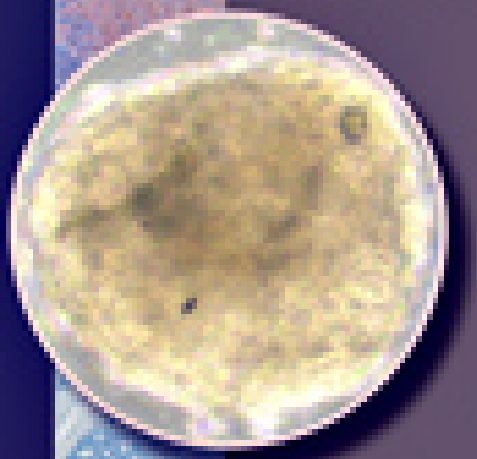
Cancer Stem Cells



Edited by William L. Farrar

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NOTCH SIGNALING IN CANCER STEM CELLS

Stem cells are characterized by two unique properties: self-renewal and multilineage differentiation potential. Self-renewal provides the cell with the ability to go through numerous cycles of cell division, while maintaining a stem cell population through asymmetric cell division. For each division, a stem cell divides into two cells: another stem cell and a progenitor cell. It is thought that the stem cell retains the stem cell characteristics, while the progenitor cell can differentiate into tissue-specific cells within a limited number of cell divisions. Embryonic stem cells are active during embryonic differentiation and develop into all of the tissues in the body. Adult stem cells can be found in differentiated tissues and can differentiate into the entirety of cell types in the tissue from which they

originate. Normal stem cells are transformed into cancer stem cells by acquiring somatic mutations in oncogenes or tumor suppressor genes.[1] Cancer stem cells share stem cell properties with embryonic stem cells such as self-renewal and differentiation potential.[2] Evidence suggests that many cancers, including leukemia, breast cancer, and glioma, contain a rare population of cells that are highly tumorigenic, in contrast to the bulk of cancer cells that have a limited capacity to form tumors in vivo. Cancer stem cells proliferate slowly, have indefinite self-replication ability, and are highly resistant to chemotherapy. Although conventional chemotherapy may eradicate the majority of cancer cells, cancer stem cells are largely spared and may go on to accumulate additional somatic mutations, eventually giving rise to recurrences and metastases. On the basis of this cancer stem cell theory, therapeutic strategies that specifically target pathways for cell renewal and cell fate decision in cancer stem cells could potentially increase the efficacy of current cancer treatment and reduce the risk of relapse and metastasis. Cancer stem cells isolated as a dye-excluding side population from numerous cancer cell lines express high levels of Notch receptors.[3,4] Consequently, Notch signaling is considered one of the most attractive targets for developing therapeutics directed at cancer stem cells.

NOTCH SIGNALING PATHWAY

The Notch signaling pathway is evolutionarily highly conserved and mediates intercellular signaling.[5] Notch signaling controls cell fate decision and patterned differentiation in numerous developmental processes. The Notch transmembrane receptors are activated by cell surface ligands DSL (Delta/Serrate/Lag2) and mediate direct cell–cell communication. Notch receptors are large, single-pass, type I transmembrane proteins. Four members, Notch1, 2, 3, and 4, have been identified in mammals. Notch receptor is synthesized as a single precursor protein but is cleaved by a furin-like protease at a juxtamembrane site (S1 cleavage) within the Golgi apparatus to create a heterodimer. Notch heterodimer consists of noncovalently associated extracellular and transmembrane subunits located at the plasma membrane.[6–8] The extracellular subunit contains a variable number of EGF-like repeats that are critical for ligand binding[9,10] and a juxtamembrane negative regulatory region (NRR) consisting of three LIN12/Notch repeats (LNR1–3) and a heterodimerization domain (HD1 or HD-N). The transmembrane subunit contains a small stretch of extracellular heterodimerization domain (HD2 or HD-C), a single-pass transmembrane domain, and an intracellular region that contains ankyrin repeats[11] involved in signal transduction.[12] In addition, this intracellular region contains nuclear localization signals (NLS) and a transactivation domain (TAD), followed by a PEST region rich in Proline, Glutamate, Serine, and Threonine residues that is involved in protein degradation (Figure 8–1).

In mammals, five Notch ligands have been identified: two Jagged (JAG1 and JAG2) and three Delta-like (Dll1, Dll3, and Dll4). All five ligands share a conserved

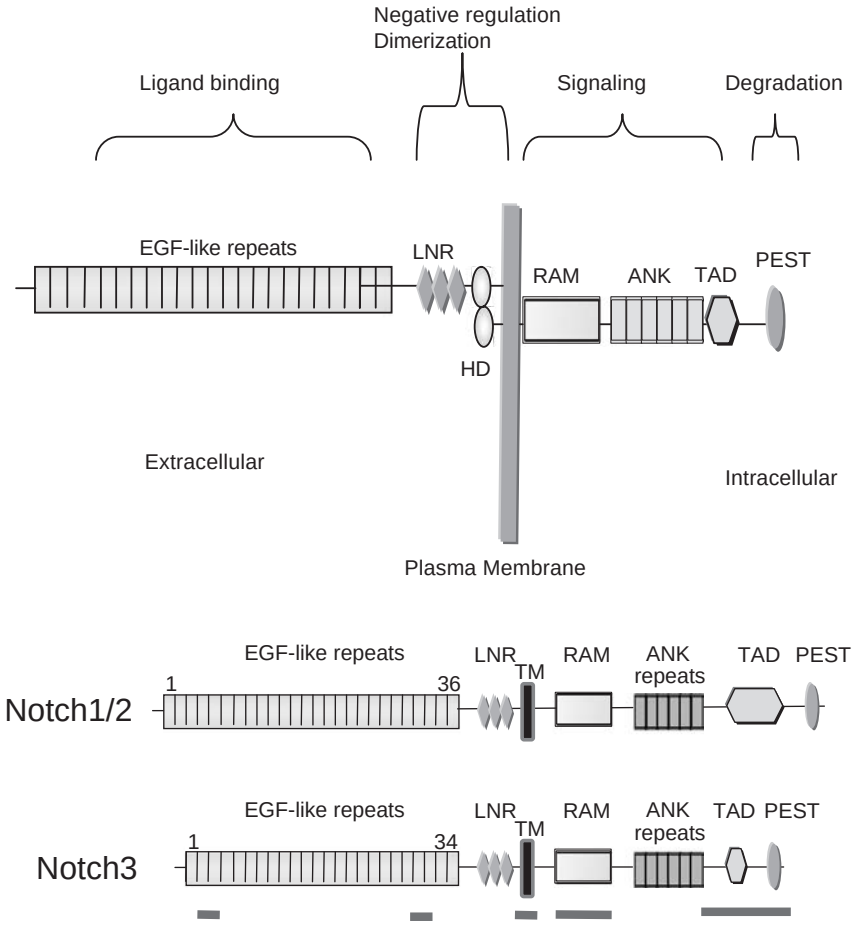

Figure 8–1: Diagram of Notch receptor. All Notch receptors consist of two subunits on the cell surface. RAM, CSL-interacting domain; ANK, ankyrin repeats; TAD, trans-activating domain; Pro-Glu-Ser-Thr (PEST), degradation motif. *See color plates*.

N-terminal DSL motif essential for binding to Notch receptor. Binding of DSL ligand to the N-terminal EGF-repeat region of the Notch extracellular subunit initiates a conformational change in the Notch receptor and triggers two sequential cleavages within the transmembrane subunit that are catalyzed by tumor necrosis factor-α converting enzyme (TACE) (S2 cleavage) at the extracellular surface, and by γ-secretase (S3 cleavage) at the intramembrane region. The latter cleavage releases Notch intracellular cytoplasmic domain (NICD) from the plasma membrane. NICD then shuttles into the nucleus, where it interacts with DNA-binding factors CSL (CBF1/Suppressor of Hairless /Lag1). In the absence of NICD, CSL acts as a repressor through interaction with corepressors and histone deacetylase.[13] Interaction of NICD and CSL disrupts the repressor complex and recruits coactivators, including mastermind-like 1 (MAML1) and histone acetyltransferase, which act in concert to activate Notch target gene expression (Figure 8–2).

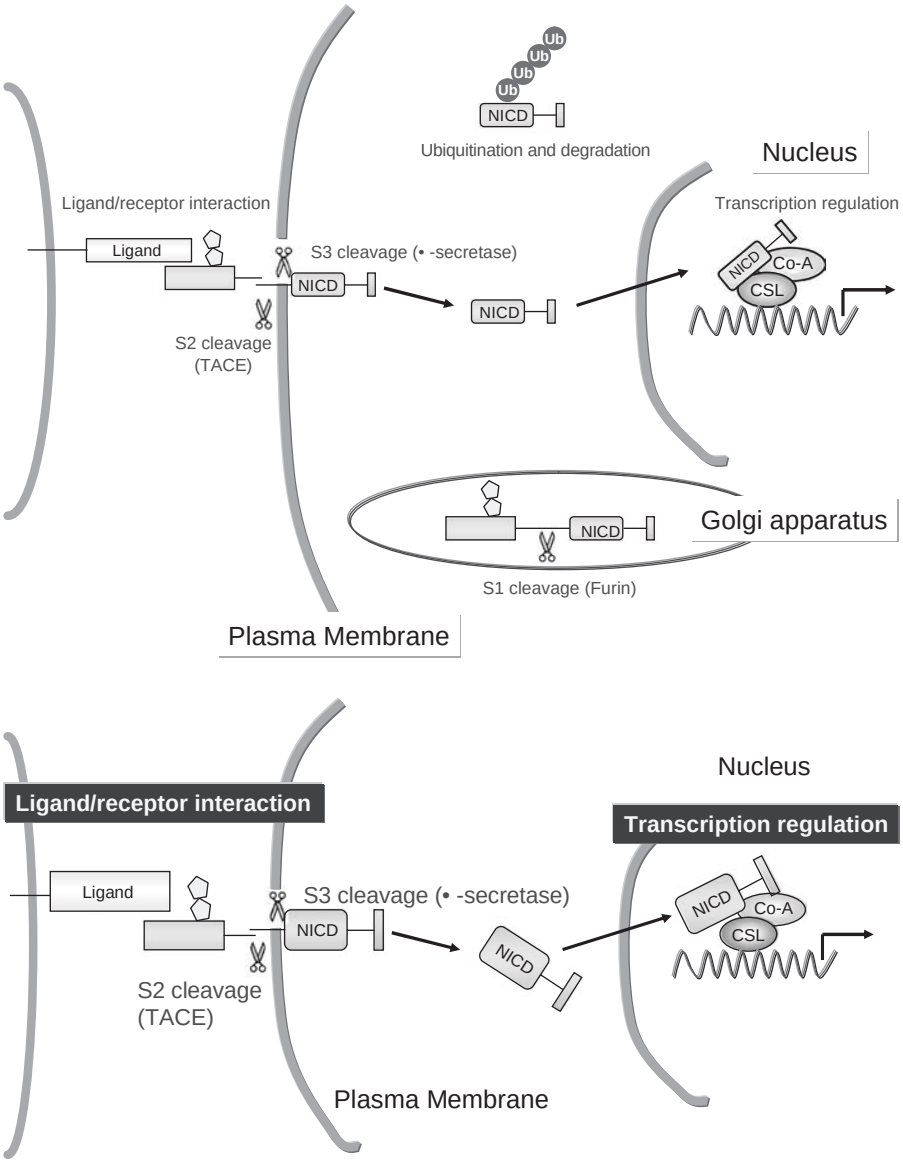

Figure 8–2: Notch pathway elements. The Notch ligands have a large extracellular region composed of a DSL domain and a series of EGF-like repeats. The Notch receptor is expressed on the plasma membrane as a heterodimer, which is generated during its translocation from the cytoplasm to the membrane by cleavage of the precursor protein by the convertase furin, and is glycosylated by Fringe. Upon ligand binding, Notch undergoes two consecutive cleavage steps: one by the protease TACE and one by a γ-secretase that results in the release of NICD and its nuclear translocation. NICD interacts with the transcription factor CSL, dissociating CSL from corepressor molecules (CoR) and recruiting coactivators (CoA) such as mastermind and histone acetyl transferase, giving rise to a transcription activation complex. Potential steps for therapeutic targeting of Notch signaling include receptor/ligand interaction, S1–S3 cleavages, transcription regulation, and protein degradation. *See color plates.*

Recent studies have demonstrated that the NRR maintains the "off" state of Notch receptors prior to ligand-induced activation. In human T-ALL, somatic mutations were frequently found in HD1 and HD2 domains of the NRR region. These mutations caused ligand-independent S2 cleavage and subsequent S3 cleavage, ultimately releasing NICD. Somatic mutations in the PEST domain that caused a premature stop, and thus resulted in deletion of the PEST domain, were also identified in T-ALL. Loss of the PEST domain results in increased NICD levels due to decreased proteasomal degradation.

NOTCH SIGNALING AS A CANCER THERAPEUTIC TARGET

Notch signaling plays an important role in cell fate decision and cell renewal. Deregulated expression of Notch receptors, ligands, and target genes has been found in a wide variety of hematological malignancies and solid tumors, including breast, ovarian, cervical, lung, head and neck, pancreas, and medulloblastoma. Therefore Notch inhibition may represent a promising treatment for cancer. In addition to the oncogenic potential, the Dll/Notch pathway is clearly required for cardiovascular development and is involved in angiogenesis. For example, genetic deletions of several Notch ligands, receptors, and downstream target genes cause vascular defects. Of note, Notch ligands, especially Dll4, were found to be strongly expressed in tumor endothelial cells, and inhibition of Dll4 has been shown to be a potential antitumor therapeutic strategy.

The main pharmacological strategies for targeting components of the Notch signaling pathway include blockage of receptor/ligand interactions and inhibition of the release of NICD. For example, (1) small compounds employed as inhibitors have been shown to prevent S1, S2, or S3 enzymatic cleavages, which are essential steps for activation of Notch signaling; (2) recombinant ligand proteins have been developed that act as competitive inhibitors for receptor binding; and (3) monoclonal antibodies have been developed to block activation of Notch receptor signaling. Potential strategies for future Notch-based targeted therapy include suppressing the protein levels of Notch components as well as inhibiting CSL/Notch transcription (Figure 8–2). In the following, we will briefly discuss the therapeutic perspectives of each strategy.

Blocking proteolytic cleavage events

Because the activation of Notch signaling involves three proteolytic cleavages of Notch receptor, blocking the enzyme responsible for each cleavage event may suppress the generation of NICD and thus block Notch signaling.

Blockade of S1 cleavage

Furin belongs to the family of pro-protein convertases that process precursor proteins into biologically active products.[14] Furin cleaves full-length Notch in the trans-Golgi network, which generates the mature heterodimeric cell surface receptor.[6,7] An inhibitor of furin proteases, AT-EK1, was found to inhibit the

generation of mature heterodimer.[15] AT-EK1 treatment was also found to decrease the level of CSL-luciferase reporter activity in a dose-dependent manner, indicating that the formation of a mature heterodimer is required for Notch signaling.[15]

Blockade of S2 cleavage

TACE is a protease in ADAM (A disintegrin and metalloprotease) protease family that is involved in the S2 cleavage of Notch receptor. It is thought that ligand binding to Notch receptor induces TACE cleavages, which remove the extracellular domain of the transmembrane subunit of Notch receptor. TACE inhibitors have been developed previously. For example, BB3103 was found to inhibit ligand-induced activation of a CSL-responsive-reporter construct.[16]

Blockade of S3 cleavage

The most well-known small compound inhibitors of the Notch signaling pathway belong to the γ-secretase inhibitors (GSI), which block the S3 cleavage and inhibit the release of the intracellular domain of Notch.[17] Gamma-secretase is a large, integral membrane protease complex that includes a catalytic subunit and accessory subunits. The gamma-secretase complex catalyzes the intramembranous proteolysis of several membrane proteins, among which the Notch receptors and the beta-amyloid precursor peptide are of paramount therapeutic relevance. Promising results have been shown in in vitro and in vivo models. For example, in APC mutant mice, GSI treatment reduced expression of the Notch target gene Hes1 and converted proliferative adenoma cells into mucus-secreting goblet cells.[18] In a Kaposi's sarcoma (KS) mouse model, intratumoral injection of GSI inhibited tumor growth via decreasing proliferation and increasing apoptosis.[19] Accumulating preclinical evidence has led to the opening of phase I/phase II dose escalation clinical trials of a GSI in relapsed or refractory T-ALL and breast cancer patients. GSI is relatively easy and inexpensive to produce. However, GSI treatment also causes widespread side effects, including severe diarrhea.[20] The major drawback of GSI is its nonspecificity, as it targets many membrane proteins, including Notch receptors and ligands, Erb4, syndecan, and CD44. Although decreasing the GSI dose can ameliorate diarrhea, GSI treatment is always associated with chronic side effects. Thus short-term treatment is preferable, and benefits versus side effects should be evaluated carefully for each patient in future clinical trials.

Blocking receptor/ligand interaction

Recombinant Notch extracellular domain protein

The Notch extracellular domain is composed of 29–36 EGF-like repeats, and a recombinant protein containing as few as two of these modules is able to interact with Notch ligands and acts as a competitive inhibitor for binding to the full-length Notch.[21] Treatment of adipocytes with recombinant protein containing EGF-like repeats 11 and 12 has been shown to block their differentiation,[21] as expected based on the known role of Notch signaling in cell fate determination of mammalian cells.

Neutralizing DLL ligands

A soluble form of the extracellular domain of Dll4 (D4ECD) was found to efficiently block the Dll4/Notch pathway. Interestingly, D4ECD increased vascular density, but the blood vessels were poorly functional, thereby accounting for the delay in tumor growth.[22] When D4ECD was overexpressed in tumor cells, it served as a soluble inhibitor of Dll4/Notch signaling.[22] When these transgenic-tumor cells were implanted into mice, the secreted soluble D4ECD reduced Notch signaling in the host and affected vascular morphologies as well as tumor growth rates. Increased vascular density with dramatic vessel sprouting and branching, which led to induced hypoxia and necrosis in the tumor, was observed in the D4ECD-expressing group.[22,23]

Neutralizing anti-Dll4 antibody

Neutralizing antibodies that bind to the Notch ligand or Notch receptor have been shown to be effective in inhibiting Notch signaling. A Dll4-neutralizing antibody was found to block the Dll4/Notch pathway.[24] Pro-angiogenesis and antitumor phenotypes were observed in tumors treated with the neutralizing anti-Dll4 antibody, similar to the Dll4-neutralizing ligand D4ECD mentioned in the previous section. Vascular targeting therapy using anti–Vascular endothelial growth factor (VEGF) can become ineffective, possibly as a consequence of the ability of tumors to adapt and become resistant to the treatment. On the other hand, Dll4-neutralizing antibody treatment can block the growth of tumors that are resistant to anti-VEGF.[24] Therefore Dll4-neutralizing antibody treatment provides an alternative option for delaying tumor growth when used in combination with anti-VEGF treatment.

Anti-Notch antibody

Monoclonal antibodies that inhibit proteolytic cleavages in Notch3 receptor have recently been developed.[25] Epitope mapping reveals that those inhibitory antibodies bind to two domains in the NRR, including LNR1 and HD2, suggesting that they clamp LNR1 and HD2 together to suppress the S2 cleavage. This finding supports the view that the NRR is important for autoinhibitory regulation.[26] Interestingly, stimulatory antibodies were also identified, and they were found to bind to linear epitope in LNR1 or HD2 of the NRR.[25] Therefore it would be interesting to analyze the conformational alteration that reveals the S2 cleavage site upon ligand stimulation and to develop strategies to either block or stimulate this process. It can be envisioned that in the future, antibodies that can modulate Notch activity are likely to have significant experimental and therapeutic potential.

Suppressing Notch protein expression

Ubiquitination controls Notch protein expression

Several distinct classes of E3 ubiquitin ligases appear to directly regulate the protein levels of Notch receptor. In mammals, the E3 ubiquitin ligase Itch has been

shown to ubiquitinate membrane-tethered Notch1.[27] Furthermore, Sel10, an F-box/WD40 repeat-containing protein that interacts with an SCF ubiquitin ligase complex, also functions as a negative regulator in the Notch signaling pathway[28] by minimizing the half-life of NICD through ubiquitination.[29,30] Genetic study of *Drosophila* has demonstrated that Numb is a negative regulator of the Notch signaling pathway.[31] The study suggested that Numb recruits components of the ubiquitination machinery to the Notch receptor, thereby facilitating Notch1 ubiquitination at the membrane, which in turn promotes degradation of the Notch intracellular domain. Immunohistochemical analysis showed that Numb expression was lost in about 50% of human breast cancers[32] and that Numb silencing increased Notch signaling in Numb-positive breast tumors.[32] Understanding the negative regulation of Notch signaling seems likely to facilitate the development of future Notch-based novel therapies.

RNA interference against components of Notch signaling

Because of promising results obtained from in vitro studies, RNA interference (RNAi) against components of the Notch signaling pathway, including both receptor and ligand, is thought to hold promise as a future novel therapy.[33–35] The advantage of the RNAi strategy is that the molecules can be designed easily and the cost is relatively low. However, clinical use of RNAi therapy is likely a long way off because there are several challenges to overcome. These include how to achieve efficient delivery of RNAi into the appropriate tissue and how to increase the stability of RNAi. In addition, there is the possibility that excess RNAi will cause untoward immune response in the host.[36,37] Therefore there is a lot to be learned on the road to developing RNAi therapy.

Inhibiting the action of NICD

NICD is responsible for triggering transcription of Notch downstream genes by forming the NICD–CSL complex and recruiting coactivators such as MAML1. Therefore strategies to interrupt the formation of the NICD–CSL complex or to block the recruitment of coactivators can potentially suppress the expression of downstream target genes.

Dominant-negative MAML1

The MAML1 binding site on NICD is essential for the transcriptional activation of Notch signaling.[38,39] Truncated forms of MAML1 that retain affinity for NICD, but lack the activator domain, competitively inhibit the recruitment of wild-type MAML1 to the NICD–CSL complex. Blocking Notch signaling by this dominant negative MAML1 suppressed primary melanoma cell growth both in vitro and in vivo.[40] Dominant-negative MAML1 peptide (62-amino-acid peptide) formed a transcriptionally inactive nuclear complex with NICD and CSL and inhibited the growth of both murine and human Notch1-transformed T-ALLs.[41] Therefore blocking the formation of a functional NICD coactivator complex can be a potential strategy for chemotherapeutic intervention.

CONCLUSION

In the past few years, evidence has accumulated that supports the cancer stem cell theory, which suggests that targeting cancer stem cells may provide a new strategy to treat human cancer. However, developing strategies to selectively inactivate or eradicate cancer stem cells appears to be challenging because Notch signaling pathways are shared by both cancer stem cells and normal stem cells. A variety of recent studies using antagonists that target these pathways suggests that it may be feasible to selectively target the cancer stem cell population. For example, Notch signaling is required for the differentiation and proliferation of cancer stem cells but is not required for the maintenance of blood-forming stem cells.[42] Exciting new findings concerning the involvement of Notch signaling in cancer stem cells have placed this signal transduction pathway in the focus of therapeutic target development in cancer treatment. However, our understanding of Notch signaling in specific cell types and diseases still remains unclear. Very little is known about the functional relationship between each Notch ligand and its Notch receptor in specific tissue microenvironments, or about the different downstream targets of each Notch receptor. Because many conundrums regarding the role of Notch signaling in both normal and cancer stem cells remain, Notch signaling and the development of strategies for the clinical targeting of the Notch pathway deserve further study.

ACKNOWLEDGMENTS

This work was supported by a New Investigator Research Grant from DoD Ovarian Cancer Research Program (T.-L.W.), a Research Scholar Grant from the American Cancer Society (T.-L.W.), and a career development award from NIH cervical cancer SPORE (T.-L.W.).

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