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Notch and breast cancer metastasis: Current knowledge, new sights and targeted therapy (Review)

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Abstract. Breast cancer is the most common type of invasive cancer in females and metastasis is one of the major causes of breast cancer-associated mortality. Following detachment from the primary site, disseminated tumor cells (DTCs) enter the bloodstream and establish secondary colonies during the metastatic process. An increasing amount of studies have elucidated the importance of Notch signaling in breast cancer metastasis; therefore, the present review focuses on the mechanisms by which Notch contributes to the occurrence of breast cancer DTCs, increases their motility, establishes interactions with the tumor microenvironment, protects DTCs from host surveillance and finally facilitates secondary colonization. Identification of the underlying mechanisms of Notch-associated breast cancer metastasis will provide additional insights that may contribute towards the development of novel Notch-targeted therapeutic strategies, which may aid in reducing metastasis, culminating in an improved patient prognosis.

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1. Introduction

Breast cancer is the most common cancer type in females, and the incidence rate has been steadily increasing worldwide over the past decade (1,2). Breast cancer-associated mortality typically results from distant metastasis, rather than from the primary tumor (3). Despite recent advances in the application of targeted therapeutic strategies, no significant improvements in the prognosis of patients with metastatic breast cancer have been achieved due to the incomplete understanding of the molecular mechanisms governing the metastatic process.

Metastasis is a complex cascade involving interactions between cancer cells and surrounding microenvironmental components, including mesenchymal cells, immune cells and the extracellular matrix (4). The first stage of breast cancer metastasis is characterized by an invasion of the basement membrane by primary tumor cells, which then become disseminated tumor cells (DTCs) (5). These cells then promote abnormal angiogenesis, intravasate into the circulatory or lymphatic system, migrate to distant organs and establish secondary tumors (6).

Accumulating evidence has indicated the important role of Notch, a highly conserved family of signaling molecules, in breast cancer metastasis. The deregulation of Notch signaling is reflected in all aspects of the metastatic processes and its role in breast cancer appears to be highly context-dependent.

2. Notch signaling

Activation of Notch signaling requires interactions between ligands on the surface of signal-sending cells and Notch receptors (Notch1-4) on the surface of signal-receiving cells. Mammalian Notch signaling comprises two pathways: The canonical pathway and the non-canonical pathway (7).

The canonical Notch ligands include two homologs in *Drosophila*, Delta and Serrate. Their counterparts in mammals are Delta-like molecules (DLLs) and Jagged (7). The Notch receptors consist of an intracellular domain (ICD), a transmembrane domain and an extracellular domain (ECD) (8). Activation of canonical Notch signaling includes receptor and ligand binding, cleavage of the ICD, translocation of activated Notch ICD (NICD) into the nucleus and binding with chorionic somatomammotropin hormone like 1 (CSL; also termed Rbp-Jκ in mice and core-binding factor in humans).

The receptor undergoes two cleavages on its S2 and S3 sites following Notch ligand-receptor ECD binding (8). Initially, the S2 site is cleaved by tumor necrosis factor-α-converting enzyme, also termed ADAM metallopeptidase domain 17, in order to release the ECD (9). Subsequently, γ-secretase cleaves the S3 site to release NICD, which requires the involvement of presenilin (10). NICD then enters the nucleus and binds to CSL within its Rbp-Jκ-associated module region to modify target gene expression, including members of the HES and HEY family (11).

However, the understanding of non-canonical Notch signaling is primitive compared with that of the canonical one. Non-canonical Notch signaling has its distinctive ligands, including Delta-like 1, an integral membrane protein, microfibril-associated glycoprotein and a secreted ligand (12). Of note, activation of non-canonical Notch signaling does not require the participation of CSL; after the binding of the ligand and receptor, NICD is released and can therefore enter the nucleus directly (12).

3. Notch and biased cell fate determinants

In the orderly development of mammary tissues, the balance between differentiation and division is achieved by asymmetric divisions (ACD), which is controlled by several lineage-specific differentiation-inducing transcription factors (13). Through ACD, the bi-potent mammary stem cells (MaSCs) divide into basal or luminal stem cells, and then become myoepithelial or ductal/alveolar cells respectively (14).

Notch signaling acts as an intrinsic regulator in the biological behavior of normal MaSCs (15). Notch signaling has been hypothesized to promote self-renewal proliferation and facilitate the myoepithelial lineage-specific commitment of MaSCs during the development of mammary glands (16). The cell fate developmental decisions of Notch signaling are negatively controlled by Numb, a protein asymmetrically located in dividing progenitor cells (17). Numb facilitates Notch ubiquitination at the membrane, promotes degradation of NICD, circumvents its nuclear translocation and inhibits activation of signaling downstream of Notch (18). However, in the case of overexpression of Notch components, the steady-state number of MaSCs may be disrupted, allowing mutant stem cells/breast cancer stem cells (BCSCs) to arise. These poorly differentiated BCSCs exhibit a high level of CD44; however, little or very low levels of CD24, resulting in a $CD44^+/CD24^{-/lo}$ phenotype (19). CD44 is a cell surface adhesion molecule that is enriched in basal-like breast cells (20). CD44 binds to hyaluronate and is associated with metastasis (21). While CD24 is a cell surface marker of differentiated breast luminal cells, cells with low expression of CD24 are usually basal-like (22). BCSCs are heterogeneous and can be subtyped into $CD44^+/CD24^{lo}$ progeny and $CD44^+/CD24^-$ progeny regarding CD24 expression (23). Compared with $CD44^+/CD24^-$ cells, $CD44^+/CD24^{lo}$ cells acquire significantly overexpressed Notch signaling components and upregulated embryonic stem cell transcription programs such as Notch1-mediated embryonic transcription factor Sox2 activation, which may aid in explaining why the $CD44^+/CD24^{lo}$ progeny exhibits greater tumor initiating ability compared with $CD44^+/CD24^-$ progeny (23). Notch1 overexpression also helps $CD44^+/CD24^-$ cells convert into $CD44^+/CD24^{lo}$ cells, and Notch4 signaling has exhibited greater efficacy, when compared with Notch1 in the formation and maintenance of BCSCs, as Notch4-knockdown completely suppresses the tumor formation while Notch1-knockdown only reduces the tumor size and number (23).

The heterogeneity of BCSCs also confers it with drug-resistant ability. Notch inhibition had little effect in the $CD44^+/CD24^-$ subpopulation. However, peptides derived from Notch and Numb can activate cell-toxic lymphocytes to eliminate BCSCs, which provides a novel insight into breast cancer treatment (23).

Previous studies (24-26) have demonstrated that the cell fate determinants are disturbed in breast cancer cells due to loss of differentiation-inducing factors, including E74-like factor 5 (ELF5), ring finger protein (RNF8) and GATA binding protein 3 (GATA3), which is attributed to genetic abnormalities in Notch signaling.

ELF5 regulates MaSCs differentiation into the alveolar and luminal lineages through the Notch signaling pathway by binding to the responsive elements within the Notch gene (24). ELF5 may also inhibit breast cancer metastasis by suppressing the activation of Slug, a transcription factor in the epithelial-mesenchymal transition (EMT) process, and loss of ELF5 provides a basis for tumorigenesis (27). NICD1 and NICD4 are hyperactivated in ELF5-null mammary epithelial cells, which may be a strong initiator for the ELF5-null breast cancer phenotype (24). RNF8 affects breast cancer development and can also regulate the basal-to-luminal cell fate as well. It has observed to interact with Notch signaling by ubiquitylating NICD1 (25). Loss of RNF8 leads to the upregulation of Notch target genes and aberrant luminal progenitor cell expansion, resulting in an increased risk of mammary tumorigenesis (25). In addition, another differentiation-inducing factor GATA3, is also directly regulated by Notch3, through the CSL-binding motif in the GATA3 promoter (26,28).

4. Notch and local invasion

Invasion of the basement membrane and mesenchyme. Specific gene programs equip cancer cells with increased mobility, which drives their migration away from primary sites (29). EMT constitutes the basis of the regulation of epithelial plasticity and cancer cell mobility. During this cascade process, epithelial cells lose their adhesion junctions and cellular polarity while acquiring mesenchymal characteristics (30). *In vitro* studies (31,32) have suggested that Notch1-knockdown reverses the Jagged1-induced EMT. These Notch1-silenced cells are capable of a less aggressive form of invasion, and may be characterized by a cobblestone-shaped phenotype rather than a spindle-like mesenchymal phenotype. Of note, numerous studies (32-37) have demonstrated that Jagged1-mediated Notch activation suppresses the levels of E-cadherin and increases the levels of the mesenchymal markers N-cadherin and vimentin, the transcription factors Slug, Snail and zinc finger E-box binding homeobox 1 (Zeb1), as well as β-catenin in breast cancer cells to promote migration and invasion. However, to the best of our knowledge, the involvement of DLL in EMT has not been reported.

Signal transducer and activator of transcription 3 (STAT3) is an important pro-EMT transcription factor mediated by

Notch (38). Notch1 has been hypothesized to activate EMT by inducing STAT3 and upregulating the expression of p65 and interleukin (IL)-1 (38). Notch2 has also been identified to promote EMT via the IL-6/Janus kinase (JAK)/STAT3 pathway in a radiation-driven model of breast cancer EMT (39). Notably, non-canonical Notch signaling was also identified to be involved in this pathway, with upregulation of IL-6 in breast cancer cells leading to the activation of JAK/STAT3 signaling (40). In the more aggressive triple-negative breast cancer, the loss of Numb leads to the activation of Notch signaling, and induces EMT and the acquirement of cancer stem cell-like properties, culminating in early relapse and metastasis (41,42). Other mobility-promoting programs, including F-actin polymerization, may also be induced by Notch1 (43).

However, Notch3 serves the opposite role in EMT by regulating estrogen receptor α (ERα). ERα is characteristic of luminal epithelial phenotype in breast cancer cells, the loss of which causes EMT and metastasis (44). Notch3 stimulates ERα expression not only by directly binding to CSL-binding elements in ERα promoter, but also indirectly by upregulating GATA-3 (an activator of ERα) (28). These two patterns result in ERα overexpression and thus suppress EMT.

Enhanced migratory ability alone, however, is insufficient to drive metastasis. Disseminating cancer cells must also invade the surrounding complex network, which primarily consists of extracellular matrix (ECM), basement membrane and mesenchyme (5). The matrix metallopeptidase (MMP) family is known to degrade the ECM and promote cancer cell invasion and metastasis (45). In breast cancer, Notch1 activation promotes the expressions of MMP-2 and -9 to break down the ECM components (46). Notch has also been demonstrated to be associated with urokinase-type plasminogen activator (uPA), which is an ECM-degradation enzyme associated with poor outcome, and a high risk of metastasis and recurrence (47). Under normal conditions, uPA induces a plasminogen proteolytic sequence. However, in breast cancer, uPA works with MMPs to erode the microvasculature and degrade the ECM to facilitate tumor cell metastasis. uPA receptor (uPAR) is highly expressed in malignant tissues and tends to be located at the leading edge or invasion front (48). Upon binding to uPA, the receptor converts plasminogen to plasmin, then degrades ECM through MMP (48). A precious study placed Notch upstream of the uPA cascade (49). A positive association has been observed between Jagged1 and uPA in various breast cancer cell lines, and Notch1-knockdown reduced uPA levels (49). Furthermore, Notch may directly regulate uPA transcription via centromere-binding factor 1 binding sites within the uPA promoter and enhancer. The subsequently activated uPAR then cleaves ECM-associated signaling molecules, including fibronectin and the laminin receptor (49,50).

Hypoxia. Hypoxia is a term for a low-oxygen environment, and may be the result of leaky vasculature and a lack of blood supply, and is important for tumor progression (51). Hypoxia-inducible factor 1α (HIF-1α) promotes metastasis and is associated with poor prognosis (52-54). Accumulation of HIF-1α and HIF-2α enhance Notch signaling (both receptors and ligands) as well as the expression of the downstream genes HES1 and HEY1; HIFs and mastermind-like protein (MAML)1, a key Notch co-activator, form a complex with NICD to recruit other Notch co-activators, including p300, indicating a HIF/MAML1/Notch axis under hypoxia (33). Hypoxia stabilizes HIFs through this signaling cascade, resulting in elevated Notch (33).

Hypoxia-induced Notch activation causes EMT in breast cancer (55). Furthermore, NICD directly binds to the Snail-1 promoter (56). In addition, Notch potentiates HIF-1α to bind to the lysyl oxidase (LOX) promoter and then stabilizes the secretion of Snail-1 and tissue inhibitor of metalloproteinase (TIMP)4, leading to EMT and metastasis (57,58). Consistent with its ability to simulate ECM degradation, LOX correlates with estrogen receptor (ER)-negative breast cancer, which is more likely to metastasize to the bone compared with ER-positive breast cancer (59). The ability of LOX to induce metastasis indicates that it may serve as a novel target to prevent bone metastasis of breast cancer.

Interaction between breast cancer cells and stromal cells. The tumor microenvironment, which primarily consists of mesenchymal cells and immune cells, is central to the progression of breast cancer (60). Solid experimental evidence has indicated that cancer-associated fibroblasts (CAFs) secrete cytokines to support breast cancer cells and protect them from host surveillance (61). CAFs secrete ADAM10-rich exosomes, which in turn were recently identified to be associated with loss of TIMP family member expression, to potentiate cell motility and aldehyde dehydrogenase (ALDH) expression through Ras homolog family member A and Notch, respectively (62). Silencing of Notch effector Rbp-Jκ, combined with down-regulation of the tumor suppressor p53, induces a senescent phenotype and the expression of CAF effector genes (63).

Immune regulation also serves an important role in breast cancer progression. $CD8^+$ T cell infiltration, together with type 1 interferon, activates innate immunity, acting as an anti-tumor mechanism in breast cancer (64). It has been reported that Notch signaling controls $CD8^+$ T cell activation through the binding of DLL1 with Notch1 or Notch2 (65). Notch1 has a crucial role in the immune-suppressive tumor microenvironment and the inhibition of Notch1 leads to recruitment of active $CD8^+$ T cells and a decrease of immune suppressive cells, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (66). However, Notch2 contributes to the anti-tumor response, and the deletion of $CD8^+$ T cell specific Notch2 in mice results in increased tumor size and decreased survival in tumor-bearing mice (67). Thus, Notch signaling has a dual role in regulating the tumor immune response, as it may exert oncogenic and tumor suppressive functions. Notch signaling may also act as a transcriptional regulator in the differentiation of tumor-associated macrophages (TAMs). TAMs may recruit Tregs and MDSCs and also suppress $CD8^+$ T cells (68). CSL deletion in monocytes inhibits not only differentiation, but also the antigen-presenting function of TAMs, restraining the immune-suppressive function of TAMs (69). Of note, overexpression of NICD has been reported to suppress the function of TAMs and then repress tumor growth, indicating that the effects of Notch signaling on TAMs may depend on the extent of Notch signaling (70).

Angiogenesis. Breast cancer cell multiplications requires a lot of nutrition, as the original blood vessels at the site of the

tumor are insufficient to the amount of nutrition for the rapid growth of breast cancer cells (71). Therefore, breast cancer cells exhibit an angiogenic phenotype that allows new blood vessels to branch and create a massed vascular network (72). In addition to transport nutrition, these immature and highly permeable new blood vessels also provide an efficient route of exit for breast cancer cells to leave the primary site and enter the circulation, which can then elicit metastasis (73). In the process of angiogenesis, Notch ligands, together with vascular endothelial growth factor (VEGF), the strongest mitogenic factor, stimulates the formation of vascular endothelial cells to establish a neovasculature (74), which then promotes breast cancer metastasis.

In vascular endothelial cells, the ends of vessel sprouts are termed tip cells and the other cells are called stalk cells (75). These cell types are essential for vessel polarity and barrier function of vessels (76). This endothelial cell specification is regulated by Notch signaling during tumor angiogenesis process, with the two types of Notch ligands exerting the opposite effects (77).

In normal conditions, VEGF receptor (VEGFR) signaling serves as an initiator in tip cell formation, while DLL4 serves an inhibitory role (78). Upregulation of DLL4 by VEGF/VEGFR in endothelial tip cells suppresses the tip-like phenotype; therefore, the single tip cell can be selected from among many candidate vascular endothelial cells and form the new vessel sprout (78-80). To avoid the excessive tip cell formation and immoderate angiogenesis, high level of DLL4 signals are sent to the adjacent cells (stalk cells) through Notch1, which then inhibits the expression of VEGFR in stalk cells and induces vascular network quiescence (81). Jagged-1, another type of ligand, is not directly associated with sprouting angiogenesis, but shifts the balance between DLL4/Notch and VEGFR signaling (82). Jagged-1 primarily exists in stalk cells and can antagonize DLL4/Notch signaling in stalk cells to ameliorate the low VEGF response, thus activating stalk cells to promote angiogenesis (80). These processes are mediated by the glycosyltransferase Fringe family, which results in Notch binding to DLL4 more easily; however, impedes its ability to bind to Jagged-1 (83). However, in metastatic breast cancer, overexpression of Jagged-1 transforms angiogenesis from physiological to pathological patterns that favors metastasis. This causes excessive angiogenesis and even gives rise to a new hybrid tip/stalk phenotype (84,85). Therefore, it is well demonstrated that hybrid tip/stalk phenotype leads to the formation of new sprouts; however, new blood vessels produced under these conditions exhibit poor perfusion with high microvessel density, which is what metastatic DTCs require. These pathological blood vessels confer great plasticity to the leading cell that have are capable of exchanging its position with adjacent stalk cells rapidly, thus creating a fast but chaotic and dense vascular network route for a large number of DTCs to exit the primary sites (84).

Demethylases and Notch-associated proteases also dynamically participate in angiogenesis in breast cancer, and the overexpression of the lysine demethylase 2A (KDM2A) in human breast cancer is associated with a worse outcome. Jagged1 is essential for KDM2A-driven tumor angiogenesis and acting as a direct target of KDM2A (86). Inhibition of KDM2A in breast cancer cells blocks Notch activation and endothelial cell tube formation (87). Proteases including MMPs are able to make space for angiogenesis and lymphangiogenesis (45). In addition, uPA and uPAR may combine to activate VEGF (49).

Intravasation. Notch signaling modulates the ability of breast cancer cells to cross mesenchymal and endothelial barriers (87). Integrins are associated with normal mammary epithelial cells, as well as with breast cancer cells (88). A feedback loop has been reported between integrins and Notch, and is characterized by activated Notch signaling controlling β1 integrin affinity, while β1 integrin inhibits the expression of Notch (89,90). β1 integrin cooperates with Notch to promote the transendothelial migration of breast cancer cells, which is characterized by enhanced polarity reversal and adhesion to the blood vessel wall (91,92).

Aberrant Notch activation stimulates endothelial cells to promote breast cancer intravasation. Vascular cell adhesion molecule-1 may be subverted by Notch1 to enhance the adhesion of tumor cells and neutrophils to endothelial cells, thus favoring the dissemination of tumor cells (93). As stated previously, breast cancer cells may also recruit factors including MMPs that increase vascular permeability and thus promote intravasation (Fig. 1).

5. Notch and survival in circulation

Breast cancer cells detach from primary sites and then enter the circulation, becoming circulating tumor cells (CTCs) (94). CTCs must first survive in the bloodstream prior to arriving at distant organs (95).

Anti-apoptotic effects. Apoptosis negatively regulates tumor progression by preventing overgrowth. This process depends on the coordination of numerous ligands and receptors, including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL-receptor1 and 2, also termed DR4 and DR5 (96). Administration of γ-secretase inhibitors (GSIs) may lead to a marked upregulation of DR4 and DR5, increase the sensitization of breast cancer cells to TRAIL-mediated apoptosis (96), activate the caspase system (i.e. caspase-8) (97), promote mitochondrial membrane leakiness and further induce apoptosis. This Notch-mediated anti-apoptosis function may depend on activator protein (AP)1, which is a dimeric transcription factor complex activated by c-Jun N-terminal kinase (JNK) (96). Blocked by GSIs, Notch fails to be activated, which increases the levels of AP1 and JNK (96,98,99), thus activating DR4 and DR5 (100). A recent study indicated that Notch4, but not Notch1, is involved in the sensitization of breast cancer cells to TRAIL-induced apoptosis (101). Alternatively, inhibition of β1 integrins may sensitize tumor cells to TRAIL-induced apoptosis, which is mediated by Notch (102). The GSI/TRAIL combination also decreases several survival factors, including survivin and B-cell lymphoma 2 (96). Furthermore, different types of breast cancer cell differ in their response to such inhibition. For example, ER-negative breast cancer cells are more sensitive to GSI/TRAIL synergism compared with ER-positive cells (96).

AKT impedes DNA damage-induced apoptosis via inhibition of apoptosis signal-regulating kinase 1, which in

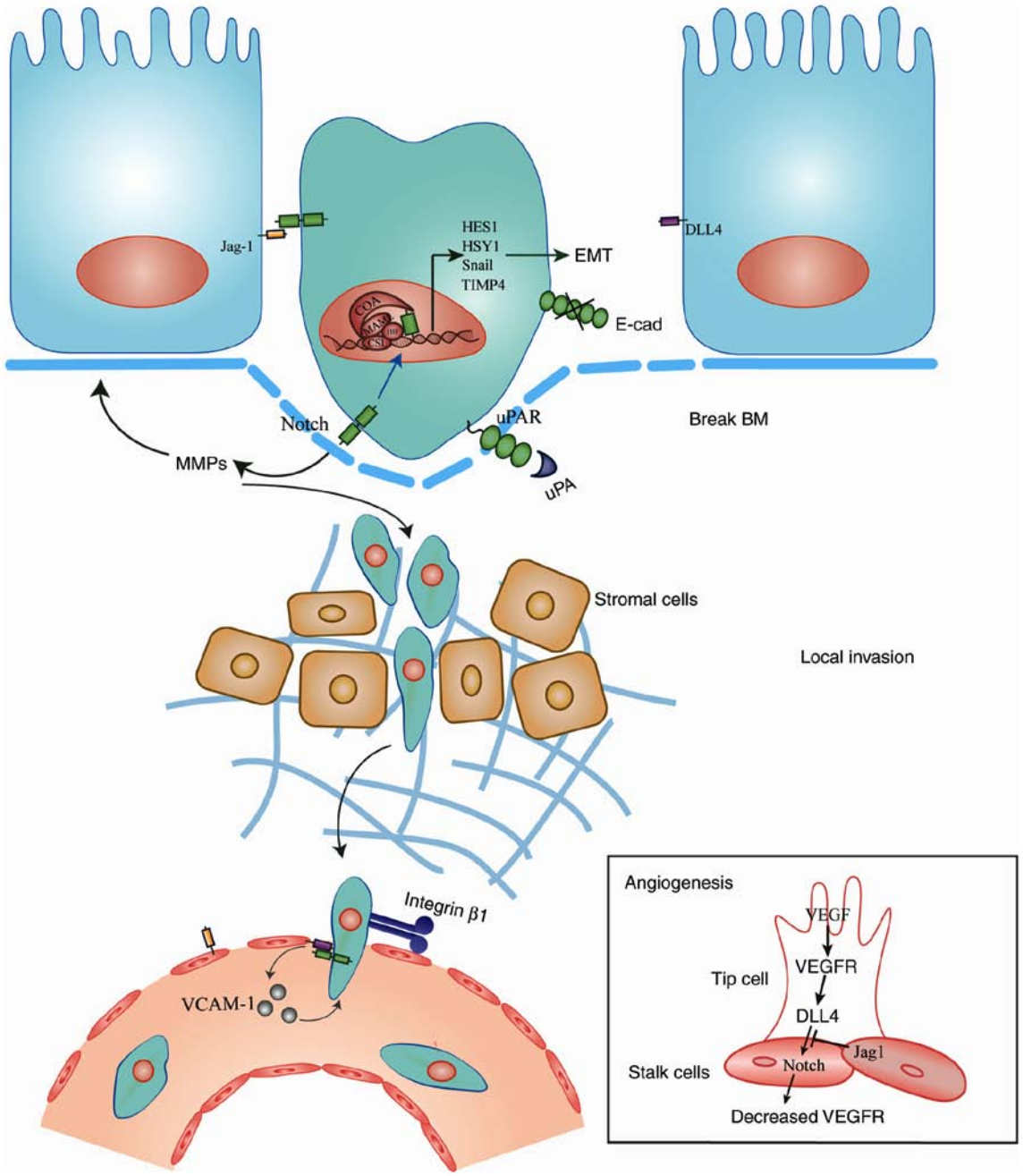

Figure 1. BC DTCs detach from primary sites and intravasation. BC DTCs obtain increased motility to break the BM and overcome ECM with Notch-associated processes such as hypoxia and EMT, as well as enzymes, including MMPs and uPA. Notch takes part in angiogenesis to sprout new vessels and BC DTCs can enter the bloodstream through these vasculatures. BC, breast cancer; DTCs, disseminated tumor cells; ECM, extracellular matrix; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen; uPAR, urokinase-type plasminogen receptor; BM, basement membrane; TIMP4, tissue inhibitor of metalloproteinase 4; EMT, epithelial-mesenchymal transition; DLL4, Delta-like molecule 4; VCAM-1, vascular cell adhesion protein 1; Jag-1, Jagged-1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

turn prevents JNK-mediated activation of p53 (103), leading to an aberrant increase of mammary progenitor cells (104). Substantial evidence has demonstrated that impairment of Notch signaling may inhibit AKT activity and sensitize cells to apoptosis (103,105). It has also been reported that the addition of DAPT, a GSI, improves the anti-tumor efficacy of RY10-4, an anti-breast cancer drug, due to the accessorial restraint on AKT phosphorylation exhibited by DAPT, which reduces the survival of breast cancer cells (106).

Notch-mediated regulation of AKT contributes to tumor cell survival through multiple pathways. AKT is hypothesized to increase MMP production via several downstream target proteins, including nuclear factor (NF)-κB and mammalian target of rapamycin (mTOR) (106). Li *et al* (46) suggested that Notch1 inhibition enhances protein phosphatase 2A (PP2A) activity and downregulates NF-κB, which may be restored by the PP2A inhibitor okadaic acid (OA). Treatment with OA also upregulates VEGF, MMP2 and MMP9, suggesting a key role of PP2A in the Notch/AKT/NF-κB axis (107). mTOR also takes part in AKT-mediated tumor cell survival, a mechanism contributing to chemoresistance (108). Its downstream effector, eukaryotic initiation factor 4E, is crucial for mTOR-mediated

inhibition of p53 and may reverse p53-mediated cytotoxicity (109). Furthermore, Notch activation enhances the activity of MDM2, an E3 ubiquitin-protein ligase, to also degrade p53 (110). Apart from these indirect Notch/p53 signaling pathways, Notch also directly binds to the amino terminus of p53 without the presence of AKT, thus inhibiting p53 phosphorylation and DNA-binding activity (111).

Chemoresistance. To overcome the threat of chemotherapy, CTCs must employ several sophisticated approaches. To contend with chemotherapeutic agents for breast cancer, CTCs may acquire morphological and functional endothelial features characteristic of tumor vascularization (112). DLL3 and Notch4, with their downstream targets p65 (an NF-κB subunit) and Zeb1, are overexpressed in tumor-derived endothelial cells during chemotherapy (113). Silencing of Notch4/DLL3 may decrease the functionality of tumor-derived endothelial cells and endothelial markers (113). The expression of VEGFR3, an important factor in tumor angiogenesis, is significantly upregulated in patients receiving chemotherapy. Notch4/DLL3 silencing also suppress the expression of VEGFR3 transcripts, indicating that breast cancer chemotherapy triggers the formation of functional tumor-derived endothelial vessels by regulating Notch and VEGF signaling (113).

Patients with ER-positive breast cancer with high levels of ALDH1 and Notch4 exhibit poor prognosis following anti-estrogen treatment (114). Although short-term treatment suppresses tumor cell proliferation, it also increases CTC activity through Jagged1/Notch4 activation, as the administration of Notch inhibitors attenuates drug resistance and improves patient outcomes (114). Long-term hormonal therapies may reduce ERα expression and increase the levels of IL-6, thus enhancing the self-renewal properties of hormonal therapy-resistant ER-dependent, as well as ER-independent tumor cells. Subsequently, IL-6 may cause a departure from metabolic dormancy induced by mitochondrial activation through an IL-6/STAT3/Notch3 transduction pattern, hence leading to the acquirement of resistance (115). Blocking IL-6 reduces the levels of STAT3/Notch3 in breast cancer cells, resulting in increased sensitivity to hormonal therapy such as tamoxifen (115). In addition, STAT1 may also facilitate the expansion of therapy-resistant breast cancer cells via Notch3 (116).

Of note, multiple courses of treatment for patients with ER-positive/human epidermal growth factor receptor 2 (HER2)-negative breast cancer may endow their CTCs with HER2 expression (117). A further study demonstrated that breast cancer CTCs that underwent this transformation maintain discrete HER2-positive and HER2-negative subpopulations (118). Of note, HER2-positive and HER2-negative breast cancer CTCs may spontaneously interconvert; however, have different functions. HER2-positive CTCs acquire a stronger proliferation potential and a higher lung metastasis frequency but are no more sensitive to HER2-targeted therapy, while HER2-negative CTCs exhibit an increased expression of Notch1 but a resistance to chemotherapy. Therefore, dual treatment in Notch inhibitor-sensitive HER2-negative/Notch-positive and chemotherapy-sensitive HER2-positive/Notch1-negative CTCs may be a reasonable approach (118).

6. Notch and secondary colonization

Prior to establishing metastases in secondary organs, breast cancer CTCs release factors, including MMPs, and gather bone marrow-derived hematopoietic precursor cells to combine with perivascular fibroblasts and fibronectin to form the pre-metastatic niche (29). Once CTCs extravasate and colonize the niche, they become micrometastases (29).

The mechanism underlying metastatic organotropism remains largely elusive; specifically, no rationale for the propensity of breast cancer to metastasize to the bone, lung and liver has been proven thus far. However, evidence has revealed that bone metastasis of breast cancer may arise with the help of Notch. In bone, breast cancer cells may employ numerous signaling pathways, including Notch, to mediate osteoblast activation and differentiation (119). Bone marrow osteoblasts produce transforming growth factor-β, which increases the levels of the Notch signaling proteins Notch3 and Jagged1, thus promoting the secretion of osteoblast-derived IL-6 and osteoblast differentiation (120,121). Inhibition of Notch signaling via knockdown of Notch3 or treatment with a GSI markedly decreases breast cancer bone metastasis (Fig. 2) (120,121).

KiSS1, a metastasis suppressor gene, is downregulated in breast cancer secondary tumor sites (122). By enhancing the activation of inhibitor of NF-κB, KiSS1 prevents NF-κB binding to the promoters of pro-inflammatory and pro-metastatic genes, thus potentially competing with Notch (122). Furthermore, KiSS1 encodes a COOH-terminally amidated active peptide, metastin (123). Of note, metastin only affects secondary tumor sites but not primary lesions (123).

Nm23 is also a suppressor of breast cancer metastasis (124). The Nm23 protein, particularly the Nm23-H1 isoform, has three major targets: ATP citrate lyase (involved in metabolism), aldolase C (involved in hypoxia) and kinase suppressor of Ras (involved in regulation of mitogenic activity) (124). Furthermore, Nm23 may suppress several metastasis-associated factors, including Smoothened (a key receptor in Hedgehog signaling) and pleiotrophin (29). Treatment with non-steroidal anti-inflammatory drugs may upregulate Nm23 expression, thus inhibiting Notch/HES1 and reducing CTCs (125,126). Ignesti *et al* (127) suggested that loss of *Drosophila awd*, a homolog of Nm23, may inhibit Notch signaling following S2 cleavage.

Expression of tenascin C (TNC), an ECM protein located in the stem cell niche, is an effective biomarker for breast CTCs that have infiltrated the lung (128). TNC enhances the level of musashi homolog 1 (MSI1), a regulator of Notch signaling, and thus confers enhanced migratory and invasive properties to breast CTCs (128). High levels of MSI1 and Jagged1 are indicative of a poor prognosis (129,130). Notably, cancer-induced sprouting neovasculature may induce tip cells to secret periostin [POSTN; Notch1 associates with POSTN at epidermal growth factor repeats (131,132)] to bind to TNC and then ECM, and facilitate TNC deposition on the ECM and its incorporation into the ECM (133).

It is incumbent on DTCs to expand and establish new colonies; otherwise, these cells enter dormancy, which is defined as growth arrest, a balance between proliferation and apoptosis (134). Dormant breast DTCs in the lung may be experiencing an absence of uPA- and α5β1 integrin-triggered

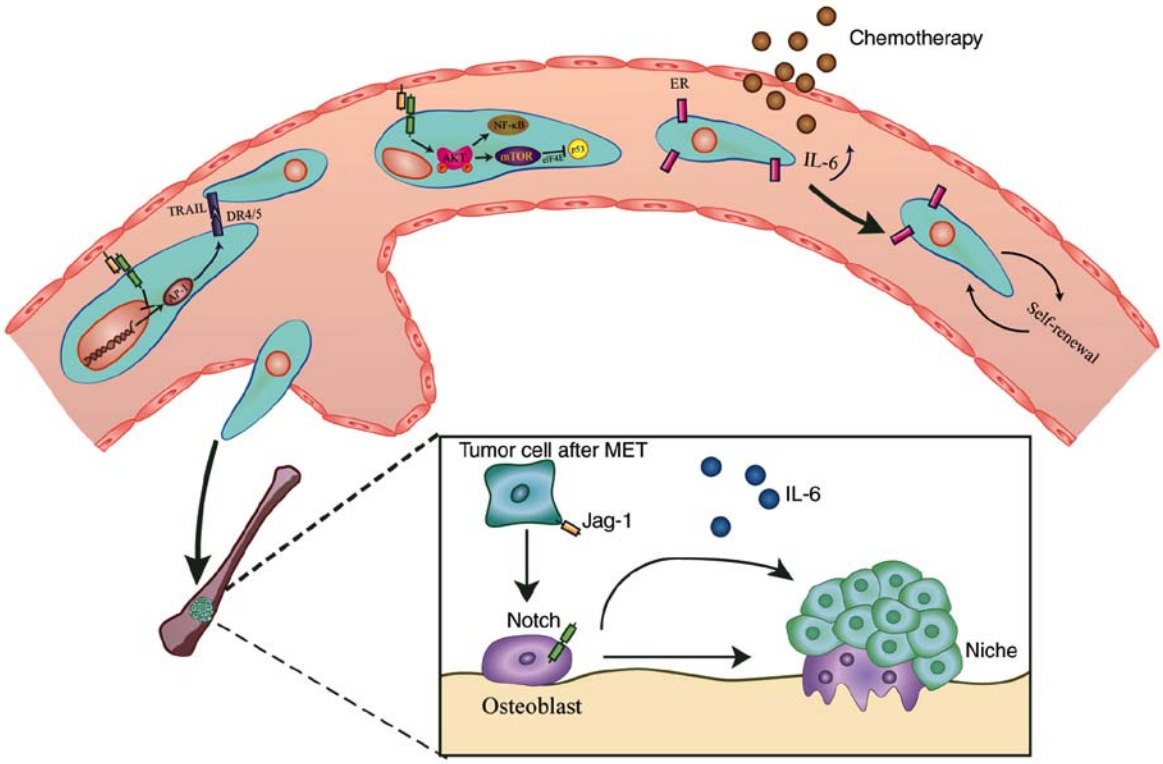

Figure 2. BC CTCs survives in the bloodstream and colonize in bone. Notch helps BC CTCs to survive from TRAIL apoptosis systems and the tumor suppressor gene p53. Chemotherapies increase the IL-6 level by the IL-6/STAT3/Notch3 axis to enhance the self-renewal properties of ER-positive BC. Notch also facilitates bone metastasis of BC by regulating osteoblasts activation and differentiation. BC, breast cancer; CTC, circulating tumor cell; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Jag-1, Jagged-1; IL, interleukin; ER, estrogen receptor.

proliferative signaling (135). Re-activation of dormant cells calls for increased uPAR-α5β1 integrin complexes and activation of upstream Notch signaling (135). Notch3 is responsible for the stability of mitogen-activated protein kinase phosphatase-1 (MKP-1), a widely expressed phosphatase (136). A previous study demonstrated that the levels of Notch3 and MKP-1 are relatively low in dormant tumors, resulting in high levels of phosphorylated p38, a target of MKP-1 that contributes to the maintenance of dormancy (137).

7. Notch-associated microRNAs in breast cancer metastasis

MicroRNAs (miRNAs) are a series of endogenous small single-stranded non-coding RNAs that are ~18-24 nucleotides in length (138). miRNAs regulate the expression of endogenous genes by complementary base pairing at the transcriptional or post-transcriptional levels (139). Over the past decade, the abnormal expression of miRNAs has been observed in nearly all malignant tumor types; therefore, miRNAs are considered to be an emerging oncology research direction (140). Certain miRNAs have been reported to be associated with Notch signaling, while a number of them are aberrantly expressed in breast cancer and are therefore associated with promoting metastasis.

The miR-34 family member miR-34a is highly expressed in normal mammary tissues; however, it is significantly downregulated in breast cancer tissues (141). miR-34a has been demonstrated to function as an important tumor suppressor by regulating a variety of tumor progression steps, including cell proliferation, invasion and apoptosis, and Notch, which is a target gene of miR-34a (142). In metastatic breast cancer cells, overexpression of miR-34a significantly increases the protein level of tumor suppressor gene p53 and decreases the expression of Notch1, thereby inhibiting cell proliferation, invasion and inducing apoptosis (143,144). Additionally, miR-34a can sensitize metastatic breast cancer cells to paclitaxel and adriamycin (chemotherapeutic drugs for breast cancer) partly by downregulating Notch1 expression (142,145). miR-34a and miR-34c, another member of miR-34 family, have been reported to prevent self-renewal and differentiation of BCSCs (146,147). Their expression is also at a very low level in BCSCs. Overexpression of miR-34a and miR-34c suppresses stemness by targeting Notch1 and Notch4, respectively (145,147). Two prognostic factors miR-1179 and miR-3178 are downregulated in breast cancer and have both been demonstrated to target Notch signaling. miR-1179 is a newly identified miRNA in 2018 (148). Clinicopathological analysis revealed that decreased miR-1179 expression in breast cancer was correlated with advanced clinical stage and lymph node metastasis (149). Upregulated miR-1179 suppresses the breast cancer vitality and motility, by inhibiting the expression of Notch1, Notch4 and their downstream Hes1 (149). miR-3178 is a prognostic factor, particularly in TNBC, and its ectopic overexpression can inhibit metastasis by blocking Notch1-induced EMT (150). In addition, miR-9 can reduce metastatic behaviors in TNBC by targeting Notch1 (151).

However, miRNAs do not all function as tumor suppressors in breast cancer, some have been observed to also promote metastasis. Notch3 can inhibit EMT in breast cancer and directly target miR-221/222 (152). By directly binding to

the 3'-untranslated region of Notch3 and inhibiting its translation, miR-221/222 exerts an oncogenic role by promoting EMT (152). miR-146a is also upregulated in BCSCs, and activates Notch signaling by targeting the Notch suppressor Numb (153,154).

8. Application of Notch signaling in the clinical treatment of breast cancer

Current treatments for metastatic breast cancer (BC) are predominantly palliative with little clinical efficacy (155). Encouragingly, numerous studies have focused on the treatment of metastatic BC via targeting Notch signaling. γ-secretase inhibitors exhibit great potential, for example, PF-03084014 (Pfizer Oncology), a small molecule selective noncompetitive and reversible GSI, displays synergistic activity with docetaxel and has demonstrated significant antitumor activity in a patient with triple-negative BC (156). In addition, a potent non-competitive oral GSI, MK-0752 (MERK), has been evaluated for the treatment of metastatic BC via induction of G0/G1 arrest (157). Monoclonal antibodies against DLL4, including REGN421/SAR153192 (Regeneron Pharmaceuticals), OMP-21M18, OMP-59R5 and OPM-52M51 (OncoMed Pharmaceuticals), which target Notch 2/3 and Notch 1 receptors, have also been investigated in clinical trials (158). Additionally, BXL0124, a Gemini vitamin D analog, has been demonstrated to be effective in suppressing $CD44^{+}/CD24^{-/lo}$ BCSCs in basal-like BC through HES1-mediated Notch1 inhibition (159). Although numerous drugs are in development, significant challenges still exist before a Notch-targeted therapeutic strategy can be clinically applied. For example, patients with BC receiving continuous doses of MK-0752 at 450 mg/daily present with symptoms of toxicity and fatigue (160). Furthermore, gastrointestinal toxicity is also a major side effect in patients treated with GSIs (161).

In recent years, cancer immunotherapy has demonstrated striking improvements in long-term survival (162), which has had a large impact on conventional systemic cancer therapy. Studies have revealed a key role of Notch in breast cancer immunotherapy. Notch1 depletion improves the efficacy of anti-tumor drugs, nivolumab (anti-programmed death-1 (PD-1) antibody) and ipilimumab (cytotoxic T cell-associated antigen-4 (CTLA-4) antibody) (66). PD-1 and CTLA-4 are inhibitory receptors on the surfaces of T cells, which can abrogate T cell activation when binding to ligands (163), and BC cells can express their ligands (PDL1 and B7 for PD-1 and CTLA-4, respectively), thereby deactivating cytotoxic T cells and attenuating the immune response.

9. Conclusion

Breast cancer is therapeutically challenging due to its distant metastasis. Recurrence at distant organs suggests that the dissemination of tumor cells may occur at very early, typically asymptomatic stages. Notch signaling modulates breast cancer metastasis in many links, and different receptors and ligands serve distinct roles (32-34,36-43,46). Notch3, however, can exert oncogenic or anti-oncogenic functions in cancer progression in a context dependent manner. By modulating GATA3 and ERα, Notch3 suppresses EMT and metastasis in breast cancer (28,44). Notch3 is also negatively associated with chemoresistance, and it has been reported that the overexpression of Notch3 results in low degree of breast cancer chemoresistance (164). Of note, breast cancer metastasis exhibits organotropisms (165), and Notch3 has been reported to be associated with bone metastasis. Notch3 enhances bone metastasis by increasing the secretion of transforming growth factor β1 by osteoblasts, thus activating the colony formation of breast cancer cells (120).

In order to promote primary tumor cell dissemination, Notch signaling can either trigger or inhibit EMT by interacting with downstream effectors (28,38-44), then regulating the invasion of breast cancer cells through the mesenchyme and basement membrane (62-70). With the help of the neovascular network, Notch signaling further initiates anti-apoptotic (96-100) and chemoresistant (114-116) characteristics in circulation and secondary colonization, which facilitate metastasis to distant organs. However, the mechanism of metastasis remains elusive. For example, certain patients carrying DTCs never develop metastasis, while other patients with large metastases do not present with DTCs at the time of primary tumor detection.

Another topic of interest in breast cancer research is exosomes, which are cell-derived vesicles that contain various biomolecules of their cell origin, such as DNA, RNA and proteins (166). It has been demonstrated that exosomes can regulate therapy resistance of breast cancer via exosomal RNA (exoRNA) transferring from stromal cells of the tumor microenvironment to breast cancer cells (116). The exoRNA can activate the RIG-I receptor (a subtype of pathogen recognition receptor) on breast cancer cells to induce STAT1 expression, which then facilitates Notch target genes expression in breast cancer cells, resulting in the upregulation of Notch3 signaling and an increase in chemoresistant BCSCs (116). In addition, exosomes have also been observed to enable organotropic metastasis by preparing a pre-metastatic niche, which is achieved through the fusion of the specific integrin (ITG) and organ-specific resident cells (167). Exosomes have also been shown to correlate with immune modulation and apoptosis in breast cancer (168). Therefore, the interaction between exosomes and Notch should be the focus of additional investigations, and exosomes may be a potential research target in breast cancer in the future.

Investigation of only one signaling pathway is also insufficient for the development of appropriate therapy, since the activation of associated pathways as well as the cross-talk between Notch and other signaling pathways are of critical importance in breast cancer metastasis. Specific aspects that will be important to consider include TNC stimulation of Notch and WNT signaling to balance dormancy and activation (128). Furthermore, Notch is also associated with the Hedgehog signaling pathway, which then regulates the tumor immunity response (169,170). However, the complicated mechanisms of metastasis reveal just the tip of the iceberg, and the presently available knowledge of Notch signaling and BC metastasis is insufficient.

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Authors' contributions

YZ was responsible for the conception and design of the review, and the drafting of the manuscript. ZX and XG were responsible for collecting the evidence and revising the manuscript. XX and LX gave final approval of the present version of the manuscript to be published. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Competing interests

The authors declare that they have no competing interests.

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