

Review

Exploitation of the Notch Signaling Pathway as a Novel Target for Cancer Therapy

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Abstract. *The Notch signaling pathway appears to be responsible for maintaining a balance between cell proliferation and apoptosis and thus it has been suggested that Notch may play an important role in species development and in the development and progression of several malignancies. Therefore, the Notch signaling pathway may represent a novel therapeutic target, which could have the highest therapeutic impact in modern medicine. This review describes the mechanisms of signal transduction of the Notch signaling pathway and provides emerging evidence in support of its role in the development of human malignancies. Further attempts have been made to summarize the role of several chemopreventive agents that could be useful for targeted inactivation of Notch signaling, which could become a novel approach for cancer prevention and treatment.*

Notch signaling is involved in cell proliferation and apoptosis which affects the development and function of many organs. Notch genes encode proteins which can be activated by interacting with a family of its ligands. To date, four vertebrate Notch genes have been identified: *Notch-1-4*. In addition, five ligands, Dll-1 (Delta-like 1), Dll-3 (Delta-like 3), Dll-4 (Delta-like 4), Jagged-1 and Jagged-2, have been found in mammals (1, 2). Although these four Notch receptors show subtle differences in their extracellular and cytoplasmic domains, they are very similar. The extracellular domain of Notch possesses many epidermal growth factor (EGF)-like repeats, which participate in ligand binding. The amino-terminal EGF-like repeats are followed by cysteine-rich Notch Lin12 repeats

(N/Lin12) that prevent signaling in the absence of the ligand. The cytoplasmic region of Notch conveys the signal to the nucleus; it contains a recombination signal-binding protein 1 for J-kappa (RBP-J)-association molecule (RAM) domain, ankyrin repeats, nuclear localization signals (NLS), a trans-activation domain (TAD) and a region rich in proline, glutamine, serine and threonine residues (PEST) sequence (3).

Notch signaling is initiated by a receptor-ligand interaction between two neighboring cells. Upon activation, Notch is cleaved, releasing the intracellular domain of the Notch (ICN) through a cascade of proteolytic cleavages by the metalloprotease tumor necrosis factor- α -converting enzyme (TACE) and γ -secretase. The first cleavage is mediated by TACE, which cleaves the receptor in the extracellular domain. The released extracellular domain is then trans-endocytosed by the ligand-expressing cell. The second cleavage caused by the γ -secretase activity of a multi-protein complex consisting of presenilin, nicastrin, *etc.* releases the ICN which is then ready to be translocated into the nucleus for transcriptional activation of Notch target genes (1, 2, 4). Therefore, inhibiting γ -secretase function prevents the cleavage of the Notch receptor, blocking Notch signal transduction. In the absence of ICN cleavage, transcription of Notch target genes is inhibited by a repressor complex mediated by the CSL (CBF1, suppressor of hairless, Lag-1). When ICN enters the nucleus, it recruits transcription activators to the CSL complex and converts it from a transcriptional repressor into an activator, which activates the Notch target genes (1, 2). A few Notch target genes have been identified, some of which are dependent on Notch signaling in multiple tissues, while others are tissue specific. Notch target genes include the *Hes-1* (hairly enhance of split-1), *nuclear factor-kappa B* (NF- κ B), *cyclin D1* and *c-myc* (1, 2, 5).

Notch Signaling in Cancer

Notch signaling plays important roles in maintaining the balance between cell proliferation, differentiation and apoptosis (1). The *Notch* gene is abnormally activated in

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many human malignancies. It has been reported that the function of Notch signaling in tumorigenesis can be either oncogenic or antiproliferative, and the function is context dependent (1, 6). In a limited number of tumor types, including human hepatocellular carcinoma and small cell lung cancer, Notch signaling is antiproliferative rather than oncogenic (7-9). However, most of the studies have shown an opposite function of Notch in many human carcinomas, including pancreatic cancer (1, 2, 8-11). It has been reported that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer (8-15).

Moreover, high-level expression of Notch-1 and its ligand Jagged-1 is associated with poor prognosis in breast and prostate cancer. Specifically, patients with tumors expressing high levels of Jagged-1 or Notch-1 had a significantly poorer overall survival compared with patients expressing low levels of these genes. Moreover, a synergistic effect of high-level Jagged-1 and high-level Notch-1 co-expression on overall survival was observed (16). Notch-1 is an important prognostic marker in T-cell acute lymphoblastic leukemia (T-ALL) and its predictive value could be even further increased if co-evaluated with other T-cell-related regulatory genes (17). Jagged-1 is highly expressed in metastatic prostate cancer as compared with localized prostate cancer or benign prostatic tissues. Furthermore, high Jagged-1 expression in a subset of clinically localized tumors was significantly associated with recurrence, suggesting that Jagged-1 may be a useful marker in distinguishing indolent vs. aggressive prostate carcinomas (18). Multiple oncogenic pathways, such as NF- κ B, Akt, Sonic hedgehog (Shh), mammalian target of rapamycin (mTOR), Ras, Wnt, epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) signaling have been reported to cross-talk with the Notch pathway and thus it is believed that the cross-talk between Notch and other signaling pathways plays an important role in tumor aggressiveness.

Notch and NF- κ B Signaling

The molecular mechanism(s) by which Notch signaling induces tumor growth has not been fully elucidated. Notch-1 has been reported to cross-talk with another major cell growth and apoptotic regulatory pathway, namely NF- κ B. NF- κ B plays important roles in the control of cell growth, differentiation, apoptosis, and inflammation (19, 20). NF- κ B mediates survival signals that inhibit apoptosis and promote cancer cell growth. The activation of NF- κ B involves the phosphorylation of I κ B (I-kappa-B), an inhibitory binding partner of the NF- κ B complex, for ubiquitination and degradation through the proteasome degradation pathway.

This allows the translocation of NF- κ B into the nucleus where it activates the transcription of genes. A key regulatory step in the NF- κ B pathway is the activation of a high molecular weight IKK (I κ B kinase) complex in which catalysis is thought to be *via* kinases, including IKK α and IKK β , which directly phosphorylate I κ B proteins (20).

Notch-1 has been reported to strongly induce NF- κ B2 promoter activity in reporter assays (21) and to induce the expression of several NF- κ B subunits (22). Notch ligands activate NF- κ B in human keratinocytes and the down-regulation of Notch-1 results in lower NF- κ B activity. The levels of basal and stimulation-induced NF- κ B activity were found to be significantly lower in mice with reduced Notch levels (23, 24). Vilimas *et al.* examined a murine model of T-ALL induced by overexpression of ICN and found that the NF- κ B target genes were up-regulated in the ICN-transformed cells. In these cells, and in human cell lines derived from spontaneous T-ALL, ICN interacts with the IKK signalosome, increasing its I κ B α kinase activity (25). It has recently been discovered that in colorectal carcinoma cells, nuclear IKK α phosphorylates SMRT (silencing mediator of retinoid and thyroid hormone receptor) not only in association with NF- κ B but also in association with CSL (26). Constitutive levels of Notch activity are essential to maintain NF- κ B activity in various cell types. Indeed, we have found that down-regulation of Notch-1 reduced NF- κ B activity. In contrast, overexpression of wild-type Notch-1 cDNA enhanced NF- κ B activity (27). We also found that down-regulation of Notch-1 caused attenuation of NF- κ B consistent with the down-regulation of NF- κ B downstream genes such as *vascular endothelial growth factor (VEGF)* and *matrix metalloproteinase-9 (MMP-9)*, resulting in the inhibition of cancer cell invasion through matrigel (27).

However, several groups have reported that NF- κ B can also regulate Notch expression (28, 29). The observations reported in the literature so far offer a complex and incomplete picture of the interactions between these two key cell fate-determining pathways. As is becoming increasingly clear in the case of other pathways, these interactions can be cooperative or antagonistic and multiple levels of feedback are possible depending on the context. The physiological relevance of these interactions needs to be thoroughly investigated. However, it can be safely stated that those planning to manipulate the Notch-signaling pathway for experimental or therapeutic purposes would do very well to examine the possible effects on NF- κ B signaling pathway and *vice versa* (30).

Notch and Akt Signaling

Akt (also known as protein kinase B) is an evolutionarily conserved serine/threonine kinase. Three isoforms, Akt1, Akt2 and Akt3, are expressed in mammals. Akt is activated

by phosphatidylinositol 3-kinase (PI3K), which transmits signals from cytokines, growth factors and oncoproteins to multiple targets, including Akt. Activation of PI3K localizes Akt to the plasma membrane *via* the pleckstrin homology domain of Akt, where Akt is activated by phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ (31). Akt plays a critical role in mammalian cell survival signaling and has been shown to be activated in various malignancies (31-33). Activated Akt functions to promote cell survival by inhibiting apoptosis through inactivation of several pro-apoptotic factors including Bcl-xL/Bcl-2-associated death (BAD), forkhead transcription factors and caspase-9 (34-36). Several studies have also shown that Akt regulates the NF- κ B pathway *via* the phosphorylation and activation of molecules in the NF- κ B pathway (37, 38).

Recently, Notch has been shown to regulate the Akt pathway. Liu *et al.* have reported that Notch-1 activation enhanced melanoma cell survival and such effects of Notch signaling were mediated by activation of the Akt pathway and the mitogen-activated protein kinase (MAPK) pathway (39). Palomero *et al.* found that Notch-1 induced up-regulation of the PI3K-Akt pathway *via* Hes-1, which negatively controls the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in T-ALL. The loss of PTEN and constitutive activation of Akt in T-ALL induced increased glucose metabolism and bypassed the requirement of Notch-1 signaling to sustain cell growth (40). Moreover, Palomero *et al.* identified loss of PTEN as a critical event leading to resistance to Notch inhibition, which caused the transfer of the phenomenon of “oncogene addiction” from the Notch-1 signaling to the PI3K/Akt signaling pathway (41, 42). Emerging evidence suggests that expression of the Notch ligand Jagged in human keratinocytes and cervical cancer cell lines leads to Akt phosphorylation and induction of a frank PI3K-dependent epithelial-mesenchymal transition (EMT) phenotype characterized by enhanced motility, morphological changes, E-cadherin down-regulation and up-regulation of vimentin and fibronectin (43). These observations also suggest that there is an urgent need for simultaneous inhibition of both pathways as a means of improving therapeutic efficacy for the treatment of most human malignancies.

Notch and mTOR Signaling

The mammalian target of rapamycin (mTOR) pathway has been reported to cross-talk with the Notch pathway (44-46). mTOR regulates translation rates and cell proliferation in part by phosphorylating two major targets, the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the ribosomal protein S6 kinases (S6K1 and S6K2). Upon phosphorylation, 4E-BP1 is released from eIF4E, allowing eIF4E to assemble with other translation

initiation factors to initiate cap-dependent translation. The eIF4E is thought to enhance the translation of transcripts possessing either complex 5'-untranslated region secondary structure and/or *via* upstream open reading frames, which often encode proteins associated with a proliferative response. S6K1 directly phosphorylates the 40S ribosomal protein S6, and then promotes ribosome biogenesis (47). mTOR exists in two distinct complexes, mTORC1 and mTORC2, within the cells: mTORC1 consists of mTOR, G-protein β -subunit-like protein (G β L), raptor and proline-rich Akt substrate of 40 kiloDaltons (PRAS40); and mTORC2 contains mTOR, G β L, rictor and stress-activated protein kinase-interacting protein 1 (SIN1). The raptor-containing complex is sensitive to rapamycin and regulates cell growth and proliferation in part through phosphorylating S6K and 4E-BP1. The rictor-containing complex is not sensitive to rapamycin (48).

The mTOR protein kinase has emerged as a critical player for controlling many cellular processes, such as cell growth and cell division, by receiving stimulatory signals from Notch and PI3K (44-46). Notch receptor activation induces the expression of specific target genes *Hes-3* and *Shh* through rapid activation of cytoplasmic signals, including Akt, mTOR and signal transducer and activator of transcription 3 (STAT3), and thereby promotes the survival of neural stem cells (44). Inhibition of tumor protein p53 by ICN mainly occurs through mTOR using the PI3K/Akt pathway, as rapamycin treatment abrogated ICN inhibition of tumor protein p53 and reversed the chemoresistance in breast cancer and T-ALL (46). Further, ectopic expression of eIF4E inhibited p53-induced apoptosis and conferred protection against p53-mediated cytotoxicity to a similar extent as that of ICN overexpression, but it was not reversed by rapamycin, which indicated that eIF4E is the major target of mTOR in Notch-1-mediated survival signaling (46). Recently, Chan *et al.* reported that the mTOR pathway is positively regulated by Notch in T-ALL cells. They found that the effect of gamma secretase inhibitor (GSI) on the mTOR pathway was independent of changes in PI3K and Akt activity, but was rescued by expression of c-Myc, a direct transcriptional target of Notch, implicating c-Myc as an intermediary between Notch and mTOR (45). Moreover, T-ALL cell growth was suppressed in a highly synergistic manner by simultaneous treatment with the mTOR inhibitor rapamycin and GSI, which represents a rational drug combination for treating this aggressive human malignancy (45).

Notch and EGFR Signaling

EGFR is a transmembrane tyrosine kinase protein. After ligand binding, EGFR dimerizes, either as a homodimer or heterodimer with other members of the EGFR family. EGFR is then auto-phosphorylated or trans-phosphorylated at

specific tyrosine residues for its activation, resulting in the activation of multiple downstream signaling cascades, including PI3K/Akt, and extracellular signal-regulated kinase (ERK), ultimately leading to increased cellular proliferation and the prevention of programmed cell death. Therefore, excessive activation of EGFR-dependent pathways may have an important role in the biological aggressiveness of human cancer.

It has been reported that Notch-1 inhibition reduced *EGFR* mRNA and EGFR protein in glioma and other cell lines, whereas transfection with Notch-1 increased EGFR expression. Additionally, a significant correlation in levels of *EGFR* and *Notch-1* mRNA in primary high-grade human gliomas has been found. Subsequent experiments have shown that p53, an activator of the *EGFR* promoter, is regulated by Notch-1. These results showed that Notch-1 up-regulates EGFR expression and also demonstrated Notch-1 mediated up-regulation of p53 in gliomas (49). Recently, Zhang *et al.* demonstrated that γ -secretase regulates EGFR through releasing ICN generation, which directly binds to the *EGFR* promoter and regulates *EGFR* gene expression (50). The findings from our laboratory have shown that the EGFR inhibitor caused marked inhibition of pancreatic cancer cell growth, which was accompanied by increased apoptosis and concomitant attenuation of the Notch-1 signaling pathway (51). These results provide some clear evidence in support of an interactive role of Notch with EGFR signaling in human cancer.

Notch and PDGF Signaling

Many tumors have been shown to overexpress the PDGF family members (52-54). The PDGFs are composed of four different polypeptide chains encoded by different genes. Four PDGF family members have been identified to date, PDGF A-D. The four PDGF chains assemble into disulphide-bonded dimers *via* homo- or heterodimerization, and five different dimeric isoforms have been described to date, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. It is notable that no heterodimers involving PDGF-C and PDGF-D chains have been described (54, 55). The PDGFs have a common structure with the typical growth factor domain involved in the dimerization of the two subunits and in receptor binding and activation. PDGF-A and PDGF-B have short *N*-terminal extensions that undergo intracellular proteolytic processing for activation, while both PDGF-C and PDGF-D chains display a distinct protein domain, the so called complement C1r/C1s, Uegf, Bmp1 (CUB) domain, as part of their *N*-terminal extensions. Several reports have indicated that the CUB domain of PDGF-D has to be cleaved extracellularly to make the COOH-terminal growth factor domain active for PDGF-D binding to its cognate receptor (54, 55). PDGFs exert their

cellular effects by activating two structurally related receptor tyrosine kinases, PDGFR- α and PDGFR- β . The PDGF-A, PDGF-B and PDGF-C are secreted as homodimers or heterodimers and bind to dimeric PDGF receptors (PDGFR) composed of α - and/or β -chains, while PDGF-D specifically binds to and activates its cognate receptor PDGFR- β (56). It has been reported that PDGF signaling regulates the expression of the Notch-1 receptor in some cell lines (57). The expression of PDGF-B correlates with Notch ligand Dll-4 expression in developing retinal arteries (58). We also found that down-regulation of PDGF-D leads to the inactivation of Notch-1 and NF- κ B DNA-binding activity and, in turn, down-regulates the expression of its target genes, such as *VEGF* and *MMP-9*. Therefore, the inactivation of PDGF-D-mediated cell invasion and angiogenesis that we have reported could in part be due to inactivation of Notch-1 activity (53). These results further suggest that the combination of the inhibitors of PDGF and Notch signaling could be therapeutically useful and thus further studies are warranted in this area.

Notch and Sonic Hedgehog Signaling

The hedgehog family of growth factors activates a highly conserved signaling system for cell-cell communication that regulates cell proliferation and differentiation during development. Abnormal activation of the hedgehog pathway has been demonstrated in a variety of human tumors, including those of the skin, brain, lung and digestive tract. The hedgehog family of signaling proteins consists of secreted proteins that signal through both autocrine and paracrine mechanisms to control cell proliferation, differentiation and morphology. There are three known hedgehog ligands, Sonic (Shh), Indian (Ihh) and Desert (Dhh). The Shh is more closely related to Ihh, while Dhh is more closely related to the hedgehog of *Drosophila*. The hedgehog proteins exert their function by binding to a 12-pass transmembrane protein called patched (PTCH) (59, 60). This interaction relieves the inhibitory effect of PTCH on a serpentine protein called smoothened (SMO). The SMO is then hyperphosphorylated and has been recently shown to localize to primary cilia. This pathway ultimately concludes with the activation and repression of target genes through the Gli family of transcription factors. In mammals, there are three Gli transcription factors (Gli-1, -2, -3) that regulate the transcription of target genes (60).

Cross-talk also exists between the Notch pathway and both Wnt and Shh signaling. Notch-1 normally represses Wnt and Shh signaling, both of which are known to regulate tumorigenesis. In Notch-1 null skin, activation of Wnt and Shh pathways resulted in the development of basal cell carcinoma and squamous cell carcinoma in the mouse (61). Katoh reported that hedgehog signals result in the up-regulation of the *Jagged-2* gene, which activates Notch

signaling (62). Moreover, Hes-1, a principal downstream target of the Notch pathway, was found to be a target of Shh in mesodermal and neural cells (59). Medulloblastoma arising in heterozygous *PTCH* knockout mice displays an elevated expression of a number of Notch pathway genes, as do similar tumors arising in mice expressing an oncogenic form of *Smo* (63-65).

The Wnt signal transduction pathway also plays an important role during embryonic development, regulating cell proliferation and survival of immature cells. However, its improper function can lead to harmful consequences for humans, such as aberrant cell proliferation and therefore, cancer. The human *Wnt* gene family consists of 19 members, which regulate Wnt glycoproteins. The classical view of this pathway is that, upon binding to their receptors, Wnt proteins induce intracellular inactivation of glycogen synthase kinase-3 β (GSK-3 β), a component of the destruction complex, which also contains adenomatous polyposis coli (APC) and axin (66). This process results in the dephosphorylation and stabilization of β -catenin, a substrate of GSK-3 β , which leads to the nuclear translocation of β -catenin. In the nucleus, β -catenin acts as a transcriptional co-activator and activates genes involved in cell proliferation and survival (66).

Several recent studies have suggested clear links between Wnt and Notch signaling. Squamous cell carcinoma develops spontaneously in the epidermis of mice expressing a dominant negative form of Mastermind1, which functions as an adaptor for RBP-J dependent Notch signaling. Dominant negative Mastermind1 inhibits Notch signaling, resulting in the activation of the Wnt pathway in keratinocytes (67). Enhanced Notch signaling activity has been observed in multiple intestinal neoplasia mice, which display hyperactivation of the Wnt signal because of a mutation of the *APC* gene, suggesting that the Wnt signal is mechanistically epistatic to the Notch signal (68). Recently, an intriguing report presented evidence that Notch and Wnt pathways act in synergy to maintain the hematopoietic stem cell (HSC) pool (69). These findings suggest that Wnt and Notch signaling together could play a role in self-renewal of HSCs. Moreover, observations that Wnt3a regulates the expression of established Notch target genes and that inhibition of the Wnt signaling component of GSK-3 affects HSC fate options through mechanisms involving regulation of both Wnt and Notch target genes suggest that the two pathways belong to a network of regulatory circuits controlling the HSC pool (69-71).

Notch and Ras

Ras is a small GTPase that cycles between an inactive, GDP-bound state and an active, GTP-bound state. In mammals, Ras appears to be a central player in multiple signaling

pathways. Ras can be activated by a wide variety of upstream signals, and Ras-GTP can bind and activate multiple downstream targets (72). Ras acts primarily within a receptor tyrosine kinase (RTK)–Ras–MAPK/ERK pathway. Among many RTKs, EGFR is one major RTK that signals through Ras and ERK (72).

Stockhausen *et al.* have shown that transforming growth factor (TGF α), a known activator of RTKs and Ras signaling, can drive cell proliferation and at the same time Ras activation could induce the expression of the Notch target Hes-1 in a neuroblastoma cell line. These studies have shown that Hes-1 expression was induced simultaneously with increased ERK1/2 phosphorylation in TGF α -stimulated neuroblastoma cells, suggesting Hes-1, a key mediator of Notch signaling, can be regulated by the Ras/MAPK signaling pathway (73). In addition to Notch being a mediator of Ras signaling, there is also some evidence for Ras as an effector of Notch. Fitzgerald *et al.* have shown that transformation by Notch-4 required active Ras signaling, in particular the activity of ERK and PI3K (74). For example, in human cultured cells transformed by a combination of active Ras, SV40 and human telomerase reverse transcriptase (hTERT), Ras acts through p38 MAPK to up-regulate the expression of Dll-1 and Notch-1. Interfering with Notch signaling in this system inhibited anchorage-independent growth, suggesting that sequential signaling through Notch is critical for Ras-induced transformation (75). Similarly, Kiaris *et al.* showed the importance of Ras and Notch in cyclin D1-dependent mammary oncogenesis by transgenic expression of the Notch antagonist Deltex (76). In this mouse mammary tumor model, H-Ras and Notch up-regulated expression of cyclin D1, suggesting that the mode of cooperation might be due to convergent up-regulation of a common target (72). Collectively, emerging evidence suggests that oncogenes such as *Ras*, *cyclin D1*, growth factors and growth factor receptors lead to the activation of Notch signaling whose cross-talk with other signaling pathways results in tumor development and progression.

Notch and Cancer Stem Cells

Recently, several reports have described molecular connections between Notch regulated transcription factors and pathways in controlling stem cell function, which further suggest that a new mechanism exists in support of the claim that Notch may drive tumor growth through the generation or expansion of tumor-initiating cells or cancer stem-like cells (CSCs) (4). Stem cells are defined by their capacity for self-renewal and differentiate into the full spectrum of cells characterizing a particular organism or tissue. Stem cells are of three major types embryonic, germinal and somatic (77). The inner cell mass of the

blastocyst generates embryonic stem cells. The embryonic stem cells are omnipotent, capable of generating any cell in the mature organism and have unlimited capacity to replicate. Germinal stem cells come from the germinal layer of the embryo. These germinal stem cells differentiate to generate specific organs. Somatic stem cells have the capacity to self-renew and differentiate into all cells characteristic of a specific organ or tissue (77). Stem cells often stay at locations that are called stem cell niches. Specifically, stem cell niches are defined as particular locations or microenvironments that allow the combined properties of stem cell self-renewal and multi-potency to be maintained (78). A combination of genetic and molecular analyses has identified many factors that support stem cell niches that also control stem cell identity. These factors include components of the Notch, Wnt and Shh signaling pathways (79).

Emerging evidence suggests that the capability of a tumor to grow and propagate is dependent on a small subset of cells within the tumor, termed CSCs. CSCs have been identified and isolated from tumors of the hematopoietic system, breast, lung, prostate, colon, brain, head and neck and pancreas (80-85). CSCs are able to self-renew, differentiate and regenerate phenotypic cells of the original tumor when implanted into severe combined immunodeficient mice (84, 85). These cells are identified by specific stem cell markers, antigens, molecules and signaling pathways (86).

The pathways that regulate self-renewal and cell fate in these systems are beginning to be elucidated. Transcription factors and molecules associated with oncogenesis, such as Notch, NF- κ B, B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), Wnt, Shh and their biochemical pathways, are active only in a small minority of cancer cells where they may play key roles in determining the biological behavior of a tumor (86). Katoh reported that the balance between Wnt-fibroblast growth factor (FGF)-Notch and bone morphogenetic protein (BMP)-hedgehog signaling networks is important for the maintenance of homeostasis among stem and progenitor cells. Disruption of the stem cell signaling network results in pathological conditions, such as congenital diseases and cancer (87). In addition to pathways such as Wnt, Notch and hedgehog, known to regulate self-renewal of normal stem cells, tumor suppressor genes such as *PTEN* and *p53* have also been implicated in the regulation of cancer stem cell self-renewal (87).

Phillips *et al.* reported that cancer stem cells can be identified by phenotypic markers and their fate is controlled by the Notch pathway in breast cancer (88). Recombinant human erythropoietin receptor increased the numbers of stem cells and the self-renewing capacity in a Notch-dependent fashion by the induction of the Notch ligand, Jagged-1. Inhibitors of the Notch pathway blocked this effect,

suggesting the mechanistic role of Notch signaling in the maintenance of the CSC phenotype (88). Farnie *et al.* also provided evidence for breast cancer stem cells and their studies have consistently shown that stem-like cells and breast cancer-initiating populations can be enriched using the cell surface markers CD44+/CD24- that showed up-regulated genes including *Notch* (89). Notch signaling also promotes the formation of CSCs in human glioma. The overexpression of Notch-1 in SHG-44 glioma cells promoted their growth and colony-forming activity. Interestingly, the overexpression of ICN increased the formation of neurosphere-like colonies in the presence of growth factors. These colonies expressed nestin and also expressed neuron-, astrocyte- or oligodendrocyte-specific markers, consistent with phenotypes of neural stem cells. These data suggest potential functions of the Notch pathway in the formation of CSCs in human glioma (90). Recently, Fan *et al.* found that Notch blockade reduced the CD133-positive cell fraction almost 5-fold and totally abolished the side population, suggesting that the loss of tumor-forming capacity could be due to the depletion of CSCs. Notch signaling levels were higher in the CSC fraction, providing a potential mechanism for their increased sensitivity to the inhibition of this pathway. They also observed that apoptotic rates following Notch blockade were almost 10-fold higher in primitive nestin-positive cells as compared with nestin-negative cells. CSCs in brain tumors thus seem to be selectively vulnerable to agents inhibiting the Notch pathway (91). Moreover, Jagged-2, a Notch ligand, was found to be overexpressed in the leukemic stem cell (LSC) samples. *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT), an inhibitor of γ -secretase, a protease that is involved in Jagged and Notch signaling, inhibited LSC growth in colony formation assays (92). Taken together, these results suggested that the Notch pathway plays an important role not only in normal stem cells, but also in cancer stem cells.

Notch as a Cancer Treatment Target

A growing body of literature strongly suggests that increased expression of *Notch* genes and their ligands are detected in many human cancer cells and tissues such as those of the pancreas, breast, lung, mesothelioma, head and neck, renal, cervical, ovarian, endometrial, osteosarcoma, glioma and medulloblastoma, and leukemia (1, 2, 5, 93-95). These results clearly suggest that inactivation of Notch signaling by novel approaches would have a significant impact in cancer therapy. Moreover, current cancer therapeutics based on tumor regression may target and kill differentiated tumor cells, which make up the bulk of the tumor, while sparing the rare cancer stem cell population that must be killed for successful therapy. The cancer stem

cell model suggests that the design of new cancer therapeutics may require the targeting and elimination of cancer stem cells. Therefore eradicating cancer stem cells is increasingly being recognized as an important goal in curing cancer and thus the Notch pathway is considered an attractive target for treatment. Reducing Notch activity in cancer stem cells may promote their differentiation, thus reducing their ability to repopulate the cells forming the tumor mass. Recently, Notch was reported to regulate interleukin-10 (IL-10) production by T-helper 1 (Th1) cells (96) and suppress immunity. Since Th1 cells and their products are known to mediate antitumor responses, Notch-induced IL-10 production by Th1 cells can self-regulate Th1 cytokine production patterns leading to the suppression of Th1 cell-induced delayed-type hypersensitivity. IL-10 production can be elicited by all four mammalian Notch receptors (96), which also suggests that the inactivation of Notch could also revert immune suppression so that the cells could be killed *via* T-cell-mediated killing.

Moreover, dendritic cells (DCs) acquire the capacity for Dll-4 expression upon stimulation with Toll-like receptor (TLR) ligands and simultaneously induce IL-10 production by Th1 cells *in vitro* and *in vivo*. On the other hand, TLR ligation up-regulates the expression of Notch ligands Dll-1 or Dll-4 *via* the myeloid differentiation primary response gene 88 (MyD88) pathway which strongly inhibits Th2 cell development (97). Therefore, it is clear that the Notch pathway may play a pivotal role in the development of specific immune responses in Th1 and Th2 cells as well as DC activation and differentiation. The interruption of Notch signaling by deletion of Notch+ tumor cells may not only eliminate clonogenic tumor cells, but more importantly, serve to interrupt the tolerance of immunosuppressive factors or circuitry induced by tumor cells by the interruption of the production of IL-10 or other suppressive substances to reverse immune suppression.

Notch signaling is activated *via* the activity of γ -secretase which makes it a target in cancer therapy. Several forms of γ -secretase inhibitors have been tested for antitumor effects. For example, IL-X, an original γ -secretase inhibitor, has been shown to have Notch-1-dependent antitumor activity in Ras-transformed fibroblasts (75). Recently, the dipeptide γ -secretase inhibitor DAPT was reported to suppress medulloblastoma growth and induce G₀-G₁ cell cycle arrest and apoptosis in a T-ALL animal model (65, 98). Treatment with tripeptide γ -secretase inhibitor resulted in a marked reduction in tumor growth in cell lines and xenografts from melanoma and Kaposi sarcoma in mice (99). Dibenzazepine, one of the γ -secretase inhibitors, has been reported to inhibit epithelial cell proliferation and induce goblet cell differentiation in intestinal adenomas (68). We also found that a γ -secretase inhibitor suppressed prostate cancer cell growth (100).

Inhibitors of γ -secretase are being tested in Phase I clinical trials, suggesting that Notch signaling is an important target in cancer therapy. However, one of the major challenges is to eliminate unwanted toxicity associated with γ -secretase inhibitors, especially cytotoxicity in the gastrointestinal tract (101). Shih *et al.* reported the possible mechanisms underlying the unwanted cytotoxicity of γ -secretase inhibitors (93). Firstly, the Notch signaling pathway is known to widely participate in cellular physiology in normal tissues, including hematopoiesis and the maintenance of arterial smooth muscle, therefore, it is plausible that inactivation of γ -secretase may lead to the dysfunction of vital organs. Secondly, γ -secretase inhibitors do not exclusively target the Notch signaling pathways because γ -secretase has many substrates in addition to Notch receptors, such as several Notch ligands, v-erb-a erythroblastic leukemia viral oncogene homolog 4 (ErbB4) and CD44. Thirdly, γ -secretase inhibitors may target proteases other than γ -secretase. Therefore, γ -secretase inhibitors may have widespread adverse effects *in vivo* because proteases participate in a wide array of cellular functions (93).

Studies from our laboratory have shown that chemopreventive agents such as genistein and curcumin (non-toxic agents from dietary sources) may inhibit Notch-1 activation in pancreatic cancer cells leading to apoptotic cell death (102, 103). A Chinese herb mixture (antitumor B) also inhibited Notch expression in a mouse lung tumor model (104). Recently, resveratrol has also been shown to induce apoptosis by inhibiting the Notch pathway mediated by p53 and PI3K/Akt in T-ALL (105). These findings suggest that Notch-1 down-regulation, especially by genistein or curcumin, could be a novel therapeutic approach for the treatment of human malignancies by targeting the inactivation of Notch signaling. However, further in-depth studies including mechanistic *in vitro* studies, *in vivo* animal experiments and clinical trials are needed to fully appreciate the consequence of the down-regulation of Notch-1 signaling by non-toxic dietary chemopreventive agents. We believe that this article could stimulate further research in this field for the development of non-toxic approaches for cancer therapy by targeting Notch signaling, which is likely to eliminate not only tumor cells, but also cancer stem cells, in addition to reverting immune suppression.

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