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The Role of Stem Cells in Breast Cancer

Joanna Magdalena Zarzynska

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Abstract

A significant progress has been made in describing cellular hierarchy and the stem cell niche in the human mammary gland. Mammary stem and progenitor cells exist in two different states: epithelial-like and mesenchymal-like. Several features of the mammary stem cells predispose them to play a critical role in breast cancer initiation, progression and metastasis. Signaling pathways contributing to the self-renewal, such as Wnt, Notch, Hh and BMP, have been shown to be linked with breast cancer stem cells. Furthermore, biomarkers connected with stemness, such as CD44, CD24, EpCAM and ALDH1, have been identified and used to characterize these cells. Additionally, many different miRNA families and microenvironmental factors were shown to regulate a lot of cancer stem cells properties and maintain their stemness. All these findings have started a new era of breast cancer research. In present breast cancer, stem cells have become the targets of breast cancer therapy, although the tests are mainly on the basic stage level. Since the cancer stem cells are able to escape chemotherapy and are resistant to drugs, radiotherapy and apoptotic processes, the therapeutic targeting is mostly concentrated on the disruption of survival signaling pathways and the use of modern technology, like nanotechnology.

Keywords: cancer stem cells, breast cancer, MaSC, BCSCs, stem cell niche, miRNA, EMT

1. Introduction

As most epithelia, mammary epithelium continuously replaces dead or damaged cells during the whole life of an animal and this process called tissue homeostasis is critical for adult tissues maintenance. Typically, epithelial tissue homeostasis is maintained through the presence of stem cells (SC). They are functionally defined in connection with their ability to self-renew and differentiate into cell lineages of their original tissue [1–3]. Mammary stem cells (MaSC) are capable of generating the complex bilayer system of the mammary epithelium composed of basal (myoepithelial) and luminal (secretory) epithelial cells. In addition, there are mesen-

chymal stem cells (MSCs), representing the stromal (fat pad) part of this organ [1]. According to current knowledge, scientists had made the model of SC mitotic division, which can be symmetric or asymmetric. During symmetric division, stem cell gives two daughter stem cells and it allows for the expansion of stem cell population. When a stem cell undergoes asymmetric division, one stem cell is obtained maintaining the self-renewal properties, whereas the another cell is called a progenitor cell. Progenitor cells have a more restricted potential in terms of their renewal and differentiation. Progenitor cells also have limited proliferation capacity and can undergo senescence [1, 2].

Several features of MaSC make them plausible sites for breast cancer (BC) initiation. Breast cancer is a potentially life-threatening malignant tumor that still causes high mortality among women. Decreasing mortality rates has been achieved, that is, by efficient screening strategies [4]. Still, BC is ranked on the second place among cancer types regarding mortality [5]. It has been estimated that approximately 1.3 million females develop BC each year with around 465,000 expected to succumb to the disease [6–8]. MaSC have been postulated to underlie the cellular heterogeneity observed in human breast cancers due to their preserved replicative capacity and differentiation potential, resulting in prolonged life span and thus increased probability of harboring and accumulation of mutations [9, 10]. The cancer stem cell (CSC) fraction typically constitutes 1–5% of the tumor size [8, 11]. In the healthy human mammary gland, SC account for approximately 8% of the cells [12]. The concept of CSC has led to the development of new theoretical models explaining the cellular origin of cancer [13, 14]. One theory, called the stochastic theory, claims that every single cell can potentially become cancerous in the appropriate microenvironment. However, differentiated cells are probably unable to accumulate a sufficient number of mutations in order to become neoplastic because of their shorter life span. Second theory, called the hierarchy (CSC) theory, suggests that CSC are more likely to initiate the tumor, as they have longer life span, increased migratory and proliferative potential and advanced DNA repair mechanisms. Since it is more probable that these two models coexist, a dynamic version of the CSC model has been developed, suggesting that within the tumor hierarchy, differentiated tumor cells may undergo dedifferentiation as a result of microenvironmental influences. In addition to the generation of cells with stem-like properties, the tumor microenvironment is also involved in the preservation of the established CSC subpopulation [15, 16].

Increasing evidence demonstrates that CSC play a critical role not only in BC initiation, but also in progression and metastasis [13]. Accumulating evidence indicates that the local recurrent and/or distant metastatic tumors, which constitute the major causes of lethality in the clinic, are related to the aggressive phenotype of a small fraction of cancer stem cells, tumor-initiating cells (TICs) or cancer metastasis-initiating cells (CMICs) [17].

Breast cancer stem cells (BCSCs) are able to escape chemotherapy due to elevated expression of ABC transporters that enable them to efflux some chemotherapeutic drugs [13]. They are resistant to apoptosis (they also express high levels of anti-apoptotic proteins, such as survivin and Bcl-2) and show drug resistance [11]. In addition, the activity of BCSCs can enhance and the ratio of side population can increase after radiation treatment. Furthermore, BC has capability to resist radiotherapy [17–19]. Therefore, it has been suggested that BCSCs might be responsible for tumor regrowth and the development of drug resistance [2, 13, 17].

Identification of BCSCs represents a major step forward in elucidation of the BC tumor hierarchy and has started a new era of breast cancer research. Still, in present, there is no uniform approach, which would allow for a quick and simple detection of BCSCs in solid tumors. Therefore, a lot of scientific studies are focused on targeting BCSCs in BC therapy in different ways, using the current knowledge about those cells. For example, BCSCs are characterized by the activation of stemness-related pathways, such as the Notch and Wnt pathways and by the expression of certain stem cell markers. Since CSC are highly resistant to chemotherapy, additional treatment of BC patients with BCSC-specific drugs and inhibitors, which target the Wnt or Notch pathway, respectively, will be required [2].

2. The concept of stem cell hierarchy in the mammary gland

The mammary epithelial tissue forms a highly organized branched bilayered ductal network consisting of basal myoepithelial cells and luminal (secretory) epithelial cells [1, 20]. Distinct markers characterize luminal and basal cells. Luminal cells express cytokeratins (CKs) 8/18 and 19, as well as other molecular markers, such as MUC1, GATA3 and CD24. Basal myoepithelial cells express CK14 (50 kDa), CK5 (58 kDa) and CK17 (46 kDa), as well as smooth muscle actin (SMA) and vimentin [21]. Numerous scientific reports have provided evidence of existence of a much more complex mammary epithelial hierarchy, which is responsible for tissue growth and maintenance during periods of development and homeostasis [20]. Mammary cell proliferation, turnover and tissue regeneration are functions of MaSC [21, 22]. To present the idea in a simplified model, progenitor cell lineages are derived strictly from bi-potent or multi-potent stem cells. Then, they divide and differentiate into the epithelium of adult mammary gland composed of both matured luminal and basal cells [23] (**Figure 1A**). The scientists have identified different subpopulations of cells in human and mouse mammary gland, using cell sorting techniques [20]. Subsets of mammary epithelial cells (MEC) were characterized using different surface markers. Accordingly, CD24 and EpCAM are known to be the luminal cell markers and CD49f and CD29 are the basal cell markers. This diversification is invariably used in classifying of luminal and basal MEC populations.

The perspective of MaSC isolation, which then were be able to give rise to an entire mammary epithelial tree upon transplantation of a single stem cell [24, 25] and the phenotypic identification of several mammary epithelial progenitor cell (MaPC) populations [26, 27], has enhanced our current understanding of the differentiation hierarchy [28]. Furthermore, *in vivo* genetic tracing experiments have shown that both cell types contribute to morphogenesis in puberty and pregnancy and ductal maintenance in the adult gland [28].

To characterize MaSC, a clear distinction between normal stem cells and tumor stem cells must be made. Emerging evidence suggests that normal breast cells, as well as breast cancer stem and progenitor cells, exist in two different states, epithelial-like and mesenchymal-like [27, 29, 30] (**Figure 1B**). Recent studies revealed that in the case of human BCSCs, epithelial-like stem cells express aldehyde dehydrogenase (ALDH$^+$), whereas mesenchymal-like stem cells are characterized by CD44$^+$/CD24$^-$ surface expression [29, 31–33].
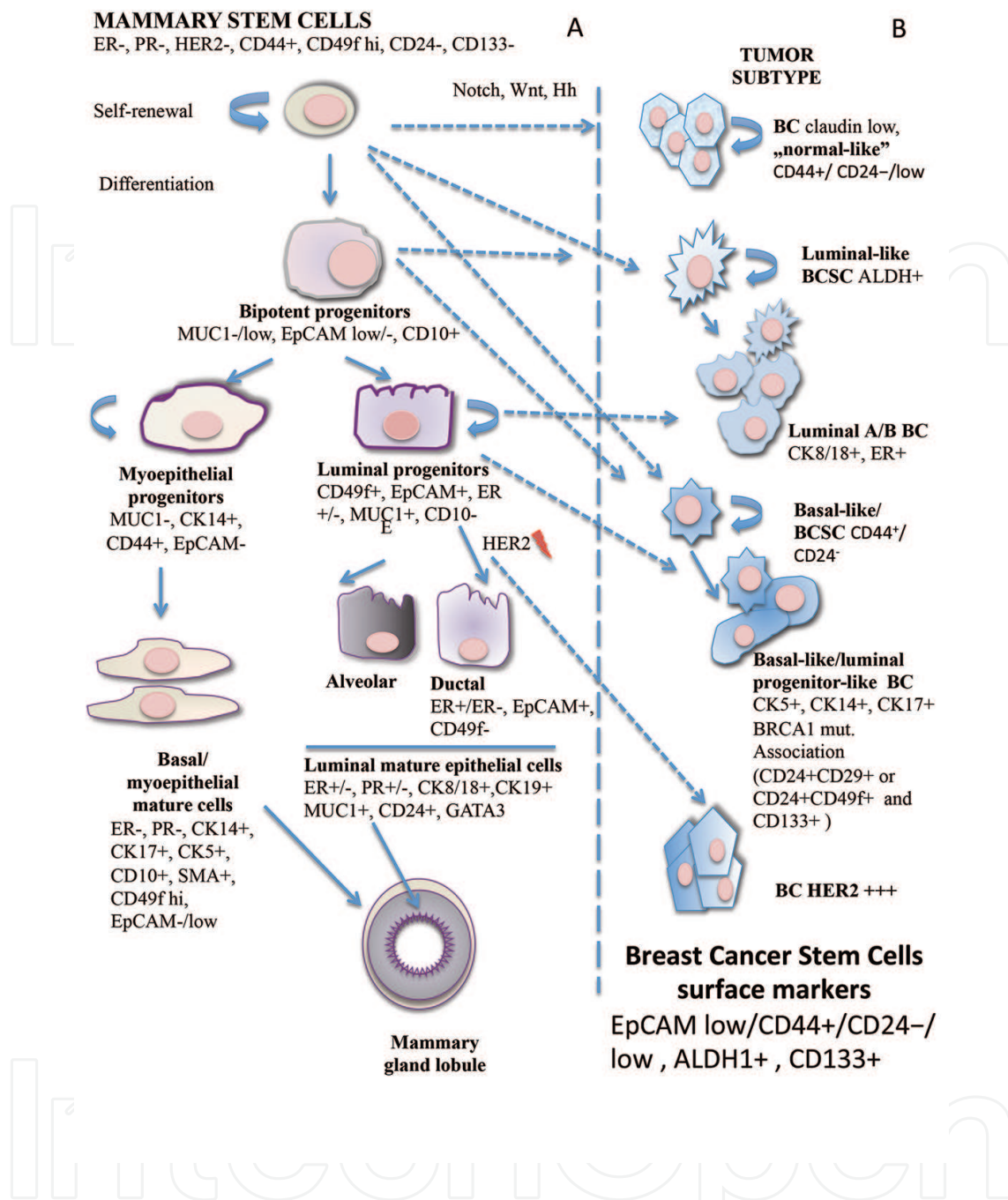
Figure 1. The simplistic draft of hierarchical model of human mammary gland stem cells (A) and correlation of stem cells with breast cancer (BC) subtypes (B). Bi-potent or multi-potent stem cells (with self-renewal ability) give rise to lineage-restricted bi-potent progenitor cells. These progenitors then divide and differentiate into the mature luminal (ductal and alveolar) and basal cells of the adult mammary epithelium. Cells are characterized with expression of different surface markers—which allow for phenotypic identifying of the subpopulations. Normal mammary stem cells (MSC) must be distinguished from tumor stem cells (BCSCs). Deregulation of MaSC self-renewal may contribute to preneoplasia of mammary gland. In particular, deregulation of conserved signaling pathways, such as Wnt, Notch and hedgehog, is linked with oncogenesis. Breast tumors are divided into hypothetical subtypes according to different profiles and different origins of cells. We can find following subtypes: normal-like/claudin low, luminal and basal-like and overexpressing HER2. Luminal progenitors cells (A and B) are mostly associated with good prognosis, those with HER2 overexpressing, also with luminal features, but usually associated with poor survival. Basal-like (the most heterogeneous) origin from luminal progenitors cells and those tumors are the most aggressive and with tendency to exhibit triple-negative phenotype. Additionally, those tumors are highly associated with BRCA1 gene mutations.

3. MaSC and BCSC markers

The approaches to BCSC isolation at present include the following: surface marker sorting, aldehyde dehydrogenase activity assay, flow cytometry sorting side population, etc. [8]. CD44, CD24 and ALDH1 are the most commonly used biomarkers to identify the BCSC fraction [31]. Two proteins, CD44 and CD24, were found in 2003 to be useful markers to distinguish tumor-initiating cells (TICs) from non-tumorigenic cells in BC [2].

CD44 (hyaluronan-binding transmembrane protein) is expressed in different isoforms and can have different glycosylation patterns [34]. Its smallest form (CD44s) is expressed in many cells, whereas its variant forms (CD44v) are particularly found in cancer cells. CD44v is involved in EMT, cellular migration, transendothelial migration and extravasation and it supports many cellular activities required to initiate tumor growth and metastasis [2, 34]. CD24 (heavily glycosylated membrane protein) downregulation may be required to prevent its interference with CD44-dependent invasiveness [35], though the underlying mechanism is not clear since CD24 also has tumor-promoting effects [2, 36]. The gene expression profile associated with CD44$^+$/CD24$^-$ cells was demonstrated to correlate with a worse prognosis in BC [33] and approximately one-third of all circulating BC cells in the blood of BC patients is CD44$^+$/CD24$^-$ [37]. CD44$^+$/CD24$^-$ phenotype of cell surface markers has an increased ability to form tumors in immunosuppressed mice than the bulk of the tumor cells [38]. Maycotte et al. had analyzed CD24 and CD44 expression in MCF7 and MDA-MB-468 cell lines using assay based on flow cytometry. Analysed cells showed different levels of autophagic flux ("autophagic flux" is defined as the activity of autophagic degradation, which comprises autophagosomes formation, transportation of substartes and lysosomal degradation) [39]. CD24 expression was decreased in cells with low autophagic flux in both cancer cell lines. Similar results were obtained in cells expressing shRNA for ATG7 or BECN1, as these cells also showed low expression of CD24, whereas the expression of CD44 remained stable. Presented results indicate that cells with decreased autophagic activity have declined CD24 expression. These results suggest that autophagy can selectively regulate CSC maintenance in autophagy-dependent breast cancer cells. It has been widely predicted that a quality control mechanism, like autophagy, is important for maintaining normal and cancer stem cell homeostasis [7, 38].

Palmer et al. [40] proposed a stem gene pluripotentiality signature as an indicator of the tumor grade in a variety of solid tumors, including BC. In addition to tissue samples, BCSC subpopulations have also been identified *ex vivo* within individual cultured BC cell lines. In triple-negative BC cell lines, CD44$^+$/CD24$^{-/low}$ BCSCs were further classified into two subcategories: the CD44^{high}/CD24$^-$ mesenchymal-like basal B and the CD44^{high}/CD24^{low} epithelioid basal A, which displayed stronger tumor-initiating properties [15].

Recent data suggest that CD44 and CD24 may not be sufficient to distinguish the cancer cell subpopulation with CSC/TIC activity, so other proteins, like ALDH1 (aldehyde dehydrogenase 1) and EpCAM (epithelial cell adhesion molecule), may also be required for cancer cells to develop tumor-initiating potential [2]. Members of ALDH1 family ALDH1A1 and ALDH1A3 are thought to be the most important for stem cell activity in cancer cells [41]. Recently, ALDH1 expression has been linked to the expression of RhoC [15, 42], a GTPase

known to be involved in metastasis. ALDH1-positive breast cancer cells could be identified by the ALDEFLUOR assay and they showed stem-like and tumor-initiating activities [15]. In the abovementioned experiment of Palmer et al. [40], distinct ALDEFLUOR-positive subgroups with stem cell characteristics have been shown to exist in eight BC cell lines and a 413 gene-specific molecular signature characterizing these BCSCs was determined by microarray analysis.

EpCAM, a transmembrane protein, was considered to be a cellular adhesion molecule until it was discovered that it is able to activate c-myc involved in maintenance of stemness [36]. The level of EpCAM expression may be critical for defining stem cells. Recent reports demonstrated that BCSC activity is associated with low EpCAM expression, whereas luminal or basal cells showed either high or no expression of EpCAM, respectively [43].

The aforementioned epithelial-like and mesenchymal-like BCSCs have been shown to interconvert from one type to another, presumably depending on the tumor phase and requirements [31]. The use of CD49f as an additional marker for the detection of BC cells lacking CK8/18/19 expression has been shown to possibly enhance the detection of circulating tumor cells (CTCs) involved in EMT-associated processes, such as drug resistance and metastasis [44]. CD44$^+$/CD24$^-$ cells express epithelial-mesenchymal transition (EMT) genes [17], display a quiescent phenotype and are localized in the tumor periphery, possibly promoting tumor spreading. The characteristic pattern of surface markers expression (CD44$^+$/CD24$^{-/low}$) was found mostly in molecular subtype of breast tumors presenting low expression of claudin. It is accompanied by EMT-associated genes, like N-cadherin, FoxC2 and Zeb [17]. In contrast, ALDH1+ cells are situated within the tumor. They are typical epithelial cells, expressing mesenchymal-epithelial transition (MET) genes and high rate of proliferation, which can influence tumor progression. All these subpopulations are similarly expressing a large number of genes, which were confirmed by high-throughput gene expression profiling (microarray analyses). BCSCs are suggested to have hallmarks of both types of normal MaSCs, epithelial (EpCAM+/CD49f+) and mesenchymal (EpCAM−/CD49f+). According to research results, BCSCs with phenotype ALDH1+/CD44+/CD24- are more aggressive and exhibit big metastatic potential. In the immunosuppressed mice, it was possible to induce tumor growth using just a few ALDH1+/CD44+/CD24- cells [31].

In human breast tumor cells, phenotype CD44$^+$/CD24^{low} is connected with basal-like tumors, in particular with inherited BRCA1 BC. Additionally, the cells are expressing the CD49f marker and their status is CK5/14^{high} EGFR^{high} and ER^{low}, PR^{low}, HER-2^{low}. It is worth noting that basal-like tumors are often linked to poorer prognosis. The occurrence of the CD44+/CD24^{low} phenotype was found to be lower in tumors of luminal type and particularly HER-2+ tumors, irrespective of ER status [11]. Results of a different study demonstrated the presence of BCSC subtypes in a CTCs population, in peripheral blood samples taken from 30 patients. In total number of 1439 CTCs, 35% of the CTCs in 2/3 patients displayed the CD44$^+$/CD24$^{-/low}$ phenotype, while 17.7% CTCs selected in seven patients revealed phenotype ADLH1^{high}/CD24$^{-/low}$ [45].

β1-integrin subunit (CD29) has also been implicated in the phenotypic characterization of BCSCs. It has been shown that BRCA1 mutant cancer cell lines contain CD24$^+$CD29$^+$ or CD24$^+$CD49f$^+$ cells, with increased proliferation and colony-forming ability [15].

In BCTCs epithelial markers expression is routinely detected and therefore, many isolation techniques are based on the use of specific antibodies, like EpCAM and MUC1. For example, for EpCAM identification, the most popular tests are Cellsearch™ system (Veridex LLC, Raritan, NJ, USA) approved by the US Food and Drug Administration, the herringbone chip, the AdnaTest breast cancer detection kit, fluorescence-activated cell sorting (FACS) analysis and the microfluidic technology. Apart from the peripheral blood, BCSCs have also been isolated directly from the primary or metastatic tumors of breast cancer patients [31].

Other techniques used for stem cell isolation are 3D cultivation in cell cultures spheroids. Stem cells are detectable by light microscopy as small and light cells (SLC) and have the ability to maintain DNA staining (using BrdU) due to their low proliferative activity [46]. However, it was shown that only 15% of [^{3}H] thymidine-positive cells are also positive for one of the two stem cell markers $p21^{CIP1}$ or Musahi-1 (MSi-1) [47].

The next marker worth mentioning is CD133 (prominin-1). Hematopoietic progenitors and adult stem cells normally express this transmembrane glycoprotein. It is a well-established melanoma and brain CSC marker. In addition, the expression of CD133 has been also detected in BCSCs and has been associated with resistance to chemotherapy in BC biopsies [48]. Furthermore, distinct CD44$^+$/CD24$^-$ and CD133$^+$ subpopulations with CSC characteristics have been detected in BRCA1 breast tumors, while CD44^{pos}CD49f^{hi}CD133/2^{hi} cells were characterized by xenograft-initiating capacity in estrogen receptor–negative BC [15]. Co-expression of stem (ALDH1) and EMT (TWIST) markers has been demonstrated in CTCs from patients with early and metastatic BC. The majority of CTCs expressing the SC marker CD133 also co-expressed the EMT marker N-cadherin and vice versa. The expression of CD133 in CTCs of BC patients has been suggested to promote chemoresistance [15]. Basal-type breast tumors with elevated SLUG expression were shown to overexpress stem-like genes, including CD133 [20]. Additional studies revealed that BC overexpressing SLUG display increased proportions of CD44$^+$/CD24$^-$ CSCs, suggesting that transcriptional programs induced by SLUG promote stemness [49].

Activation of some genes is proposed to be associated with stem cell phenotypic characteristics, for example, *Sox2* gene (a transcription factor involved in the maintenance of pluripotency of undifferentiated embryonic stem cells) [15]. Activation of this gene is typical for early steps of BC development and characterizes tumors with basal-like phenotype. Increased expression of Sox2 is analyzed as prognostic predictor of BC. Also, mutations in p53 are representative for BC with stem cell-like patterns. It is suggested that loss of p53 function promotes dedifferentiation and is positively selected during tumor progression [15, 50].

4. The role of microenvironment in BC progression: stem cell niche

Stem cell niche refers to a microenvironment in which stem cells reside. The anatomical niche for SC is composed of different compartments [51]. Signals from surrounding cells (stromal cells, a specific type of fibroblast which interacts with the stem/progenitor cells via surface receptors, gap junctions, cytokines, growth factors, hormones, etc.) and extracellular matrix

(ECM) seem to be involved in regulation of SC activity, regulation of SC renewal and survival [1]. Since mammary gland is an endocrine-responsive organ, many hormonal factors are analyzed also in connection with stem cells, for example, the biological influence of E2 and P on the compartment of stem and progenitor cells is largely unknown. However, it is assumed that the stem cells are estrogen receptor (ER) negative, whereas the progenitor cells are ER positive [2]. The role of BRCA1 gene in human ER⁻ stem/progenitor cell differentiation into ER + luminal epithelial cells has been revealed in the latest scientific findings [11]. ER⁻ stem cell transition into ER⁺ progenitor cells is precluded by BRCA1 deletion. Studies demonstrated that women with heterozygous mutations in the BRCA1 gene are more susceptible to breast and ovarian cancers and the tumors formed were mostly of basal-like phenotype, showing characteristic deficiency of ER, PR and HER-2 receptors.

As mentioned above, deregulation of the microenvironmental homeostasis of normal SC is suggested to contribute to their neoplastic transformation [52]. The activation of the EMT program has been associated with the acquisition of SC traits by normal and neoplastic cells [15]. Transcription factors involved in EMT (*e.g.* Snail, Twist and Zeb) have also been found to induce SC properties in human mammary carcinoma cells [15]. Environmental cues from signaling molecules, which induce EMT in BC such as IL-6, can promote pluripotency in breast cancer cells *via* a positive feedback loop including NF-kB, Lin28 and Let-7 miRNA [15].

5. miRNA and stem cells in breast cancer

MicroRNAs are negative regulators of genes, repressing expression at the posttranscriptional level [53]. They also regulate various properties of CSC, including self-renewal, differentiation, proliferation and fate determination, by affecting several key signaling pathways at the molecular level. Many different miRNA families have already been connected with suppressing/promoting cancer cells. For example, miR-125a is known tumor suppressor in bulk tumor cells of BC origin [53, 54]; however, it has been shown that miR-125a plays a different role in breast epithelial SC, which is cancer promotion [53]. MicroRNA profiling of BCCSs indicated that miR-200c, miR-203 and miR-375 expression was significantly inhibited, whereas the expression of miR-125b, miR-100, miR-221 and miR-222 was most notably enhanced [55]. Expression analysis of miRNAs in both normal mouse and human mammary tissue has revealed three conserved clusters of miRNAs, miR-200C-141, miR-200b-200a-429 and miR-183-96-182, that appear to be downregulated in MaSC and putative BCSCs [56, 57]. In humans, miR-93 level was significantly higher in luminal progenitor cells than in the MaSC-enriched population and overexpression of this miRNA biased these cells toward a luminal fate [58].

MiR-200 family serves as a key mediator of CSC due to its prominent role as an EMT regulator. These family members are downregulated in BCCSs due to epigenetic alternation, in comparison with non-tumorgenic cancer cells [59]. Downregulation of miR-200 expression expands the SC compartment and promotes BC progression. The tumor suppressor p53, which can activate miR-200c by direct binding to miR-200c promoter sites, is reported to regulate both EMT and CSCs [60]. Similar results were obtained in the case of miR-22, a strong inhibitor of miR-200 promoter demethylation, which is connected with tumor invasiveness and

metastatic properties [59]—therefore, miR-22 is a crucial epigenetic modifier and promoter of EMT and cancer stemness toward metastasis [61]. In addition to miR-200 family, miR-21 and MiR-302/369 have also been proposed to regulate EMT and CSC. In BC, the depletion of miR-21 expression leads to reversal of EMT and decreased CSC numbers through inactivation of AKT/ERK pathway [60]. MiR-302/369 cluster members can directly target EMT genes, like TGF-beta receptors or the RhoC and the downregulation of miR-302/369 promotes the switch of fibroblasts into somatic stem cells [60].

miRNAs can also regulate the breast cancer cell interactions with other cells by affecting certain genes, for example, Tac1 gene, linked to BC, regulates breast cancer cell interaction with the mesenchymal stem cells. Three miRNAs—miR-130a, miR-206 and miR-302a—have been shown to regulate Tac1 expression and their action against Tac1 may affect quiescence of breast cancer cells in the marrow cavity [11].

6. Signaling pathways regulating MaSC and contributing to the etiology of breast cancer

Wnt (wingless), Hh (hedgehog), Notch and BMP/TGF-β (bone morphogenetic proteins/transforming growth factor β) signaling pathways contribute to the self-renewal of stem and/or progenitor cells in a variety of organs. When deregulated, these pathways can contribute to oncogenesis [59].

The Notch pathway has been shown to play a particular role in MaSC expansion [62, 63] and promotes BC progression by supporting EMT [11, 64]. Overexpression of the Notch pathway components has been linked to decreased survival of BC patients [65]. In a large proportion of BCs, epigenetic mechanisms that activate Notch signaling were related to the role of miR-146a, which targets NUMB, a negative regulator of Notch [59]. Inhibition of Notch1 with specific antibodies significantly reduced the CD44$^+$CD24$^{-/low}$ subpopulation (BCSCs) and diminished the incidence of brain metastases from BCC.

β-Catenin, a downstream target of Wnt signaling pathway, has been identified as a crucial survival signal for MaSC and a balance modulator between differentiation and stemness in adult stem cell niche in the mammary gland [59]. Overexpression of Wnt in mouse mammary glands can also lead to increased mammary tumor formation. Such tumors contain cells of both basal/myoepithelial and luminal phenotypes, suggesting an origin from a common precursor [11, 59].

In the hedgehog pathway, Patched (PTCH) transmembrane protein is a receptor for the hedgehog family of signaling molecules (Sonic-Shh, Indian-Ihh and Desert-Dhh) [59] and has been connected to early embryonic tumorigenesis [11]. PTCH constitutively represses Hh pathway activity through its interaction with a transmembrane protein Smoothened (SMO) [59]. Overexpression of these pathway components, that is, Shh, Ptch1 and Gli1, has been found in majority of human BCs.

Furthermore, studies demonstrated that EMT stimulation by TGF-β co-occurs with BCSC formation [66]. BCSCs with CD44$^+$/CD24$^{-/low}$ phenotype show increased expression of many

genes which are known to be TGF-β targets and they are typical for mesenchymal and migratory cell type. In one of the experiments, when MDA-MB-231 cells (model of BC) were injected to athymic mice, the change in TGF-β actions was observed. The cancer-promoting actions (tumorgenic and metastatic) of TGF-β were counteracted by BMP7 or BMP2/7 heterodimer [59], which diminished Smad signaling pathway activity and increased cancer cell invasiveness. Additionally, the activity of pro-survival and anti-apoptotic pathways is often increased in CSCs. Typically, for example, JAK/STAT pathway is highly activated [59].

7. Ways of targeting cancer stem cells: pharmacological agents

Although targeting BCSCs brings hope for future treatment of BC and is widely tested on the basic research level, a disproportionally limited number of clinical trials evaluating the effect of treatment on the expression of BCSC biomarkers are in progress [31].

Among the tested treatment approaches are those regulating the activity of signaling pathways. The targeting of BCSCs involves the disruption of BCSC survival signaling pathways (i.e., Notch, HER2, hedgehog, Wnt, PI3K/Akt/mTOR, interleukin 8, TGF-beta) [31]. Targeting Notch signaling has become a promising field in the treatment of stem cells in breast cancer. By inhibiting the Notch pathway, the CSC population can be reduced along with improved responses to chemotherapy [67]. Several inhibitors of Wnt signaling molecules are under investigation with reference to several cancers [68]. For example, inhibition of the Notch signaling pathway by γ-secretase inhibitors (GSI) has been shown to reduce the pool of BCSCs [15, 62]. GSI and other drugs that interfere with the Notch pathway are currently under consideration as new options to treat BC [65]. Because there is a link between the Notch and Her2-dependent pathways [69], blocking either of them was found to affect CSC survival. Hence, Her2 inhibitors, such as trastuzumab, may be potential additional drugs suitable for targeting CSC [70]. Several scientific groups have exploited cyclopamine (SMO signaling inhibitor), to inhibit the Hh cascade, thereby inhibiting the growth, invasion and metastasis of breast, prostatic, pancreatic and brain malignancies both *in vitro* and *in vivo* [71]. PKF118-310, an inhibitor of Wnt signaling pathway, was recently reported to eliminate BCSCs in a HER2 overexpressing mouse model. Vismodegib, GDC-0449, a hedgehog inhibitor, can block tumor growth in tamoxifen-resistant BC xenografts [31]. Everolimus (RAD001), an inhibitor of PI3K/Akt/mTOR pathway, halted tumor growth of SC in primary breast cancer cells and cell lines and was particularly effective when administered in combination with docetaxel [72].

The resistance of BCSCs to chemotherapeutic drugs leads to the reconstitution of the initial tumor cell population and disease progression [15]. Conventional therapies targeting the tumor bulk have proven insufficient for the eradication of CSC. For example, conventional therapies based on mitotic interference of taxanes (paclitaxel and docetaxel) [73] do not target the subpopulation of quiescent CSC in a tumor. Bhola et al. [74] reported that paclitaxel increased IL-8 expression by autocrine TGF-β signaling and enriched CSC. Interestingly, Gupta et al. reported that SAL, a polyether antibiotic widely used in veterinary medicine, is a potent agent able to selectively target BCSCs and to inhibit mammary tumor growth in vivo [43]. Since autophagy promotes the maintenance of BCSCs [75], SAL can inhibit autophagy

and lysosomal proteolytic activity in both BCSCs and cancer cells [76]. It also acts as an inhibitor of potassium ionophore in Wnt signaling.

Another therapeutic approach is blocking the ABC transporters expressed in most CSC [13]. For instance, tyrosine kinase inhibitors (TKIs) act by binding to ATP and preventing it from binding to the ATP-binding site of several oncogenic tyrosine kinases. It has been reported that some TKIs, such as nilotinib (Tasigna), can efficiently reduce the activity of ABCB1 and ABCG2 transporters. Apatinib (YN968D1) was tested on breast cancer cell lines and in xenograft models of breast cancers overexpressing ABCG2 and/or ABCB1. In combination with paclitaxel, it significantly increased the activity of paclitaxel in the animal models. The therapeutic use of ABC transporters inhibitors has failed so far because of the toxicity issues [13].

One of the most recent innovative approaches in breast cancer therapy is the recruitment of normal stem cells for the eradication of tumor cells. It has been pointed that mesenchymal stem cells (MSCs) have "tumor tropism," which means that they show the ability of migration not only toward the sites of inflammation or injury, but also importantly to the tumor microenvironment.

Other tested options include the following: targeting of CSC metabolic pathways, the use of miRNAs, the use of small inhibitors as salinomycin, cancer immunotherapy, drugs involved in the treatment of noncancer diseases and nanotechnology (nanodrugs can easily accumulate within tumor sites due to their enhanced vascular permeability) [31].

8. Conclusions

Scientific findings from breast cancer studies have revealed that the SC content in breast tumor correlates with its invasiveness and the outcome of the disease. The resistance of BCSCs to chemotherapeutic drugs and other conventional BC therapies has led scientists to move toward establishment of novel therapeutic approaches. Current knowledge about BCSC characteristics and regulators still allows only for evaluation of those therapies on an experimental level of preclinical studies. The most efficient cancer treatment protocols remain to be established on the basis of simultaneous targeting of BCSCs and bulk tumor cells. Therefore, there is still a great need for profound studies, which would extend our knowledge about stem cells and the interplay between these cells and tumor microenvironment. Looking at the practical aspects of BCSC usage one of the biggest challenges that still need to be resolved is the isolation of their population from the patients' blood.

Abbreviations List

ABC transporters	ATP-binding cassette transporters
AKT/ERK	Protein kinase B/extracellural signal-regulated kinase
ALDH	Aldehyde dehydrogenase

ATG7	Autophagy-related protein 7
BC	Breast cancer
Bcl-2	B-cell lymphoma 2 protein
BCSCs	Breast cancer stem cell
BECN1	Beclin1
BMP/TGF-β	Bone morphogenetic proteins/transforming growth factor β signaling pathway
BMP2	Bone morphogenetic protein 2
BMP7	Bone morphogenetic protein 7
BRCA1	Breast cancer 1 gene
CD133	Prominin-1
CD24	Cluster of differentiation 24
CD29	Integrin beta 1
CD44	Cell surface glycoprotein/hyaluronan-binding transmembrane protein
CD49f	Integrin alpha 6
CK	Cytokeratin
CMIC	Cancer metastasis-initiating cell
CSC	Cancer stem cells
Dhh	Desert signaling molecule (hedgehog family)
E2	Estrogen
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting
FoxC2	Forkhead box protein C2
GATA3	Trans-acting T-cell-specific transcription factor GATA-3
Gli1	Glioma-associated oncogene homolog 1 (zinc finger protein)/glioma-associated oncogen1

GSI	γ-Secretase inhibitors
HER-2	Human epidermal growth factor receptor2/ERBB2
Hh	Hedgehog signaling pathway
Ihh	Indian signaling molecule (hedgehog family)
IL-6	Interleukin 6
JAK	Janus kinase
Let-7	Let-7 family, microRNA precursors
Lin28	Lin28-homolog A
MaPC	Mammary epithelial progenitor cell
MaSC	Mammary stem cells
MCF7	Human breast adenocarcinoma cell line (Michigan Cancer Foundation-7)
MDA-MB-468	Breast adenocarcinoma cell line/ATCC HTB-132; triple negative
MEC	Mammary epithelial cells
MET	Mesenchymal-epithelial transition
MSi-1	Musahi-1
mTOR	Mammalian target of rapamycin kinase
MUC1	Mucin 1
NF-kB	Nuclear factor kappa light-chain-enhancer of activated B cells
P	Progesterone
PR	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
PTCH	Patched transmembrane protein
RhoC	Small signaling G protein, Ras homolog gene family, member C
SC	Stem cells
Shh	Sonic signaling molecule (hedgehog family)
shRNA	Small hairpin RNA
SLC	Small and light cells
SLUG	Snail2 and zink finger protein
SMA	Smooth muscle actin

SMO	Smoothened transmembrane protein
Sox2	SRY—(sex determining region Y)—box2
Src	Protooncogene non-receptor tyrosine-protein kinase Src
STAT	Signal transducers and activators of transcription
TGF-beta	Transforming growth factor beta
TIC	Tumor-initiating cells
TKIs	Tyrosine kinase inhibitors
TWIST	Twist-related protein and transcription factor
Wnt	Wingless signaling pathway
Zeb	Zink finger transcription factor

Author details

Joanna Magdalena Zarzynska

Address all correspondence to: joanna_zarzynska@sggw.pl

Department of Food Hygiene and Public Health Protection, Faculty of Veterinary Medicine, Warsaw University of Life Sciences-SGGW, Warsaw, Poland

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