1A1, DLGAP5↓ Decreased mammosphere formation	Kim et al[213]
BEZ235	Synthetic chemical	*In vitro*, HCC1143, SUM149	PI3K/mTOR inhibition RNAseq and immunofluorescence: $CK19^{hi}/Vim^{hi}/CK14^{lo}$	Risom et al[214]
		In vitro, HCC1143	Induction of $CK19^{hi}/VIM^{lo}/CK14^{lo}$ enrichment in HCC1143 cell line. De-enrichment of $VIM^{hi}/CK14^{lo}$ population	Risom et al[214]
Bisphenol A	Synthetic chemical	*In vitro*, MCF10A	↓BMP4 mediated stem cell maintenance and ↑ myoepithelial differentiation	Clément et al[215]

Agent	Classification	Experimental Model	Significance	Ref
BXL0124	Synthetic chemical	*In vitro*, SUM159	mRNA level: OCT4, CD44, LAMA5, NOTCH↓ Differentiation marker: CK14, SMA↑, CK18, CK5↓ Decreased mammosphere formation Differentiation induction of CSC	Shan et al[166]
CHM-09	Synthetic chemical	*In vitro*, MDA-MB-231	EGFR Tyrosine kinase inhibitor Differentiation marker: N-cadherin↓, E-cadherin↑ Induction of CSC apoptosis and cell cycle arrest Increased mesenchymal epithelial transition	Manupati et al[216]
Citral	Natural product	*In vivo*, 4T1 cells injected BALB/c mice (50 mg/kg, given orally for 28 days)	*In vivo* efficacy: primary and secondary xenograft tumor size↓; number of $ALDH^+$ tumor cells↓	Nigjeh et al[217]
Curcumin	Natural product	*In vitro*, MCF-7, MDA-MB-231	Gene expression: OCT4, NANOG, SOX2↓ Decreased mammosphere formation Differentiation induction of CSC: $CD44^+/CD24^-$↓	Hu et al[218]
		In vitro, SUM149, MCF10A, MCF-7	Gene expression: ALDH1A3, PROM1, TP63, ITGA6↓ Decreased mammosphere formation	Colacino et al[219]
CWP/ICG001	Small molecule	*In vitro*, MCF-7, MDA-MB-231	Induction of Sam68-CBP complex, leading to disruption of CBP/β-catenin Differentiation induction of CSC Increased CSC apoptosis	Benoit et al[220]
Dasatinib	Synthetic chemical	*In vitro*, paclitaxel-resistant SUM159	mRNA level: CDH2, FN1, SNAI1, ZEB1, TP63, SMA↓, E-cadherin↑ protein level: p-Src↓ 3D culture of SUM159. Dasatinib treated group showed formation of round, acinar-like structure Decreased mammosphere formation Differentiation induction of CSC Increased MET	Tian et al[221]
Diallyl Trisulfide	Natural product	*In vitro*, MCF-7, SUM159	Gene expression level: CD44, ALDH1A1, NANOG, OCT4↓, Wnt/β-catenin signal↓ Reduced CSC viability	Li et al[222]
Digitoxin	Natural product	Patient tumors, patient tumor derived xenograft (pretreated cells with 20 nM for 17 days *in vitro* followed by tail vein injection into NSG mice)	Increased intracellular calcium, dissociated cell tight junction → altered DNA methylation profile and downregulated target genes of OCT4, SOX2, NANOG, SIN3A *In vivo* efficacy: delayed metastatic outgrowth over the course of 72 days post-injection	Gkountela et al[223]
Disulfiram	Synthetic chemical	*In vitro*, MDA-MB-231	Protein level: STAT3, cyclinD, Survivin, ALDHA1↓, Caspase-3↑ Cell surface marker: $CD44^+/CD24^-$↓ Increased CSC apoptosis Decreased mammosphere formation	Kim et al[224]
Doxorubicin	Synthetic chemical	*In vitro*, Hs578T	Decreased proliferation, aggregation and mammosphere formation of stem-like cells Affects the balance between self-renewal and differentiation	Tudoran et al[225]
EC-70124	Synthetic chemical	*In vitro*, HS578T, BT549, MDA-MB-231, HCC3153	Gene expression: PI3K/mTOR, JAK/STAT ↓ Cell marker: CD44, ALDH1, CD49f, CD133↓ Differentiation induction of CSC Decreased EMT	Cuenca-López et al[226]

Agent	Classification	Experimental Model	Significance	Ref
Efatutazone	Synthetic chemical	*In vitro*, MCF10A, MCF10DCIS; *In vivo*, MCF10DCIS xenograft (30 mg/kg, oral gavage for 3 weeks)	PPARγ agonist mRNA level: hFABP4, CK8↑, CK6a, CK6b, CK17↓ Differentiation induction of CSC: upregulation of PPARγ responsive genes in epithelial and stromal components; *In vivo* efficacy: delayed tumor progression; induced differentiation: lipid droplets↑, CD44 and p63 staining↓, CK8 staining↑; FABP4 and PLIN2 mRNA↑	Ory et al[177]
Entinostat	Synthetic chemical	*In vitro*, MDA-MB-231, SUM149 and HCC1937; *In vivo*, MDA-MB-231 xenograft, Entinostat (2.5 mg/kg) 5 days/week (oral), ATRA (5 mg/kg) 5 days/ week (intraperitoneal) and Doxorubicin (2 mg/kg) once a week (intravenous) for 4 weeks	HDAC inhibition In combination with ATRA and doxorubicin: decreased tumor sphere formation, $CD44^+/CD24^-/EpCam^+$↓ Induced differentiation markers: RAR-β, ELF3, DHRS3, basal lineage markers (CK5 and CK15), luminal markers (ER, PR) and CK19, epithelial cell-specific genes (CLDN 1, 3, 4, 7), Occludin and E-cadherin↑ and vimentin↓; *In vivo* efficacy: Tumor-initiating frequencies↓	Merino et al[227]
Flubendazole	Synthetic chemical	*In vitro*, MDA-MB-231, BT-549, MCF-7, SK-BR-3; *In vivo*, MDA-MB-231 xenograft (25 mg/kg/day, given intraperitoneally for 16 days)	Gene expression: MYC, OCT4, SOX2, NANOG cyclinD1↓ Oil red O staining↑, $CD44^+/CD24^-$↓ Decreased mammosphere formation Differentiation induction of CSC: β -catenin, N-cadherin, vimentin↓, CK18↑ Reduced CSC self-renewal; *In vivo* efficacy: tumorigenicity↓	Hou et al[178]
Graphene Oxide	Synthetic chemical	*In vitro*, MCF7	CSC signaling pathway: Wnt, NOTCH, STAT1/3, Nrf2↓ Cell surface marker: $CD44^+/CD24^-$↓ Differentiation induction of CSC Decreased mammosphere formation	Fiorillo et al[228]
Helichrysetin	Natural product	*In vitro*, MCF10A, MCF10DCIS, MCF10CA	Differentiation induction of CSC: ID2↓ Decreased mammosphere formation Decreased CSC self-renewal	Liu et al[229]
IM-412	Synthetic chemical	*In vitro*, MDA-MB-453, MDA-MB-231	FGFR1/3 inhibition Protein level: Smad2/3, p38/MAPK, Akt, JNK↓ Reduced EMT Differentiation induction of CSC: Inhibition of TGF-β pathway	Jung et al[230]
Ivermectin	Synthetic chemical	*In vitro*, MDA-MB-231	Gene expression: SOX2, NANOG, OCT4 ↓ Cell surface marker: $CD44^+/CD24^-$↓ Decreased CSC viability Decreased CSC self-renewal	Dominguez-Gomez et al[231]
K252	Small molecule	*In vitro*, MDA-MB-468	Differentiation induction: ERN1 inhibition Confocal microscopy of CK5 and CK8 expression showing ERN1 and ALPK1 Knockdown induces luminal differentiation (CK5−/ CK8+) in MDA-MB-468 Reduction of colony forming unit of anchorage independent growth of TNBC cell lines Protein level: β-casein↑	Strietz et al[232]
Laminin	Endogenous	*In vitro*, LM05-E	Gene expression: SOX2, NANOG, OCT4 ↓ Protein level: p-ERK↑ Reduced CSC viability Differentiation induction of CSC Decreased mammosphere formation	Berardi et al[233]

Agent	Classification	Experimental Model	Significance	Ref
Lovastatin	Synthetic chemical	*In vitro*, MDA-MB-468, MDA-MB-231	Gene expression: Hippo, Notch, Wnt↓ Protein level: Yap/Taz protein↓, vimentin↓/E-cadherin↑ Differentiation induction of CSC Reduced EMT	Koohestani mobarhan et al[212]
Metformin	Synthetic chemical	*In vitro*, MDA-MB-231, MCF-7	RNA expression/ Differentiation induction: miRNA-708 ↑, CD47↓ Protein level: CD47↓	Tan et al[234]
Nobiletin	Natural product	*In vitro*, MCF7	CD36 inhibition Gene expression: SOX2, OCT4, NANOG↓ Decreased mammosphere formation	Sp et al[235]
Ouabain	Natural product	Patient tumors, patient tumor derived xenograft (0.67 mg/kg/day, given intraperitoneally for 3 weeks)	Increased intracellular calcium, dissociated cell tight junction → altered DNA methylation profile and downregulated target genes of OCT4, SOX2, NANOG, SIN3A *In vivo* efficacy: spontaneous metastasis formation↓	Gkountela et al[223]
Palbociclib	Small molecule	*In vitro*, MCF-7, MCF10DCIS	CDK4/6 inhibition Long term suppression of P63 Immunohistochemistry in DCIS Mammosphere formation↓ Differentiation induction of CSC: NELL2↑	Kietzman et al[236]
PD98059	Synthetic chemical	*In vitro*, MDA-MB-231	MAPK inhibition Differentiation marker: N-cadherin↓, E-cadherin↑ Induction of CSC apoptosis and cell cycle arrest Increased MET	Manupati et al[216]
Prolactin	Endogenous	*In vitro*, SKBR-3 and BT-474; *In vivo*, SKBR-3 xenograft (0.1 μg/g each second day, given intraperitoneally for 5 weeks)	mRNA level: ALDHA1, ALDHA3, CD44↓ Cell surface marker: ALDH↓ Decreased mammosphere formation; *In vivo* efficacy: tumor growth and Ki-67 staining↓	Hachim et al[237]
P123	Peptide (1.9kDa)	*In vitro*, CSC cells	BMP signal agonist Cell surface marker: $CD44^+$ population↓, E-$cadherin^+$ population↑in BCSC	Bosukonda et al[238]
Quercetin	Natural product	*In vitro*, MCF-7	PI3K/Akt/mTOR signal inhibition Decreased mammosphere formation Cell surface marker: $CD44^+/CD24^-$↓	Li et al[239]
Quisinostat	Synthetic chemical	*In vitro*, MDA-MB-231, MDA-MB-468, HCC38, MCF-7	HDAC Class I and II inhibition Cell surface marker: $CD44^+/CD24^-$↓ Decreased CSC viability (in combination with doxorubicin)	Hii et al[240]
RANK-Fc	Synthetic chemical	*In vivo*, MMTV-PyMT transgenic mice (10 mg/kg, subcutaneous, three times a week for 4 weeks (for passage 1) and 2 weeks (for passage 2))	Luminal epithelial phenotype (TFAP2B, SPDEF, and TFAP2C targets) ↑ *In vivo* efficacy: tumor initiating-ability↓, secondary mammosphere formation derived from treated tumors↓, tumor cell differentiation↑, lactogenic differentiation↑, $Sca1^-$/lo CSC population↓	Yoldi et al[241]
Resveratrol	Natural product	*In vitro*, MDA-MB-231	SIRT1 induction Cell surface marker: $CD44^+/CD24^-$↓ Differentiation induction	Deus et al[242]
Rosiglitazone	Synthetic chemical	*In vitro*, Py2T cells derived from MMTV-PyMT tumors and MTfIECad cells derived from MMTV-Neu tumors; *In vivo*, Py2T xenograft, high dose group: PD98059 (5	EMT transdifferentiation of breast cancer cells into adipocytes: lipid droplets of fluorescent Nile Red Co-treatment with PD98059 Differentiation induction of CSC: E-cadherin↑;	Ishay-Ronen et al[243]

Agent	Classification	Experimental Model	Significance	Ref
		mg/kg) and Rosiglitazone (16 mg/kg) daily; low dose group PD98059 (2 mg/kg) and Rosiglitazone (16 mg/kg) daily, given intraperitoneally) for 14 days.	*In vivo* efficacy: FABP4 and adiponectin expression↑ Development of unilocular lipid droplets in tumor cells Total tumor mass and tumor invasion↓	
Salinomycin	Synthetic chemical	*In vitro*, MCF-7	Decreased mammosphere formation	Wang et al[244]
SCH772984	Synthetic chemical	*In vitro*, MCF-10A, MDA-MB-231, MDA-MB-436	ERK inhibition Cell surface marker: ALDH↓ Protein level: p21↑	McGrail et al[245]
Seocalcitol	Synthetic chemical	*In vitro*, SUM-1315, BT-549, BT-20, SUM-159PT, MDA-MB-468, MFM-223, CAL-148	Vitamin D receptor signal activation Cell surface marker: ALDH↓ Decreased mammosphere formation Differentiation induction of CSC	Thakkar et al[246]
Silibinin	Natural product	*In vitro*, MDA-MB-468	Gene expression: CD133, ALDH, C-MYC, NANOG, KLF4, SOX2↓, GATA3, BRCA1↑ Cell surface marker: ALDH+/CD133, ALDH+/CD44, CD133/CD44↓ phenotype of MDA-MB-468 in 2D and 3D culture: increase in size and spindle shape in treatment group. BRCA1 upregulation in 3D culture group with treatment Differentiation induction of CSC Decreased mammosphere forming size	Abdollahi et al[247]
Simvastatin	Natural product	*In vitro*, MDA-MB-468, MDA-MB-231	Gene expression: Hippo, Notch, Wnt↓ Protein level: Yap/Taz protein↓, vimentin↓/E-cadherin↑ Differentiation induction of CSC Reduced EMT	Koohestani mobarhan et al[212]
Trametinib	Synthetic chemical	*In vitro*, SUM159, HCC1143	MEK inhibition $CK19^{hi}$/ $CK14^{hi}$/VIM^{lo}; Drug Tolerant Progenitor cells enrichment Produced cells with large cytoplasmic volume	Risom et al[214]
T315	Synthetic chemical	*In vitro*, MDA-MB-231, SUM159; *In vivo*, SUM159 xenograft (50 mg/kg, twice daily, oral gavage for 11 days)	ILK inhibition, Decreased NOTCH1 signaling, Decreased mammosphere formation; *In vivo* efficacy: $ALDH^+$ population and mammosphere formation of dispersed tumor cells↓, tumor initiating ability↓	Hsu et al[248]
Vitamin D	Natural product	*In vitro*, SUM159	mRNA level: OCT4, CD44, LAMA5, NOTCH↓ Differentiation marker: CK14, SMA↑, CK18, CK5↓ Decreased mammosphere formation Differentiation induction of CSC	Shan et al[166]
Withaferin A	Natural product	*In vitro*, SUM159 and MCF-7; *In vivo*, MMTV-*neu* mice (0.1 mg/mouse, three times per week, given intraperitoneally for 28 weeks)	Inhibited self-renewal of BCSC: decrease in mammosphere number Inhibition of stemness markers: ALDH1 and $CD44^+$/$CD24^-$/ESA^+↓, Decreased in stemness-related genes: OCT4, SOX2 and NANOG↓; *In vivo* efficacy: mammosphere number and size↓, ALDH1 activity↓, tumor burden↓	Kim et al[249]
4a1	Synthetic chemical	*In vitro*, MDA-MB-231	HEXIM induction Protein level: HEXIM1, p27↑, NANOG↓ Nile red staining increased in 4a1 treatment	Ketchart et al[250]
5-aza-2'-deoxycytidine	Synthetic chemical	$CD44^{hi}$/$CD24^{low}$ expressing CSC isolation from primary malignant breast tumor from patient	mRNA level: p15, p16, BRCA1, BRCA2, p53↑ cell surface marker: $CD44^+$/$CD24^-$↓ protein: ABCG2↓	Phan et al[251]