

## Review

# Breast cancer stem cells: a moving target for cancer nanomedicine

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## Abstract

The identification of so-called cancer stem cells (CSCs) has sustainably changed our views on cancer by adding hierarchical principles, where tumor cells emerge from a founder population similar to steady-state regenerative processes in normal tissues. The rare founder population of CSCs is thought to be responsible for the recurrence of treatment-resistant tumors and metastatic spread and thus has been declared as the number one target for the next generation of anti-cancer drugs. Here, we will review the state of the art in research on breast cancer stem cells (BCSCs), for which a huge amount of data has accumulated in the past few years. Initial studies have suggested that the CD44+/CD24- profile and epithelial-to-mesenchymal transition (EMT) are associated with BCSCs, which has resulted in the recent identification of first compounds with BCSC-eliminating properties. In this early phase, however, it remains mostly unclear, to which extent these new compounds may exert toxicity to normal stem cells, since a substantial part targets molecular pathways critical for normal stem cell function. Moreover, these new drugs often require combination with conventional chemotherapeutics potentially posing new challenges to nanomedicine in

circumventing toxicity and enabling targeted delivery. Most recent data further suggests that normal breast cancer cells might be able to re-create BCSCs and that additional, yet undiscovered kinds of BCSCs may exist. This points to future escape mechanisms. As a consequence, another broad future field of nanomedicine might be finding new drugs via systematic screening approaches. Collectively, this area provides ample possibilities for both traditional and novel nanomedical approaches.

**Keywords:** breast cancer stem cells; cancer nanomedicine; cancer stem cells; cancer treatment.

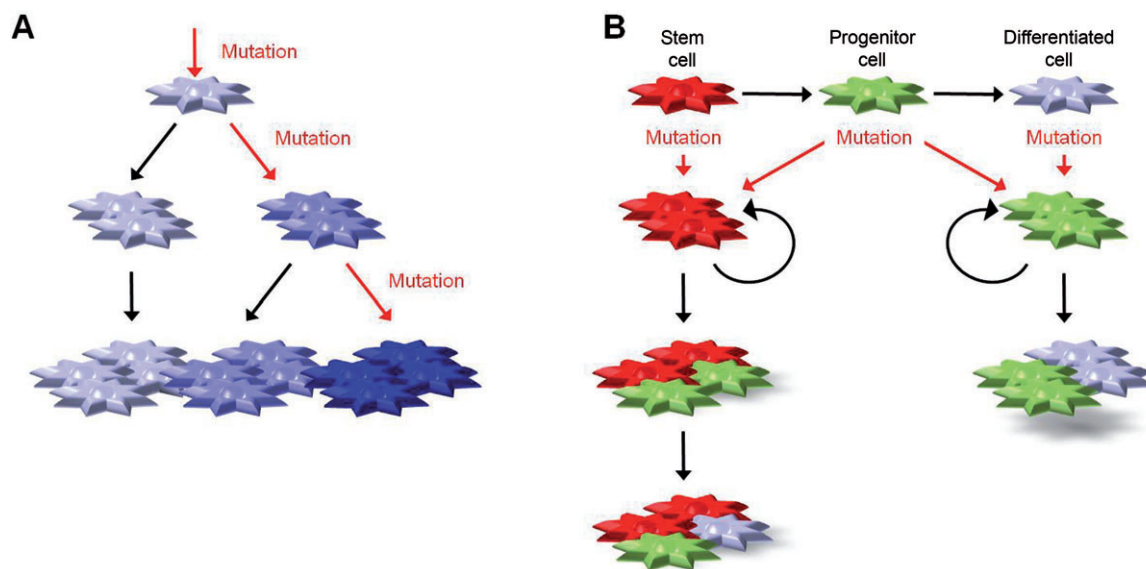
## Introduction

Cancer remains a global burden with 7.6 million (13%) of all deaths in 2008 worldwide (1). Standard therapies such as surgery, radiation- and chemotherapy have led to steady progress in cancer treatment but encounter limitations with regards to treatment resistance, recurrent metastatic cancer, and personalization of cancer therapy. Nanomedicine, via modulation of size and surface properties at the nanoscale, aims at generating improved therapeutic and diagnostic devices. Traditional nanomedicine approaches, using passive targeting mechanisms, have proven useful to enhance the efficacy of conventional drugs, for example by allowing the delivery of increased doses of doxorubicin to tumors as liposomal formulation in Doxil [reviewed in (2)]. New future challenges, however, are posed by the knowledge accumulating in the past few years.

Heterogeneity, not only between tumors of the same type in different patients, but also of cancer cell populations within the same tumor of an individual patient is a well-known phenomenon. One of the consequences of intratumoral heterogeneity is that only a small fraction of about 2% of the cancer cells may have invasive potential and probably even less may finally result in the successful establishment of metastases. Traditionally, the heterogeneity within a tumor has been thought to result from the acquisition of mutations and clonal evolution of co-existing cancer cell populations (Figure 1). This model has had some shortcomings, however. Acquiring an invasive/metastatic phenotype *a priori* is not necessarily subjected to positive selection as it does not offer immediate advantages to the tumor compared to enhanced proliferation and angiogenesis. Similarly, there is no forcing need for the cancer cells to develop drug resistance unless a drug is applied. Another conceptual hurdle has been posed by the fact that epithelial cells commonly have a rapid turnover, leaving a relatively small time window to acquire multiple genetic changes for conversion to cancer cells.

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**Figure 1** Cancer stem cell vs. clonal selection concept. A) The clonal selection theory assumes that a given cell gains tumorigenic potential by initiating mutations. Further consecutive mutations give rise to heterogeneous tumors, in which subpopulations of cancer cells may coexist with different molecular profiles that offered growth and survival advantages. B) The cancer stem cell theory suggests that mutations acquired by different cell types within the normal regenerative hierarchy may produce heterogeneity and different cancer cell compositions. Mutations in a stem cell may give rise to heterogeneous tumors that in part retain differentiation potential, but have a dominant stem cell phenotype due to an expanded, self-renewing stem cell population. Mutations in progenitor cells could theoretically lead to conversion to stem cells or, alternatively, give rise to an expanded progenitor population that still reflects differentiation to a certain degree but has a dominant progenitor component. Mutations in differentiated cells may convert them to progenitor cells, which are self-expanding and possibly maintain some potential for differentiation. Theoretically, differentiated cells may also convert to stem cells by mutation (not shown), which, however, would require more extensive reprogramming. The cancer stem cell concept does not rule out the clonal selection theory as the accumulation of further mutations may result in clonal selection at any stage.

The so-called cancer stem cell (CSC) concept does not oppose the clonal selection theory, but provides important expansions. Normal tissues undergo steady-state regeneration, which is directed by hierarchical mechanisms. Starting point are adult stem cells, which are capable of both self-renewal and asymmetric division. The latter gives rise to so-called progenitor cells, which are partly committed, i.e., can only produce certain cell lineages, which finally comprise the differentiated cells. Self-renewal attaches a long lifespan to the stem cells, providing a larger time window for critical mutations to accumulate. The two major possibilities are that either an adult stem cell directly acquires a critical cancer-causing mutation (3–5) or that one of its descendants first acquires changes that allow for self-renewal, followed by cancer-causing mutations as discussed further below. The result is a cancer cell, which shows the properties of self-renewal and asymmetric division, i.e., a CSC. As the CSC continues to produce offspring in a hierarchical fashion, this adds to intratumoral heterogeneity (Figure 1).

A hierarchical organization with few percent of stem cell-like cells within a tumor may explain why it is only a small subset of the cancer cells giving rise to metastatic spread. It is well known from the hematopoietic system that stem cells can home to the bone marrow. Vice versa bone marrow-derived stem cells have also been found to migrate actively to sites of tissue destruction in other organs, where they can give rise to non-hematopoietic structures (6–9), and stem/progenitor cells

within a given organ can be recruited to predefined sites and/or sites of tissue damage (10–12). Collectively, this means that stem cells per se have an inherent migratory and invasive potential, reflecting their natural role in regenerative processes.

The so-called side population of cells has been identified based on increased efflux of the Hoechst 33342 dye and has been proposed to coincide with the hematopoietic stem cell population (13). Consecutive studies indicated that side populations mark also stem cells in other tissues, including the mammary gland, as well as in different tumors. The multidrug-resistance gene ABCG2, a transmembrane transporter for various compounds, is responsible for dye efflux and accordingly has been considered as one of the first CSC markers (14–18) because dye efflux characterizes the side population. The prevalence of such transporter systems in the membrane of stem cells and CSCs represents one factor contributing to the high level of resistance to conventional anti-cancer drugs. A second factor is the low division rate of normal stem cells (NSCs) and CSCs, as many conventional drugs preferentially act on rapidly dividing cells. Stem cells represent a slow-dividing or quiescent population and, for example in the mammary gland epithelium, express the cell cycle inhibitor CDKN1A, coding for the tumor suppressor p21 (19–21). In fact, there are examples, suggesting that CSCs are not eliminated by standard chemotherapeutic drugs, but even expanded (22–24).

This is actually also reflected at the level of resistance to irradiation. Both NSCs and CSCs have been demonstrated to display enhanced radioresistance and irradiation caused a selective expansion of the CSCs (25–28). Seemingly, this is based on a more efficient DNA repair response in NSCs and CSCs (28–31). Further, there are some first indications that the activation of a DNA damage response and of DNA repair mechanisms might be coupled to the stem cell state. BRCA1 has been shown to play a role in the regulation of mammary stem/progenitor cell fate and its inactivation results in the accumulation of progenitor cells (32, 33), while Oct4 and Sox2, two decisive proteins in the maintenance of stemness, are activated by a complex of DNA repair proteins, which turned out to be necessary for the pluripotency of embryonic stem (ES) cells (34).

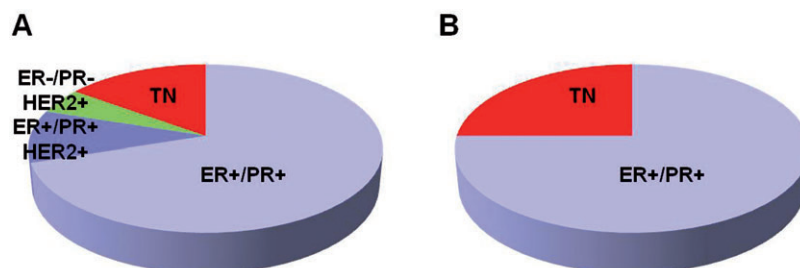
The shortcomings of conventional cancer therapies thus can, at least in part, be explained by the existence of a special, rare subpopulation of cancer cells with stem cell-like properties. Besides capability for self-renewal and asymmetric division, the CSCs share other features with NSCs, including migratory/invasive potential, which is related to metastatic spread, and quiescence, resulting in decreased sensitivity to chemo- and radiotherapy. Chemo- and radiotherapy in turn may result in an undesirable expansion of the CSCs. This is highly reminiscent of processes taking place in wound healing after tissue damage as has been exemplified for bone marrow-derived hematopoietic stem cells (35). To efficiently eliminate the CSCs and to prevent their expansion may require new drugs and novel drug designs. However, as discussed in this review, using breast cancer stem cells (BCSCs) as the example, there are some particular hurdles to be overcome. This includes generating the required specificity to not cause damage to NSCs and the still increasing gain of basic knowledge with regards to CSCs, indicating that these cells might be more heterogeneous or flexible than originally anticipated.

## Breast cancer subtypes

For the discussion of BCSCs and the design of BCSC-targeting nanodrugs, it is of critical importance to be aware of the existence of different breast cancer subtypes, potentially emerging from and containing different kinds of stem/progenitor

cells. Traditionally, i.e., based on immunohistochemical techniques, breast tumors have been subdivided into estrogen/progesterone receptor positive (ER+/PR+), HER2/Neu positive (HER2+), and triple-negative (TN), i.e., ER-/PR-/HER2-subtypes. This distinction has proven useful for prognosis and choice of treatment strategies. ER+ breast cancer is treated with drugs antagonizing estrogen action, such as tamoxifen, fulvestrant, or aromatase inhibitors, while targeted therapy with antibodies against HER2 is the treatment of choice for HER2+ breast cancer, whereas TN breast cancer is treated by chemotherapy (36). TN breast cancer comprises about 15% of the cases, while ER+ breast cancer is the most common type, accounting for about 80% of the breast cancer cases. About 15% of the tumors are HER2+ breast cancer, which partly overlap with ER+ breast cancer, i.e., 10% of the total breast cancer cases are ER+/PR+ and HER2+, while 5% are ER-/PR-/HER2+ (Figure 2) (37, 38). ER+ breast cancer is associated with an overall 5-year survival of 90%, compared to 80% for TN breast cancer (36–38), with distant metastases representing the major cause of death (36). For the HER2+ breast cancers, the overall 5-year survival rates compare to these of the ER+ and TN breast tumors, depending on whether the HER2+ tumors are hormone receptor positive or negative, respectively. Based on the less favorable prognosis, TN breast cancer is commonly defined as the more pressing clinical problem. However, in numerical terms ER+ breast cancer causes about 3-fold more cancer deaths than TN breast cancer, i.e., an estimated 23,050 vs. 8644 breast cancer deaths (Figure 2), if extrapolated from the estimated numbers for 2011 in the US (36).

mRNA expression profiling has led to a more sophisticated definition of breast cancer subtypes based on their molecular profiles. The first study, analyzing the levels of about 8000 mRNAs in breast tumors from 42 patients, resulted in a distinction of the former TN breast cancer into basal-like and normal-like breast cancer, while HER2+ and ER+ breast cancer comprised separate subtypes, of which the latter was referred to as luminal (i.e., epithelial)-like subtype (39). The luminal subtype has consecutively been defined further to consist out of a luminal A and a luminal B subtype, based on the analysis of larger numbers of breast cancer cases (40). Herschkowitz and colleagues identified later another basal-like breast cancer subtype, called the claudin-low subtype, characterized by



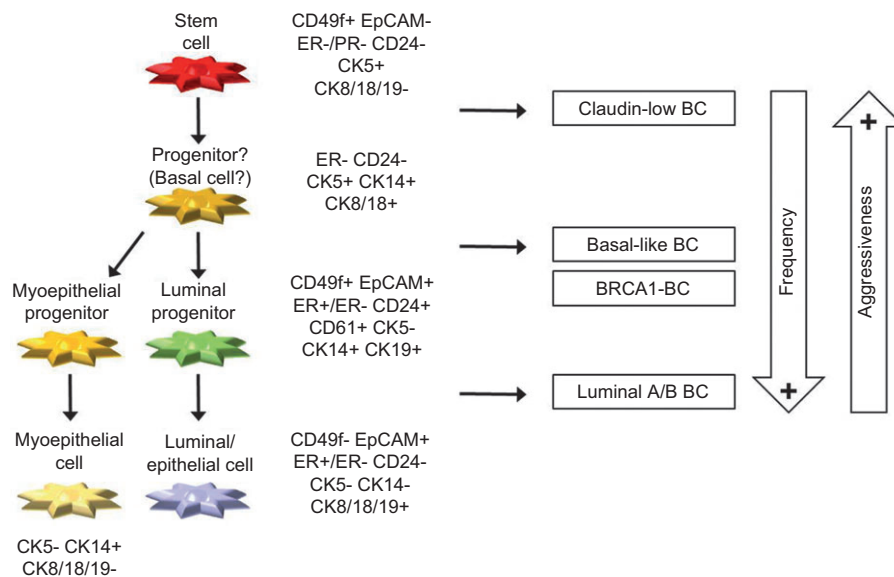
**Figure 2** Frequency of breast cancer types and contribution to mortality. A) ER+/PR+ breast cancer comprises about 80% of the cases, including 10%, which are additionally positive for HER2 (HER2+). Another 5% of the cases are ER-/PR-/HER2+, while about 15% are ER-/PR-/HER2- (i.e., triple negative; TN). B) While ER+/PR+ breast cancer has a better prognosis than TN breast cancer, it accounts for about 75% of the mortality from breast cancer compared to an approximately 25% contribution of TN breast cancer.

low expression of Claudins 3, 4, 7, Occludin, and E-cadherin and the presence of additional lymphocyte and endothelial signatures (41).

### Relation of breast cancer subtypes to the mammary stem cell hierarchy

The classification into claudin-low, normal-like, basal-like, HER2+, luminal A, and luminal B breast cancer subtypes has assisted in developing first models, relating breast cancer to the hierarchical events driven by stem cells in the normal mammary gland. This allowed assigning the breast cancer subtypes to individual stem and progenitor cell types (Figure 3) (32, 33, 42). Transplantation experiments in mice first suggested that the dye-excluding side population, comprising 0.2%–0.5% of the cells in the mammary gland, contains mammary stem cells capable of regenerating mouse mammary glands upon transplantation of about 2000–5000 cells. However, it seemed that mammary gland cells depleted for the side population likewise retained regenerative potential (17). Shackleton and colleagues (43) used fluorescence-activated cell sorting (FACS) with defined cell surface markers for the separation of the mouse mammary gland cells. These and consecutive studies identified a cell population positive for CD24 and with high levels for integrin  $\alpha 6$  (CD49f) and

integrin  $\beta 1$  (CD29), i.e., CD24<sup>+</sup>/CD49f<sup>hi</sup>/CD29<sup>hi</sup> cells, as the mammary stem cells (43, 44). According to these analyses, a single cell is sufficient for regeneration of a fully operative mammary gland, and – unlike the majority of the breast cancers – these cells lack ER as well as PR and additionally are HER2-, while expressing the epidermal growth factor receptor (EGFR). Collectively, the data led to the conclusion that basal-like breast cancer emerges from mammary gland stem cells (44). The scheme was slightly revised based on studies of BRCA1 mutated breast tumors. Mutations in BRCA1 were previously known to compromise DNA double strand repair and to exert a strong predisposing effect for familial breast cancer (45–47). The analyses indicated that BRCA1 surprisingly is also involved the fate decision of mammary stem cells because BRCA1 mutant breast tissues contained a significantly increased number of integrin  $\beta 3$  (CD61) positive luminal progenitor cells (32, 33). This not only linked DNA-repair to stem cell processes, but also resulted in a reclassification. The basal-like breast tumors are now thought to emerge from luminal progenitors and not from mammary stem cells (also often referred to as basal cells), as suggested by the authors and supported by comparing the genome-wide mRNA expression profiles of breast cancer subtypes with sorted mammary gland cell populations (33, 48). According to the present scheme, the claudin-low breast cancer subtype is closest related to the mammary stem cell, followed by what



**Figure 3** Relationship between stem cell hierarchy and breast cancer subtypes [adapted from ref. (33)]. The mammary stem cell may give rise to an intermediate, which some authors referred to as “committed progenitor” (33). This type possibly corresponds to what others referred to as “basal cell”, but the distinction is somewhat unclear up to now, as well as the distinguishing marker profiles. Alternatively, the two cell types could also represent the quiescent and the activated state of the mammary stem cell. According to molecular profiles and marker characteristics, the claudin-low breast cancer (triple-negative) subtype may emerge from this stage because it is closest related to the mammary stem cell profile. At the next level, the tree diverges into myoepithelial and luminal progenitors. The latter are thought to represent the cell type, from which other triple-negative, i.e., other basal-like and BRCA1 deficiency-caused breast cancer subtypes emerge. According to this scheme, luminal breast cancers result from more differentiated cells. The tumors can perhaps be considered as being trapped at different locations in this linear hierarchy. The model creates a match between the relative abundance of the cell types in the mammary gland and the frequency of the breast cancer subtypes, i.e., an increase from top to bottom. The aggressiveness of the tumors may inversely be correlated. Various marker combinations have been used in past studies and the exact configurations are still somewhat elusive because other studies, for example, identified CD24<sup>+</sup>/CD49f<sup>hi</sup>/CD29<sup>hi</sup> cells as mammary stem cells (43, 44).



has been designated as the “normal-like” breast cancer subtype. Basal-like breast cancer, including the BRCA1-mutated tumors emerge from the luminal progenitor cells, while luminal breast cancer type A and B derive from stages between luminal progenitor and mature luminal cells (Figure 3). As a consequence also some breast cancer cell lines have been reclassified to claudin-low tumors according to their expression profiles. It is, however, not yet quite clear, where the HER2+ breast cancer subtype fits into this scheme.

Traditionally, cytokeratins have been used as markers for discerning between the different populations (49–53). According to this, mammary stem cells are positive for cytokeratin 5 (CK5+) but negative for luminal cytokeratins (CK8/18/19-) and may develop via a double-positive intermediate (CK5+ and CK8/18/19+), which in addition expresses the myoepithelial cytokeratin 14 (CK14+) to cells of the myoepithelial (CK5-, CK8/18/19-, CK14+) and the luminal lineage (CK5-, CK8/18/19+, CK14-; Figure 3).

Hence, human breast cancer subtypes could be classified according to their molecular match to the different developmental stages of the mammary gland hierarchy, which was enabled by molecular dissection of the mammary gland stem and progenitor cells. The claudin-low breast cancer subtype locates closest to the mammary stem cell, while luminal breast cancer resides closest to the mature luminal cell type. This model is in good agreement with the frequency of the breast cancer subtypes. The frequency is lowest for claudin-low breast cancer, representing a less frequent subclass of the 15% TN breast cancers and thought to match best to mammary stem cells that have an estimated frequency of <1% in normal tissue. Luminal breast cancer makes up 80% of the cases, which corresponds to the high prevalence of luminal progenitors and mature luminal cells in the normal mammary gland. The correlation would fit both stem cell models of the emergence of breast cancer. Rare cell types would be predicted to give less frequently rise to tumors, supporting the direct emergence of CSCs from NSCs. However, a conversion event, which sets the cells back one level, e.g., mature luminal cells towards the direction of luminal progenitors, would be expected to be more likely than a conversion of mature luminal cells to mammary stem cells, because the latter would require more drastic molecular changes and reprogramming. In general, however, the subdivision indicates that the aggressive claudin-low breast cancer represents a considerable small fraction of breast cancer cases. Lessons learned from PARP-inhibitors, primarily targeting the BRCA1-negative breast cancer patient group, which is equal to or larger than the claudin-low group, taught that such small patient groups provide extraordinary hurdles for drug development in late clinical phases (54).

### Discovery and general properties of breast cancer stem cells

Back in 2003, Al-Hajj and co-workers reported that breast tumors contain breast cancer cell populations with different tumorigenic potential upon transplantation into mice

(55). Cells sorted from 9 different breast tumors, including 8 metastases and 1 primary tumor, efficiently created tumors in mice, when either positive for CD44 (CD44+) or negative for CD24 (CD24-). Among the CD44+/CD24- cells, epithelial cell adhesion molecule positive cells (EpCAM+, also referred to as ESA+) retained tumorigenic potential, while EpCAM-/CD44+/CD24- cells did not. Only about 2% of the breast cancer cells displayed the EpCAM+/CD44+/CD24- profile, which had an estimated 50-fold enrichment for BCSCs, indicating that the BCSCs are similar scarce in tumors as the NSCs in the normal mammary gland. The study pinpointed the two main criteria still used nowadays for BCSCs. Firstly, tumors resulting from EpCAM+/CD44+/CD24- cells created also EPCAM-, CD44-, and CD24+ cells, indicating asymmetric division events and potency to give rise to heterogeneous offspring. Secondly, the EpCAM+/CD44+/CD24- cells could be isolated from the tumors grown in mice and gave rise to tumors with the same properties upon serial transplantation, suggesting that they possess self-renewal activity. Unfortunately, the study did not provide information about the breast cancer subtype the cells for the experiments were derived from, i.e., whether these were ER+ and/or TN breast cancer. However, this initial report triggered that strong emphasis was put on the CD44+/CD24- profile in the vast majority of the downstream studies. Indeed, initial typing of breast cancer cell lines demonstrated that TN breast cancer cell lines contained a high percentage of CD44+/CD24- cells, opposed to ER+ breast cancer cell lines. Most results discussed in this review have thus been generated by using cell lines with a high frequency of CD44+/CD24- cells, such as SUM149, SUM159, MDA-MB231 and HCC1954, which overlap with the BCSCs identified by positivity for ALDH1 activity (ALDH1+) via ALDEFLUOR staining (56, 57). Of note, however, also CD44-/CD24+ breast cancer cells are ALDH1+ (57). ALDH1+ cells were more prevalent in TN breast cancer cell lines, albeit some ER+ breast cancer cell lines likewise displayed high frequencies. Sorting and consecutive molecular profiling of ALDH1+ breast cancer cells identified CXCR1, the IL-8 receptor, as being upregulated in this population, and CXCR1-inhibition by either an antibody or a small chemical compound resulted in a reduction of the BCSCs (56, 57). The data suggested that different BCSC-enriched populations may exist in breast cancer.

Normal mammary stem cells (MaSCs) and BCSCs have been shown to produce floating spheres, so-called mammospheres, when cultured under special, non-adherent conditions (53, 58). The number of mammospheres is proportional to the number of MaSCs and BCSCs generated under these conditions, because the mammospheres have a clonal origin. Further, the mammospheres can be dissociated and serially cultured, which results in a relative increase of MaSCs and BCSCs with each passage and demonstrates capacity for self-renewal. Mammospheres have been shown to be enriched for CD44+/CD24- cells and have readily been obtained from both breast cancer cell lines and primary breast tumors, albeit the latter gave rise to lower success rates (58). The quantification of mammospheres is taken as the surrogate in vitro assay for determining the number of BCSCs. Accordingly, resistance

to anoikis, i.e., resistance to apoptosis under anchorage-independent growth conditions, has turned out as another property of MaSCs and BCSCs.

Populations enriched for BCSCs are able to give rise to tumors in mice, when injected at cell numbers as low as 200–1000 cells (55, 56, 58, 59), which corresponds to a potency increased by a factor of about 1000 compared to unsorted bulk tumor cells. BCSCs, like CSCs of other cancer types, display a high degree of resistance to radio- and chemotherapy (26, 60–62), and have been shown to possess an increased metastatic potential (63–65). Paclitaxel has even been demonstrated to result in a 3-fold expansion of BCSCs (24).

Taken together, these studies provided initial data on stem cell-like subpopulations in breast cancer. Self-renewal, drug and radio-resistance and high potential for metastatic spread raised the possibility that therapeutic targeting of this specific subpopulation may lead to major improvements in sustainable breast cancer treatment. Accordingly, strong focus was set on exploring, which pathways could serve as starting points for drug development.

### BCSC-Relevant pathways and the role of epithelial-mesenchymal transition

Besides BRCA1, already discussed above, most of the key proteins and pathways formerly shown to play a role in either mammary gland development or the genesis and progression of breast cancer have meanwhile been connected to BCSCs. Based on its importance for mammary gland development, Liu and co-workers analyzed the role of the Hedgehog signaling pathway in MaSCs and BCSCs (66), indicating that the receptors PTCH1 and SMO, the ligand IHH, and the transcription factors GLI1 and GLI2 are strongly upregulated in mammospheres vs. differentiated mammary gland cells. Agonists of this pathway increased and antagonists decreased mammosphere formation as well as mammary gland outgrowth in a mouse transplantation model, which was mediated by the epigenetic regulator BMI1. The same pathway components were found to be upregulated in CD44+/CD24- BCSCs (66). The transcription factor GATA3 is critical for the differentiation of mature cells from integrin  $\beta$ 3-positive (CD61+) luminal progenitor cells. Its inactivation was shown to result in accumulation of luminal progenitors and accelerated breast cancer progression in mice (67, 68). GATA3 induces a reversion of epithelial-mesenchymal transition (EMT), which is associated with the suppression of breast cancer metastasis (69). EMT is a complex process, which cumulates in the conversion of cells from an epithelial to a more basal-like phenotype and is associated with acquiring many attributes ascribed to BCSCs, such as invasiveness, drug and radio-resistance, potential for self-renewal and induction of BCSC marker profiles.

Upon hyperactivation, the WNT/ $\beta$ -catenin pathway was shown to result in radiation-induced progenitor cell enrichment in the mouse mammary gland (25). Consecutively, the analysis of mouse models of Wnt pathway-induced breast

cancer suggested that these tumors may likewise emerge from CD61+ luminal progenitor cells and hyperactivation of WNT signaling was linked to basal-like breast cancer and induction of an EMT via the EMT-promoting transcription factors SLUG (SNAIL2) and TWIST (70, 71). The cytokines IL-8 and IL-6 linked inflammation to the emergence and/or maintenance of BCSCs. IL-8 caused an expansion of ALDH1+ BCSCs, while inhibition of CXCR1, its receptor, caused a reduction (56, 57). IL-6 was shown to increase the mammosphere-forming capacity of normal mammary gland epithelial cells and of a breast cancer cell line, which required signaling via Notch3 (72). Inactivation of p53 may result in IL-6 transcriptional activation, which in turn switches on the transcription of CD44 (73). Further, IL-6 action resulted in an EMT in vitro (74).

Activation of HER2 (epidermal growth factor receptor 2; EGFR2) was demonstrated to increase the number of ALDH1+ cells and mammosphere formation of both normal mammary gland epithelial and breast cancer cells as well as the in vivo tumorigenicity of breast cancer cells (75). Subsequently, it was shown that these functions may incorporate HER2-mediated activation of the Notch1 receptor (76). However, Harrison and colleagues suggested that Notch4 rather than Notch1 is responsible for BCSC maintenance, as only Notch4 inactivation resulted in a substantial BCSC reduction (77). The EMT-promoter ZEB1 was identified as co-activator of Notch signalling, which is negatively regulated by members of the mir-200 microRNA family, known to counteract EMT (78). MiR-200c itself inhibits the stem cell phenotype in MaSCs and BCSCs via suppression of BMI1, which results in loss of mammary gland formation and tumorigenicity, respectively (79). Vice versa, mir-34c was found to suppress EMT and BCSC self-renewal via targeting of Notch4 (80). The well-known tumor suppressor p53 was recently shown to inhibit the BCSC phenotype via inhibition of Notch signaling and positive regulation of the EMT-inhibiting mir-200c (81, 82). The miRNA let-7, well known for its role in developmental processes, has been shown to decrease BCSC self-renewal by inhibition of HRAS and to promote differentiation by negatively regulating HGMA2 (83). Inactivation of PTEN, another tumor suppressor frequently mutated in human cancers, promoted the formation of MaSCs and BCSCs (84). Liu and co-workers reported that activation of the oncogenes HRAS and MYC elicits an EMT in breast epithelial cells, which could be partly reverted by activation of the tumor suppressor p21 (CDKN1A) (85). EMT induced by any of the two transcription factors SNAIL1 or TWIST1 or by addition of TGF- $\beta$ 1 was shown to induce the CD44+/CD24- profile in mammary epithelial cells and to increase their mammosphere-forming capacity as well as to promote the occurrence of *bona fide* BCSCs (86).

Hence, several studies linked induction of an EMT to the maintenance and/or expansion of MaSCs and BCSCs positive for the CD44+/CD24- profile, and the induction of an EMT seems to incorporate most of the pathways and key proteins known to play a role in breast development and/or tumorigenesis.

## Drug discovery and development approaches

Drug discovery and development approaches to target BCSCs mainly concentrated on interfering with the afore-mentioned pathways and with EMT. To this end, few systematic drug screens have been performed. One of the earliest efforts screened a library of 16,000 compounds for differential killing of immortalized human breast epithelial (HMLE) cells, which were engineered to be either positive or negative for E-cadherin (CDH1) (24). The ratio behind this was that loss of E-cadherin is causing an EMT and this rendered the breast epithelial cells similar resistant to conventional chemotherapeutics as inactivation of E-cadherin in tumorigenic HMLE cells did. This identified salinomycin as compound, which selectively eliminated both normal and tumorigenic breast cells that have undergone EMT due to E-cadherin inactivation (24). However, as far as analyzed, the effects of salinomycin were at least equivalent, if not more pronounced, on normal mammary epithelial cells that underwent EMT. Because an EMT might occur during the emergence of basal cells, which fulfill regenerative functions in the mammary gland, devices for selective delivery to breast cancer cells would likely be required. As a first step to convert BCSC-targeting to a nanomedical approach, Zhang and co-workers recently constructed micelles for passive delivery of salinomycin, which—in combination with actively targeting paclitaxel-delivering micelles—showed good efficacy in preclinical models (87).

Bandyopadhyay et al. made use of a combination of a TGF $\beta$  type I receptor kinase inhibitor, which blocks an EMT, and doxorubicin. The combination prevented the emergence of doxorubicin-resistant breast cancer cells due to doxorubicin-induced EMT (88). In addition, Oak and colleagues reported that use of the HER2-blocking antibody trastuzumab, which targeted the HER2+ breast cancer cells, was efficient in vitro in combination with salinomycin, which targeted HER2-BCSC-like cells (89). However, salinomycin, an antibiotic only approved for use in animals, is controversially discussed because it may exert unacceptable toxicity in humans (90). It functions as inhibitor of MDR1 (ABCB1; P-glycoprotein) (91), which acts as transporter to shuttle drugs out of cells, but possibly has also important functions in normal stem cells. This defines a demand for smart nanomedical devices that would allow for a BCSC-selective targeting to reduce harm caused to normal stem cells.

Notch and  $\gamma$ -secretase inhibitors, displaying reasonable effects in preclinical models (92) are under consideration for therapeutic targeting of BCSCs, but so far without groundbreaking success in clinical trials (93). Further, the Akt inhibitor perifosine has been shown to reduce mammosphere formation in vitro (84), but so far lacks striking evidence for efficacy in clinical trials, including various cancer types (94–99), and/or exerted unacceptable side effects (100) when used for single agent treatment. However, there are initial indications that combinatorial treatment with perifosine might exert beneficial effects (101, 102).

Besides that several other strategies have been proposed based on preclinical data, including inhibition of WNT signaling via antibodies (103) or other WNT pathway inhibitors

(104), combinatorial metformin/doxorubicin treatment (105, 106), focal adhesion kinase (FAK) inhibition (107), application of the apoptosis inducer TRAIL in combination with either c-FLIP inhibition (108) or cisplatin (109), therapeutic application of BMP2/7 (110), blocking of CD44 (111), or the therapeutic application of miRNAs, e.g., let-7 (112), to mention only few examples.

Systematic screens for BCSC-eliminating drugs recently experienced acceleration. A smaller library, comprising 280 compounds of the NIH clinical collection, was screened for agents that would selectively eliminate CK5+ breast cancer cells and recovered retinoids with corresponding activity (113). Zhou and colleagues sorted side population cells from the MCF7 breast cancer cell line, which were then propagated further in mammosphere culture. This resulted in the enrichment of CD44+/CD24- cells and in increased drug resistance (114), probably pointing to an EMT. Using about 2000 compounds of the NCI diversity set compound library, a differential screen for compounds killing these cells vs. adherently growing breast cancer cells was performed, which identified 8-quinolinol as active compound. As other compounds that target cancer cells with EMT, it exerted poor anticancer activity in mouse models when given alone, but potentiated the effect of paclitaxel (114). One of the most comprehensive screens so far scanned 300,000 compounds for their activity on differential killing of normal mammary epithelial cells with knockdown of CDH1, which identified acyl hydrazones as active agents (115).

Collectively, these attempts reveal a recurrent motif. Drugs intended to target cancer cells that have undergone an EMT commonly do not work in single agent therapies, but require the action of conventional chemotherapeutics to first induce an EMT in the cancer cells. The combinatorial treatment may then be more effective than chemotherapy alone. Recent data may expand this principle to antiangiogenic drugs, which result in an expansion of BCSCs via hypoxia-induced EMT, so that it was proposed that antiangiogenic therapy in conjunction with targeting of EMT-BCSCs could represent a therapeutic concept (116). Getting the combined toxicity of such treatment strategies under control will provide interesting challenges for the conception of novel nano drug delivery devices and will probably require active rather than passive targeting.

## Shortcomings and open questions

While this area presented rapid progress from the discovery of BCSCs to the implementation of first clinical trials, there is also a number of aspects that require critical consideration and indicate that BCSCs are not yet understood to a full extent. Several groups raised doubts about the importance of EMT and CD44+/CD24- cells based on studies of primary breast cancer tissues. CD44 expression was inversely correlated with invasive properties of luminal breast cancer (117). A higher frequency of CD44+/CD24- cells may favor distant metastasis, but was not associated with clinical outcome (118). The ALDH1 status but not the CD44+/CD24- status was found to be associated with prognosis and treatment resistance (119). Aulmann and colleagues found that the CD44+/CD24- population was depleted

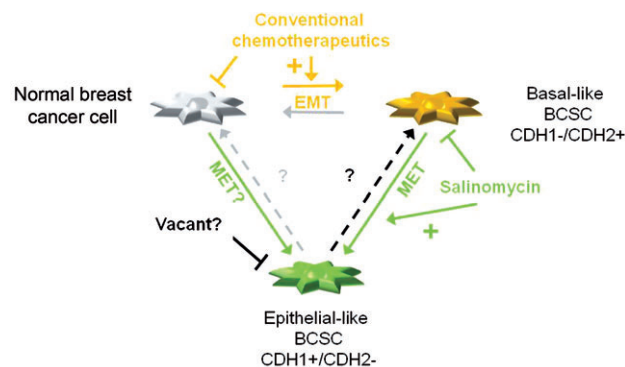


and not enriched in breast cancer by conventional chemotherapy (120). Kok et al. derived a molecular signature from mammospheres of ER+ breast cancer and found that this is associated with a good instead of a poor prognosis in primary tumors (121). Shipitsin and colleagues reported that, while CD44 is a relevant marker for BCSCs, metastases appear to be CD24-positive and not CD24-negative (122). Similarly, there are also reports that link expression of the EMT inducer BMI1 to a good instead of a poor prognosis in primary breast cancer (123). However, in 2010, a meta-analysis came to the conclusion that both the ALDH1+ and the CD44+/CD24- status are significantly associated with poor overall survival in breast cancer patients (124). By contrast, a recent study again came to the conclusion that the CD44-/CD24+ phenotype is a marker for poor prognosis (125). Controversial results extend also to the action of some drugs. A preclinical study pointed to potential opposite effects of the  $\gamma$  secretase (Notch signaling) inhibitor RO4929097 because it caused an expansion instead of a reduction of the BCSC population upon treatment, as well as radioresistance (126).

There is evidence that an EMT and the corresponding marker profiles, including the CD44+/CD24- status, are associated with basal-like (i.e., TN) breast cancer, especially with the malignant but rare claudin-low breast cancer type (127–130), so that discrepancies in primary tumors may perhaps resolve, if luminal-like and basal-like breast cancer are considered separately.

Others raised the question as to whether as skewed image might have emerged, because the CD44+/CD24- human breast cancer cells may simply better engraft in immunocompromised mice (130, 131). Yoo and Hatfield reported that, when using mouse cancer cell lines for implantation into mice, all randomly selected clones were able to support outgrowth of tumors (132). Opposed to this, however, Zhang and colleagues identified CD29 high/CD24 high breast cancer cells as BCSCs via studies in the p53 null mouse model of breast cancer (133). Further, Matilainen and co-workers demonstrated that cells of the mouse breast cancer cell line 4T1 grown as mammospheres have different tumorigenicity in mice. Remarkably, in this case a low mammosphere-forming potential was associated with the emergence of BCSC markers and metastatic spread in vivo, drawing an opposite image compared to what has been anticipated before (134). In fact, using the luminal breast cancer cell line MCF7, Kim and colleagues recently showed that not cells with EMT-like characteristics, but with luminal traits are the more aggressive tumor-forming cell population. Opposite to EMT-like MCF7 cells, these luminal-like cells did not give rise to increased mammospheres in vitro (135).

Taking these findings together, the question has to be raised as to whether there exist yet undiscovered BCSC populations, which are epithelial (luminal)-like and possess different properties than the ones characterized so far (Figure 4). This may include low or no mammosphere-forming potential and different marker profiles, so that these may have escaped previous studies. In fact, when breast cancer-bearing mice were treated with salinomycin, and the remaining tumors were subsequently analyzed, CDH1-positive breast cancer cells were found, indicating that a new BCSC population may have emerged by



**Figure 4** Drug actions and BCSC plasticity. Conventional chemotherapeutics eliminate the normal “bulk” breast cancer cells but simultaneously promote an EMT, resulting in an expansion of basal-like BCSCs, which are, for example, negative for E-cadherin and positive for N-cadherin (CDH1-/CDH2+). Salinomycin eradicates basal-like BCSCs, but possibly produces a new type of salinomycin-resistant, more epithelial-like BCSCs, prospectively with CDH1+/CDH2- profile. It is not yet known, whether other treatment strategies, e.g., inhibition of Wnt, Notch, or Hedgehog signaling would produce similar effects. The hypothetical epithelial-like BCSCs are not yet characterized. It is neither known whether there are drugs that may target this type, nor whether these cells can convert into the other two cell types.

reversion of EMT (24). Because drug discovery so far concentrated on targeting breast cancer cells that have undergone EMT, *de novo* approaches may be required that define and characterize further potentially existing BCSC populations as basis for new discovery strategies.

Further complicating the situation, Chaffer et al. recently demonstrated that normal mammary epithelial cells and conventional (non-BCSC) breast cancer cells can spontaneously convert to stem-like cells (136), which could mean that upon selective therapeutic targeting of BCSCs these can eventually be replenished from non-stem-like breast cancer cells. This could provide a conceptual basis to explain the lack of efficacy when using BCSC-targeting compounds in single agent treatment strategies. As a consequence, it is presently thought that the future therapies need to target both cell types, the BCSCs and the non-BCSCs, within a given breast tumor.

Finally, a shortcoming has so far been that virtually all drug discovery approaches target pathways that are equally important for BCSCs and MaSCs. Search strategies concentrated on targeting cells that underwent EMT and not on targeting BCSCs vs. MaSCs. As a consequence, the therapeutic approaches pursued so far eliminate normal stem cells in addition to BCSCs, which could, in the long-term, turn out as a drawback unless nanomedicine is able to provide smart devices that allow for highly selective delivery to cancer cells. Recognizing this shortcoming, Sachlos and colleagues just reported on the first differential screen for drugs that would target tumorigenic vs. normal stem cells. Screening of a set of about 2500 compounds surprisingly recovered, among others, a dopamine receptor antagonist, exerting such desirable differential activity (137). There is presently data missing on the clinical relevance of the dopamine receptor in breast cancer, however.



## Conclusions

The cancer stem cell concept is able to explain various phenomena met in breast cancer, including treatment resistance, tumor relapse, metastatic spread, and the heterogeneity of breast cancer subtypes, which may reflect tumor cells locked at different stages of a hierarchical development. The drugs and the treatment approaches nowadays under consideration for targeting BCSCs generally also target processes important for steady-state regeneration by MaSCs and prospectively also by normal stem cells in other tissues. Thus, one of the main objectives in future nanomedicine stays providing smart devices for cancer-specific delivery. There is, however, evidence that BCSCs represent a flexible, moving target, as these can be re-created from conventional cancer cells. For this reason, the nanodevices may need to combine classical chemotherapeutics with BCSC-targeting drugs, so that nanomedicine will have to find solutions for delivery to the right cell types and for dealing with the combined toxicity of these drugs.

Caused by the history of discoveries, the present strategies concentrate on targeting BCSC-like cells that underwent an EMT, which may provide means to treat the less frequent, albeit more aggressive basal-like, especially the so-called claudin-low, breast cancer subtypes. Contradictory data has been recovered regarding the relevance of EMT in the more frequent luminal-like (ER+) breast cancer subtypes and there are initial indications that (an)other type(s) of BCSCs may exist with more epithelial-like features. As a consequence, nanomedicine will prospectively also have to recover novel drugs and treatment approaches, which either target such cancer cell populations or exert a broader activity against several types of BCSCs.

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