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Breast cancer stem cells: A new challenge for breast cancer treatment

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1. ABSTRACT

The biggest challenge for cancer research is relapses that occur in patients undergoing chemotherapy and radiotherapy, suggesting that some cells in tumors escape targeted treatment. Key questions are why relapses occur and why current therapies fail to remove all cancer cells. The cancer stem-cell hypothesisis based on the fact that not all cells within a tumor are similar. Other than tumorigenesis and metastasis, cancer stem cells have some properties that are similar to those of normal stem cells, such as self-renewal and differentiation. Accordingly, breast cancer stem cells may arise from mutation of normal mammary stem cells or progenitor cells. Cancer stem cell regulation involves several factors, such as Wnt, Notch, and Hedgehog, mutations of which endow cancer stem cells with the capacity for self-renewal. Moreover, epithelial mesenchymal transition and microRNAs recently have been shown to regulate the "stemness " of cancer cells. Targeting cancer stem cells could prevent relapse and provide new hope for cancer prevention.

2. INTRODUCTION

Despite all the progress made toward treating breast cancer, it still remains one of the leading causes of cancer-related deaths among women, causing more than 40,000 deaths per year (1) in the United States. Moreover, relapses in patients following surgery and chemotherapy, indicating the existence of specific breast cancer cells that are refractory to chemotherapy and capable of surviving for prolonged periods. Breast cancer is a complex and heterogeneous disease; not all cancer cells have similar potential for proliferation and differentiation. We still do not understand the intricate mechanisms involved in cancer progression. Recently, the cancer stem-cell (CSC) hypothesis has evolved to explain the heterogeneity, complexity, and recurrence of cancer after chemotherapy. In this review, we will focus on the importance of cancer stem cells in breast cancer progression.

3. CONCEPT OF CANCER STEM-CELL THEORY

The classical view of carcinogenesis supports the idea that any cell within a tissue can be transformed to a tumorigenic cell and that each cell within a tumor has the same tumorigenic potential (2). If the classical view were correct, all cancer cells would have similar potential to grow tumors. Thus, few cancer cells could grow into new tumors. In addition, the classical model cannot explain the heterogeneity within cancers. Another model of carcinogenesis is the cancer stem-cell (CSCs) model, which is based on three observations: first, tumors arise from single cells; second, not all cells within tumors are similar (3); and third, large numbers of cells are required to grow a tumor (4). Thus, the CSCs model not only contradicts the classical model, but supports hierarchy in cancer cells (5). However, these two models are not mutually exclusive. Tumor growth can be initiated by CSCs and further accumulation of mutations will lead to the development of more aggressive CSCs, driving tumor growth.

3.1. What are cancer stem cells?

As their name indicates, cancer stem cells have stem-cell-like properties; CSCs are a population of cancer cells that have the ability to self-renew and differentiate into multiple cell types , thus generating the diverse cells that comprise a tumor (6). Although, in general, stem cells maintain a balance between differentiation and self-renewal, depending on the environmental conditions (7), this balance is lost in cancer stem cells.

3.2. Where do cancer stem cells come from?

It is well known that the accumulation of various mutations converts normal somatic cells into cancer cells. In some cases, CSCs arise from the mutation of normal stem cells. Interestingly, stem cells not only have the highest proliferative potential, but also have the longest life span, giving them the opportunity to accumulate genetic mutations (6). Some CSCs arise from mutations in restricted progenitors or differentiated cells that have acquired the properties of cancer stem cells, such as the capacity for self-renewal (8) . One good example of this category occurs in hematopoietic malignancies, in which various fusion products, such as the MOZ-TIF2 (9), MLL-AF9 fusion products, confer stem-like properties on committed progenitor cells, leading to the generation of CSCs.

3.3. Identification of cancer stem cells

The first solid proof of existence of cancer stem cells was provided by Dick *et al* in acute myleoid leukemia (AML) (5), where the development of acute myeloid leukemia (AML) from progenitor cancer cells in immunodeficient mice was demonstrated (5). These cells were initially termed as ***leukemia-initiating cells***, since they have the capacity to differentiate and self renew. Hierarchy within these cells was also demonstrated, since these initiating cells were able to differentiate *in vivo* into leukemic blast cells. These leukemia initiating cells were found positive for CD34 and negative for CD38 and these glycoproteins served as cell surface markers for these initiating cells.

3.4. Stem cells in breast tissue

The first demonstration of the existence of stem cells in rodent mammary glands, Kordon and colleagues (10) demonstrated the ability of multipotent stem cells to recapitulate entire mouse mammary glands. The self-renewal property of stem cells was also demonstrated by serial transplantation of mammary epithelia, which repopulated mouse mammary glands. Until recently, purification of stem cell isolation has been difficult due to lack of good cell-surface markers. However, Visvader *et al*. (11), using fluorescence-activated cell sorting and different cell-surface markers, have identified a subpopulation of mammary glands that can reconstitute a complete mammary gland *in vivo*. In this subpopulation, $Lin^-CD29^{hi}CD24^+$, Lin^- indicated that cells were negative for endothelial marker ($CD31^-$) and hematopoietic antigens ($CD45^-$ and $TER119^-$). The Lin^- population was further screened for high expression of cell-surface markers CD29 and CD24. It was concluded that cells having a phenotype of the $Lin^-CD29^{hi}CD24^+$ population are multipotent and self-renewing. Several studies have demonstrated similarities in the expression of genes in normal mammary stem cells and breast cancer stem cells (BCSCs) (12,13) and that BCSCs express the same cell-surface markers as do mammary stem cells (6). Hence, the transformation of normal stem cells into cancer stem cells may drive the growth of tumors.

4. BREAST CANCER STEM CELLS AND CANCER:

Clarke (13) has demonstrated the presence of stem cells in breast cancer. Among the many breast cancer cells, only a few are tumorigenic; i.e., capable of forming tumors. These tumorigenic cells generate both other tumorigenic cells and nontumorigenic ones, which form the major mass of tumor . The tumorigenic cells are identifiable by their higher expression of cell-surface marker protein CD44 (14) and their low expression or lack of CD24 (15). Both CD24 and CD44 are involved in cell adhesion, cell migration, and metastasis (16) (17). It has been found that as few as 100 cells that are $CD44^+$and CD 24 (low phenotype) are sufficient to form tumors and can be serially passaged in immunocompromised mice, indicating that this population has tumor- initiating cells (15) (16). These tumorigenic populations generated tumorigenic as well as nontumorigenic cell populations. Thus, elucidation of breast cancer stem cells has driven a new era of research in breast cancer.

4.1. Role of breast cancer stem cells in metastasis

Metastasis, the leading cause of death among cancer patients (18), is a complex process involving various cellular processes, including cell migration, invasion, adhesion, growth, and division. Stem cells have been thought to responsible for metastasis due to the fact that not all breast cancer cells within a tumor possess the same metastatic potential, and only a small subset of cells home to specific sites in the body, (6). CSCs do possess tumorigenic, invasive and migratory characteristics necessary to induce metastasis. Moreover, BCSCs possess an increased ability to survive in foreign microenvironment (19). CSCs are likely to have a major role in tumor metastasis and recurrence after treatment (6). In agreement

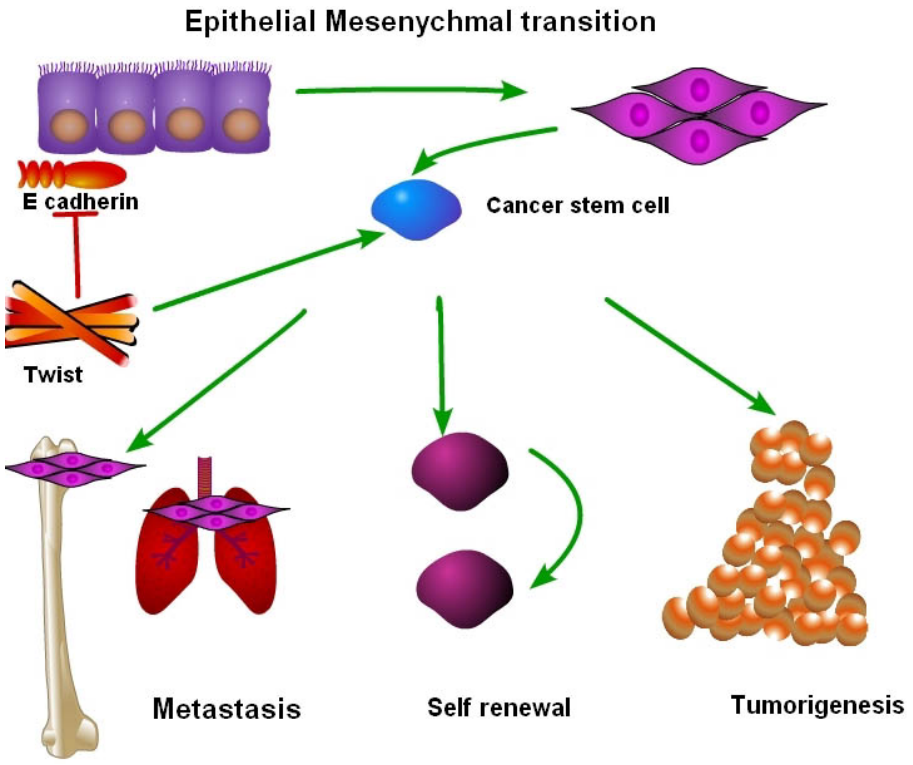

Figure 1. EMT driven "stem cell" and "tumorigenic" characters of BCSCs: The epithelial-mesenchymal transition in epithelial cells leads to various stem-cell properties such as self renewal, metastasis, and tumorigenesis.

with this, the bone marrow of cancer patients has been demonstrated to have disseminated CD44 (+) and the CD24 (-/low) phenotype (20) , which corresponds to the characteristics of breast cancer stem cells. These cells are present in both primary tumors and distant metastases. BCSCs have higher expression of CD44 than other tumor cells do. Also, it has been found that metastatic breast cancer cells adhere to endothelial cells of bone marrow via CD44 (21).

Charafe-Jauffret *et al.* (22) have identified BCSCs from different breast cancer cell lines based on the expression of aldehyde dehyrogenase and demonstrated that these BCSCs are responsible for mediating metastasis. Molecular profiling was done to identify different genes that are important in stem-cell function. CXCR1/IL-8RA was identified as one of those genes. CXCR1 is a receptor that binds CXCL8 and the chemokine IL-8,which is important in stimulating the self-renewal of BCSCs (22). In a different study, the same investigators found that BCSCs can be inhibited by blocking the CXCR1 receptor. The efects of CXCR1 are mediated by the FAK/AKT/FOXO3A pathway (23). Blocking CXCR1 by a CXCR1-specific antibody results in reduction of tumor growth and metastasis. Another chemokine receptor, CXCR4, has been shown to be crucial in key steps of metastasis, such as chemotaxis and homing. Through interaction with its ligand CXCL12/SDF-1α, CXCR4 is a major factor in breast cancer metastasis (24). Also, CXCR4 expression levels have been shown to correlate with the presence of metastatic prostate and breast cancer (25). Schabath *et al.* have established a relationship between BCSCs and CXCR4. CD24, a cell-surface protein not expressed by BCSCs, attenuates the function of CXCR4 in breast carcinoma (26), especially affecting the ability of cells to metastasize. It can thus be concluded that lack of CD24 expression in breast cancer stem cells makes these cells more migratory due to the influence of CXCR4 (26).

4.2. Epithelial mesenchymal transition and breast cancer stem cells

Epithelial mesenchymal transition (EMT) is a dynamic process that cells acquire normally during embryonic development, as well during metastasis, to increase their motility. Indeed, EMT has been demonstrated to be a prerequisite for cancer metastasis (27). A link has been shown between EMT and the acquisition of epithelial stem-cell properties in human mammary epithelial cells (HMLEs) (28). It has been found that the induction of EMT in HMLE cells results in various cancer stem-cell traits such as mammospheres, efficient soft agar colony formation, and tumor formation. Moreover, EMT markers are expressed in mouse and human mammary glands, as well as mammary carcinomas (28). It has been demonstrated that activation of the Ras-MAPK pathway in human mammary epithelial cells results in EMT-driven "stem-cell" and "tumorigenic" characters of BCSCs (29) (Figure 1).

Cells that have undergone EMT via immune response have a phenotype similar to that of BCSCs are more tumorigenic. These cells can re-establish tumors and

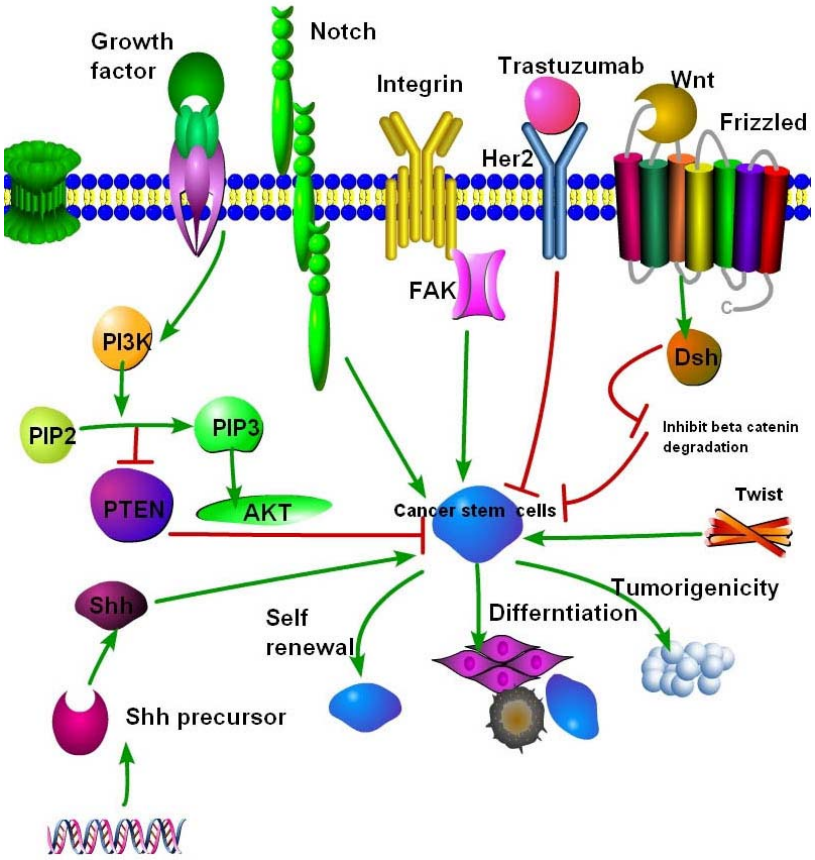

Figure 2. Different signaling pathways that regulate breast cancer stem cells (BSCSs) are shown. Self-renewal is one of the most important properties of stem cells. Gene mutations such as Her2, FAK, Notch, PTEN, Wnt, and Hh signaling pathways contribute to the self-renewal of breast cancer stem cells. Her2 overexpression in breast cancer cell lines increases the number of cancer stem cells. Trastuzumab specifically targets HER2-positive breast tumors.

have shown increased resistance to chemotherapy and radiation, indicating that EMT can produce BCSCs (30). Thus, EMT has been shown to endow normal and transformed mammary epithelial cells with mesenchymal and stem-cell properties, such as the ability to self-renew and to initiate tumors. The connection between EMT and stem cells may have numerous implications with regard to the progression of breast tumors. In addition to conventional chemo- and radiation therapies, which target bulk tumors, targeting specific molecular pathways associated with EMT in BCSCs populations may ultimately provide an effective strategy for treating breast cancer.

5. SELF-RENEWAL IS ONE OF THE MOST IMPORTANT PROPERTIES OF STEM CELLS

In the remaining sections of this review, we will describe different and important gene mutations that contribute to the self-renewal of breast cancer stem cells.

5.1. Her2

Human epidermal growth-factor receptor-2 (Her2) is a member of the ErbB family of receptor tyrosine kinases and has a key role in breast cancer progression. Overexpression of Her2 is correlated with a particularly aggressive clinical phenotype (31). Her2 overexpression in breast cancer cell lines increases the number of cancer stem cells (32). Moreover, breast cancer stem cells have been found to have increased Her2 expression (33). Trastuzumab and Lapatinib, two prescription drugs that are used to treat breast cancer specifically target HER2-positive breast tumors. These drugs were found effective in targeting Her-2 over expressing cancer stem cells, as evident by decrease in sphere forming efficiency and serial translatability. However, Her-2 overexpressing cancer stem cells have been shown to be sensitive to these drugs (33) (Figure 2).

Nakanishi *et al* (33) have demonstrated the expression of a side-population (SP) of cells in luminal-type breast cancer and the association of these cells with tumor-initiating cells (34). SPs were obtained by selecting for the expression of various surface antigens, including HER2, CD44, and CD24. It was found that HER2 expression was significantly correlated with the SPs. The use of different pharmacological modulators to evaluate the effects of HER2 signaling on SPs showed that the numbers of cells in SPs decreased in the presence of HER2 signaling inhibitors such as AG825 or trastuzumab. This indicates that HER2

contributes to the SP phenotype. In summary, HER2 has an important role in BCSCs, which may account for the poor responsiveness of HER2-positive breast cancer to chemotherapy, as well as the aggressiveness of cancer.

5.2. FAK

Focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, mediates intracellular signaling by integrins. Integrins are a class of cell-surface receptors for extracellular matrix; they regulate different cell functions by mediating signals either from inside to outside or outside to inside of cells. FAK gets activated upon binding of components of extracellular matrix (ECM) and its activation is enhanced by co activation of many extracellular stimuli such as G –protein coupled receptor, cytokines and inflammatory mediators (35). FAK is overexpressed in breast cancer. This increased FAK expression correlates with metastatic disease and poor prognoses (36). Upon activation by integrin, FAK undergoes phosphorylation, forms a complex with Src, and regulates downstream signaling.

Deletion of FAK in mouse mammary epithelium suppressed mammary tumorigenesis by effecting breast cancer cells/ progenitor cells (37). Moreover, it has been shown that FAK-targeted mice result in decreased self-renewal of mammary cancer stem cells *in vitro,* as well as tumorigenicity and impaired maintenance *in vivo*. Thus, FAK may serve as a novel target for regulating BCSC populations.

5.3. Notch

Notch receptor signaling pathways are important in normal breast development, as well as in the development and progression of breast cancer (38). In mammals, four notch receptors (notch1-4) interact with surface-bound or secreted ligands, including Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1, and Jagged 2. Modifier proteins from the Fringe family (Lunatic, Manic, and Radical Fringe) modulate these interactions between notch receptors and various ligands (39). Upon ligand binding, Notch receptors are activated by serial cleavage events involving members of the ADAM protease family, followed by intramembranous cleavage regulated by gamma secretase (presenilin), an enzyme that is necessary for Notch processing.

Harrison *et al.* (38) have evaluated the role of Notch receptors in regulating "stemness" in breast cancer cell lines and human tumor samples,demonstrating that Notch activity is higher in stem-cell-enriched populations than in differentiated populations. Moreover, pharmacologic or genetic inhibition of Notch1 or Notch4 reduced tumor formation *in vivo*. Interestingly, Notch 4 inhibition more strongly inhibited tumor growth. These studies show that Notch4-targeted therapies will be more effective in suppressing breast cancer recurrence. Hirose *et al.* (40) have shown that inhibition of notch signaling in Her-2-negative breast-cancer-initiating cells inhibits survival signals and thus Notch represents a therapeutic target for her 2 negative breast initiating cells (40) (Figure 2).

5.4. Twist

Twist, a transcription factor of the basic helix-loop-helix class protein, down-regulates E-cadherin and is involved in metastasis (41). Vesuna *et al.* (42) have demonstrated the function of Twist in regulating breast cancer stem cells. Overexpression of Twist in breast cancer cells produced a phenotype of cancer stem cells characterized by high expression of CD44, low or no expression of CD24, increased aldehyde dehydrogenase 1 activity, and high expression of ABCC1 transporter (42). Furthermore, these investigators have shown as few as 200 cells overexpressing twist forms tumor in the mammary fat pads of mouse. They have also demonstrated that Twist transcriptionally regulates CD24, thus Twist is important in the regulation of BCSCs.

5.5. PTEN

The deletion of phosphatase and tensin homolog (PTEN), which is a dual lipid and protein phsophatase, acts as a tumor suppressor. PTEN is mutated in various cancers, including breast cancer (43). PTEN acts as a lipid phosphatase to dephosphorylate phosphatidylinositol (3-5)-trisphosphate (PIP3), antagonizing the PI3-K/Akt pathway. Deletion of PTEN results in increased activation of the PI3-K/Akt pathway, which correlates with poor prognosis in breast cancer patients (44). A recent study by Hasan Korkaya *et al* have demonstrated the role of the PTEN/Akt pathway in the regulation of normal and malignant mammary stem/progenitor cell populations (45). PTEN knockdown leads to Akt-driven enrichment of normal and malignant human mammary stem/progenitor cells via activation of the Wnt/beta catenin pathway. Moreover, Korkaya *et al.*, have demonstrated that the Akt inhibitor perifosine targets the tumorigenic cell population in breast tumor xenografts (45). These studies indicate the possibility that the PTEN/PI3-K/Akt/beta-catenin pathway can be used therapeutically as a means of targeting BCSCs.

5.6. Wnt

Wnt (Wingless-Int) signaling is involved in regulating the self-renewal and differentiation of a variety of stem cells (Figure 2). Lindvall *et al.* (46) found that Lp6, the Wnt coreceptor, is required for normal mouse mammary development. One recent study has provided a link between the Wnt signaling pathway and EMT (46) (47). Using animal models, DiMeo *et al* (47) have shown that several components of Wnt signalling, such as WISP2, FZD9, LRP5, HoXD4, HUNK, and others are overexpressed in early lung metastases and that this overepression correlates with basal-like breast cancer. They demonstrated that inhibition of wnt signaling leads to decrease in the capacity of cancer cells to self renew and tumorigenesis. It has been shown that EMT leads to acquisition of stem cell characteristics. It was found that inhibition of wnt signalling also resulted in the suppression of EMT transcription factor such as Twist and slug.

5.7. Hedgehog

Hedgehog (Hh) is a developmental pathway that directs growth and embryonic patterning. It is also implicated in cancer (48). BMI-1, a well-studied polycomb group of proteins, is important in the self-renewal of stem cells. The role of Hh signaling and BMI-1 in regulating the self-renewal of normal and malignant human mammary stem cells has been demonstrated (49). The hedgehog

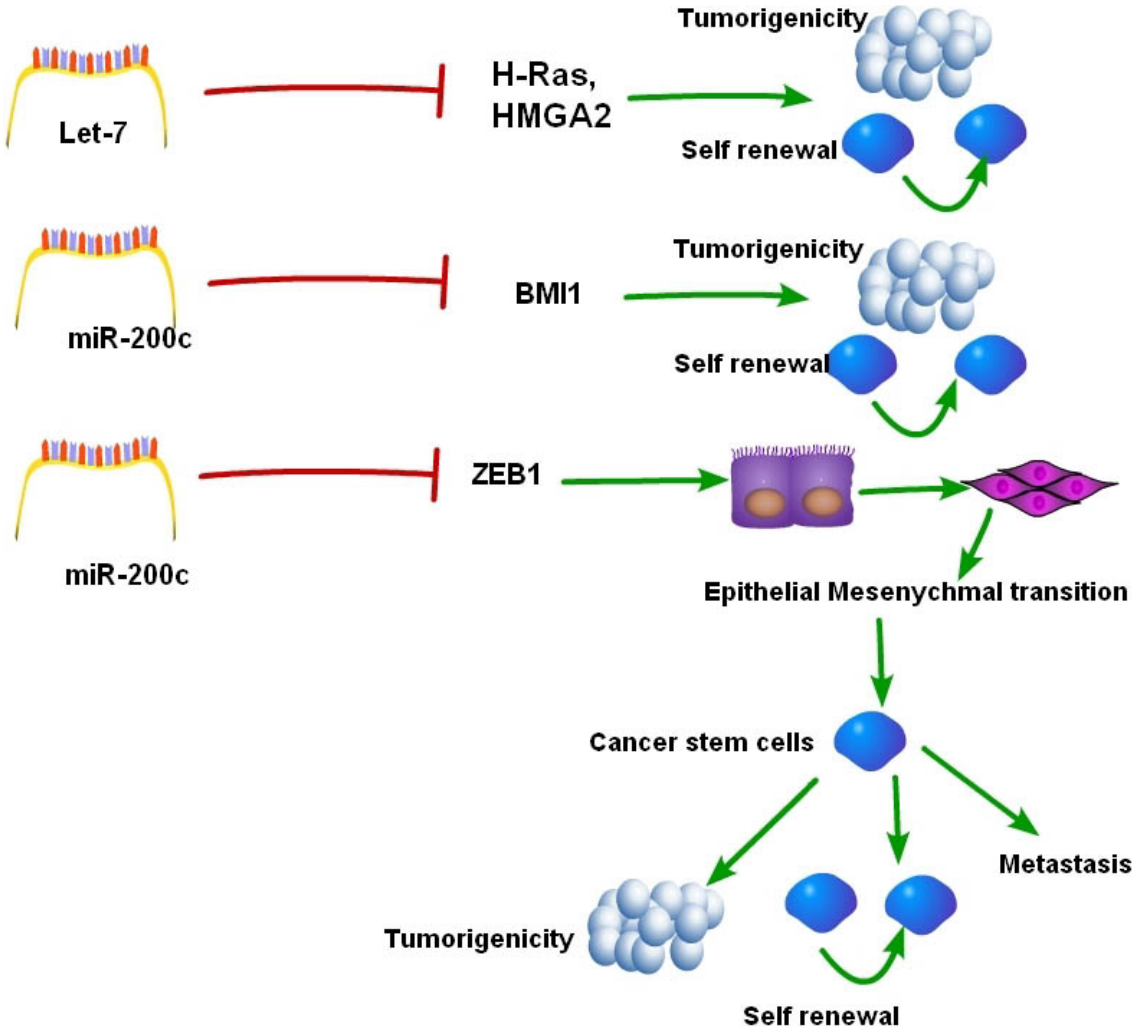

Figure 3. MicorRNAs are important in regulating cancer stem cells. Let-7 inhibits expression two oncogenes, RAS and HMGA, to regulate BCSCs, thereby also regulating tumorigenicity. miR-200c targets BMI1, which is a regulator of stem cell self-renewal. miR-200C inhibits normal mammary ducts and tumor formation driven by BCSCs *in vivo*. miR200c also regulates E-cadherin expression and EMT by repressing ZEB1, and thus regulates tumor formation.

signaling components, including PTCH1, Gli1, and Gli2, are highly expressed in mammospheres but, during the differentiation state, these components are down-regulated. These effects are mediated by the polycomb group of protein BMI-1. Hence, therapeutic manipulation of the Hh pathway is of great therapeutic value in cancer biology, and thus several preclinical studies and clinical trials underway in a range of malignancies.

6. ROLE OF miRNAS IN BREAST CANCER STEM CELLS OR TUMOR-INITIATING CELLS

MiRNAs are posttranscriptional regulators that have been found to have a role in stem cells (50) and carcinogenesis. In the first study elucidating the role of miRNA in breast cancer stem cells, Yu *et al.* (51), using microarray analysis, showed that miRNAs, including the let-7 family, display a noticeable regulation in parental SKBr3 cells that have not been enriched for CSCs as compared to the SK-Br-3rd cell line, which was selectively enriched by chemotherapy for breast cancer stem cells. Let-7 silencing contributes to the "stemness" in breast cancer stem cells. Yu *et al.* (49)found that let-7 inhibits two oncogenes, RAS and HMGA2. In nonenriched parental SK-Br3 cells, H-Ras and HMGA2 levels were low, but knockdown of let-7 increased their expression levels. In SK-Br-3rd cells enriched for CSCs, levels of H-RAS and HMGA2 were elevated, but these levels were decreased by forced expression of let-7. Hence, this study highlights the role of let-7 in regulating breast cancer stem cells (Figure 3).

Shimono Y *et al* have demonstrated a molecular link between BCSCs with normal breast stem cells (52) and

demonstrated the differential expression of 37 microRNAs in human BCSCs and nontumorigenic cancer cells. They found that three clusters of microRNA, miR-200-c-141, miR-200b-2001-429, and miR-183-96-182, were down-regulated not only in BCSCs, but also in mammary stem/progenitor cells, and embryonal carcinoma cells. Moreover, they found that miR-200c targets BMI1, a known regulator of stem-cell self-renewal. miR-200C inhibits mammary duct formation and tumor formation driven by BCSs *in vivo*. Hence, miR-200c connects BCSCs with normal stem cells (52).

It should be noted, however, that few original studies have been done on miRNAs in CSCs. Moreover, miR200c regulates E-cadherin expression and EMT via repressing ZEB1 (53).These studies suggest that the miR-200 family miRNAs are important regulators of multiple stem-cell functions that control both EMT and self-renewal. Hence, functional studies of specific miRNAs within the CSCs will benefit the development of novel therapeutic methods to target CSCs.

7. CONCLUSION

Delineating these specific populations within breast cancer and designing therapeutic measures against these cancer stem-cell populations would be helpful in preventing relapses of breast cancer. Most clinical treatments operate under the assumption that all cancer cells have equal malignant potential. Thus, these treatments shrink the bulk of tumor cells but often fail to eliminate cancer stem cells, resulting in the recurrence of tumors. Hence, therapies targeting the cancer stem-cell maintenance pathway will eliminate the tumor-forming cells and possibly be curative.

One major challenge facing BCSC research has to do with the development of appropriate model systems for the isolation and characterization of BCSCs. Several lines of evidence have highlighted the importance of the tumor microenvironment in determining CSC properties (54). There is an urgent need to develop model systems in which the tumor microenvironment can be accurately recapitulated. Usually BCSCs have been found within malignant tumors. However, Xu *et al.* (53) have shown that BCSCs can be isolated and form benign tumors (55). This study challenges the link between CSCs and malignancy. Drugs targeting various pathways regulating stem-cell renewal, such as the Her2, PTEN, Notch, Wnt, Hedgehog pathways, can serve as targets to treat breast cancer. In summary, research on BCSCs is in the nascent stage. Further studies are needed to unravel the mystery of these chemo-resistant cancer stem cells.

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