Notch signaling: targeting cancer stem cells and epithelial-to-mesenchymal transition

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Abstract: Notch signaling is an evolutionarily conserved pathway involved in cell fate control during development, stem cell self-renewal, and postnatal tissue differentiation. Roles for Notch in carcinogenesis, the biology of cancer stem cells, tumor angiogenesis, and epithelial-to-mesenchymal transition (EMT) have been reported. This review describes the role of Notch in the “stemness” program in cancer cells and in metastases, together with a brief update on the Notch inhibitors currently under investigation in oncology. These agents may be useful in targeting cancer stem cells and to reverse the EMT process.

Keywords: Notch signaling, EMT, cancer stem cells, mesenchymal stem cells, metastases, Notch inhibitors

Introduction

The Notch pathway is one of the most intensively studied candidate therapeutic targets in cancer stem-like cells (CSCs), and several investigational Notch inhibitors are being developed. Notch signaling has been reported to promote the self-renewal of CSC in several malignancies and to participate in tumor–stroma and tumor–endothelium interactions in CSC niches in primary and metastatic tumors.1,2 However, successful targeting of Notch signaling in CSCs will require a clear understanding of Notch regulation and the context-dependent interactions between Notch and other therapeutically relevant pathways. Understanding these interactions will increase our ability to design rational combination regimens that are more likely to prove safe and effective for primary and metastatic tumors. Additionally, to determine which patients are most likely to benefit from treatment with Notch-targeting therapeutics, reliable biomarkers to measure Notch pathway activity in CSCs from specific tumors will have to be identified and validated.

Notch receptors and ligands

Mammals express four transmembrane Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4)3 and five canonical transmembrane ligands (Delta-like [DLL] 1, DLL 3, DLL 4, Jagged-1, and Jagged-2) (Figure 1).4–7 Delta family ligands differ from Jagged family ligands because their smaller extracellular domains can mediate Notch activation in trans (from cell to cell) and Notch inhibition in cis (on the same cell). The relative affinity of Notch receptors for Delta and Jagged family ligands is controlled by receptor glycosylation, and specifically by the addition of Fuc-GlcNac (fucose-N-acetylgalcosamine) moieties by a fucosyltransferase and Fringe family
Notch in ligand-dependent activation, while ADAM17 may (ADAM10 or ADAM17). Separation allows a second cleavage (S2) by a disintegrin with the ligand into the ligand-expressing cell. Subunit engagement, N extracellular (N EC), consisting of Notch extracellular (N EC) and Notch transmembrane domain. Mammals contain four Notch receptors (Notch-1–4) and five ligands (Jagged-1/2, homologous to Serrata in Drosophila) and DLL 1, 3, and 4 (homologous to Delta in Drosophila).

Abbreviations: ANK, ankyrin repeats; CR, cysteine-rich domain; DLL, Delta-like; DOS, Delta and OSM-11-like protein domain; DSL, Delta, Serrata, and LAG-2 domain; EGF, epidermal growth factor; LNR, cysteine-rich Lin 12-Notch repeats; P, P eST domain; R, RAM domain; TAD, transactivation domain; N I, Notch intracellular domain; N EC, Notch extracellular domain; N TM, Notch transmembrane domain.

Figure 1 Schematic representation of Notch receptors (A) and Notch ligands (B) in mammals.

Notes: Mammals contain four Notch receptors (Notch-1–4) and five ligands (Jagged-1/2, homologous to Serrata in Drosophila) and DLL 1, 3, and 4 (homologous to Delta in Drosophila).

N-acetyl-glucoaminidyl-transferases. Cell-to-cell contact is generally necessary for the activation of Notch signaling. This usually results in coordinated modulation of genes involved in cell fate determination, such as proliferation, survival, or differentiation.

Notch receptors undergo three proteolytic cleavages. First, Notch precursor proteins are processed in the trans-Golgi apparatus. A single polypeptide precursor is cleaved (S1) by a furin-like convertase to produce the mature Notch receptor, which is a heterodimer consisting of Notch extracellular (N EC) and Notch transmembrane (N TM) subunits. Mature receptors are trafficked to the plasma membrane, where they await engagement with membrane-associated ligands. Upon ligand–receptor engagement, N EC is dissociated from N TM to be endocytosed with the ligand into the ligand-expressing cell. Subunit separation allows a second cleavage (S2) by a disintegrin and metalloproteinase domain-containing protein 10 or 17 (ADAM10 or ADAM17). ADAM10 is thought to cleave Notch in ligand-dependent activation, while ADAM17 may participate in the less clearly understood process of ligand-independent activation. The S2 cleavage releases a short extracellular peptide and generates a short-lived intermediate that is cleaved again (S3) by the γ-secretase complex. The S3 cleavage releases the intracellular portion of Notch (N IC)9 N IC translocates to the nucleus and binds to the CBF-1-Suppressor of Hairless/Lag 1 ([CSL] also known as RBP-jx), a constitutive transcriptional repressor, displacing corepressors and recruiting coactivators such as Mastermind-like (MAML) proteins, homologous to Drosophila Mastermind. The Notch–CSL–MAML complex in turn recruits multiple transcriptional regulators forming the “Notch transcriptional complex” (NTC).10,11 Notch activates many genes associated with differentiation and/or survival, including the Hairy/Enhancer of Split (HES) family and Hairy/enhancer-of-split related with YRPW motif-like protein (Hey) family of basic helix-loop-helix transcription factors,12 cyclin D1,13 and c-Myc (Figure 2).14 The genomic sites at which Notch activates transcription vary from cell to cell, and quite likely among different Notch paralogs. Other transcriptional regulators influence transcriptional regulation by Notch-1.15–17 The close-range cell–cell interaction necessary for Notch activation may be one of the signals whereby intercellular signals trigger epithelial-to-mesenchymal transition (EMT) in the tumor microenvironment.

Notch signaling, EMT, and cancer stem cells

Many human cancers are thought to contain populations of cells that display stem cell-like properties. These properties include self-renewal, which drives tumorigenesis; resistance to cell death, which drives tumor progression; and differentiation, which contributes to cancer cell heterogeneity. There is increasing evidence that these CSCs mediate tumor metastasis and, by virtue of their relative resistance to chemotherapy and radiation therapy, may contribute to treatment failures and relapses following therapy.18

Self-renewal and cell fate determination of normal stem cells are regulated by both cell-autonomous (intrinsic) and non-cell-autonomous (extrinsic) pathways. The dysregulation of these pathways resulting in stem cell expansion may be a key event initiating carcinogenesis. Developmental pathways such as Notch play an important role in normal stem cell functions and are frequently deranged in cancers.19–22 Deregulated expression of Notch proteins, ligands, and targets, including overexpression and activation of Notch, has been described in a multitude of solid tumors, including cervical,23 head and neck,24 endometrial,25 renal,26 lung,27 pancreatic,28 ovarian,29...
Notch-1 has oncogenic effects. This has been demonstrated in most cases, however, inappropriate activation of Notch signaling has been reported in some hematological malignancies, including Hodgkin lymphomas, anaplastic large-cell non-Hodgkin lymphomas, some acute myeloid leukemias (AMLs), B-cell chronic lymphoid leukemias (B-CLLs), and multiple myeloma (MM). Dysregulation of Notch signaling has been reported in some hematological malignancies other than T-ALL. These include Hodgkin lymphomas, anaplastic large-cell non-Hodgkin lymphomas, some acute myeloid leukemias (AMLs), B-cell chronic lymphoid leukemias (B-CLLs), and multiple myeloma (MM) (for a recent review, see Pancewicz and Nicot). In most cases, inappropriate activation of Notch signaling is oncogenic. In some cases, however, loss of function of Notch-1 has oncogenic effects. This has been demonstrated in the epidermis and, more recently, in a subset of head and neck squamous carcinomas. Notch signaling is essential to the orderly differentiation of squamous epithelia, and loss of Notch-1 causes loss of barrier in such epithelia. This in turn triggers an inflammatory response and cytokine cascade that may favor transformation. However, in the case of CSC, the literature supports a role of several Notch paralogs in the maintenance and survival of these cells. Extrinsic signals that regulate stem cell behavior originate in the stem cell microenvironment. Although there is still relatively little detailed information on the composition and function of cancer stem cell microenvironments in different malignancies, it is clear that tumor growth and metastasis are highly dependent on the tumor microenvironment. This microenvironment is comprised of tumor-associated fibroblasts, endothelial cells, adipocytes, and several types of immune cells, all of which have been demonstrated to play roles in tumor growth and metastasis. Several studies have demonstrated that loss of epithelial phenotype through EMT can promote the acquisition of a stem-like phenotype and drug resistance. Notch signaling regulates both the formation of CSCs and the acquisition of the EMT phenotype, which are associated with drug resistance. An epithelial gene signature has been associated with sensitivity to the epidermal growth factor receptor inhibitor erlotinib in lung cancer cells. Similar results have been reported in head and neck squamous cell carcinoma and hepatocellular carcinoma with gefitinib and cetuximab. Conversely, EMT has also been shown to promote resistance to conventional therapeutics, including paclitaxel, vincristine, and oxaliplatin. Recent studies have shown links between EMT and gemcitabine-resistant pancreatic cancer, oxaliplatin-resistant colorectal cancer, lapatinib-resistant breast cancer, and paclitaxel-resistant ovarian carcinoma. Therefore, elucidating mechanisms that govern the acquisition of EMT in cancer cells would likely be useful for devising targeted therapeutic approaches to overcome or prevent resistance to conventional cancer therapeutics.

Notch activation triggers mesenchymal transformation not only in epithelial but also in endothelial cells. These changes include downregulation of endothelial markers (vascular endothelial-cadherin, tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domain [Tie]1, Tie2, platelet-endothelial cell adhesion molecule-1, and endothelial nitric oxide synthase) and upregulation of mesenchymal markers (α-SMA, fibronectin, and platelet-derived growth factor receptors). Jagged-1-mediated stimulation of endothelial cells induces phenotypic and functional changes consistent with EMT. Notch also cross-talks with several transcription and growth factors relevant to EMT, including Snail, Slug, and transforming growth factors.
Epithelial cells from a primary prostate tumor can undergo EMT with activation of embryonic programs of epithelial plasticity, including Notch, and switch from a sessile, epithelial phenotype to a motile, mesenchymal phenotype. Growth factors and molecular alterations that contribute to EMT induction in primary tumors have been identified as important stimulators of skeletal metastasis formation. Aberrant expression of EMT markers N-cadherin, vimentin, platelet-derived growth factor-D, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Notch-1, and zinc finger E-box-binding homeobox (ZEB)1 was observed in primary prostate cancers and bone metastatic lesions. Notch-1 was highly expressed in bone metastases compared to primary prostate cancers, suggesting that Notch-1 could play a role in prostate cancer bone metastasis through the induction of an EMT phenotype (Figure 3). Recent data from Zhu et al support this model, showing that Jagged-1 expression increases dramatically in high-grade and metastatic prostate cancers compared to primary lesions. Furthermore, Notch signaling is often and aberrantly activated by hypoxia, which induces EMT during tumor progression. Bone is one of the most frequently targeted organs for breast cancer metastasis, and regions of the bone are known to be hypoxic. This hypoxic niche in

Figure 3: Role of Notch in tumor metastasis as an inducer of EMT.

Notes: Epithelial cells can undergo EMT with activation of embryonic programs of epithelial plasticity, including Notch. Aberrant expression of EMT markers N-cadherin, vimentin, platelet-derived growth factor-D, NF-κB, Notch-1, and ZEB 1 has been observed in metastatic lesions, together with high Notch receptor and ligand expression. Notch signaling is often and aberrantly activated by hypoxia that induces EMT during tumor progression. Thus, the hypoxic niche promotes EMT and self-renewal of breast CSCs, suggesting a critical role of Notch-induced EMT in tumor progression and metastasis.

Abbreviations: CSC, cancer stem-like cell; EMT, epithelial-to-mesenchymal transition; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ZEB, zinc finger E-box-binding homeobox; NIC, Notch intracellular domain; MAML1, Mastermind-like protein 1; CSL, CBF-1-Suppressor of Hairless/Lag1 (also known as RBP-jk).
bone microenvironment is believed to promote self-renewal of hematopoietic stem cells. Xing et al have also shown that Jagged-2 was upregulated in bone marrow stroma under hypoxia, which significantly promoted EMT and self-renewal of breast CSCs, suggesting a critical role of Notch-induced EMT in tumor progression and metastasis.

**Notch signaling and mesenchymal stem cells**

Mesenchymal stem cells (MSCs) are multipotent cells with non-hematopoietic origin that constitute a minor population (0.01%) of nucleated cells in bone marrow. MSCs are a potential source of stem cells for cellular and genetic therapy, and can differentiate into multiple lineages such as chondrocytes, osteocytes, adipocytes, myocytes, and astrocytes. Recently, MSCs were found to play an important role in the tumor-supporting stroma. MSCs are known for their active mobilization from bone marrow and migration to sites of injury. Reports have suggested that bone marrow-derived MSCs are preferentially recruited to tumor stroma when compared to normal stroma, mainly by inflammatory factors in the tumor microenvironment. These reports increased interest in understanding the potential role of MSCs in tumor progression. MSCs are recruited to the tumor microenvironment in response to various cytokines, which are secreted by tumor cells and their associated stroma and act as precursors for pericytes and carcinoma cancer-associated fibroblasts. MSCs promote tumor cell proliferation indirectly through their immunosuppressive properties and directly through cancer cell supportive properties. An earlier study from Sanchez et al suggest that, under nutrient-deprived conditions, the MSCs associated with tumor stroma undergo autophagy, secreting antiapoptotic factors that protect breast cancer cells embedded in the stroma. These studies suggest that targeting tumor associated stromal cells along with tumor cells may provide more effective treatment strategies for breast cancer. Recent evidence also suggests that MSCs participate in tumor growth and metastasis and are the most prominent cell type within the stroma of many cancers. Subcutaneously implanted human mammary carcinomas coinfected with MSCs acquire an increased metastatic potential. An important factor in the function of tumor microenvironment is the cell–cell communication between stromal cells and cancer cells. The role of gap junctions in the transport of cellular communicators and juxtacrine regulation based on direct communication is well documented.

The role of MSCs in inducing EMT in tumor cells has been the focus of a number of recent studies. Several possible mechanisms through which MSCs play a role in tumor microenvironment have been proposed. One such mechanism is exosomes secreted by MSCs, which promote EMT in gastric cancer cells. EMT processes endow epithelial tumor cells with properties that may facilitate CSC generation and survival, ie, increased invasive ability, increased resistance to apoptotic signals, and increased ability to potentiate angiogenesis. In vivo models of EMT-derived cells in primary tumors have enhanced metastatic potential. Inflammatory cells and cytokines, hypoxia-induced increase of reactive oxygen species in mitochondria, and MSC can all effectively drive the EMT of tumor cells. Passage by neoplastic epithelial cells through an EMT event allows these cells to approach a stem cell-like state. EMT programs are known to be induced by heterotypic signals that epithelial cells receive from their microenvironment. In response to stimulation by carcinoma cells, MSCs express greatly elevated levels of prostaglandin E2 (PGE2). The resulting PGE2, together with cytokines also induced in the MSCs, contributes to the entrance of nearby carcinoma cells into a stem cell-like state. An in vitro study using coculture models of MSCs and breast cancer cells showed that EMT is stimulated by increased expression of oncogenes and other genes associated with invasion, angiogenesis, and antiapoptosis. However, the nature of these heterotypic signals and the identities of the stromal cells that release them remain poorly understood.

Notch signaling is important for MSC differentiation and related to its role in EMT. MSC modified with miR-126 release proangiogenic factors and induce expression of proangiogenic Notch ligand DLL 4, enhancing angiogenesis. Moreover, Notch signaling regulates the expression of CXCR4 in MSCs, modulating their migration. Notch-1 has also been reported to mediate the induction of Tregs by MSCs. Tregs are thought to promote tumorigenesis by dampening antitumor immune responses. As with BMP and Wnt signaling in osteogenesis, Runx2 function is also influenced by Notch signaling. Runx2 protein to repress terminal osteoblastic differentiation in vitro (Figure 4).

**Notch signaling and tumor metastasis**

Recent insights have linked Notch signaling to cancer metastasis. It is now well recognized that cancer progression not only requires deregulated signaling pathways and accumulated genetic alterations in cancer cells, but also relies on the support from tumor microenvironment. For example, in the case of breast cancer, tumor cells frequently
metastasize to the bone and brain, where tissue microenvironment enhances the metastatic growth of cancer cells by providing growth factors and ligands that activate multiple metastasis-related pathways including Notch, Wnt, and Hedgehog.109–111 Elevated expression of Jagged-1 has been associated with an increased incidence of triple-negative breast cancer (TNBC) bone metastasis and tumor cells that overexpress Jagged-1-generated severe osteolytic lesions in mouse tibiae, suggesting a potential role of tumor-derived Jagged-1 in promoting bone metastasis.106 Interestingly, the same paper also showed that breast cancer cells with a high level of Jagged-1 promoted bone metastasis by activating Notch signaling in the osteoblasts, which in turn directly enhanced osteoclast differentiation by secreting interleukin (IL)-6. These results indicate a new paradigm for Notch signaling in breast cancer metastasis in which bone stromal cells respond to tumor-derived ligands and promote osteolysis in a paracrine manner. Furthermore, it has been shown that Notch signaling is activated in endothelial cells, promoting angiogenesis after interaction with cancer cells in head and neck squamous cell carcinomas.112 These studies indicate that reciprocal interactions of tumor and untransformed stromal cells in the microenvironment play critical roles in the activation of Notch signaling. Tumor microenvironment includes not only various types of stromal and immune cells but also several homeostatic factors, such as pH and oxygen concentration.113,114 Hypoxic breast cancer cells have been shown to enhance the capillary-like tube formation of endothelial cells in a Jagged-2-dependent manner. Knockdown of Jagged-2 by siRNA in cancer cells blocks angiogenesis of endothelial cells in coculture experiments.115

The brain is a frequent metastatic site for several types of tumors, including melanoma and lung and breast cancers, and metastatic tumor cells need to adapt to this totally different microenvironment. It has been demonstrated that brain metastatic TNBC cells excessively expressed IL-1β, which stimulates the surrounding astrocytes to express Jagged-1.116 Direct interaction of the reactivated astrocytes with CSCs resulted in significantly upregulated Notch signaling in CSCs. This in turn further enhanced the self-renewal of CSC, suggesting that there is a vicious circle paracrine loop of IL-1β and Notch signaling inducing one another through direct interaction between CSCs and astrocytes. This vicious circle promotes the growth of metastatic CSCs in the brain. The blood–brain barrier-permeable Notch inhibitor γ-secretase inhibitor (GSI) Compound E can significantly suppress brain metastasis in vivo.117 These results represent a novel paradigm for the understanding of how metastatic breast CSCs re-establish a niche for their self-renewal in a glial microenvironment entirely different from their tissue of origin, opening a new avenue by which to identify a novel and specific target for the brain metastatic disease (Figure 5).

Targeting Notch signaling to reverse EMT and stemness in CSCs

Several classes of investigational Notch inhibitors have been developed. These include monoclonal antibodies against Notch receptors or ligands;118–123 decoys (soluble forms of the extracellular domain of Notch receptor or Notch ligands);124–127 blocking peptides;128 GSIs;129–135 or natural compounds.136–140 To date, GSIs are the most extensively explored. GSIs are less specific than biologics, but have the potential advantages of favorable biodistribution and pan-Notch inhibition. While γ-secretase has numerous substrates besides Notch receptors, the pharmacologic activity and toxicity of GSIs in vivo appears to be due largely to Notch inhibition.141,142 GSIs have been administered to patients in Phase I clinical trials, either as single agents or in combination with standard of care.143 As is the case for most stem cell pathway inhibitors, the development of Notch inhibitors will need to be guided by biology. Biomarkers indicative of Notch activity (and of its inhibition by investigational drugs) will have to be identified and validated in each indication. Additionally, mechanism-based combinations will have to be developed.
This implies that standard clinical trial designs with single-agent investigational drug and tumor volume as the primary end point may not be the most appropriate strategy for clinical trials of Notch-targeting agents, or, for that matter, for other CSC-targeted drugs. For example, in Her2/Neu positive BT474 xenografts, the combination of two chemically different GSIs with trastuzumab dramatically inhibited tumor recurrence, producing complete cures in most animals treated with one drug and all animals treated with another.\(^1\) GSIs given as single agents had virtually no effect on tumor volume in this experimental model, nor did they enhance tumor volume regression induced by trastuzumab. Thus, these agents prevented tumor regression with no significant effect on tumor volume. This effect is most likely due to CSC blockade, and suggests that survival-based end points may be needed in the clinic, at least for some indications. Recurrence-free survival and/or good surrogate end points predictive of survival (eg, circulating tumor cells, tumor-sphere-forming cells) are likely to be more informative. These challenges do not diminish the tremendous therapeutic opportunity offered by a pathway that is essential for EMT and CSC maintenance, angiogenesis, and, in many cases, proliferation and survival of cancer cells.

The Notch pathway has tremendous potential as a new target in cancer therapy. Importantly, Notch may be a particularly powerful target for CSCs, which are resistant to standard treatments such as chemotherapy and radiation but seem especially sensitive to inhibition of stem cell pathways such as Notch. Although several Notch inhibitors are currently at the clinic,\(^1\) Notch inhibitors used as single agents do not always yield major responses based on tumor volume in all models. Several issues remain to be addressed:

1. GSIs can affect bulk tumor cells, CSCs, stroma, and angiogenesis. The effects observed in vivo depend on the relative importance of these cellular targets in each tumor and tumor model.
2. GSIs are not pharmacologically equivalent: they have different pharmacokinetics, potencies, and off-target effects, and should not be considered equivalent.
3. At least one Notch paralog, Notch-4, has been shown to be resistant to some GSIs.\(^1\) Whether this is true of all GSIs is unclear, but Notch-4-driven tumors may be resistant to some GSIs.
4. GSIs generally have gastrointestinal toxicity, which is Notch-mediated and results from goblet cell metaplasia of intestinal stem cells. This precludes long-term, sustained administration of these drugs. Intermittent administration schedules have been used in the clinic and in preclinical models. These regimens do dramatically decrease toxicity. However, it is unknown whether intermittent inhibition of Notch signaling is sufficient to achieve an anti-CSC effect. Tumor-selective delivery systems may be necessary to achieve sustained Notch inhibition within the tumor microenvironment.
5. Combination treatments, ideally based on mechanistic information, are likely to prove more successful than single-agent regimens. For instance, glucocorticoids decrease the intestinal side effects of GSIs in T-ALL models.\(^1\) In estrogen receptor alpha (ER\(\alpha\))-positive breast cancer, combinations of Notch inhibitors with endocrine therapy have shown promise in preclinical models\(^1\) and in two early-phase clinical trials.\(^1\) In a presurgical window study, the addition of GSI MK0752 to tamoxifen or letrozole decreased Ki67 in 17/20 patients compared to endocrine therapy alone.\(^1\) In the metastatic setting, a combination of exemestane and GSI RO4929097 yielded clinical responses in seven out of 14 patients.\(^1\) In both cases, no diarrhea was observed. This may be due to the fact that endocrine therapy ameliorates the gastrointestinal toxicity of GSIs.\(^1\) Similarly, in TNBC, combinations of GSIs and taxanes have shown synergistic efficacy.\(^1\)
6. Non-GSI strategies to inhibit Notch signaling, including stapled peptides, decoys, monoclonal antibodies to Notch ligands or receptors, or inhibitors of downstream mediators may prove useful in some indications. Several classes of
Conclusion
Deregulation of Notch signaling has been associated with mobilization and spread of primary tumor cells to distant locations. EMT and mesenchymal–epithelial transition play important roles during tumor invasion, metastasis, and therapeutic resistance. EMT is also linked with the acquisition of stem cell-like characteristics. The concept of EMT inducing a CSC phenotype provides a possible mechanistic basis for metastasis, chemoresistance, tumor dormancy, and delayed recurrence. Notch signaling is one of a handful of embryonic pathways that control the generation and self-renewal of CSCs, at least in part through EMT. Significant efforts are underway to develop pharmacologic inhibitors of Notch signaling that can inhibit EMT and/or eradicate CSCs in common human malignancies.

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