REVIEW ARTICLE

Signaling Factors Involved in Self-Renewal of Breast Cancer Stem-like Cells

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Abstract

Since cancer stem-like cells (CSC) was identified in acute myeloid leukemia (AML) in 1997, the involvement of CSC has been reported in various cancers including glioma, breast cancer, lung cancer, intestine cancer, skin cancer, etc. Cancer cells are speculated to originate from the small CSC population; CSC also display chemotherapeutic resistance and radio-resistance. Hence, control of the CSC population may lead to the development of therapeutic strategies for inhibiting tumor growth, recurrence, or metastasis. To study CSC and their population dynamics, the flow cytometry analysis, tumor sphere culture and organoid culture would lead to the development of effective tools. Based on *in vitro* methods, characteristic signaling pathways in CSC were reported using these techniques, e.g., Wnt, Notch, and Hedgehog pathways. Furthermore, *in silico* analysis has revealed that key growth factors expressed via activation of nuclear factor κB (NF-κB) plays a role in CSC self-renewal. This review summarizes how CSC are distinguished from non-CSC and how CSC retain their self-renewal capacity through signaling or growth factors in an autocrine or paracrine manner in breast cancer.

Keywords

Cancer stem-like cells, breast cancer, signaling

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Introduction

Postoperative or post-treatment survival is greater among breast cancer patients than among those with other types of cancer. However, recurrence and metastasis to the bones, lungs, and brain are possible after a latent period of 5–10 years.[1] Various reports suggested that the cancer stem-like cells (CSC) are found in breast cancer and are considered to deter the prognosis of events including tumor recurrence and metastasis.[2–5] Tumors are speculated to comprise a heterogeneous cell population constituted by CSC, transit-amplifying (TA) cells, and terminally differentiated cells.[6,7] CSC can undergo self-renewal, symmetric cell division, and can yield terminally differentiated cells and somatic stem cells via asymmetric cell division[8,9] In this heterogeneous cell population, CSC, a small cell population, occupies the highest position in tumor hierarchy. With slow cell cycles and high anti-oxidative capacity compared with non-CSC, CSC are resistant to conventional chemo- and radiotherapy targeting proliferating cancer cells.[10–12] Despite large cancer cell populations being eliminated through chemotherapy, only a few CSC may survive and cause tumor recurrence and metastasis. Hence, it is essential to elucidate the characteristics of CSC and standardize methods of assessing CSC-enriched populations and associated culture methods. Flow cytometry analysis using known CSC-specific antibodies is a popular and simple method for assessing CSC.[13–15] The CSC population can be enriched and fractionated through flow cytometry analysis because cell membrane characteristics of CSC is analyzed in only living cells. In standardizing culture methods for CSC, tumor sphere culture and organoid culture are useful tools to assess the self-renewal capacity of CSC *in vitro*.[16,17] Sphere culture has been used to assess the survival and self-renewal capacity of neural stem cells in culture.[18]

CSC-derived tumor spheres are obtained through floating cell cultures in sphere culture medium (SCM) containing neural stem cells and a cocktail of growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and hormones. Because CSC are resistant to loss of anchorage dependence (anoikis), only CSC can grow under the serum-free and non-adherent conditions.[19,20] Hence, tumor sphere forming ability correlates with self-renewal capacity of CSC *in vitro*. On the other hand, organoid culture, that is three dimensional (3D) culture system, has been used as the study of cellular differentiation and morphogenesis from stem and progenitor cells containing with gut or mammary gland.[21–23] Organoids are expanded in collagen gel or matrigel that based on growth medium containing EGF, TGFβ-antagonist Noggin and the Wnt-agonist R-spondin1 as growth stimuli, and ROCK inhibitor Y-27632 to avoid anoikis.[24] In CSC study, organoid culture also is a helpful method for assessing the maintenance of tumor formation because tumors are able to be reconstructed from a small number of tumor cells *in vitro*.[25,26] Moreover, organoid culture is able to be applied to drug screening due to obtaining many clonal organoids.[27] Accordingly, organoid culture will be hopefully lead to not only assess the CSC capacity but also develop anti-CSC agents.

Controlling the CSC activity would influence the inhibition of tumor growth, recurrence, or metastasis. To establish the strategy to target CSC, it is important to identify the signaling pathways activated in CSC by using the flow cytometry and the CSC culture system. Recently, it continues to become clear that several potentials signaling pathways are activated in CSC. This review discusses signaling pathways in breast CSC and the possibilities for development of novel strategies for targeting breast CSC.

The Characteristic of CSC in Breast Cancer

Breast CSC were first reported in 2003 by Al-Hajj *et al.*.[28] They reported enrichment of breast CSC in a $CD44^{high}/CD24^{low/-}/Lineage^{-}$ cell population (so-called CSC population) derived from clinical samples subjected to flow cytometry, and this cell population had high tumor-initiating activity when inoculated into the mammary fat pads of immunodeficient mice. After that, by using staining of the cell surface antigen and the flow cytometry analysis in a similar way, specific markers expressed in cell surface of breast CSC were found. Epithelial cell adhesion molecule (EpCAM), CD10, β1 integrin (CD29), α6 integrin (CD49f), and CD133 may be used as cell surface markers of breast CSC.[28–31]

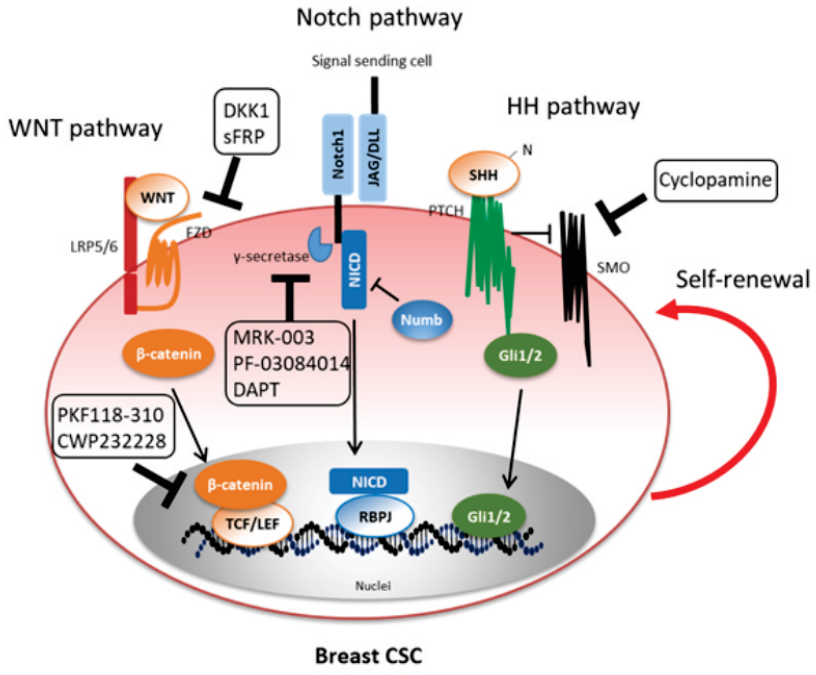

Figure 1. Mechanisms of self-renewal of breast CSC through activation of Wnt, Notch, and HH pathways. Hyperactivity of multiple signal pathways retains the characteristics and self-renewal ability of breast CSC. Several antagonists of signaling pathways inhibit breast CSC self-renewal. Abbreviations: LRP5/6, low-density lipoprotein receptor-related protein 5/6; FZD, Frizzled; TCF, T cell factor; LEF, Lymphoid enhancer-binding factor; JAG, Jagged; DLL, Delta-like; NICD, Notch intracellular domain; RBPJ, Recombining binding protein suppressor of hairless; SHH, Sonic hedgehog; PTCH, Patched;

SMO, Smoothened.

Breast CSC populations are maintained by molecules secreted from themselves (autocrine signaling) or their micro environment, i.e., the so-called CSC niche (paracrine signaling), e.g., endothelial cells, immune cells, and cancer-associated fibroblasts (CAFs).[32,33] These molecules intricately interact and regulate breast CSC self-renewal in an autocrine/paracrine manner; it has been difficult to understand distinctive signal pathways in breast CSC. However, over the past several years, potential signaling pathways activated in breast CSC have been elucidated primarily through *in silico* analyses. These include common pathways between cancer and somatic stem cells; however, it has been speculated that CSC are caused by abnormal and hyperactivation of various signaling pathways. Hence, we shall focus on the aberrantly activated signaling pathways, including Wnt, Notch, and Hedgehog pathways, for self-renewal of breast CSC (Figure 1). Moreover, we revealed that signaling pathways induced by key growth factors neuregulin 1(NRG) and insulin-like growth factor 2 (IGF2) to regulate self-renewal capacity of breast CSC.[34,35] Inhibition of those signaling pathways by reagents may probably help develop novel therapy for eradication of breast CSC.

Key Factors for Breast CSC Maintenance

Wnt pathway

The Wnt pathway regulates various functions of normal or tumor stem cells, through two pathways: canonical and non-canonical pathways.[36,37] In the canonical pathway, binding of Wnt ligands to a dual receptor complex comprising the WNT co-receptors LRP5 or LRP6 and the Frizzled family (FZD1-10), a seven transmembrane domain receptor, initiates Wnt–beta-catenin signaling. The non-canonical pathway includes the planar cell polarity (PCP) pathway and Ca^{2+} pathway, which does not involve beta-catenin. This review refers to the canonical Wnt pathway. The Wnt pathway is involved in mammary gland development and carcinogenesis in mice or human.[38,39] For example, *Axin2* is a direct target gene of the Wnt pathway though beta-catenin. A few of Axin2-positive stem cells generate basal and luminal alveolar cells in adult virgin mice[40], while Wnt-induced Axin2 activates Snail-induced epithelial–mesenchymal transition (EMT), resulting in breast cancer cell invasion and progression[41]. In studies on CSC, the expression of phosphorylated beta-catenin was lower in the side population (SP), which is a flow cytometry method for detecting stem cells or CSC based on the ability to efflux the fluorescent dyes, compared with the non-SP.[42] When the staining intensity of cytoplasmic and nuclear beta-catenin was measured, immunostaining intensity was higher in the SP than in the non-SP, and nuclear accumulation of beta-catenin was significant in the SP. Hence, the Wnt pathway may activate in CSC. In another group, overexpression of sFRP1 or DKK1, negative regulators of the Wnt pathway, or shRNA-mediated knockdown of LRP6 reduced tumor sphere forming ability, tumor growth, and metastasis in basal-like breast cancer.[43] However, overexpression of Twist in immortalized human mesenchymal epithelial cells (HMLE) induced EMT and tumor sphere formation.[44] Because Twist, an EMT-related transcription factor, upregulates *Axin* mRNA, a beta-catenin/TCF-LEF target gene, and inhibition of tumor sphere formation through treatment with recombinant sFRP1 or DKK1; this indicated that EMT-induced pathway contains beta-catenin-dependent Wnt pathway in autocrine signal and active migration and self-renewal of breast CSC. MicroRNAs (miRNAs) are important to understand the regulation of WNT signaling. Some miRNAs were reported to be regulators of CSC signaling. The high expression of miR-142 and miR-150 were observed in human breast CSC, and that miR-142 directly targets *APC* mRNA and those inducing the suppression of tumor sphere forming activity and tumor growth[25]. Another study reported that Let-7c expression was inversely correlated with estrogen receptor α (ERα) expression and Wnt activity.[45] Let-7c targets 3'UTR of *ERα* mRNA and inhibits breast CSC self-renewal though the APC/β-catenin/TCF pathway. Selectively targeting occurs through the Wnt pathway, e.g., PKF118-310 or CWP232228 as chemical antagonists of the Wnt pathway, inhibited breast CSC tumor sphere forming ability and tumorigenesis in a murine model.[46,47] Those may be candidate therapeutic agents for breast CSC maintenance through Wnt pathway.

Notch Pathway

The Notch pathway plays an important role in the development of breast CSC and their characteristics[48]. In mammals, Notch receptors comprise 4 subtypes (Notch1–4), which are activated by binding with five ligands (DLL, delta-like 1, 3, and 4; JAG, jagged 1 and 2) from

signal sending cells, leading to the expression of basic helix-loop-helix (bHLH) transcription factors containing HES family or HEY family.[49] It is known that the Notch pathway-related genes of mammary development correlate with breast cancer cells.[50] Hence, the high expression of Notch receptor correlates with poor prognosis of breast cancer patients.[51] In breast CSC, the Notch pathway induces hyperactivation of aldehyde dehydrogenase 1A1 (ALDH1A1), a marker of stemness, through the induction of deacetylase sirtuin 2 (SIRT2), which causes tumorigenesis and tumor growth.[52] Moreover, the Notch pathway in breast cancer promotes CSC self-renewal, which enhances glucose uptake and aggressive hormone-independent tumor growth *in vivo*[53]; hence, the Notch pathway is associated with CSC characteristics in cancer metabolism. Recently, the Notch pathway-related proteins containing with gamma (γ)-secretase, enzyme complex related to Notch cleavage, and Notch receptors were reported to be remarkable therapeutic targets for CSC self-renewal. Treatment of MRK-003, a γ-secretase inhibitor and an antagonist of the Notch pathway, inhibit tumor sphere forming ability and the differentiation of progenitor cells.[54] Mice administered MRK-003 displayed apoptosis and the differentiation in ERBB2 breast cancer mouse model. PF-03084014, other γ-secretase inhibitor, used in combination with docetaxel, an anti-cancer reagent, reversed these effects and demonstrated early-stage synergistic apoptosis.[55] PF-03084014 used in combination with docetaxel reduces the $ALDH^{high}$ and $CD133^{+}/CD44^{+}$ CSC populations and tumor progression in the xenograft model. Other groups reported that breast cancer cells treated with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), an inhibitor of the Notch pathway, decreased the CSC population and knock-down of Notch1 or Notch4 suppressed tumor sphere forming efficiency and tumor progression.[56] Furthermore, brain metastatic human breast cancer cells treated with DAPT had reduced $CD44^{high}/CD24^{low}$ CSC populations compared with non-treated cells and inhibited brain metastasis in an experimental murine model.[57] These results suggest that the Notch pathway can regulate the characteristics of breast CSC containing self-renewal and metastatic potential.

Hedgehog Pathway

The Hedgehog (HH) pathway plays a key role in various biological processes, such as cell differentiation, proliferation, and growth in normal or tumor cells.[58] The HH pathway is activated by binding of ligands to the Patched (PTCH) receptor and subsequently alleviating inhibition of Smoothened (SMO).[59,60] Activation of SMO results in subsequent regulation of the expression of Gli transcription factors that are responsible for cancer cell proliferation, apoptosis, and invasion.[61–64] Overexpression of molecules related with the HH pathway is observed in CSC during chronic myeloid leukemia[65], medulloblastoma[66], skin cancer[67], etc. In breast cancer, the HH pathway activated in CSC and the mechanism underlying CSC self-renewal has been revealed recently. Using breast tumor derived from xenografts, Liu *et al.,* first reported that the expression level of *PTCH1, Gli1*, and *Gli2* mRNA, which are associated with the HH pathway, affects the CSC population as compared with the non-CSC population[58]. Similarly, the expression level of PTCH1, SMO, Gli1, and Gli2 proteins increases in tumor spheres than in adherent cells in the breast cancer cell line MCF7. In addition, p63 is the sister homolog of p53 and a master regulator of normal epithelial stem cell maintenance[68]. p63 directly regulates the expression of *Shh, Gli2*, and *Ptch1,* leading to tumorsphere formation in transgenic mice with conditional overexpression of the ErbB2 oncogene.[69] Cyclopamine (CP), an antagonist of the HH pathway, is an effective inhibitor of self-renewal of breast CSC.[70] Furthermore, FOXC1 binds directly to Gli2 and regulates ALDH activity and tumor sphere forming ability via activation of SMO-independent HH signaling in basal-like breast cancer. Basal-like breast cancer, lack expression of hormone receptors and ErbB2 receptor, is associated with poor prognosis in breast cancer.[71] Inhibition of the FOXC1/Gli2 pathway using anti-HH inhibitors can improve basal-like breast cancer treatment.

Growth factor Signaling Pathway through Nuclear Factor-Kappa B (NF-κB)

Currently, hyperactivation of NF-κB caused by growth factor stimulation plays a key role in CSC self-renewal. NF-κB activation increases in $CD24^{-}$/$Lineage^{-}$ cell population compared with the other cell population, as revealed through *in silico* analyses using breast cancer cell lines[14]. Furthermore, the NF-κB pathway induced to express several inflammatory cytokines, containing Interleukin-6 (IL-6), IL-8, and CCL5 in breast cancer cells[32]. Because inflammatory cytokines function as paracrine factors secreted from their CSC niche cells, these observations suggest that NF-κB activation is a key factor for self-renewal of breast CSC and

maintenance of those niches. Further, I introduce growth factors we have found as factors activating NF-κB in breast CSC.

Neuregulin1(NRG)

NRG, also known as Heregulin (HRG), functions as a ligand of ErbB3/HER3 and activates the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway or the MEK/Erk signaling pathway.[16,72] The expression of NRG is reported to correlate with clinical prognosis and chemotherapeutic resistance in several types of cancer, especially HER2-positive breast cancer. Our groups reported that NRG stimulation in breast cancer cell line MCF7 increased to form tumor spheres and expresses the stem cell marker Nanog in NRG-treated spheres; thus, NRG may be related with initiation and undifferentiation of CSC.[16] Moreover, NF-κB was remarkably activated in NRG stimulation via the PI3K/Akt signaling pathway, leading to the formation of tumor spheres in primary breast tumor cells and tumor-initiating activity in xenograft models. The activated NRG/PI3K/NF-κB axis can induce IL8 mRNA expression, a regulator of self-renewal in BCSC-enriched populations. Other groups reported that NRG treatment for breast cancer cell lines increase the $CD44^{high}$ cell population[73] and induction of EMT through activation of Snail expression or phosphorylated Smad2 via the PI3K/Akt pathway.[74] These findings suggest that NRG through the PI3K/Akt/NF-κB signaling pathway has a significant effect on the maintenance and self-renewal ability of breast CSC. Cytokines secreted owing to NRG stimulation may function as autocrine factors and paracrine factors to stimulate to CSC niche.

IGF2

IGF2 is a member of the insulin family and binds to IGF-1 receptor (IGF-1R) homodimers or IGF-1R and insulin receptor (IR) heterodimers.[75,76] Recent studies reported that the IGF-1R signaling pathway contributes CSC characteristics in several types of cancer. In lung adenocarcinoma, IGF-1R signaling pathway is required for chemotherapeutic resistance by altering chromatin states or acquisition of CSC characteristics.[77] Similarly, IGF-1R accumulation induces the expression of FOXO3a protein (not the PI3K signaling pathway), which confers radioresistance in glioma stem cells.[78] We reported that the IGF2/IGF-1R signaling pathway plays a key role in establishing tumor spheres of primary breast cancer cells[34]. IGF-1R was specifically upregulated in CSC-enriched populations in freshly obtained primary breast cancer cells. In addition, the IGF2/IGF-1R/PI3K signaling pathway induced the expression of the inhibitor of DNA binding protein 1 (ID1). Inhibitor of DNA-binding 1 (ID1) is a member of ID family proteins (ID1 ~ ID4) and is reported to function as master regulators for stemness of normal tissues or tumors.[79] ID1 appeared to operate as a transcript regulator via upregulation of *IGF2* mRNA itself, thereby probably leading to tumor sphere formation. In chemotherapy, treatment with an anti-human IGF1/2 antibody (KM1468) blocked tumorigenesis derived from the $IGF\text{-}1R^{high}$ CSC-enriched population in a patient-derived xenograft (PDX) model. Hence, it is important that NRG1 stimulation may trigger a IGF2-ID1-IGF2 positive feedback circuit for the maintenance for stemness of breast CSC (Figure 2). On the other hand, fusion genes involved in tumor progression were recently identified in several types of tumors in lung cancer.[80] The CD74-NRG1 fusion gene has been reported by several groups and its role of in lung and breast cancer has been investigated[81,82]; recently, the relationship between this fusion gene and CSC has been reported[35]. Overexpression of CD74-NRG1 in lung or breast cancer cell lines enhanced tumor sphere forming ability and tumor initiation in xenograft models. CD74-NRG1 expression promoted the expression of the secreted IGF2 and phosphorylation of its receptor, IGF-1R through the PI3K/Akt/NF-κB signaling pathway, leading to the formation of tumor spheres. This study suggests that CD74-NRG1 fusion gene expression probably contributes to CSC maintenance via the IGF2/IGF-1R signaling pathway.

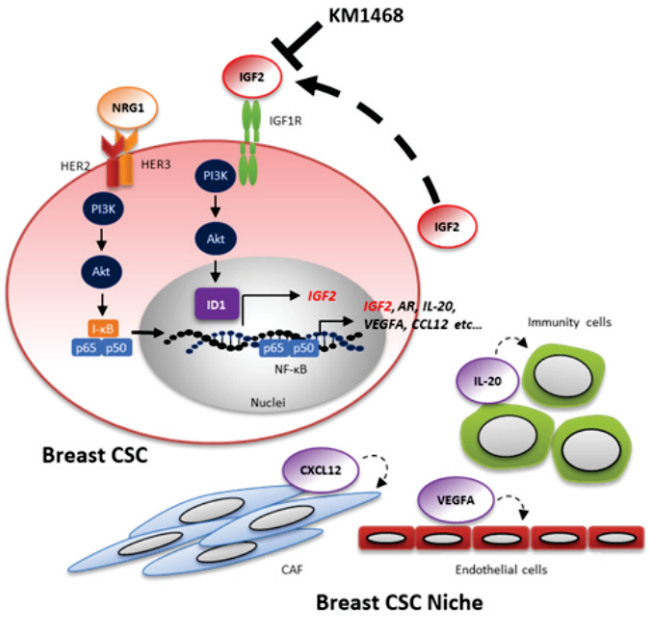

Figure 2. Model of molecular mechanisms that stabilize the stemness of breast cancer cells. The HER2/HER3-PI3K-NF-κB pathway may trigger a IGF2-ID1-IGF2-mediated positive feedback circuit as a fundamental mechanism of stabilization of stemness. In addition, the HER2/HER3-PI3K-NF-κB pathway also leads to the production of many soluble factors that may regulate the surrounding CSC niche cells: cancer-associated fibroblasts (CAF), endothelial cells, immune cells, etc. KM1468 is human anti-IGF1/2 antibody and inhibits tumor initiating activity in breast cancer. This figure is adapted from Tominaga et al.[34] Abbreviations: NRG1, Neuregulin1; PI3K, Phosphoinositide 3-kinase; NF-κB, nuclear factor-κB; I-κB, inhibitor of NF-κB; IGF2, Insulin growth factor 2; IGF1R, Insulin growth factor 1 receptor; ID1, inhibitor of DNA binding protein 1; AR; Amphiregulin; IL-20, Interleukin 20; VEGFA, vascular endothelial growth factor A; CCL12, Chemokine (C-C motif) ligand 12

Conclusion

This review is focused on breast CSC maintenance, including self-renewal ability through activation of signaling pathways in breast cancer. Robust autocrine or paracrine signaling, containing Wnt, Notch, Hedgehog and growth factor signaling pathways, is essential for the maintenance of breast CSC. Actually, multiple signaling pathways are complex and have cross-talk each other in cancer cells. For example, on cell lines from gastric adenocarcinomas, the expression of sFRP1 was induced by activated Hedgehog pathway, which indicated that HH pathway induced Wnt pathway inactivation[83]. Other group suggested that sonic hedgehog, HH pathway agonist, and Jagged 2 (JAG2), a Notch receptor, were able to reduce activated beta-catenin through SMO, leading to suppressing beta-catenin transcription in tongue cancer[84]. Since CSC might be thought to remain by activated or inactivated multiple signaling pathways in recurrence of breast cancer, it would be important to reveal cross-talk between signaling pathways on CSC to understand the mechanism of tumor recurrence.

In addition, growth factor-induced signaling pathways contribute to not only maintenance of breast CSC behavior, but also activation of CSC niches. Our reports using patient-derived breast cancer cells obtained from surplus surgical tumor tissues indicate that inflammatory cytokines and chemokines secreted from CSC niches regulate CSC maintenance through NF-κB activation.

To identify the specific signal pathway of CSC may probably help establish anti-cancer agents that do not affect normal tissues and somatic stem cells. Since it is possible to activate multiple and complex signaling pathways in CSC by various factors, it may be necessary to concurrently inhibit candidate targeting molecules, e.g., HER2/3, NRG, IGF-1R, IGF2, and ID1 in case of breast cancer. However, unresolved questions regarding how CSC are stimulated from their niche for the maintenance of their properties persist. Future studies are needed for novel therapeutic strategies to inhibit the bidirectional signaling pathway between CSC and their niche.

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