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**Title**

Breast cancer stem cells: Multiple capacities in tumor metastasis.

**Permalink**

<https://escholarship.org/uc/item/8rk3985z>

**Journal**

Cancer letters, 349(1)

**ISSN**

0304-3835

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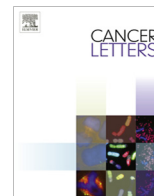
**Publication Date**

2014-07-01

**DOI**

10.1016/j.canlet.2014.03.036

Peer reviewed



## Mini-review

## Breast cancer stem cells: Multiple capacities in tumor metastasis

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## ARTICLE INFO

## Article history:

Received 27 December 2013

Received in revised form 27 March 2014

Accepted 28 March 2014

## Keywords:

Breast cancer

Cancer stem cells

HER2

Therapeutic resistance

EMT

Metastasis

## ABSTRACT

Breast cancer is the leading cause of cancer death among women worldwide. Accumulating evidence indicates that the local recurrent and/or distant metastatic tumors, the major causes of lethality in the clinic, are related to the aggressive phenotype of a small fraction of cancer cells loosely termed as cancer stem cells (CSCs), tumor initiating cells (TICs), or cancer metastasis-initiating cells (CMICs). Breast cancer stem cells (BCSCs) are shown to exhibit unique growth abilities including self-renewal, differentiation potential, and resistance to most anti-cancer agents including chemo- and/or radiotherapy, all of which are believed to contribute to the development and overall aggressiveness of the recurrent or metastatic lesions. It is in the urgent need not only to further define the nature of heterogeneity in each tumor but also to characterize the precise mechanisms governing tumor–host cross-talk which is assumed to be initiated by BCSCs. In this review, we will focus on recently identified key factors, including the BCSCs among circulating tumor cells, interaction of BCSCs with the host, epithelial mesenchymal transition (EMT), tumor microenvironment, the intrinsic resistance due to HER2 expression, potential biomarkers of BCSCs and cancer cell immune signaling. We believe that new evidence coming from both bench and clinical research will help to develop more effective approaches to control or significantly reduce the aggressiveness of metastatic tumors.

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## Introduction

Breast cancer is genetically and clinically a heterogeneous disease [1–3], and metastatic lesions are the leading cause of death in patients [4–7]. Accumulating evidence suggests that the tumor bulk of breast cancer contains a heterogeneous tumor cell population that is derived from a subset of cells that show the characteristics of stem cells, termed as tumor-initiating cells or cancer stem cells (CSCs) [8,9]. The whole course of tumor metastasis is a complex procedure requiring the most aggressive cancer cells rather than all tumor cells to be able to survive the long time circulation and to form new local lesions by extravasation and migration. Accumulating evidence indicates that CSCs play a key role in not only the original tumorigenicity but also in their ability for local invasion and migration [10–12]. Overlapping with some features of normal stem cells, CSCs are shown to be resistant to proapoptotic factors, rendering them a formidable adversary to current anti-cancer modalities [13–15]. Extensive studies have demonstrated that breast cancer stem cells (BCSCs) exhibit the ability to metastasize to specific parts of the body and are believed to be a cause for

metastatic lesions. Although it is expected that the tumor heterogeneity and BCSCs may be the last obstacles for effective breast cancer treatment, the molecular insights and potential specific biomarkers for the therapy-resistant BCSCs need to be further elucidated before potential clinical benefits could be achievable.

## The concept of CSCs and self-renewal

The concept of the CSC was first hypothesized in the 20th century by Bonnet and Dick in their studies of human acute myeloid leukemia (AML) [16]. Their study indicated the presence of a unique cellular hierarchy in AML, reflecting the similar order identified in normal hematopoiesis. Leukemic stem cells identified in this hierarchy, originally termed as CSCs, were categorized as CD34<sup>+</sup>/CD38<sup>-</sup>. Recent studies demonstrate that most CD34<sup>+</sup> AMLs are derived from progenitor cells but not hematopoietic stem cells [17]. Subsequently, the CSC concept has been described and extended to many solid tumors, including breast cancer, prostate cancer, colorectal cancer, lung and brain cancers [16]. In particular, breast cancer is shown to be heterogeneous, and the tumor bulk is derived from BCSCs [18–20].

The CSC theory challenges the traditional concept of tumorigenesis. Studies show that some CSCs may be derived from normal

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stem cell transformation, leading to tumor growth [21–27]. Other studies indicate that mesenchymal stem cells can accelerate cancer cell metastasis [28–32]. It is even proposed that normal stem cells promote the process of tumorigenesis, tumor metastasis, as well as CSCs dynamic change [28]. In 2003, Al-Hajj and colleagues reported for the first time that breast cancer can originate from BCSCs [33]. They identified and isolated a small subset of cells within primary breast cancer cells of which a few cancer cells were able to form palpable tumors in the mammary fat pad of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Such cells express CD44 or CD44 with epithelial specific antigen (ESA), but not CD24, consistent with the phenotypic characteristics of mammary stem cells with multipotent differentiation ability [33,34]. Nevertheless, although these results demonstrate the possibility that BCSCs originate from the normal mammary stem cells, it is generally accepted that further genomic profiling and comparison of the normal stem cells versus BCSCs should provide insightful information on the aggressive phenotype of BCSCs.

CSCs are featured by their potential to self-renew and initiate tumors in immunocompromised mice upon serial passage. Several studies reveal that CSCs are responsible for tumor metastasis and the recurrent lesions following chemotherapy and radiotherapy [35–37]. The tumor microenvironment plays a significant role in regulating chemoresistance and radiation resistance via inflammatory cytokines to stimulate CSC self-renewal, which may promote tumor proliferation and metastasis [36,38]. Different cells in the tumor microenvironment produce several factors, including PDGF, IGF, Notch, Wnt, MMPs, and Hedgehog (Hh) to regulate tumor growth, invasion, and metastasis [39–43] (Wnt, Notch and Hh are shown in Fig. 1). Among them, Wnt is known to cause self-renewal of CSCs at early phases and plays a significant role in the initiation and maintenance of CSCs [44–46]. Hh signaling of CSCs was shown to be active not only in a pancreatic xenograft mouse model [47] but also in regulating the maintenance of human leukemic stem cells [48,49]. Ablation of the Hh pathway effector smoothed (Smo) caused elimination of chronic myeloid leukemia (CML) stem cells [48]. In addition to the Hh pathway, activated Notch signaling was shown in colon CSCs [50]. Antibodies targeting Delta-like 4 ligand (DLL4), another downstream effector of Notch signaling, inhibited growth of human colon cancer in xenograft mouse model [50]. Therefore, similar to Notch inhibition, therapeutic agents identified to inhibit these self-renewal signaling pathways may have potential effectiveness to decrease the number of CSCs and suppress tumorigenicity and metastasis.

### BCSCs and circulating tumor cells (CTCs)

A subpopulation of circulating tumor cells (CTCs) identified in patient blood has been hypothesized to initiate metastatic carcinoma [51–54]. However, although the presence of increased CTCs is an indicator of poor prognosis and cancer progression, the phenotype of CSCs (or metastasis-initiating cells; MICs) among CTCs has not been elucidated. Since CTCs are able to initiate metastatic growth in distant organs, resembling the same behavior as MICs [55], it is reasonable to believe that stem cell-like CTCs are a subpopulation of cells with metastatic ability to migrate to distant sites from the primary tumor [56]. A recent study showed that MIC-containing CTC populations originating from primary human luminal breast cancer expressing EPAM, CD44, CD47, and MET caused lung, liver, and bone metastasis in mice [51]. In a small patient cohort exhibiting tumor metastasis, the population of EPAM<sup>+</sup>CD44<sup>+</sup>CD47<sup>+</sup>MET<sup>+</sup> correlated with increased metastasis and low overall survival [51]. An additional study identified BCSCs in a CTC population among patient peripheral blood samples [55]. This study shows that among a total of 1439 CTCs, 35% of the CTCs

in 20 out of 30 patients exhibited the BCSC CD44<sup>+</sup>/CD24<sup>-low</sup> phenotype, whereas 17.7% of the CTCs identified in seven patients were ADLH1<sup>high</sup>/CD24<sup>-low</sup> [55]. Such data support the conclusion that functional BCSCs and MICs within CTCs may lead to early diagnosis and treatment of metastatic breast cancer. However, more detailed studies on the characteristics of CTCs are needed to elucidate molecular mechanisms of tumor metastasis initiated by dissemination, seeding, and engraftment of CTCs.

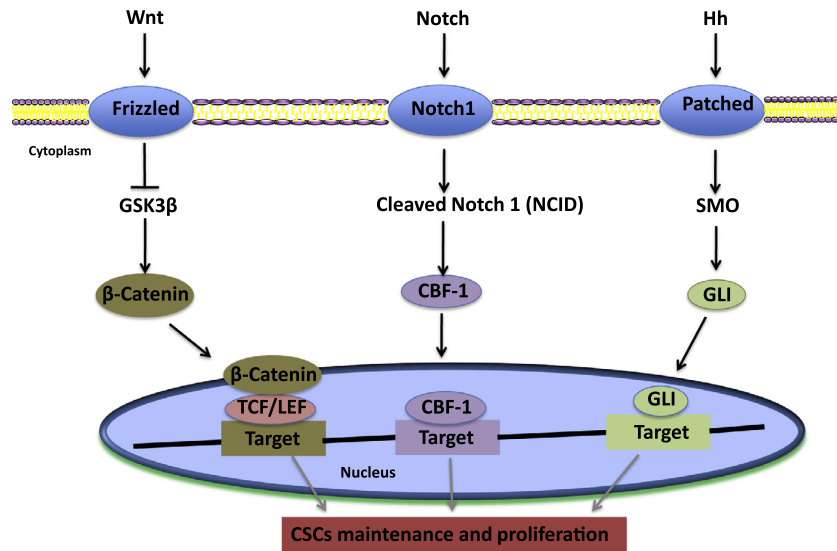
### BCSCs and EMT

The epithelial–mesenchymal transition (EMT) is a phenotypic process converting polarized and adjacent epithelial cells to mesenchymal cells conferring motile and migratory properties [57]. It has been proposed that CSCs in primary tumors can metastasize to distant tissues or organs to disseminate and form metastatic colonies via EMT [58]. A recent study has revealed a dynamic in vivo pattern of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer [59]. The CD44<sup>+</sup>/CD24<sup>-</sup> subpopulation in RAS or HER2 overexpressing tumor cells is considered to be the phenotype with increased EMT potential [60,61]. The gene expression signature, CD44<sup>+</sup>/CD24<sup>-low</sup>, is also found to occur mainly in human breast tumors of the recently identified “claudin-low” molecular subtype [62]. This subtype is characterized by expression of many EMT-associated genes, such as FoxC2, Zeb, and N-cadherin [62,63].

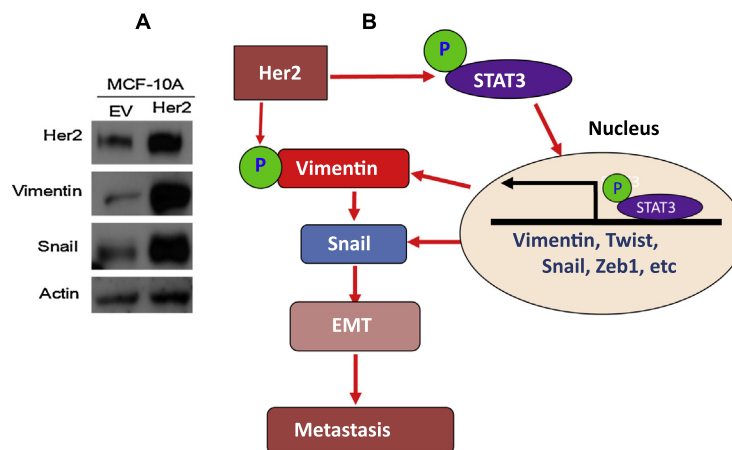
EMT may serve as a critical step to the metastatic processes of BCSCs [61,64]. Vimentin, one of the key genes regulating EMT and tumor aggressiveness in breast cancer [65,66] is shown to be indicative as a poor prognostic factor for triple-negative breast cancer [67] and upregulated in normal human breast epithelial cells overexpressing HER2 [64,68]. We found that HER2 was able to activate Stat 3 signaling pathway [3] and EMT-associated genes, such as vimentin (Fig. 2A). These results, together with enhanced EMT-associated genes, suggest that HER2-associated activation of Stat3 signaling can induce EMT of normal mammary epithelial cells such as MCF-10A cells, and contributes to metastatic BCSCs, for which a signaling pathway is proposed here shown in Fig. 2B. However, the occurrence of EMT is not acquired spontaneously but regulated by microenvironment signaling. Regulation of EMT plasticity is most likely dependent on normal cells in the tumor microenvironment, including cancer-associated stromal cells, such as infiltrating immune cells, fibroblasts, and endothelial cells [69]. During tumorigenesis, changes in immunological signaling networks occur not only in epithelial tumor cells but also in the proximal tumor-associated stromal cells. Interestingly, several cytokines, chemokines, or growth factors were identified in mammary tumor stroma compared with normal mammary stroma [70]. Further investigation is needed to clarify our understanding of how BCSCs and each of above tumor-associated factors influences cancer cell development, EMT plasticity and tumor aggressiveness.

### CSCs, tumor microenvironment, and metastatic lesions

Here we would like to illustrate the mechanism of interplay of tumor–tumor microenvironment by showing the case of bone marrow vascular and endosteal niches for hematopoietic stem cells (HSCs) which is known to contribute to the tumor microenvironment of CSCs [71–73]. In the vascular niche, HSCs monitor hematopoietic function and promote rapid responses to hematopoietic demand. Endosteal HSCs regulates other cells, including mesenchymal stem cells, CXCL12-abundant reticular cells, and chimeric antigen receptor (CAR) cells. The endosteal niche is primarily controlled by osteoblasts expressing cytokines, growth factors, and many adhesion molecules, which are critical for maintenance of



**Fig. 1.** Self-renewal of CSCs is driven by signal transduction pathways including Wnt, Notch, and Hedgehog. Transcriptional factors including TCF/LEF, CBF-1 and GLI following the activation of Wnt, Notch1 and Hh respectively, work cooperatively to organize the self-renewal and cell proliferation of CSCs; Wnt activation inhibits GSK3 $\beta$  causing the activation of  $\beta$ -catenin to control the Wnt pathway, which together with CBF-1 and GLI helps maintain the CSC phenotype.



**Fig. 2.** HER2 may activate both STAT3 and vimentin that in turn induces EMT of human mammary epithelial cells to become aggressive and metastatic tumorous cells. (A) Induction of vimentin and snail by overexpression of HER2 in normal human breast epithelial MCF-10A cells (EV, empty vector; unpublished results). (B) Proposed HER2-mediated activation of EMT signaling pathways in breast cancer development and aggressive growth. EMT-associated genes including vimentin and snail are upregulated in human breast epithelial MCF-10A cells due to HER2-overexpression via HER2 gene transfection, which may mimics the feature of HER2-expressing BCSCs [3].

CSCs. Once CSCs metastasize to the bone marrow HSC niche, they may stay dormant or undergo G0 cell cycle arrest and become quiescent. After asymmetric cell division, stem cell progeny may return to their niches and form metastatic bone lesions where they colonize and replace the bone marrow HSCs; prevent the bone marrow HSC colonization and implantation; and directly compete with bone marrow HSC niches [74].

The exosomes and tumor microenvironment play a major role in cancer development, metastatic lesions, and therapeutic resistance [75–77]. Comparison of comprehensive gene expression profiles of normal breast tissue versus invasive breast carcinomas detected extensive gene expression changes in breast cancer progression which include many secreted proteins and receptors [77]. Exosome vesicles are shown to be released from metastatic tumors and at their surface contain a large number of proteins and lipid components closely related to their origins [78]. Exosomes detected from stromal cells and cancer cells are required to establish the metastatic tumor lesions [78]; the melanoma-derived exosomes promote tumor metastasis by receptor tyrosine kinase MET [75]. Additionally, mouse models of lung cancer metas-

tasis show that CSCs, as the seeds of metastasis, are directly involved in pre-metastatic niche formation and express tenascin C (TNC) [79], which enhances the expression of musashi homolog 1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) to regulate CSC activity in the tumor microenvironment. Therefore, MSI1-mediated regulation of the Notch signaling pathway and LGR5-mediated regulation of the Wnt pathway are both critical for controlling CSCs metastasis [79]. Also, breast cancer cells are shown to be able to induce adjacent fibroblasts expressing MMP-2 and MMP-9 to promote the formation of the metastatic microenvironment [43]. These data, again, support the concept that the local tumor microenvironment plays an important role in the execution of tumor metastasis.

#### BCSCs, HER2 status, and metastasis

In clinic, about 28% of breast cancer patients are diagnosed as HER2-positive based on HER2 transcription and gene dosage. Without HER2 disruption, these tumors are resistant to routine

anti-cancer therapy [3] indicating limited application of anti-HER2 modalities in breast cancer patients [22]. It has been shown that HER2 overexpression promotes the enrichment of stem cells in both normal and malignant cells [80] and the function of HER2 in breast cancer patients with amplified HER2 gene expression is unclear [3]. We reported that a small fraction of MCF7 breast cancer cells derived from long-term irradiation showed a heterogeneous population and some isolated clones from this surviving tumor cell population are highly radioresistant [81] with enriched with HER2-positive BCSCs [3]. Creighton et al. found that the residual breast tumor cell population survived after chemotherapy treatment was enriched with both tumor-initiating and mesenchymal features [62]. They further defined a gene expression signature common to expression of both CD44<sup>+</sup>/CD24<sup>-/low</sup> and EMT-associated genes [82]. Furthermore, Magnifico et al., showed that compared to parental breast cancer cells, HER2 was highly expressed in BCSCs, rendering them sensitive to Herceptin treatment [82]. However, HER2 protein level could be enhanced at the transcriptional level [3] and Feng et al. illustrated that HER2 protein level can also be linked to post-transcriptional processes, a modification that is mediated by the RNA binding protein Lin28 [83]. These results implicate an important role of HER2 status that varies among tumors and affects the therapeutic efficacy. Therefore, HER2 upregulation in BCSCs and signaling pathways causing HER2 regulation should also be taken into account in treatment design even for “HER2-negative” tumors. In vitro experiments showed that radiation can promote the enrichment of BCSCs through NF- $\kappa$ B that binds to HER2 promoter region to enhance HER2 gene transcription [84]. It should be emphasized that radiation-induced BCSCs repopulation may not be related only to HER2 positive tumor cells, but also to HER2<sup>-/low</sup> breast cancer cells since HER2 may be induced by transcription without enhancing HER2 gene dosage by therapeutic conditions in HER2<sup>-/low</sup> tumors [84,85]. In fact, radio-resistance of MCF7 and MDA-MB-231 clones, derived from long-term irradiation, was associated with significant increase of HER2 expression [3,84]. These studies highlight a potential critical pattern of dynamic alterations of key genes in tumor cells under genotoxic therapeutic conditions, such as ionizing radiation used in radiotherapy. In consistency with these data, Malik et al. found that the bone marrow microenvironment affects HER2 expression in MCF7 cells, suggesting that microenvironment aids in the establishment of tumor cells in the host [83]. In addition, a pathway of HER2–STAT3 axis is suggested as a key signaling pathway for therapy-resistant breast cancer cells [3]. Thus, a potential dynamic change of HER2 status (both gene dosage and protein levels) and its down-stream signaling networks, especially in BCSCs, should be taken into account.

### Potential biomarkers for BCSCs

In light of accumulating evidence supporting BCSC-mediated metastasis, increasing efforts have been made to define sensitive and reliable biomarkers for BCSCs that are able to document metastatic lesions. For example, breast and pancreatic cancer cells expressing CD44 increase metastatic ability [86]. However, this does not simply mean that only the CD44<sup>+</sup>/CD24<sup>-/low</sup> BCSCs are able to metastasize, several BCSC phenotypes are found to co-exist in a tumor and each phenotype shows its own unique metastatic potential and sensitivity to therapeutic regimens [87]. Although no hierarchy of metastatic BCSCs exists, studies have shown that BCSCs can establish focal metastasis, which are prone to metastasize to the lung [35]. On the other hand, different subsets of BCSCs are likely associated with the growth and metastasis of the primary cancers, suggesting that BCSCs exhibit characteristics of heterogeneity and their specific cell surface biomarkers need to be

identified. Cells bearing the CD44<sup>+</sup>/CD24<sup>-/low</sup> surface marker profile exhibit aggressive behavior and poor prognosis [35]. However, the mechanisms by which CD44<sup>+</sup>/CD24<sup>-/low</sup> cells promote metastasis are not fully understood.

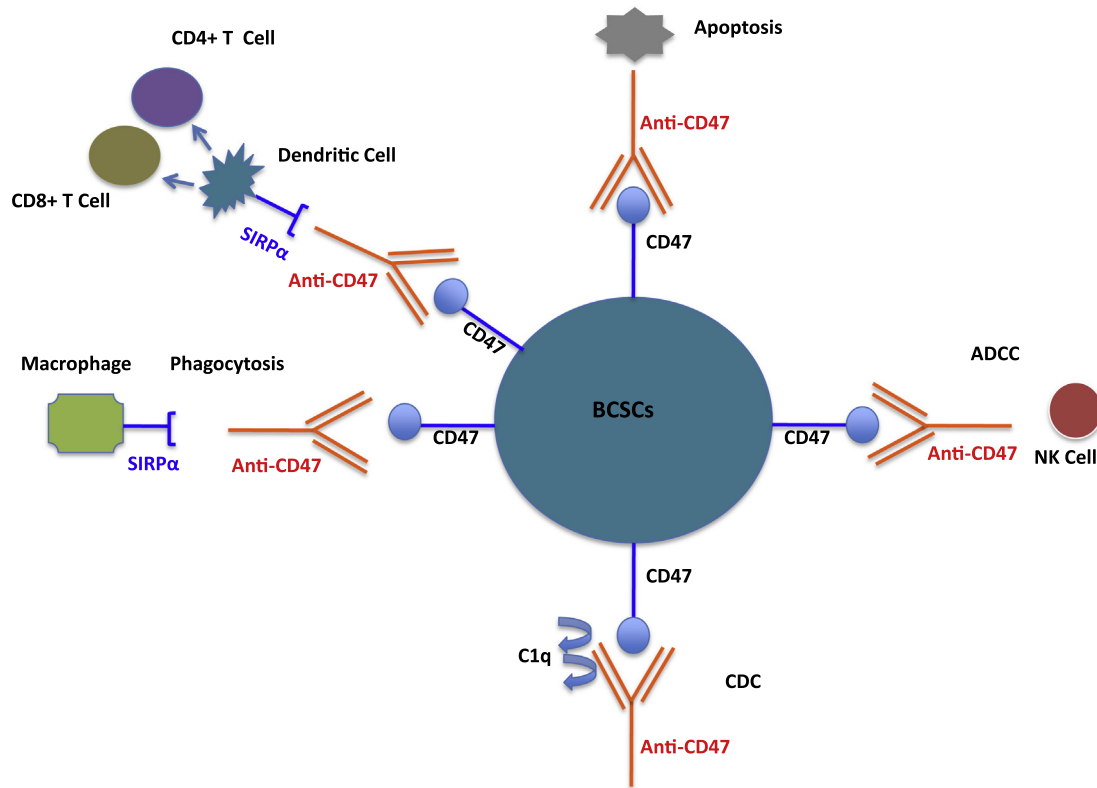
CD44 is an adhesion receptor on the cell surface, mediating the interaction between cells and the extracellular matrix. The main ligands of CD44 are hyaluronidase and osteopontin (OPN), both of which are also expressed in target tissues such as bone, liver, lung, and brain. OPN is localized on the luminal surface and plays an important role in cell adhesion, cytokine and growth factor production, as well as in the regulation of the immune system. Tumors in majority (71%) of breast cancer patients with early bone metastasis contain the CD44<sup>+</sup>/CD24<sup>-/low</sup> cells and CD44 adheres to bone marrow endothelial cells where OPN is expressed [88]. Therefore, it is reasonable to propose that increased OPN expression in the metastatic environment may enhance the capacity of BCSCs and tumor invasion and progression. In addition, OPN stimulates VEGF expression and induces the activation of endothelial cells, leading to vascular proliferation and resulting in tumor progression and cell migration [89]. The coordinative secretion of OPN and CD44 may therefore impact cancer cell migration to the specific sites of cancer metastasis [90].

Co-overexpression of OPN with chemokine receptor CXCR4 exhibits a high bone metastasis in breast cancer. CXCR4 can induce migration and homing of hematopoietic and other progenitor cells. It also interacts with its ligand CXCL12 (SDF-1) in cancer cells. Under this rationale, CXCR4 overexpression may be used as a monitoring index of lymph node negative and hormone receptor positive breast cancer recurrence [91]. CD24, also a key cell surface protein and cell lineage marker of hematopoietic cells, negatively regulates CXCR4 and restricts cancer cell metastasis by antagonizing CXCR4 chemotaxis. CD44 promotes the proliferation of progenitor cells by inducing the survival pathways mediated by integrin, which can also be activated by OPN. Therefore, the CD44<sup>+</sup>/CD24<sup>-/low</sup> subsets with a high migration activity probably resulted from increased CXCR4 due to decreased CD24 expression needs to be further explored.

### BCSCs immune evasion and potential CD47-SIRP $\alpha$ pathway

Accumulating evidence demonstrates that the immune system plays a significant role in preventing tumor initiation and controlling tumor growth [92–94]. Many cancers have evolved diverse mechanisms to evade such monitoring. Tumor surveillance, the targeting and elimination of cancer cells by the innate and adaptive immune system have been studied and macrophages and other phagocytic cells are shown to play a key role in regulating tumor growth through phagocytic clearance [95]. With the capacity of antigen presentation, phagocytosis, and cytokine production, the macrophages are believed to play a fundamental role in pathogen defense and inflammatory responses. The adaptive immune response is well established to exhibit an important role in anti-tumor immunity through macrophages [96]. Previously, it was thought that tumor growth was enhanced by tumor-associated macrophages (TAMS) [97]. More recently, TAMS, including protumorigenic or anti-tumorigenic macrophages, were shown to be able to present antigens, produce inflammatory cytokines, initiate angiogenesis, and tolerate cytotoxic activity [98]. In physiological conditions, however, the macrophage phagocytosis is significant for clearing damaged or foreign cells. Pro-phagocytic signals on the target cells enable macrophage-mediated phagocytic engulfment and clearance. Contrasted to the role of cytokine production and antigen presentation by macrophages on tumor growth, the importance of macrophage phagocytosis in tumor pathogenesis is relatively unexplored. Tumors including breast cancer may evade





**Fig. 3.** Targeting the CD47-SIRP $\alpha$  signaling pathway to eliminate cancer stem cells. An anti-CD47 antibody inhibits the CD47-SIRP $\alpha$  interaction, leading to macrophage phagocytosis of BCSCs. The anti-CD47 antibody is shown to eliminate BCSCs via antibody Fc-dependent mechanisms involving NK cell-mediated ADCC or CDC. In addition, anti-CD47 treatment may lead to phagocytic uptake of BCSCs by dendritic cells, resulting in antigen presentation to CD8<sup>+</sup>T and CD4<sup>+</sup>T cells and enhancing an anti-cancer adaptive immune response.

TAMS through the expression of anti-phagocytic signals, including CD47 and CD200 [92]. CD47 is a widely distributed membrane protein that interacts with the signal-regulatory protein  $\alpha$  (SIRP $\alpha$ ), an inhibitory receptor on myeloid cells that gives a so-called do-not-eat-me signal in tumor cells. Thus, multiple strategies may be developed to modulate CD47-SIRP $\alpha$  signaling in reducing the aggressiveness of BCSCs. Utilizing an anti-CD47 antibody, or an anti-SIRP $\alpha$  antibody, or a recombinant SIRP $\alpha$  protein to inhibit CD47-SIRP $\alpha$  interaction, leads to macrophage phagocytic engulfment of tumor cells [92,99,100]. The anti-CD47 approach may help to eradicate aggressive tumor cells via Fc-dependent mechanisms involving NK cell-mediated antibody dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [101] and by specifically stimulating a caspase-independent mechanism [102]. In addition, anti-CD47 treatment may also enhance the phagocytic uptake of cancer cells by dendritic cells, resulting in antigen presentation to CD8<sup>+</sup>T or CD4<sup>+</sup>T cells thus stimulating an anti-cancer adaptive immune response [103]. Based on these results, we propose a potential signaling network of CD47-SIRP $\alpha$  in therapy-resistant cancer stem cells shown in Fig. 3.

### Perspective

Continued efforts in dissecting the key characteristics of the BCSCs and their interaction with tumor microenvironments will shed new light on the mechanisms underlying breast tumor aggressive phenotype and reveal future therapeutic targets. Although several biological features of BCSCs have been identified, many unclear areas in BCSCs-associated tumor aggressiveness, especially in clinical studies, remain to be explored. Nevertheless, in an era of increasingly personalized cancer diagnosis, identifica-

tion of BCSC biomarkers will provide useful information to invent targets for both prognosis and intervention of breast cancer.

### Conflict of Interest

None declared.

### Acknowledgements

We apologize for not being able to cite all important works due to space restrictions. We would like to thank Dr. Dong Lin for providing immunoblot data; Dr. Andrew Vaughan, Dr. Guochun Jiang and Mr. Robert Li for helpful discussions and reviewing the manuscript. Research in the laboratory of the authors has been supported by NIH NCI Grants CA133402 and CA152313.

This paper is dedicated to the memory of Dr. Terry D. Oberley.

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