Review Article

Stem Cell INVESTIGATION

Cancer stem cells as a potential therapeutic target in breast cancer

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Abstract: Cancer stem cells (CSCs) are defined as the small population of cancer cells that have stem cell properties as in hierarchically organized tumors. They are considered as the source of tumor initiation and maintenance. These cells are highly resistant to current cancer treatment and may be responsible for the disease recurrence after therapy as well. Therefore, considerable efforts have been made to elucidate the molecular and pathological properties of the CSCs to develop effective therapies targeting CSCs. A growing body of experimental evidence has revealed that therapeutic targeting CSCs may offer a new strategy for patients with breast cancer (BC). In this review, we summarized the evidence for existence of CSCs, followed by an overview of their molecular biomarkers, signaling pathways and potential therapeutic strategies to target these CSCs in BC.

Keywords: Cancer stem cells (CSCs); breast cancer (BC); biological markers; molecular targeted therapy

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Introduction

Breast cancer (BC) is one of the leading causes of death among females globally (1). Although increased rates of early diagnosis of BC led to a significant reduction in mortality in recent years, many patients have recurrent BC (2,3). Thus, there is an urgent need to improve our understanding of the mechanisms of BC progression.

In recent years, experimental data indicate that BC is composed of heterogeneous cell populations with different biological properties (4-6). The tumorigenic process is preserved by a minor subset of cells in the tumor called cancer stem cells (CSCs) (7,8). CSCs are defined by their ability by their ability for self-renewal and multipotency (9-11). These features displayed by CSCs are important for a better understanding of the BC initiation and progression. Accumulating evidence suggests the presence of CSCs in BC, which may contribute to the failure of existing therapies to consistently eradicate malignant tumors (12,13). Therefore, therapeutic targeting of CSCs may provide a novel strategy that is more effective than the current drugs targeting the bulk mature cancer cells in treatment of BC.

This review will discuss the evidence for the existence of CSCs, novel molecular biomarkers and self-renewal pathways related to CSCs, as well as the possibility of targeting CSCs as a potential therapeutic strategy for BC.

The CSCs hypothesis

To fully appreciate the theory of CSCs, it is essential to understand the basic concepts of the biology of normal stem cells. Stem cells from normal tissues are characterized by the following essential properties: self-renew, giving rise to daughter cells that have limited proliferative potential and intended to differentiate, and the number of stem cells in normal tissues must be under strict genetic control in order to prevent uncontrolled expansion (14-16). Understanding of the basic biology of stem cells is crucial for the development of CSCs hypothesis. The emerging and controversial CSCs theory proposes that there is a small fraction of cancer cells which constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor (10,17,18). These cells with the properties resembling stem cells are called CSCs (9,18,19). Currently, the widely accepted definition of a CSC is a cell within a tumor that possesses the capacity to self-renew

and to cause the heterogeneous lineages of cancer cells that comprise the tumor (10).

The CSCs hypothesis has gained wide attention in recent years after the identification of subpopulations of tumor-initiating cells in hematological malignancies and solid cancers such as breast, colon, pancreas, lung, prostate and brain cancers (20-26). Serial xenotransplantation of a putative CSCs-enriched population in immunodeficient mice is the primary assay used in demonstrating CSCs (10). Tumorigenic capacity of stem cells can also be characterized based on the expression of defined cell surface makers and intracellular enzymes such as aldehyde dehydrogenase (ALDH) (10,27).

In BC, CSCs are identified by the presence of cell surface marker protein CD44, with low levels of CD24 (6,28,29). Breast stem cells can be easily identified by their ability to grow in serum-free suspension cultures which called mammospheres, an *in vitro* alternative test for self-renewal (30,31). Besides, they could also be identified by the ability of retaining bromodeoxyuridine or H3-thymidine (32,33). In BC, the CSCs hypothesis could have profound implications in the prevention, detection and treatment of the disease (34). In addition, the heterogeneity of BC is attributed by some researchers as a function of CSCs (35). It is also suggested that the CSCs hypothesis could be incorporated into the molecular staging of BC (36,37). Growing evidence suggests that CSCs may be responsible for therapy resistance and relapse of BC. For instance, a higher proportion of $CD44^+$ $CD24^{-/low}$ cells of BC are associated with shorter relapse-free and overall survival with increased distant metastases (38-41).

Biomakers of CSCs in BC

Distinct and specific surface biomarker phenotypes can be used to distinguish CSCs from other tumor cells and normal stem cells. Currently, the most common method used to identify CSCs is fluorescence-activated cell sorting based on cell surface markers or intracellular molecules.

CD44

CD44 is a type I transmembrane glycoprotein that binds hyaluronan and a variety of extracellular as well as cell-surface ligands (42,43). The molecule exists in multiple spliced forms and shows enormous variability in glycosylation (42-44). The CD44 protein contains four major domains, including the conserved extracellular hyaluronan-binding and variably spliced regions, the transmembrane sequence, and the intracellular cytoskeletal-signaling domain (44,45). CD44 plays an important role in adhesion, motility, proliferation and cell survival (46). It's a useful marker for identifying CSCs in breast tumors as well as in various other tumors (6,24,47-49). It's reported that ESA^+ $CD44^+$ $CD24^{-/low}$ Lin^- cells were identified as breast CSCs (6). They found that, this population has a greater capacity for tumor formation in immunodeficient mice compared to other cell populations (6).

CD24

CD24 is a heavily and variably glycosylated 35-60 kDa GPI-linked sialoprotein that is expressed on B cells, T cells, and keratinocytes (50). It is also a marker for exosomes released into the urine and amniotic fluid. CD24 binds to P-Selectin on activated platelets and vascular endothelial cells (51,52). The expression of CD24 is a hallmark of a wide range of epithelial cancers and has recently been used as an indicator of metastasis (53-55). The presence or absence of CD24 on the cell surface has been used as a marker for putative CSCs, which seems to be tissue specific (56). The $CD44^+$ $CD24^{-/low}$ population of cancer cells were defined as breast CSCs (6). They were found to have increased adhesion, invasion, and migration characteristics when compared with CD24 expressing cells (54,57). Recent reports showed that breast CSCs have a mesenchymal phenotype (58). Also, transformed BC cells could be able to switch between the mesenchymal and epithelial phenotypes (58).

ALDH activity

ALDH belongs to the ALDH family which is a group of enzymes involved in oxidizing a wide variety of intracellular aldehydes into their corresponding carboxylic acids (59). There are different isoforms of ALDH and ALDH1 is a detoxifying enzyme responsible for the oxidation of aldehydes intracellular. The Aldefluor assay system has been developed to detect the activity of the ALDH1 isoform (60). ALDH1 activity showed to be increased in CSCs and has since been successfully used to isolate CSCs in different cancers (61-65). Normal and cancer human mammary epithelial cells with increased ALDH activity have stem/progenitor properties by utilizing *in vitro* and *in vivo* experimental systems (13). In breast carcinomas, high ALDH activity identifies the tumorigenic cell fraction, which is capable of self-renewal and of generating tumors

that recapitulate the heterogeneity of the parental tumor (13,66). Also, it showed that ALDH1 was a predictor of poor clinical outcome of BC patients (64). In conclusion, ALDH1 activity has been widely used as a functional stem cell marker to isolate CSCs in BC.

Side population (SP)

Hoechst 33342 is a DNA dye historically used for flow cytometric analysis of the DNA content of live cells (67). Hoechst is able to penetrate intact cell membranes and it could be also transported out of cells by ATP-binding cassette (ABC) transporters (68). SP cells can be identified using dual wavelength flow cytometry combined with Hoechst 33342 dye efflux. These cells have been detected in various human solid malignant tumors including BC (69-71). It's reported that SP cells have increased resistance to chemotherapeutic agents and apoptotic stimuli (72,73). Also, SP cells have increased migratory potential and thus may play an important role in the metastatic spread of BC (74). However, recent studies have shown arising problems in using SP cells as a CSCs fraction because of conflicting results due to cross-contamination of the SP and non-SP fractions (75).

Other biomarkers

Additional markers useful in characterizing breast CSCs were recently reported. CD133, identified for breast CSCs isolated from cell lines generated from mouse mammary tumors, is a known marker of CSCs in several solid tumors (76-78). An additional marker, PROCR, identified using gene expression profiling of primary BCs, is also a known marker of hematopoietic, neural, and embryonic stem cells (79,80). Other surface markers such as CXCR4 and ABCG2 may be associated with CSC characteristics. CXCR4 was reported to promote metastasis in BCs (81). Recently, a highly tumorigenic subpopulation expressing $PROCR^{+}ESA^{+}$ was identified (82), and which may provide a CSC molecular signature in BC.

Key signaling pathways

The signaling pathways that regulate self-renewal and differentiation of CSCs are not well understood. However, it seems that there are some overlap in the key signaling pathways between CSCs and normal adult stem cells. Here we summarized some of these signaling pathways such as Wnt/β-catenin, Hedgehog (Hh), and Notch signaling that play a vital role in regulating BCSCs.

Wnt/β-catenin signaling pathway

The Wnt signaling pathway is critical for the regulation of embryogenesis, cell fate determination, self-renewal and differentiation of stem cells (83). It causes an accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional coactivator of transcription factors that belong to the TCF/LEF family (84). In the absence of Wnt signal, β-catenin is targeted by coordinated phosphorylation by CK1 and the APC/Axin/GSK-3β complex leading to its ubiquitination and proteasomal degradation. However, in the presence of Wnt ligand, the co-receptor LRP5/6 is brought in complex with Wnt-bound Frizzled. This leads to activation of Dishevelled (Dvl) by sequential phosphorylation, poly-ubiquitination, and polymerization, which displaces GSK-3β from APC/Axin through an unclear mechanism. In addition, it allows β-catenin to accumulate and localize to the nucleus and subsequently induce a cellular response via gene transduction alongside the TCF/LEF transcription factors (84). Wnt/β-catenin signaling is implicated in the maintenance of CSCs of a variety of cancers including BC (83,85). For example, upregulation of β-catenin in stem cell survival pathway was shown to mediate the resistance of mouse mammary stem/progenitor cells to radiation (73). MMTV-Wnt1 transgenic mice could develop premalignant mammary hyperplasia with elevated stem cell numbers, and their subsequent carcinomas contain a CSC population defined by methods similar to those applied to human BCs (86). It's also reported that Wnt proteins could act directly on mouse mammary stem cells to promote their self-renewal or expansion (87).

Notch signaling

Notch signaling is an evolutionarily conserved pathway in multicellular organisms that regulates cell-fate determination during development and maintains adult tissue homeostasis (88). The Notch transmembrane signaling proteins are expressed in both stem cells and early progenitor cells. It has four different notch receptors, referred to as NOTCH1-4, which play an important role in normal breast development, cell fate, and stem cell self-renewal (89). Aberrant Notch signaling has been implicated in the development and progression of both preinvasive

ductal carcinoma in situ and invasive BC (88,90). Notch signaling pathway is believed to be dysregulated in CSCs, ultimately leading to CSCs uncontrolled self-renewal. For example, it was shown that Notch signaling play an important role in the self-renewal function of malignant breast CSCs (88,91). Notch 4 is critical for normal mammary development, which could suppress differentiation of breast epithelial cells *in vitro* and development of normal mammary glands while promoting the development of mammary tumors *in vivo* (91). These observations suggest that alterations of Notch 4 signaling might be involved in the transformation of normal mammary stem cell to CSCs.

Hh signaling

Hh signaling pathway is a highly conserved pathway that plays a critical role in embryonic growth and cell fate determination during development (92). Vertebrates consist of three main Hh homologs: Indian hedgehog (Ihh), Desert hedgehog (Dhh) and Sonic hedgehog (Shh). Pathway activation is initiated by binding of one of the three Hh homologs to Patched (Ptch), an Hh receptor necessary for proliferation, differentiation and cell fate (93). Hh signaling is triggered by binding of ligands with transmembrane receptor Ptch and is subsequently mediated by transcriptional effectors belonging to the Gli family, whose functions is tuned by a number of molecular interactions and post-synthetic modifications (93). Hh signaling pathway is another major pathway that is involved in breast stem cell self-renewal (94). It's reported that the Hh pathway takes part in regulating self-renewal of normal and malignant human mammary stem cells (92). Accumulating evidence also suggests that inhibition of Hh signaling in breast tumors may interfere with the maintenance of a putative CSCs subpopulation (94). Human breast CSCs, as identified by the $CD44^+$ $CD24^{-/low}$ Lin^- phenotype, show increased gene expression of PTCH1, GLI1 and GLI2 compared to remaining tumor cells isolated from primary BCs (95). Additionally, it has been found that inhibition of Hh signaling increases the response of cancer cell lines to classical chemotherapies (96).

Breast CSCs as therapeutic targets

Accumulating studies have demonstrated a small subpopulation of CSCs exist in the cancer cell population. CSCs have powerful self-renewal capacity and tumor-initiating ability, and are resistant to conventional cancer treatment such as chemotherapy and radiation (9). These conventional anticancer therapies are effective at debulking the tumor mass but spare the relatively quiescent CSCs, which are responsible for cancer recurrence. So it is necessary to develop therapeutic strategies acting specifically on CSCs. Therapeutic targeting of CSCs may therefore provide a novel strategy that is more effective than the current drugs targeting the bulk mature cancer cells in treatment of BC. Numerous therapeutic approaches aiming at eradicating CSCs have been developed in recent years such as targeting molecular markers and key signaling pathways, as well as inducing the differentiation of BCSCs.

The first approach is to target molecular markers of CSCs. CD44 is a CSCs surface marker and is upregulated in invasive breast carcinoma (6). It's reported that targeting CD44 with the specific antibody P245 significantly inhibited the growth of human BC xenografts (97). Treatment with this antibody prevents tumor relapse after chemotherapy-induced remission in a basal-like human BC xenografts (7,97). Moreover, in the treatment of MCF-7 BC, an anti-CD44 antibody-conjugated gold nanorod has been used to target and photo-ablate $CD44^+$ cells, which display significant CSC characteristics (98,99).

A second approach is to target key signaling pathways of CSCs. The stem cell signaling pathways play important roles in CSCs renewal and maintenance such as Notch, Wnt/β-catenin and Hh pathways. Small molecules of gamma secretase inhibitors (GSI) or Notch 4 neutralizing antibody have been shown to reduce the population of CSCs (100). GSIs are currently undergoing clinical trials for the treatment of advanced BC. It showed that oral GSI was well tolerated at a weekly dosing, but no clinical benefit was observed in patients with BC (101). Furthermore, inhibition of Wnt signaling by dietary polyphenols curcumin and piperine has been shown to decrease mammosphere formation and percentage of ALDH1-positive cells (102). Some studies also demonstrate that inhibitors of Wnt/β-catenin signaling eradicated breast tumor-initiating cells *in vitro* and *in vivo*, which provide a compelling rationale for developing such antagonists for BC therapy (103). Finally, recent studies demonstrate that Hh signaling pathway plays an essential role in maintaining the $CD44^+$ $CD24^{-/low}$ subpopulation, and this pathway might represent a new candidate for BC therapy targeting CSCs (104).

Inducing differentiation of BCSCs is another approach to target CSCs. It will result in the loss of the potential to self-renewal in the CSCs. Enforced expression of let-7 miRNA induced differentiation of $CD44^+$ $CD24^{-/low}$ CSCs

and inhibited their ability to form tumors in mice (105). Most recently, Gupta *et al*. used a high-throughput screening approach to determine the anticancer activity of approximately 16,000 compounds. It was identified that salinomycin could selectively target $CD44^+$ $CD24^{-/low}$ CSCs (106). Treatment of mice with salinomycin induced epithelial differentiation of tumor cells and resulted in inhibition of tumor growth (106). These findings suggest that inducing differentiation of CSCs might be a promising approach for breast cancer therapy.

Conclusions

In summary, recent studies have identified a small population of highly tumorigenic cells with stem cell properties in human BC that are referred to as BCSCs. They are considered to be the source of tumor initiation and maintenance. Also, growing evidence suggests that CSCs may be responsible for therapy resistance and relapse of BC. Current treatments of BC have shown efficacy in removing the bulk of differentiated cancer cells while failing to eliminate the BCSCs, targeting BCSCs might be a promising approach to treat BC metastasis and relapse.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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