

NOTCH SIGNALING IN TUMORIGENESIS AND METASTASIS OF BREAST CANCER

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Notch signaling regulates a variety of cellular processes, including stem cell maintenance, cellular differentiation, proliferation and apoptosis. Aberrant Notch signaling has been associated with several human cancers, including acute T-cell lymphoblastic leukemia, cervical and lung carcinoma, and neuroblastoma [1]. Notch signaling has been highlighted as a pathway involved in the development of the breast and is frequently dysregulated in invasive breast cancer [2].

Notch genes encode large transmembrane proteins that act as receptors for the Delta, Serrate, Lag-2 (DSL) family of ligands. In mammals, there are four Notch proteins (Notch1-Notch4) and five ligands (Delta-like-1, Delta-like-3, Delta-like-4, Jagged-1, and Jagged-2). Ligand binding to Notch, whether on the same cell or between adjacent cells, leads to two proteolytic cleavages, one outside and one within the transmembrane domain, which releases the Notch intracellular domain (NotchIC). NotchIC subsequently translocates into the nucleus, and interacts with members of the CSL (CBF1, suppressor of Hairless, Lag-1) family of transcription factors, resulting in transcription of Notch target genes including those belonging to the HES and HRT(HEY) families [3, 4]. These target genes are critical for maintenance of stem cells.

NOTCH IN MAMMARY TUMORIGENESIS

Murine mammary tumorigenesis: The first indication that Notch signaling might play a role in mammary tumorigenesis came from the finding that the mouse mammary tumor virus (MMTV) provirus was inserted within the Notch4 gene in 20% of tumors in Czech II mice [5]. Provirus insertion causes expression of a constitutively active form of a truncated

Notch protein consisting of the transmembrane and intracellular domains. Similarly, in c-ErbB2 transgenic mice infected with MMTV, some of the mammary tumors exhibit MMTV integration into the Notch1 gene, resulting in expression of constitutively active Notch1 protein [6]. Transgenic mice expressing activated Notch1, Notch3, or Notch4 have impaired development of mammary glands, and generate mammary tumors [7, 8].

Human breast cancer: Involvement of Notch signaling in murine mammary tumorigenesis and its association with various human cancers clearly raises the possibility that aberrant Notch signaling could play a role in human breast cancer. Elevated expression of Jagged1 and Notch1 in breast cancer is observed in a wide variety of human breast carcinomas and is correlated with poor prognosis [2, 9-11]. Overexpression of constitutively active Notch4 in normal human breast epithelial cells induces transformation *in vitro* [12]. The incidence of HER2-negative breast cancer is 70-80% in primary and metastatic disease. Notch3 signaling appears to have a unique cell proliferative and anti-apoptotic role in HER2⁻ cancer, but not in Herceptin-sensitive HER2⁺ breast cancer [13], raising hope for anti-Notch3 therapy as an alternative strategy for HER2⁻ disease.

Importantly, more than 50% of human breast tumors express reduced protein levels of Numb, a negative regulator of Notch signaling, and reduced Numb expression is correlated with breast tumor grade and proliferation rate [14]. However, there might be additional function of Numb as a tumor suppressor in breast cancer. A recent study indicates that Numb forms a tricomplex with p53 and the E3 ubiquitin ligase HDM2, thereby preventing ubiquitination and degradation of p53. In primary breast tumors, loss of Numb expression causes decreased p53 levels and increased chemoresistance [15].

Cancer stem cells: Cancer stem cells represent a new paradigm in tumor biology. Cancer stem cells can both initiate tumor growth, and then re-initiate tumor expansion after therapy, causing relapse [16]. Cancer stem cells isolated as a dye-excluding side population from numerous cancer cell lines express high levels of Notch1 [17]. Notch signaling has also been implicated in the regulation of self-renewal of normal mammary stem cells [18]. In human breast cancer cells, the tumorigenic cancer stem cell has been identified as CD44⁺CD24^{-/low}Lineage⁻ [19, 20]. As few as 100 cells with this phenotype are able to form tumors in mice. Decreasing Notch signaling by treatment with γ -secretase inhibitors or Notch4

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neutralizing antibody inhibits tumorigenic mammosphere formation in primary breast cancer cells, suggesting that Notch signaling plays a key role in self-renewal of breast cancer stem cells [21].

NOTCH AND HYPOXIA

Hypoxic environments contribute to cancer progression. Hypoxia promotes stem cell renewal *in vitro* as well as *in vivo*. Hypoxia-inducible factor 1 α (HIF-1 α) interacts with Notch1 intracellular domain to augment the Notch downstream responses in hypoxia [22].

In breast cancer, HIF-1 α stabilization by hypoxia causes HIF-responsive gene induction and tumor progression. The 66-kDa isoform of the SHC gene (p66Shc) is induced by the exposure to hypoxia, and it controls the expression of Notch3. p66Shc/Notch3 interplay modulates self-renewal and expression of a hypoxia survival gene, carbonic anhydrase IX, in breast cancer mammospheres [23]. High serum levels of IL-6 correlate with poor prognosis in breast cancer patients. IL-6 triggers Notch3-dependent upregulation of the carbonic anhydrase IX gene and promotes a hypoxia-resistant/invasive phenotype in human breast carcinoma cells [24].

NOTCH AND WNT

Signaling pathways involving Wnt, Notch, and Hedgehog all play major roles in mammary gland development and tumorigenesis. Aberrant expression of several Wnts and other components of this pathway in human breast carcinomas has been reported. Ectopic expression of Wnt1 is sufficient to transform primary human mammary epithelial cells, resulting in tumorigenic conversion. The Wnt1-transformed cells have enhanced expression of the Notch ligands Dll-1, Dll-3, and Dll-4, and increased Notch signaling is also required for the tumorigenic phenotype [25].

POSSIBLE ROLE OF NOTCH IN BONE METASTASIS

Bone marrow is the gateway for establishment of painful osteolytic breast cancer metastasis. 70-90% of women dying of breast cancer have osteolytic bone metastasis. Bone metastasis is a leading cause of pain, disability, pathological fractures, nerve compression, and hypercalcemia.

Notch in the Bone Marrow Niche: Bone marrow is the home for hematopoiesis, and it also provides a fertile soil for cancer metastasis. The invasive malignant cells and hematopoietic stem cells share much homology in migration and signaling machinery. Osteoblasts of bone marrow were identified as the obligate niche for support of long-term hematopoietic stem cells, providing the Notch ligand Jagged1 and other factors under regulation by bone morphogenetic protein (BMP) and PTH/PTHrP signaling [26, 27]. Increasing evidence suggests that the osteoblast niche inhibits drug-induced apoptosis and confers *de novo* drug resistance in multiple myeloma cancer cells [28]. This type of paracrine Notch signaling in metastatic breast cancer cells could explain their predisposition to bone metastasis.

TGF- β : Evidence for the role of transforming growth factor- β (TGF- β) signaling in cancer metastasis has recently

been documented specifically in breast cancer. TGF- β promotes bone metastasis of breast cancer by increasing PTHrP production in tumor cells (Fig.1). Blockade of TGF- β signaling decreases PTHrP production and bone metastasis, and prolongs the survival of tumor-bearing mice [29]. Similarly, PTHrP-neutralizing antibody greatly decreases osteolytic bone metastasis [30]. In addition, TGF- β enhances the gene expression of IL-11 and CTGF, which may contribute to osteolytic bone metastasis [31].

TGF- β and Notch signaling converge in the regulation of a number of developmental processes. Cross-talk occurs between the two pathways. TGF- β increases the expression of Hes-1, a direct target of Notch, in several cell types [32]. TGF- β induces the interaction of the intracellular domain of Notch1 with Smad3, an intracellular transducer of TGF- β signaling. In breast cancer, epithelial-mesenchymal transition (EMT) is correlated with the highly aggressive metastatic spread of these tumors. TGF- β -induced EMT is blocked by RNA silencing of the Notch target gene Hey-1 and the Notch ligand Jagged1, and by chemical inactivation of Notch [33]. TGF- β is one of the most abundant factors in bone matrix, and it is critical to discover how the autocrine Notch signaling in breast cancer cells contributes to the pathophysiology of TGF- β -mediated bone metastasis [34-37] (Fig.1).

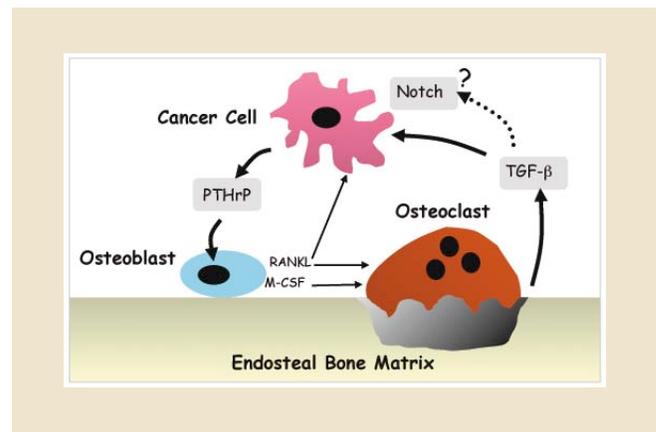


Figure 1. Mechanism of TGF β -stimulated bone metastasis of breast cancer. Bone-derived TGF β and tumor-derived PTHrP play major roles in the “vicious cycle” of osteolytic bone metastasis [34-37]. TGF β acts directly on tumor cells, and enhances the production of PTHrP, which then increases osteoclast activity by way of osteoblastic expression of RANKL and M-CSF. A possible role of the Notch pathway in breast cancer metastasis to bone is indicated downstream of TGF β signaling.

Animal model to study bone metastasis: Animal models are important tools to investigate the pathogenesis and develop treatment strategies for bone metastasis in humans. Rats, mice, dogs, and cats often develop spontaneous mammary carcinoma, but bone metastasis is rare. Bone metastasis in breast cancer xenograft models has been achieved with intracardiac injection of MDA-MB-231 cells, a human breast cancer cell line [38]. MDA-MET, a subline of MDA-MB-231, was selected for its strong propensity for bone metastasis [39]. Inoculation of MDA-MET cells with ultrasound guidance in athymic mice repro-

ducibly generates osteolytic lesions, providing a useful tool to study the requirements for Notch signaling in bone metastasis of breast cancer (Fig.2).

SUMMARY

Notch signaling regulates tumorigenesis of breast cancer cells. Along with TGF- β and Wnt signaling, a likely role exists for Notch pathway regulation of both the self-renewal of breast cancer stem cells, and the promotion of osteolytic bone metastasis. Characterization of these biological processes should help to identify new therapeutic targets in human breast cancer.

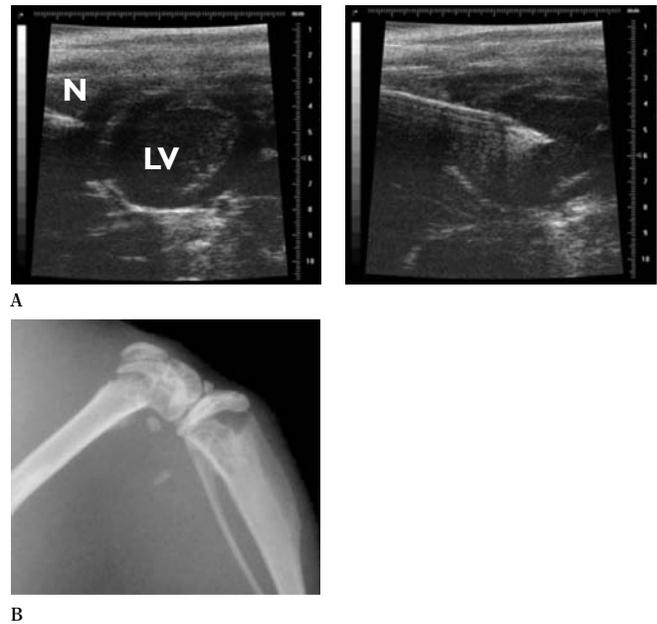


Figure 2. Intracardiac injection of MDA-MET cells with ultrasound guidance. (A) MDA-MET cells were injected into left ventricle (LV) of 7-8 week old athymic mice. Left panel: two-dimensional ultrasound image showing the needle tip (N) approaching the LV; Right panel: entry of the needle tip into LV, allowing delivery of tumor cells into the arterial circulation. (B) Osteolytic lesion of femur and tibia 3 weeks after injection appears to be dependent on Notch signaling in the breast cancer cells.

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