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Notch signaling and its role in breast cancer

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1. ABSTRACT

The Notch signaling plays a key role in cell differentiation, survival, and proliferation through diverse mechanisms. Thus, alterations of the Notch signaling can lead to a variety of disorders including human malignancies. In this review, we will focus on recent advancements in identification of aberrant Notch signaling in cancer, and the possible underlying mechanisms in breast cancer. We will also highlight the therapeutic potential of targeting Notch for cancer treatment.

2. INTRODUCTION

As an evolutionarily conserved pathway, Notch signaling regulates various physiological processes, including stem cell maintenance, differentiation, proliferation and apoptosis. In mammals, loss of Notch signaling leads to embryonic lethality as a result of severe defects in somitogenesis, angiogenesis, cardinogenesis and neurogenesis (1). Deregulation of Notch has been associated with numerous human cancers. For example, the constitutive Notch signaling caused by point mutation or chromosomal translocations in T-cell leukemia has been well established. Accumulating evidence strongly indicates that aberrant Notch signal has a tumor promoting function in breast. Since several excellent reviews (1-6) have been published regarding Notch signaling as well as its role in mammary development and tumorigenesis, in this review, we will focus on how Notch signaling and its interaction with other signaling pathways contribute to breast cancer development. We will also discuss a potential utility of the Notch pathway as a therapeutic target and a prognosis marker for breast cancer. Finally, we will also summarize

Table 1. Structure characteristic of mammalian Notch receptors and ligands

	Name used in this review	EGF-like repeats	Distinct domain	Common domain
Receptor	Notch 1,2	36	TA	LNR; RAM; CDC10 repeat; NLS; PEST
	Notch 3	34	-	
	Notch 4	29	-	
Ligand	Dll1,4	8	-	DSL
	Dll3	6	-	
	Dlk	5	-	
	Jag-1,-2	16	VWC	

the recent findings of the role of Notch signaling in cancer stem cells.

3. NOTCH SIGNALING PATHWAY

3.1. Notch receptors and ligands

In mammals, the key components of Notch pathway comprise four transmembrane receptors (Notch 1-4) and six ligands (Dll1, Dll3, Dll4, Dlk and Jag-1,-2) (see Figure 1 and Table 1). Each Notch receptor is synthesized as a full-length precursor protein consisting of extracellular, transmembrane and intracellular domains. Within the Golgi apparatus, the precursor protein is cleaved into two associated peptides and subsequently presented on the cell surface as a heterodimer (7, 8). One subunit of the heterodimer contains most of the extracellular domain, which comprises a set of epidermal growth factor (EGF)-like repeats responsible for ligand binding, followed by three cysteine-rich Lin12/Notch repeats (LNR); the other contains the rest of the extracellular domain, the transmembrane domain and the intracellular domain, which harbors a RAM domain (which binds Lag-1), six Ankyrin (also known as CDC10) repeats flanked by nuclear localization signals (NLS), and a transactivation domain (TA domain) containing amino acid sequence PEST near the C-terminus. Through non-covalent linkage, the two subunits form a complete functional receptor. Similar to Notch receptors, Notch ligands also contain a set of EGF-like repeats related to a single cysteine-rich motif at their extracellular domain of DSL (Delta, Serrate, Lag-2). On the other hand, jagged proteins possess additional von Willebrand factor type C domain (VWC domain), which distinguishes them from Delta like ligands (9).

3.2. Notch signaling activation

Notch signaling is normally activated by ligand-receptor binding between two neighboring cells. This interaction leads to two proteolytic events as part of the activation mechanism. The first cleavage occurs near the extracellular side of the plasma membrane by the tumor necrosis factor-alpha-converting enzyme (TACE), a disintegrin-metalloprotease (10). The second cleavage occurs within the transmembrane domain mediated by a γ-secretase complex (11), resulting in the release of Notch intracellular domain (N-ICD). Then, the N-ICD translocates to the nucleus where it forms a complex with the ubiquitously expressed transcription factor CSL (CBF1, Suppressor of Hairless, Lag-1). In the absence of N-ICD, CSL functions as a transcriptional repressor due to its association with co-repressors (12-14). When N-ICD enters the nucleus, it disrupts the repressor complex, and converts CSL into a transcriptional activator by recruiting co-activators, such as Mastermind-like (MAML) and the histone acetyltransferase, to activate transcription of Notch target genes (15-17) (Figure 2).

3.3. Notch signaling targets

Transcriptional repressors of the Hes and Hey families, which play an important role in cell differentiation, are well-known direct target genes of CSL (18). These proteins belong to the basic helix-loop-helix family of transcription factors with a basic domain binding to specific sequences in the promoter region of target genes, and repress the transcription by recruiting a set of co-repressors or sequestering transcriptional activators. In addition to particular differentiation-related factors, the transcriptional targets of Notch signaling also include cell cycle regulators (e.g., p21 and cyclin D1) (19, 20), transcription factors (e.g., c-Myc, NF-κB2) (21, 22), growth factor receptors (e.g., ErbB2) (23, 24), and regulators of apoptosis (25). This may explain why Notch pathway is involved in a variety of cellular events and why deregulation of Notch pathway can lead to disruption of cell-cycle, apoptosis control and tumor formation.

4. NOTCH SIGNALING IN TUMORIGENESIS

Notch signaling was first linked to tumorigenesis through identification of the t(7:9)(q34;q34.3) chromosomal translocation in a subset of human pre-T-cell acute lymphoblastic leukemias (T-ALL) (26). This translocation leads to the juxtaposition of Notch 1 and T-cell receptor β (TCR-β) promoter, which results in a truncated form of Notch 1 such that it is expressed in a TCR-β regulated manner, and constitutively activated independent of ligand stimulation (Figure 3). In addition, activating mutations in Notch 1 independent of the chromosome translocation have been found in more than 50% of human T-ALL cases (27). In mouse mammary tumors, mouse mammary tumor virus (MMTV), which is widely used to identify oncogenes, frequently targets Notch 1 (23, 28) and Notch 4 (29-32). Similar to the t(7;9) translocation in T-ALL, this virus insertion causes constitutive expression of Notch 1 or Notch 4 intracellular domains and augmented activation of target genes in a ligand-independent manner (Figure 3). Overexpression of intact Notch receptors has also been documented in cervical, pancreatic, endometrial, renal, lung, colon, and head and neck carcinomas (33-36), as well as in Hodgkin's and large cell anaplastic lymphomas (37). These findings highlight the linkage between the activation of Notch pathway and tumor development, suggestive of an oncogenic role of Notch. Given that a large body of information on Notch in cancer, we will discuss several

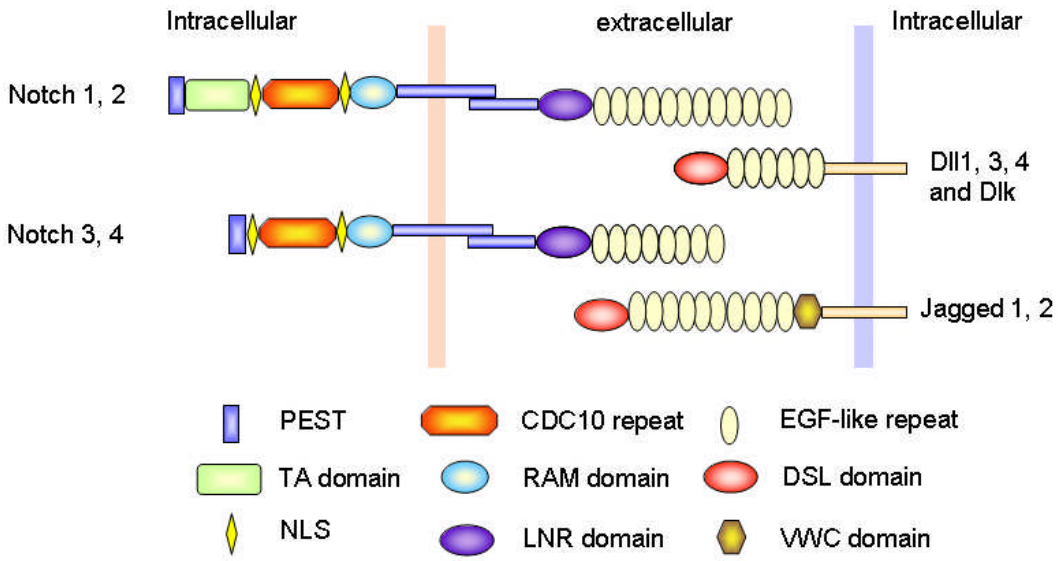

Figure 1. Mammalian Notch receptors and ligands. The full-length receptors are expressed on the cell surface as heterodimers, which consist of non-covalently associated extracellular and transmembrane Notch subunits. All Notch receptors contain epidermal growth factor (EGF)-like repeats and Lin12/Notch repeats (LNR) at extracellular domains; a RAM domain, CDC10 repeat flanked by nuclear localization signals (NLS), and PEST sequence at transmembrane domain. Notch 1 and 2 receptors contain an extra transactivation (TA) domain. Both Delta-like and Jagged ligands are transmembrane proteins containing a DSL domain followed by EGF-like repeats. There is an additional von Willebrand factor type C (VWC) domain in Jagged ligands.

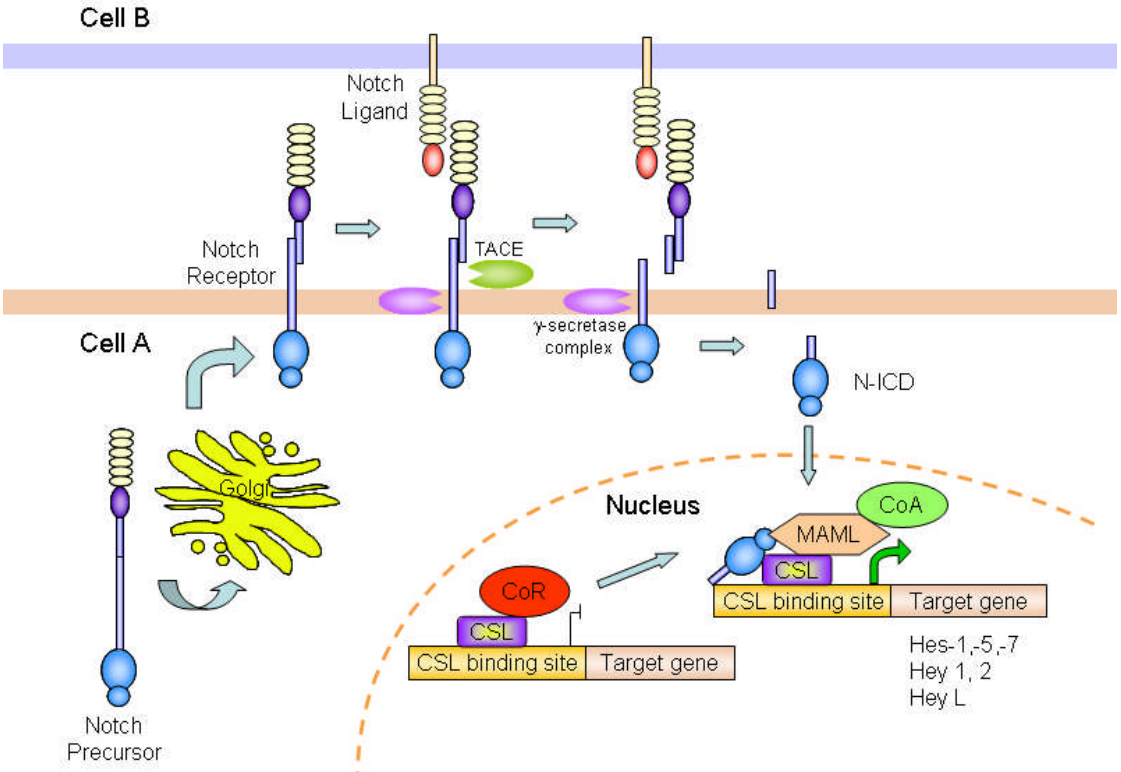

Figure 2. Schematic diagram of Notch signaling pathway. Each Notch receptor is synthesized as a single precursor protein following the cleavage into a heterodimer within the Golgi apparatus, then transports on the cell surface. Upon ligand binding, the receptor undergoes consecutive cleavage by TACE and γ-secretase, which liberates the Notch intracellular domain (N-ICD). N-ICD translocates into the nucleus, interacts with the transcription factor CSL, thereby displacing co-repressors (CoR) and recruiting co-activators such as MAML. This results in the activation of downstream target genes including the Hes and Hey famili

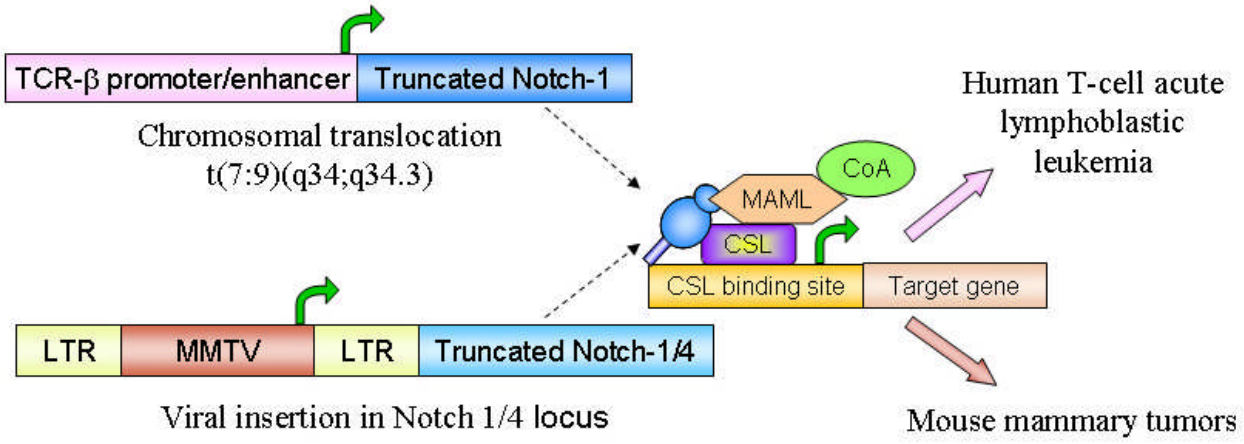

Figure 3. Ligand-independent Notch activation in tumor development. Chromosomal translocation t(7;9) leads to juxtaposition of a truncated Notch 1 gene with the TCR-β promoter/enhancer. This results in ligand-independent, constitutive activation of intercellular Notch 1 and human T-ALL. In analogy with the t(7;9) translocation in T-ALL, mouse mammary tumor virus (MMTV) insertions can take place in the Notch 1 or Notch 4 genes. This leads to constitutive expression of Notch 1 or Notch 4 intracellular domains and development of mammary tumors. This figure is adapted from (4).

relatively well-characterized examples of how Notch is involved in tumorigenesis, such as promoting cell proliferation, rescuing cell from programmed cell death (apoptosis evasion), assisting angiogenesis, invasion and metastasis, as well as its interactions with viral oncogenes. Then, we will turn to the complexity of Notch pathway for its role in tumorigenesis by introducing several reports that certain carcinomas can arise through downregulation of Notch pathway. Finally, we will briefly discuss the interplay between Notch pathway and epigenetic silencers in cancer.

4.1. Cell proliferation

Cancer is a disease of uncontrolled cell proliferation. Since the proliferative behavior of cancer cells is so aberrant, promoting cell proliferation has long been used as one of the criteria to define an oncogene. Indeed, the vast majority of oncogenes are upregulated or overexpressed in various cancers, which also applies to Notch pathway. Not only Notch receptors, but also its ligands are overexpressed in numerous cancers, while downregulation of Notch pathway in these cancers results in cell growth inhibition and apoptosis (28, 37-44). The mechanisms underlying Notch-mediated cell proliferation appear to be cell type specific, depending on which cellular pathways Notch interacts with, such as Ras/MAPK (45), PKC/NF-κB (46), Wnt (47), TGFβ (48) and Sonic Hedgehog (Shh) (49). More details will be given in Section 4.

4.2. Cell survival (apoptosis evasion)

Organisms possess the inherited ability to block the development of cancer through the actions of alarm system, which can cause cells to enter quiescence or apoptosis in the event that the machinery regulating cell proliferation is malfunctioning or the cell is exposed to various stresses. On the other hand, cancer cells evolve numerous ways to inactivate the apoptotic machinery to survive and thrive, including the activation of the Akt/PKB pathway, increase in anti-apoptotic Bcl2-related proteins and the inactivation of p53. One of the key roles of Notch in promoting tumor progression is to facilitate tumor cells escape from apoptosis.

For instance, increased CSL-dependent Notch signaling is sufficient to transform normal breast epithelial cells by the suppression of apoptosis (50). In pancreatic cancer, downregulation of Notch 1 inhibits cell growth and induces apoptosis, which is associated with the decreased expression of cyclin A, cyclin D1, cyclin-dependent kinase 2, Bcl2 and BclX(L), as well as up-regulation of p21 and p27 (51). Notch pathway can also enhance tumor cell survival through the activation of mitogen-activated protein kinase (MAPK) and Akt pathways as evidenced in melanoma cell lines (52). Both MAPK and Akt pathways are activated in melanoma cells following Notch 1 pathway activation. Inhibition of either the MAPK or Akt pathway reverses the Notch 1 signaling-induced tumor cell progression. As a downstream target of Notch, nuclear factor-kappa B (NF-κB), which is a critical regulator of cell proliferation and apoptosis, provides another mechanism by which Notch rescues tumor cells from apoptosis (51, 53). In addition, Notch 1 has been shown to inhibit p53-mediated apoptosis in immortalized epithelial cells (51) and T-cell lymphoma (54). A recent study further demonstrates that intracellular Notch 1 inhibits p53 by suppressing its activating phosphorylations at Ser^{15}, Ser^{20}, and Ser^{392} as well as nuclear localization. Apparently, this inhibition of p53 is mediated through *m*ammalian *t*arget *o*f *r*apamycin (mTOR) dependent PI3K-Akt/ PKB pathway (55).

4.3. Angiogenesis

Angiogenesis is critical for tumor growth and progression and it relies on interactions between tumor cells, endothelial cells (ECs), and stromal cells. Various angiogenic molecules produced by either tumor cells or tumor stromal cells can directly bind to their cognate receptors on ECs and thus initiate angiogenesis (56). For

instance, tumor cells secrete soluble pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), to recruit endothelial cells from surrounding host tissues to form blood vessels. Often, these vessels are abnormally shaped and leaky, and can have tumor cells incorporated into their walls (57).

Notch proteins have an important role in the regulation of tumor angiogenesis. Notch 1 and Dll4 have been reported to be induced by VEGF (58), suggesting that pro-angiogenic factors activate Notch signaling to promote angiogenesis. The recent discovery of Dll4 as an endothelial-specific Notch ligand further extends our understanding of the linkage between Notch and tumor angiogenesis. In primary endothelial cells, Dll4 mRNA levels are induced by either VEGF or hypoxia and are highly expressed in the vasculature of human clear-cell renal cell carcinomas and breast cancer, which is positively correlated with VEGF expression at mRNA level. On the other hand, its expression is undetectable in normal kidney or breast samples. Meanwhile, silencing Dll4 in endothelial cells leads to inhibition of cell proliferation, migration and network formation (59), all of which are crucial for angiogenesis. In a xenograft study with human breast MCF-7 cell line, which does not express Dll4, reveals that tumors derived from MCF-7 express high levels of mouse Dll4 within their vasculature (60).

Moreover, Notch signaling from tumor cells can trigger the Notch activation of neighboring ECs through ligand Jag-1 and, consequently, promote tumor angiogenesis (61). It was shown that the expression of Jag-1 was increased in a MAPK-dependent manner in head and neck squamous cell carcinoma (HNSCC) cells stimulated with growth factors. As a consequence, Notch signaling in co-cultured human dermal microvascular endothelial cells (HDMECs) is activated, leading to the formation of capillary-like sprouts. However, this effect is abolished by knockdown of Jag-1; moreover, HNSCC cells overexpressing Jag-1 form larger tumors with increased vascularization. In human HNSCC tissue samples, high Jag-1 expression correlates with an increase in microvessel formation (61). These results highlight a possible novel mechanism of juxtacrine signaling from tumors to the surrounding vasculature.

4.4. Cell invasion and metastasis

Tumor metastasis is a major cause of death of cancer patients and it occurs through a series of steps, including cell invasion, degradation of basement membranes, and the stromal extracellular matrix. Notch signaling plays a critical role in the pathogenesis and progression of human malignancies, but the precise role and mechanism of Notch 1 for tumor invasion remains elusive. A recent study by Wang *et al* provides a new insight into how Notch participates in cell invasion and metastasis (62). Because NF-κB, VEGF, and matrix metalloproteinases (MMPs) (a family of related enzymes that degrade extracellular matrix) are critically involved in the processes of tumor cell invasion and metastasis (63-66), the role and mechanism(s) by which Notch 1 regulates these important metastasis elements have been extensively investigated. The down-regulation of Notch 1 reduces NF-κB DNA-binding activity as well as expression of its downstream targets VEGF and MMP-9 (67). Knockdown of Notch 1 by small interfering RNA (siRNA) decreases cell invasion, whereas Notch 1 overexpression leads to increased tumor cell invasion. Taken together, it appears that Notch 1-induced cell invasion is in part due to activation of the NF-κB pathway and its target genes, such as MMP-9 and VEGF. Clearly, further in-depth studies are warranted to better understand the molecular mechanism regarding the cause and effectrelationship between Notch 1 and NF-κB during Notch 1-induced cancer cell invasion.

4.5. Notch and viral oncogenes

In addition to gene mutation, viral infection can also cause cancer. A well-known example is the cervical cancer, which is caused by human papillomavirus (HPV) through its oncogenic protein E6 and E7. The following are two examples that the Notch pathway interacts with viral oncoproteins and contributes to tumorigenesis.

4.5.1. EBV viral protein EBNA2 and Notch

EBV infection is associated with several human malignancies, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and lymphomas in the immune-compromised patients. It also has been found that EBV has the ability to immortalize B cells. A key viral protein for immortalization is the transactivator EBNA2 which controls expression of several viral and cellular genes. EBNA2 is tethered to promoters by interacting with the cellular repressor CSL (68). This resembles the physiological activation of CSL-repressed promoters by intracellular Notch receptors (N-ICD). Since EBNA2 and N-ICD have been shown to be partially interchangeable in regard to activation of target genes in B cell lines and modulation of differentiation processes, it is conceivable that EBNA2 is a biological equivalent of an activated Notch receptor (68, 69), and that the mimicry of Notch signal transduction is involved in EBV-driven immortalization (70). However, although the cellular Notch protein and EBNA2 share several biochemical and functional properties, such as interaction with CSL and the ability to activate transcription of a number of cellular and viral genes, N-ICD still cannot completely replace the EBNA2 function. This has been shown in studies where in the absence of functional EBNA2, N-ICD cannot maintain cell proliferation and cell immortalization (71, 72).

4.5.2. HPV viral protein E6/E7 and Notch

Cervical carcinoma evolves slowly from cervical intraepithelial lesions (CIN) to invasive carcinomas. Approximately 99% of cervical carcinoma is directly associated with the presence of high-risk HPV (73). The viral oncogenes E6 and E7 have been shown to disrupt cell cycle regulation by targeting p53 and pRb (74, 75). However, the disruption of cell cycle and apoptosis control through inactivation of p53 and Rb is not sufficient for transformation. In general, at least one other event is required for full cellular transformation. The Notch 1 protein appears to be at the center of this complex interplay, because Notch 1 receptor expression is increased during the progression from cervical intraepithelial lesions

(CIN) to invasive cervical carcinoma. In one study mouse and human primary cell lines were transfected with HPV16 E6 and E7 to determine whether HPV E6 and E7 expression causes upregulation of Notch 1 expression. Of interest, Notch 1 expression and its activity were directly upregulated by E6 and E7 through both transcriptional and post-transcriptional mechanisms (76). Moreover, a protein involved in Notch processing, Presenilin-1 (PS-1), was also upregulated by E6 and E7. Downregulation of Notch 1 expression in a human cervical carcinoma cell line expressing E6/E7 can cause marked inhibition of proliferation *in vitro* and tumorigenicity *in vivo* (76). These data suggest that E6- and E7-mediated upregulation of Notch signaling may contribute to disruption of regular cell growth in cervical cancer (76). Moreover, activated Notch 1 signaling has been shown to synergize with E6 and E7 in transformation of immortalized epithelial cells and lead to the generation of resistance to anoikis (77). This resistance to anoikis by activated Notch 1 is mediated through the activation of Akt/PKB, a key effector of activated Ras in transformation (77), suggesting that activated Notch signaling may serve to substitute for the lack of activated Ras mutations in the majority of human cervical neoplasms. A more recent study further demonstrates that activated Notch 1 inhibits p53-induced apoptosis and sustains transformation by HPV E6 and E7 through a PI3K-PKB/Akt-dependent pathway (78) and thus, activation of Notch signaling may serve as an additional mechanism to inhibit wild-type p53 function in papillomavirus-associated neoplasia.

4.6. A controversial role of Notch in tumorigenesis

So far we have discussed several lines of evidence that Notch contributes to tumor progression and the possible molecular mechanisms. However, Notch pathway is far more complex than what is conceived of its role in tumorigenesis. In certain types of cancer, Notch can function as a tumor suppressor instead of an oncogene. For instance, expression of Notch 1 and its effector Hey-1 gene in human prostate adenocarcinomas are significantly down-regulated compared to normal control tissues (79). In addition, the active N-ICD is absent in human Medullary thyroid cancer (MTC) tumor tissue samples and MTC-TT cells. Activation of Notch 1 results in significant MTC cell growth inhibition, which is mediated by cell cycle arrest associated with upregulation of p21 (19). Growth suppression induced by Notch 1 activation was also reported in human tongue carcinoma cells, where Wnt-β-catenin pathway is down-regulated (80). Although activated Notch 1 cooperates with HPV E6/E7 in cell transformation, as discussed above, constitutively active Notch 1 signaling is able to cause growth suppression in HPV-positive cervical cancer cells (81, 82). This suppression of cell growth by activated Notch 1 is likely due to repression of viral E6/E7 expression through AP-1 down-regulation, resulting in increased p53 expression and blockage of pRb hyperphosphorylation (82). Alternatively, activated Notch 1 signaling suppresses activity of the helix-loop-helix transcription factor E47, via ERK1/2 activation, resulting in inhibition of cell cycle progression (81). Hence, Notch 1 downregulation appears to be important during late stages of tumor progression. Even more complex, Notch 1 and Notch 2 are shown to have antagonistic effects on the growth of the same type of tumors. In embryonal brain tumor cell lines, while Notch 2 promotes, Notch 1 inhibits cell proliferation, soft agar colony formation, and xenograft growth (83). Therefore, Notch has two faces for its role in tumorigenesis, as an oncogene or tumor suppresser, which appears to be dependent on the cellular context and the crosstalk with other signaling pathways (1).

4.7. Interplay between Notch signaling and epigenetic silencers in cancer

In addition to genetic abnormalities, epigenetic alterations of gene expression also seem to contribute to the pathogenesis of cancer. The epigenetic modifications, particularly through abnormal histone modification and aberrant DNA methylation, permit heritable gene silencing. This is responsible for the inactivation of tumor suppressor and tumor-related genes, which ultimately contribute to the initiation and progression of cancer, and to metastasis (84, 85). A recent study with genetic screening revealed an unexpected cooperation between epigenetic silencing pathways and the Notch signaling pathway during tumorigenesis. Two Polycomb group epigenetic silencers, Pipsqueak and Lola, are identified to participate in *Drosophila* eye tumorigenesis. When coupled with overexpression of Delta, deregulation of Pipsqueak and Lola induces the formation of metastatic tumors (86). Meanwhile, the expression of the gene retinoblastoma-family protein (Rbf) is downregulated, which is associated with DNA hypermethylation in these tumors (86). Together, these results establish a mechanism that links the Notch pathway, epigenetic silencing pathways and cell-cycle control in tumorigenesis (86). Given that both Notch hyperactivation and aberrant gene silencing have been identified in human cancers, these discoveries in the fruit fly may provide a useful new tool for investigations of Notch-associated cancers (87).

5. CROSS-TALK BETWEEN NOTCH SIGNALING AND OTHER ONCOGENIC PATHWAYS IN MAMMARY TUMOR DEVELOPMENT

Although the role of Notch in tumorigenesis is still controversial, evidence from *in vitro* experiments, mouse models and human tumor samples indicates that Notch plays a predominantly oncogenic role in breast cancer, which is largely through its interaction with other signaling pathways involved in mammary tumorigenesis. Here, we list several well-characterized oncogenic signaling pathways in the context of their cross-talk with the Notch pathway, leading to the mammary tumor development.

5.1. Ras signaling pathway

Ras signaling is frequently activated in breast carcinomas due to increased expression of wild-type H-Ras and/or overexpression or activation of epidermal growth factor-receptor (EGFR) family or downstream targets (88). H-Ras is thought to play a key and initial role in mammary carcinogenesis in humans (89). Of interest, Ras and Notch correlate in breast carcinomas. For instance, immunohistochemical analysis revealed that four of seven

cases of human primary breast ductal carcinoma are Ras positive and all 7 are Notch 1 positive (90). Furthermore, Ras-positive tumors show stronger and diffuse Notch 1 staining, whereas Ras-negative tumors reveal weaker Notch 1 staining (90).

Moreover, several studies suggest that the ability of activated Ras or Notch to transform cells depends on interactions with the other factors (23, 91, 92). In cultured human cells transformed by a combination of active Ras, SV40, and hTERT, Ras acts through p38 MAP kinase to upregulate expression of the intracellular form of wild-type Notch 1 (90), Notch ligand Dll1 and presenilin-1, a protein involved in Notch processing. These observations suggest that Notch signaling is among key downstream effectors of oncogenic Ras. Interfering with Notch signaling in this system inhibits anchorage-independent growth, implying that sequential signaling through Notch is critical for Ras-induced transformation (90). Similarly, in mice, overexpression of the Notch inhibitor Deltex suppresses H-Ras-induced mammary tumorigenesis. Among those with transgenic human Ras, 80% of the mice develop mammary tumors within 10 months. In contrast, only 20% of mice with both transgenic Ras and Notch antagonist Deltex develop mammary tumors in the same period. Both H-Ras and Notch 1 up-regulate expression of cyclin D1, suggesting that the mode of cooperation might be convergent up-regulation of a common target (93). As another line of evidence, both Ras pathway inhibitor and dominant negative Ras decrease the colony formation or tumor growth in immune compromised mice for mammary tumor cells overexpressing intracellular Notch 4 (Notch 4-ICD) (91).

5.2. ErbB2 signaling pathway

The human ErbB2 gene encodes a protein tyrosine kinase receptor, a member of human epidermal growth factor receptor (hEGFR) (94). Amplification and overexpression of this gene is frequent in the breast carcinoma, where overexpression directly correlates with poor patient outcomes (95, 96). Although protein overexpression could be a result of gene amplification, high levels of ErbB2 mRNA and protein in these tumors appear to derive from a single copy gene, indicating that transcriptional control mechanisms are important (96, 97). In this regard, Chen *et al* found that N-ICD cooperates with CSL bound to the ErbB2 promoter and activates its transcription (24). CLS is able to stimulate promoter activity via wild type but not mutant ErbB2 palindromic sequences, and this activity is markedly enhanced upon co-expression of N-ICD (24). In a transgenic mouse model, the MMTV/ErbB2 mice spontaneously develop mammary tumors stochastically after a long latent period, suggesting that the ErbB2 oncogene is not sufficient for tumor formation. Using provirus insertional mutagenesis approach they identified Notch 1 as a target for MMTV/ErbB2 (23).

5.3. TGFβ signaling pathway

Notch also interacts with the TGFβ pathway, which plays a dual role in tumor development. During early stages, TGFβ acts as a tumor suppressor by inhibiting proliferation; at later stages, it functions as an oncogene by promoting invasion and metastasis through epithelial-mesenchymal transition (EMT) (5). Notch signaling antagonizes or synergizes with TGFβ function, but in either case Notch promotes tumor growth. In cervical cancer cell line with spontaneously activating Notch 1, growth inhibition in response to TGFβ is diminished (48). Similarly, MCF-7 human breast cancer cells, which express activated form of the Notch 4 receptor, also are resistant to the growth-inhibition effects of TGFβ; blockage of Notch receptor processing by γ-secretase inhibitor (GSI) restores growth inhibition (98). However, Notch and TGFβ signaling may interact synergistically to promote EMT. Activation of TGFβ pathway leads to upregulation of the Notch target gene Hes-1 *in vitro* and *in vivo* due to the interactions between the TGFβ effector Smad 3 and activated Notch 1 (99). At the onset of EMT, TGFβ induces Hey-1 in mammary epithelial cells; RNA silencing of Hey-1 or Jag-1, and chemical inactivation of Notch by GSI block TGFβ-induced EMT (100).

5.4. Wnt signaling pathway

Similar to Notch 1 and 4, the Wnt gene is a preferential target of MMTV (101-103). Aberrant expression of several Wnts and other components of this pathway in human breast carcinomas has also been reported (104). Moreover, an extracellular inhibitor of Wnt signaling, secreted Frizzled-related protein 1, is down-regulated in 80% of breast carcinomas (105, 106); and the positive regulator Disheveled is up-regulated (107). These observations strongly support the notion that Wnt signaling plays an oncogenic role in breast cancer. The signaling cascade initiates by the interaction of a Wnt ligand with a seven-transmembrane-domain Frizzled receptor, which leads to the stabilization of β-catenin by inhibiting glycogen-synthase kinase-3β (GSK-3β) activity (108). Stabilized β-catenin then translocates to the nucleus, where it forms a bipartite complex with transcription factors of the T cell factor family and activates target gene expression (109).

Even more complex cross-talk occurs between Notch and Wnt pathways in mammary carcinoma. Ayyakannu *et al* nicely demonstrated that increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism (110). Increased Notch signaling in primary human mammary epithelial cells (HMECs) is sufficient to reproduce some aspects of Wnt-induced transformation. *In vitro* and *in vivo* studies further indicate that Notch signaling is required for Wnt-1-induced transformation of primary HMECs. The relevance of these findings for human breast cancer is supported by the fact that the expression of Wnt-1 and Wnt-4 and of established Wnt target genes, such as Axin-2 and Lef-1, as well as the Notch ligands, such as Dll3 and Dll4, are up-regulated in human breast carcinomas (110). One possible candidate linking between these two pathways is the kinase GSK-3β, which can phospharylate not only β-catenin but also Notch 2 and thus, inhibit downstream gene Hes-1. Wnt-1 overexpression, which represses GSK-3β, leads to the activation of the Hes1 promoter (111). Nevertheless, other evidence also suggests

Table 2. Targeting Notch pathway as potential cancer therapy

Targets in Notch pathway	Potential therapies
Extracellular Notch receptors and ligands	Monoclonal antibodies; SiRNA
Ligand-mediated receptor activation	Soluble ligands; Receptor decoys; Inhibitors of enzymes involved in cleavage of Notch receptors (eg. GSI)
Intracellular constitutive Notch activation	Intracellular Notch antagonist (eg. Numb); Disruption of Notch-CSL complex; Repression of downstream targets

an antagonistic relationship between Notch and Wnt pathways in mammary gland development. Although both Notch and Wnt promote mammary tumor development, Wnt-1 stimulates a hyperplastic phenotype with increased ductal development, whereas Notch 4 inhibits ductal development (112). Thus, the interplay between Wnt and Notch pathway merits further study in terms of mammary tumorigenesis.

5.5. HIF signaling pathway

The hypoxia response upregulates a number of pathways conducive for tumor survival, such as angiogenesis, glycolysis, growth-factor signaling, immortalization, genetic instability, tissue invasion and metastasis (113). The transcription factor hypoxia inducing factor HIF-1, composed of α and β subunits, regulates most hypoxia responses. Under normoxia, HIF-1α is subject to O_2-dependent prolylhydroxylation and ubiquitinated by the von Hippel-Lindau (VHL) complex and rapidly degraded by proteasome pathways (114, 115). Under hypoxia, limited O_2 rescues HIF-1α from prolylhydroxylation and ubiquitination dependent degradation. Instead, HIF-1α translocates to the nucleus and binds to the HIF-1β subunit and activates transcription of downstream targets (113). A recent study highlights the interaction of the Notch and HIF pathway. HIF-1α interacts with N-ICD and is then recruited to Notch-responsive promoters upon Notch activation under hypoxia condition, which increases the expression of Notch direct downstream gene Hey-2 (116). In neuroblastomas, hypoxia upregulates Notch 1 and Hes-1, which resultes in cell de-differentiation and a more aggressive tumor (117). More interesting, in the breast cancer cell line MCF-7, HIF-1α protein level increases with the overexpression of Notch 1 (118). In addition, Dll4 with high expression in breast cancer but not in normal samples, which are highly related with angiogenesis, can also be induced by HIF-1α (59).

6. NOTCH SIGNALING AS A PROGNOSIS MARKER OF BREAST CANCER

Given that Notch signaling is activated in a wide variety of human breast cancer cases, is it possible to use components of Notch pathway as a prognosis marker for breast cancer? Indeed, several studies seem to support this possibility.

Aberrant expression of Notch 1 and Notch 2 in breast cancer tissues has been reported compared with normal breast tissue. A high level of Notch 1 may be associated with a poorer outlook for the breast cancer patient, while a high level of Notch 2 correlated to a higher chance of survival (119). In another study, high levels of Jag-1 and Notch 1 are found in a subset of tumors with poor prognosis pathologic features. Moreover, a synergistic effect of high-level Jag-1 and high-level Notch 1 coexpression on overall survival is also observed (120).

In addition, regulators of Notch pathway may also be potential biomarkers for breast cancer prognosis. For instance, from 321 consecutive breast cancer cases that are characterized for the expression of Numb, a negative regulator of Notch pathway, ~50% of cases have lost Numb-mediated control of Notch signaling (121). Remarkably, a strong inverse correlation is seen between Numb expression levels and tumor aggressiveness (121). Mutations in Notch pathway also seem to correlate with patient outcomes. In this regard, two germline alterations in presenilin-2 (PS-2), R62H and R71W, are identified in breast cancer (122). Both alterations compromise PS-2 function in Notch signaling. The effect of the R71W alteration is noticeably stronger and the frequency of this allele is almost threefold higher in breast cancer than controls. These results suggest that the novel PS-2 alleles, especially R71W, may potentially confer a moderate risk of susceptibility to breast cancer (122).

7. TARGETING NOTCH SIGNALING PATHWAY AS POTENTIAL THERAPIES FOR BREAST CANCER

Since Notch signaling plays an oncogenic role in breast cancer, inhibition of Notch pathway may represent a potential therapy for this disease (123). Now it is known that the cancer-associated events might take place at several levels in Notch cascade, and a number of genetic and pharmacological strategies are either available or theoretically possible to block Notch signaling at different points of the pathway. Approaches of Notch interventions for potential therapies are listed in Table 2. The best-developed approach for Notch signaling inhibition is the small molecule inhibitor GSI. Experiments using GSI to inhibit Notch pathway *in vitro* and *in vivo* have shown promising results for its application in cancer therapy. For example, in Kaposi's sarcoma (KS), the activated forms of Notch 1, 2, 4 are overexpressed. GSI treatment reduces Notch signaling, and results in apoptosis in primary and immortalized KS cells (124); inhibition or tumor regression (124). In human mammary carcinomas, GSI treatment of Numb negative tumor cells (which lose control of Notch signaling pathway) can cause a dramatic suppression of their growth potential, accompanied by a marked decrease in Notch activity. In contrast, no significant effect was observed in Numb positive tumors cells (50). In addition, GSI may also be appreciated for its ability to inhibit tumor angiogenesis. In *in vitro* studies, GSI reduces endothelial cell proliferation without inducing cellular toxicity, and at the same time, suppresses the formation of capillary structures (125) In mouse model of human glioblastoma and human lung adenocarcinomas, GSI potently inhibits the growth and vascularization (125).

However, despite several advantages of GSIs as anti-cancer agents, the main pitfall is that they lack the specificity as they target all Notch receptors and other

transmembrane proteins. Since Notch signaling is required for many physiological processes, GSI treatment may cause broad range of toxicity. The current trend in cancer therapy is to replace systemic chemotherapy with target-specific biologicals/chemicals. It may be possible to focus on the downstream mediators of Notch signaling, or the specific ligands and/or receptors with elevated expression level rather than components of the central signaling axis. Therefore, specific inhibitors targeting individual Notch pathway components overexpressed in breast cancer need to be developed.

8. NOTCH SIGNALING AND CANCER STEM CELLS

The recent discoveries of stem-like cells in solid tumors has some intriguing implications for both understanding the molecular and cellular basis for cancer, and providing some interesting and alternative routes for treatments. Relatively dedifferentiated, stem-like side populations have been isolated from a number of solid tumors, including breast carcinomas (126). These side populations harbor unique properties such as the ability to form colonies in soft agar and seem to be major instigators of tumorigenicity *in vivo*. Cancer stem cells are also chemoresistant in many cases, presumably because of their ability to pump out cancer fighting drugs through activated Mdr1 drug pumps within their cell membranes (127). Several "stemness" genes are associated with tumor stem cells, and are believed to be responsible for the promotion of their stem-like phenotypes, as well as promoting survival and proliferation of these cells. These genes include Wnt (128), nestin (129), sonic hedgehog (130) and Notch (131).

Notch plays an active role in embryogenesis and in regulation of stem cell fate. Notch's ability to regulate various genes that affect cell cycle regulation, proliferation, and differentiation also makes it a key component in the regulation of cancer stem cells. For instance, Notch has been shown to be necessary in regulating stem cell differentiation in crypt cells in the small intestine, which has implications in colorectal carcinomas that contain cancer stem cells (132). Notch signaling is important for proper development and differentiation of mammary precursor cells (133). Notch also maintains the stem cell population by promoting self renewal in normal mammary stem cells (134). Pregnant mice with overexpressed and truncated Notch 4 receptors fail to produce differentiated secretory lobules or produce milk (133). Clinically, breast cancer stem cells are known to express high levels of Notch 1 receptor (135). These findings further support the notion that Notch is a potential therapeutic target.

9. PERSPECTIVE

Given that Notch signaling regulates various physiological processes, it is not surprising that Notch signaling plays a critical role in tumorigenesis. Despite the controversy over the role of Notch in this aspect, it seems to be clear that Notch plays an oncogenic role in breast cancer, which is likely through cooperation with other signaling pathways. Furthermore, Notch also seems to play an active role in cancer stem cells and traditional therapeutic strategies appear to be ineffective at eradicating cancer stem cells primarily due to the combination of drug resistance, high proliferative ability, and immortality. Therefore, Notch is an attractive therapeutic target, in particular for breast cancer. Although current anti-Notch agents lack specificity, we expect that novel anti-Notch agents with more specificity will be developed in the near future as we better understand the molecular mechanism of Notch signaling.

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