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Regulation of angiogenesis via Notch signaling in breast cancer and cancer stem cells

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Abstract

Breast cancer angiogenesis is elicited and regulated by a number of factors including the Notch signaling. Notch receptors and ligands are expressed in breast cancer cells as well as in the stromal compartment and have been implicated in carcinogenesis. Signals exchanged between neighboring cells through the Notch pathway can amplify and consolidate molecular differences, which eventually dictate cell fates. Notch signaling and its crosstalk with many signaling pathways play an important role in breast cancer cell growth, migration, invasion, metastasis and angiogenesis, as well as cancer stem cell (CSC) self-renewal. Therefore, significant attention has been paid in recent years toward the development of clinically useful antagonists of Notch signaling. Better understanding of the structure, function and regulation of Notch intracellular signaling pathways, as well as its complex crosstalk with other oncogenic signals in breast cancer cells will be essential to ensure rational design and application of new combinatory therapeutic strategies. Novel opportunities have emerged from the discovery of Notch crosstalk with inflammatory and angiogenic cytokines and their links to CSCs. Combinatory treatments with drugs designed to prevent Notch oncogenic signal crosstalk may be advantageous over λ secretase inhibitors (GSIs) alone. In this review, we focus on the more recent advancements in our knowledge of aberrant Notch signaling contributing to breast cancer angiogenesis, as well as its crosstalk with other factors contributing to angiogenesis and CSCs.

Keywords

Notch; Breast cancer; Tumor angiogenesis; Oncogenesis; Breast cancer stem cells

1. Introduction

The formation of new blood vessels from existing ones (angiogenesis) is a crucial requirement for the growth, progression and metastatic spread of a tumor [1]. Low oxygen

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microenvironment triggers angiogenesis in normal and pathological conditions, i.e., tumor growth [2]. The malignant cells undergo an angiogenic switch leading to secretion of angiogenic factors and proteolytic enzymes in response to hypoxia culminating in the activation of endothelial cell (EC) proliferation, migration and establishment of a robust capillary network. This irregular and ill-organized network is capable of providing the growing tumor mass with all the required metabolites. In addition, the tumor angiogenesis network also provides tumor cells with the opportunity to enter the circulation and the opportunity to form distant metastases [3]. Tumor angiogenesis is elicited and regulated by several factors. Among these factors, Notch signaling plays an important role. Notch is essential for a variety of cell fate decisions and can regulate diverse cellular biological processes especially during embryogenesis. To signal, membrane-bound Notch receptors and ligands need to be co-expressed in adjacent cells. Notch receptors and ligands are expressed in tumor cells as well as in the stromal compartment and have been implicated in tumorigenesis [4,5]. Notch genes encode transmembrane receptors that are highly conserved from invertebrates to mammals. Notch-mediated signals regulate cell-fate decisions in a large number of developmental systems [6,7]. Such signals are mainly transmitted through direct contact between adjacent cells expressing Notch receptors and their ligands. Notch receptors activated in response to ligand expressed by adjacent cells have the potential to regulate cell fate specification, differentiation, proliferation, or survival [8]. Notch signaling pathway is frequently dysregulated in several human malignancies. Over expression of Notch receptors and their ligands has been found in cervical, colon, head and neck, lung, renal carcinoma, pancreatic cancer, acute myeloid, Hodgkin, Large-cell lymphomas, as well as breast cancer [9–11]. Overall, it is well-established that Notch signaling plays an important role in tumor progression [5,12]. Signals exchanged between neighboring cells through the Notch pathway can amplify and consolidate molecular differences, and influence how cells respond to intrinsic or extrinsic developmental cues that are necessary to unfold specific developmental programs [13]. Because the same signaling pathways within different contexts can trigger a variety of cellular activities, cancer progression activities induced by Notch and its crosstalk with other signaling pathways are also context dependent. In light of several valuable reviews published on the role of Notch signaling in several types of cancer [14–17], we wish to focus this review on the more recent advancements in understanding how aberrant Notch signaling and its crosstalk with other factors contribute to breast cancer angiogenesis and CSC.

2. Structure, activation and function of Notch receptors and ligands

The Notch system in vertebrates comprises four receptors (Notch1–Notch4) and at least five ligands from the families Delta and JAG/Serrate (DSL):JAG1,JAG2, Delta-like (Dll)-1, Dll-3, and Dll-4 [10,11,13]. Ligands of Notch receptors can be divided into several groups based on their domain composition. Canonical DSL ligands (JAG1,JAG2 and Dll-1) are type I cell surface proteins, consisting of the Delta/Serrate/LAG-2 (DSL), Delta and OSM-11-like proteins [DOS, which is specialized tandem EGF repeats] and EGF motifs. The other subtypes of DSL canonical ligands include Dll-3 and Dll-4 that lack the DOS motif [18–20]. Both the DSL and DOS domains are crucial for physical binding with Notch receptor [9]. However some membrane-tethered and secreted noncanonical ligands lacking DSL and DOS

domains have also been documented to activate Notch signaling both in vitro and in vivo [19,21–26], which may explain the diverse and frequent effects of Notch signaling with the small number of canonical DSL ligands and receptors in vertebrate genomes [19].

Notch receptors belong to a large single-pass type 1 transmembrane protein family; the extracellular domain consists of 29–36 tandem arrays of EGF (epidermal growth factor)-like repeats, followed by a conserved negative regulatory region (NRR or LNR) consisting of three cysteine-rich Notch Lin12 repeats (N/Lin 12) and a heterodimerization (HD) domain [27]. Notch family members differ in the number of EGF-like repeats, however they share many similarities in structure [9,28]. EGF-like repeats mediate ligand binding, whereas NRR functions to prevent both ligand-dependent and -independent signaling [28]. The cytoplasmic portion of Notch is composed of a DNA binding protein (RBP-Jk associated molecule or RAM) domain and six ankyrin (ANK) repeats, which are flanked by two nuclear localization signals (NLS), followed by a transactivation domain (TAD) and a domain rich in proline, glutamine, serine and threonine residues (PESTs) that controls the receptor half life [9,29,30] (Fig. 1).

Membrane localization of Notch requires S1 cleavage of precursor of the Notch receptor. This event occurs in the Golgi network by the action of a furin-like convertase. Then, the two fragments are re-assembled as a non-covalently linked heterodimeric receptor at the cell surface [6]. Mature Notch receptors are heterodimers made up of an extracellular subunit, a transmembrane subunit (N^{TM}) and a cytoplasmic subunit. Activation of Notch consists of two consecutive cleavages of the transmembrane receptor upon the binding of a Notch ligand, which triggers S2 cleavage. This process takes place at the cell surface. N^{TM} subunit is cleaved by ADAM/Tumor necrosis factor- α -converting enzyme (TACE) metalloprotease family at Site 2 (located ~12 amino acids before the transmembrane domain). S2 cleavage releases the Notch extracellular domain (NECD) from the heterodimer and creates a membrane-tethered Notch extracellular truncation (NEXT), which becomes a substrate for γ -secretase. S3 is cleaved by γ -secretase at Sites 3 and 4 [31]. This last cleavage occurs on the plasma membrane and/or in endosome. The new mobile cytoplasmic subunit [Notch intracellular domain (NICD or N^{IC})] is translocated to the nucleus, where it interacts with members of the DNA-binding protein, recombination signal binding protein for immunoglobulin kappa J (RBP-Jk) or CBF1/Su(H)/Lag-1 (CSL) family of transcription factors [8]. Activated NICD–RBP-Jk complex displaces co-repressors and recruits coactivator (co-A) mediating the transcription of target genes such as Hes-1 (hairy enhancer of split), cyclin D, Hey-1 (hairy/enhancer-of-split related with YRPW motif) and others [10,11]. In the absence of NICD, CSL may interplay with the ubiquitous corepressor (Co-R) proteins and histone deacetylases (HDACs) to repress transcription of some target genes [32,33].

3. Notch signaling in vascular development

Gain- and loss-of-function mutations in humans, mice, and zebrafish have demonstrated the involvement of Notch signaling in multiple aspects of vascular development [34–37]. The vascular system comprises arteries, veins, and lymphatics which separated subdivisions into large vessels, small vessels and capillaries. These vessels primarily consist of endothelial

cells, supporting cells (smooth muscle cells, SMCs and pericytes), and surrounding matrix. Mounting evidence suggests that Notch ligands (Dll-4, Jagged-1, and Jagged-2), receptors (Notch1, 2, 3, and 4) and effectors (HERP1, 2, and 3) are involved in the development of the vascular system [37–39]. In this process Notch pathway is to identify distinct cell subpopulations from bipotential precursor cells, a process known as lateral specification or lateral inhibition [13,40,41]. Mice homozygous for null mutations of Notch (including Notch1, plus Notch4, and Jagged1) show embryonic lethality together with vascular remodeling defects [35,42]. Both Dll-1-deficient and homozygous Notch2 mutant mouse embryos showed hemorrhage, possibly resulting from poor development of vascular structures. However, Dll-1 and Notch2 were not detected in large vessels of mutant embryos [43,44]. EC-specific expression of Notch4 activated form in transgenic mice led to embryonic lethality with abnormal vessel structure and patterning. This phenotype was similar to that seen in Notch1- and Notch1/Notch4-deficient mice [45]. The similarities between the vascular phenotypes observed in knockout mice (loss-of-function) and transgenic mice (gain-of-function) suggest that a window of appropriate Notch expression levels might be needed for proper development of the embryonic vasculature.

4. Activation of Notch signaling and its target genes in breast cancer angiogenesis

Roles of Notch1 and Notch4 in angiogenesis have been established using mutant mice. Notch1 and Notch1/Notch4 double mutant embryos displayed severe defects in angiogenic vascular remodeling [35]. Among Notch receptors with potential roles in carcinogenesis and angiogenesis, Notch1 (MW: 272 kDa and NICD MW: 110–120 kDa) is relatively the best studied [46–48]. It was found earlier that Notch1, a putative collaborator of *c-myc*, was mutated in high proportion (52%) in CD4⁺CD8⁺ T-cell tumors [49]. These mutations led to high expression of truncated Notch1 proteins. The Notch1 gene was identified as a novel target for mouse mammary tumor virus (MMTV) provirus insertional activation. MMTV insertion in the Notch1 gene induced the overexpression of 5' truncated ~7 kb RNA (280 kDa mutant protein: Notch1 ectodomain) and truncated 3' Notch1 transcripts (3.5–4.5 kb) and proteins (86–110 kDa) that can transform HC11 mouse mammary epithelial cells in vitro [46]. Notch1 was further found to be a mediator of oncogenic Ras (retrovirus-associated DNA sequence kinase, small cytoplasmic GTP-binding proteins) [50]. Moreover, Notch1 and JAG1 were co-upregulated upon estrogen treatment not only in MCF-7 breast cancer cells, but also in ECs, suggesting a role of Notch1-JAG1 in angiogenesis [51].

The exact roles of Notch2 (MW: 205 kDa) and NICD (MW:110 kDa) in angiogenesis have not been determined. Notch2^{-/-} mice develop normally until E9.5, and then around E11.5 massive cell death occurs [52]. These results suggest that Notch2 plays an essential role in post implantation development in mice. The role of Notch 2 is dependent on its ankyrin repeats and is probably linked to some aspects of cell specification and/or differentiation [52]. However, Notch2 (unlike Notch1) is not essential for generating hematopoietic stem cells from ECs [53]. The development of hematopoietic cells is closely related to angiogenesis, indicating the existence of hemangioblasts and hemogenic ECs. A recent report shows Notch2 has a role in EC and vascular dysfunction. Inflammatory cytokines can

elicit a switch toward Notch2 expression over Notch4, leading to reduced Notch activity and increased apoptosis [54].

Mutations in human Notch3 (MW:244 and NICD MW:86 kDa) cause CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), a late-onset disorder causing stroke and dementia, which arises from slowly developing systemic vascular lesions ultimately resulting in the degeneration of vascular smooth muscle cell [55,56]. Notch3 expression maintains a differentiated phenotype of mural cells (smooth muscle cells, pericytes, or fibroblasts) through an autoregulatory loop that requires endothelial-expressed Jagged1 [57]. Thus, Notch3 is considered to be critical for proper angiogenesis and mural cell investment [58]. ECs and mural cell interactions support fully functional blood vessels and regulate vessel assembly and differentiation or maturation. Alterations in mural cell density and attachment to the endothelium are associated with several human diseases such as diabetic retinopathy, venous malformation, and hereditary stroke. In addition, mural cells are implicated in regulating tumor growth and have thus been suggested as potential targets in tumor antiangiogenic therapy [59]. Notch4 actions in breast cancer are also cell-context dependent. Early studies indicated that Notch4 is an EC specific homologue of Notch and it may play a crucial role in vasculogenesis and angiogenesis [60]. However, in contrast to Notch1, constitutive Notch4 activation in ECs inhibits angiogenesis in part by promoting β 1-integrin-mediated adhesion to the underlying matrix [61]. Such inhibition of angiogenesis requires the ankyrin repeats and appears to involve RBP-J κ -dependent and independent signaling [62]. On the other hand, mammary carcinogenesis is related to gain-of-function mutations of Notch4 leading to dysregulated levels of the Notch4 NICD [63,64]. Transgenic expression of the 1.8 Kb Notch4 RNA species in non-malignant human mammary epithelial cell line MCF-10A enabled these cells to grow in soft agar, suggesting Notch4 can transform MCF-10A cells [65]. Notch4 was also found to subvert normal epithelial morphogenesis and to promote invasion of the extracellular matrix. Moreover, Notch4 significantly increased the tumorigenic potential in vitro of mammary epithelial cells by changing the morphogenetic properties [66,67].

The best-characterized Notch targets are transcriptional repressors of the Hes (Hes1-7) and Hey subfamilies (Hey1, Hey2, HeyL, HesL/HeIT, Dec1/BHLHB2, Dec2/BHLHB3) [68–70]. Both Hes and Hey proteins contain a basic domain which determines DNA binding specificity, and a helix-loop-helix domain which allows the proteins to form homo- or heterodimers. In contrast, Hes6 is a novel estrogen-regulated gene and a potential oncogene overexpressed in breast cancer, with tumor-promoting and proliferative functions [71]. Other Notch target genes include proteins and factors involved in the control of the cell cycle and survival processes such as p21^{WAF1/Cip1}, a cyclin-dependent kinase inhibitor that acts as both a sensor and an effector of multiple antiproliferative signals. Notch activation contributes to contact inhibition of ECs, in part through repression of p21^{Cip1} expression [72]. *Deltex*, including *Deltex1*, *Deltex2*, *Deltex4* [73], acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats [74]. Deltex family also functions as a transcriptional regulator downstream of the Notch receptor, but has no direct role in angiogenesis [75]. Nuclear factor-kappa B (NF- κ B) (a transcriptional factor) was identified as a Notch target gene early on [76], and was later found to collaborate with Notch signaling in angiogenesis [77,78]. Dysregulation of *cyclin D1* (a mitogenic sensor and

allosteric activator of cyclin dependent kinase CDK4/6) [79] and *c-myc* (an oncogene and cell cycle regulator) is considered one of the hallmarks of many cancers [10–13,80]. Both factors are involved in angiogenesis and have been identified as Notch target genes [79,81].

Notch signaling can modulate apoptosis. NICD interacts with, and can inactivate p53 through phosphorylation [82]. Recently, Survivin, a member of the inhibitor of apoptosis family of proteins (IAP) that induces cell proliferation, was identified as a novel Notch target gene [83,84]. Notch stimulation resulted in direct activation of Survivin gene transcription through at least one RPB-J κ site in the Survivin promoter [85]. Activation of Notch directly induced the transcriptional up-regulation of Survivin in ER-breast cancer cells [86,87]. Accumulated evidence suggests that lack of Survivin in EC causes embryonic defects in angiogenesis [88]. Moreover, the knockdown of Survivin in ECs could inhibit angiogenesis [89,90].

5. Notch signaling, breast cancer stem cell and angiogenesis

A new theory about the initiation and progression of cancer is emerging from the idea that tumors, like normal adult tissues, contain stem cells (called cancer stem cells, or CSCs) and more importantly, could arise from them [91]. Genetic mutations in genes encoding proteins involved in critical signaling pathways for stem cells such as BMP (bone morphogenetic protein), Notch, Hedgehog and Wnt would allow cells to undergo uncontrolled proliferation and form tumors. Notch receptors and/or ligands were demonstrated to correlate with CSCs in several cancer types (Table 1). Notch activity had increasingly been investigated in breast CSC (BCSC) subpopulation [92–94]. Upregulated Notch expression was found in BCSC and initiating cell populations characterized by phenotypic markers CD44⁺/CD24⁻ [14], and was linked to tumor-initiating properties and CSC-like invasive features [14]. Notch1 NICD impairs mammary stem cell (CD24⁺CD29^{high}) self-renewal and facilitates their transformation through a cyclin D1-dependent pathway [95]. Moreover, Notch1 is related to BCSC self-renewal. The ErbB2 (HER2) promoter contains Notch–RBP-J κ binding sequences [96] that can be activated by Notch1 signaling and increase HER2 transcription in both mammary stem/progenitor cells [15,93] and BCSC. These Notch1 effects could impact the self-renewal properties of BCSC [97]. Expression of erythropoietin receptor (EpoR) on the surface of BCSC has been reported [98]. It has been shown that Notch1 interacts with erythropoietin (Epo) to maintain the self-renewing capacity of BCSC. In addition, recombinant human Epo (rhEpo) increased the numbers of BCSC and self-renewing activity in a Notch-dependent manner through induction of JAG1 [98].

Notch1 mRNA is primarily expressed in luminal cells of normal breast epithelium [99]. In contrast, Notch4 is mainly present in the basal cell population and in the BCSC-enriched population [92]. These data suggest that Notch1 and Notch4 may impact different subpopulation cells via distinct roles in BCSC. Secretase inhibitors, DAPT and DBZ, which preferentially affect Notch1 activity, only partially abrogated mammosphere-forming units (MFUs) and tumor formation, whereas Notch4 knockdown caused a significantly greater inhibition in MFUs than Notch1 [92]. Therefore, it was suggested that Notch4 signaling regulates the route from BCSC into progenitor populations. In contrast, Notch1 activity regulates the progenitor proliferation and luminal differentiation. Then, the single activation

of Notch1 receptor gene might not be sufficient to generate mammary carcinogenesis in mice. Conversely, activation of the Notch4 receptor inhibited mammary epithelial cell differentiation and could be sufficient for mammary carcinogenesis in mice [100].

Notch2 and Notch3 have been also linked to BCSC. Recently, a single nucleotide polymorphism (SNP) rs11249433 in the 1p11.2 region has been identified as a novel risk factor for breast cancer that was strongly associated with ER+ but not ER- cancer [101]. Notch2 expression was particularly enhanced in carriers of the risk genotypes (AG/GG) of rs11249433, that may favor development of ER+ luminal tumors and affect tumor-initiating cells [101]. Notch3 is a poor activator of Hairy/Enhancer of split 1 and 5 (Hes-1 and Hes-5), in contrast to that of Notch1 [102]. The Notch 3 intracellular domain represses Notch1-mediated activation through Hes promoters. Notch3 is critical for the differentiation of human progenitor cells to luminal lineage in vitro [99]. Notch activation leads to the formation of dimers of Hes and/or Hey proteins that repress the transcription of a variety of genes by interacting with co-repressors or sequestering transcriptional activators. Moreover, activation of canonical Notch signaling induces the maintenance of stem or progenitor cells through the inhibition of normal cell differentiation [13,103,104]. Several oncogenes, such as HER2 [105,106], Akt [107] as well as transcriptional factors, such as STAT3 [108], NF- κ B [109] were recently found to be associated with BCSC. Since Notch signaling crosstalks with these oncogenic pathways (discussed below), it could impact BCSC and breast cancer development through such crosstalks.

CSCs also have critical roles in promoting tumor angiogenesis. Strong evidence comes from studies of correlation of CSCs and VEGF/VEGFR. The VEGF expression in CD133+ glioma CSCs was up-regulated by 10–20 folds, combined with a dramatically increased vascular density identified by CD31 staining [126]. In addition, VEGF neutralizing antibody (bevacizumab) can deplete glioma CSCs-induced vascular EC migration and tube formation [126]. Recent studies further established that CXCL12 and its receptor CXCR4 may promote glioma CSCs growth and angiogenesis by stimulating VEGF production [127]. Malignant melanoma-initiating cells (MMICs) were identified by an ATP-binding cassette (ABC) member ABCB5 [128,129]. ABCB5 (+) melanoma cells have been shown to overexpress the vasculogenic differentiation markers CD144 (VE-cadherin) and TIE1 and are associated with CD31 (-) vasculogenic mimicry (VM), an established biomarker associated with tumor angiogenesis and increased patient mortality [130,131]. Induced VEGF was found in ABCB5 (+) cells that constitutively expressed VEGFR-1 but not in ABCB5 (-) bulk populations that were predominantly VEGFR-1 (-). In vivo, melanoma-specific shRNA-mediated knockdown of VEGFR-1 inhibited the development of ABCB5 (+) VM morphology and ABCB5 (+) VM-associated production of the secreted melanoma mitogen laminin [131]. Their results support the notion that not only VEGF, but also VEGFR-1 in MMIC regulates VM and is associated with laminin production and tumor angiogenesis. Additional evidence was provided in a report in which tumor with larger CSC population was found to recruit a substantially higher number of endothelial progenitor cells (EPCs), indicating that CSCs promote local angiogenesis and EPC mobilization via stimulating proangiogenic factors such as VEGF and SDF-1 [132].

To date, our understanding of the interplay between CSCs and angiogenesis is limited and continues to evolve with intense investigations. Although we believe Notch signaling is critical for both CSCs and angiogenesis, other signaling pathways which may have direct or indirect interactions with Notch are also important as discussed below.

6. Crosstalk between Notch signaling and other oncogenic pathways in breast cancer angiogenesis

In breast cancer, activation of Notch signaling can upregulate several factors that in turn transmit bidirectional signals among cancer cells expressing both ligands and receptors. Notch could also transmit signals among cancer, stroma and endothelium cells [10,133]. Therefore, it is not surprising that extensive crosstalks exist between Notch signaling and many others such as Wnt and Hedgehog signaling, growth factors, cytokines, oncogenic kinases as well as transcriptional factors.

6.1. Developmental signaling

6.1.1. Hedgehog signaling—Hedgehog is a developmental signaling pathway that plays key roles in embryogenesis, maintenance of adult tissue homeostasis, tissue repair during chronic persistent inflammation, and carcinogenesis [134–136]. More recently, hedgehog signaling has also been implicated in angiogenesis. While hedgehog signaling in adult angiogenesis may constitute a simple recapitulation of that in embryonic development, it should be appreciated that Hedgehog signaling occurs in embryonic angiogenesis in different developmental contexts [137]. Hedgehog family ligands, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh), undergo autoprocessing and lipid modification to generate mature peptides [138–140]. Genetic evidence in mice as well as molecular biological studies in human cells clearly indicate that dysregulated Hedgehog signaling can lead to mammary hyperplasia and tumor formation [141]. Notch ligand, JAG2 is induced by Hedgehog signaling during carcinogenesis [142].

Notch–Hedgehog crosstalk induces the expression of Hes3 and Shh through rapid activation of cytoplasmic signals, including Akt, STAT3 and the mammalian target of rapamycin (mTOR), promoting the survival of neural stem cells [143]. Hedgehog signals could induce Hes1 in both C3H/10T1/2 mesodermal and MNS70 neural cells [144]. In human breast cancer, dysregulated Hedgehog, together with Notch and Wnt signals, could also regulate the self-renewal and differentiation ability of BCSC [145].

6.1.2. Wnt signaling—Dysregulation of the Wnt pathway has been extensively studied in multiple diseases, including some angiogenic disorders. Wnt signaling activation is a major stimulator in pathological angiogenesis and thus, Wnt antagonists are considered to have a therapeutic role for neovascular disorders [146,147]. Some Wnt antagonists have been identified directly from the anti-angiogenic factor family [148–150]. Wnt1 expression led to subsequent activation of Notch signaling in human mammary epithelial cells (HMECs) [151]. Concomitant upregulation of the Wnt target genes *Lef1* and *Axin2* along with Notch ligand Dll-3 and Dll-4 was found in breast carcinomas, suggesting that the same process takes place in tumors [151]. The blockade of expression of Notch ligands abrogated

HMEC transformation by Wnt1, demonstrating the requirement for Notch–Wnt crosstalk during mammary tumorigenesis [151]. Notch-regulated ankyrin repeat protein (Nrarp) acts as a molecular link between Notch- and Wnt signaling in ECs to control stability of new vessel connections in mouse and zebrafish [152]. Dll4/Notch-induced expression of Nrarp limits Notch signaling and promotes Wnt signaling in endothelial stalk cells through interactions with Lymphoid enhancer-binding factor-1 (Lef1) [152]. These results suggest that the balance between Notch and Wnt signaling determines whether to make or break new vessel connections.

6.2. Growth factors

6.2.1. HER/ErbB—HER/ErbB genes (HER1/EGFR, HER3 and HER4) encode for receptor tyrosine kinase (RTK)-transmembrane proteins that, upon binding of several ligands (epidermal growth factor, EGF; amphiregulin; heregulin or *neu*-mouse and transforming growth factor alpha, TGF- α) regulate cell proliferation, differentiation and survival [153,154]. Dysregulation of EGF receptor (EGFR) by over-expression or constitutive activation can promote tumor angiogenesis and metastasis. Moreover, EGFR overexpression is associated with poor prognosis in many human malignancies including breast cancer [155,156].

Growth factors and their receptors play an essential role in regulating the proliferation of epithelial cells [157,158]. Over-expression of HER2 in human tumor cells is closely associated with increased angiogenesis and expression of VEGF-A. HER2 signaling may increase the rate of hypoxia-inducible factor 1 α (HIF-1 α) synthesis which in turn mediates VEGF-A expression [159]. On the other hand, inhibition of the VEGF pathway leads to suppression of tumor growth. The anti-HER2 antibody trastuzumab has been shown to inhibit tumor cell growth and VEGF expression [160,161].

Notch signaling could regulate HER2 activity since the HER2 promoter contains Notch-binding sequences [96]. Yamaguchi et al. [162] observed that down-regulation of Notch3 significantly suppressed proliferation and promoted apoptosis of the ErbB2-negative tumor cell lines. Magnifico et al. demonstrated that HER2-overexpressing cells displayed activated Notch1 signaling [97], and that inhibition of Notch1 signaling by small interfering RNA or γ -secretase inhibitor down-regulated HER2 expression and reduced sphere formation [97].

In contrast to HER2, the long-standing relationships between the EGFR and Notch signaling pathways, and the opposing effects exerted by these signal transduction cascades, have been well documented in various developmental settings and organisms [163,164]. Dai et al. found that forced overexpression of Notch1 by transfection increased EGFR expression in human breast cancer cells [165]. Moreover, overexpression of Notch1 reversed EGFR inhibitor-induced cell toxicity, suggesting that Notch and EGFR signaling may be positively cross-linked in human breast cancer. Dong et al. [166] further observed that inhibition of either EGFR or Notch signaling alone was insufficient to suppress basal-like breast tumor cell survival and proliferation, whereas simultaneous inhibition of EGFR and Notch signaling uncovered a lethal relationship between these two oncogenic pathways [166].

6.2.2. PDGF/PDGFR signaling—Platelet-derived growth factor (PDGF) is a potent angiogenic family of molecules comprised of four polypeptide chains encoded by different genes. PDGF-A and PDGF-B were identified earlier whereas PDGF-C and PDGF-D were discovered more recently [167–169]. The PDGF isoforms exert their cellular effects by specific binding to two structurally related tyrosine kinase receptors (α and β PDGFR). PDGF is a potent mitogen and chemoattractant for mesenchymal cells, neutrophils and monocytes [170]. Therefore, the expression of PDGF correlates with advanced tumor stages and unfavorable prognosis in human breast carcinomas [171]. PDGF produced in carcinomas is generally thought to act on the non-epithelial tumor stroma promoting angiogenesis [172].

The growing body of literature strongly suggests that a crosstalk between PDGF-D and Notch signaling occurs in cancer [173]. Dr. Sarkar's group demonstrated that down-regulation of PDGF-D leads to the inactivation of Notch1 and NF- κ B DNA-binding activity, as well as down-regulation of its target genes, such as VEGF and MMP-9 in pancreatic cancer cells [78]. Therefore, the inactivation of PDGF-D-mediated cell invasion and angiogenesis could in part be attributable to inactivation of Notch1 [78]. Additionally, down-regulation of PDGF-D also inhibited the Notch1 expression in breast cancer cells [174]. Interestingly, mRNA and protein expressions of Notch1–4, Dll-1, Dll-3, Dll-4, JAG2 as well as Notch downstream targets, such as Hes and Hey were significantly higher in PC3 prostate cancer cells expressing PDGF-D, indicating PDGF-D was correlated to Notch signaling [173].

6.2.3. TGF- β signaling—Genes encoding components of TGF- β signaling pathway, including ligands TGF- β 1 and TGF- β 2 and receptor TGFBR1 are functionally polymorphic in humans [175–177]. TGF- β can regulate such diverse processes as cell proliferation, differentiation, motility, adhesion, organization, and apoptosis. Both in vitro and in vivo experiments suggest that TGF- β can utilize these diverse programs to promote cancer metastasis through its effects on the tumor microenvironment, enhanced invasive properties, and inhibition of immune cell function [178,179]. Recent knockout studies of factors in TGF- β signaling have shown that this pathway is also indispensable for angiogenesis [180–182].

TGF- β signaling is linked to Notch in many processes. First, TGF- β can upregulate Notch ligands. JAG1 has been shown to be a TGF- β target gene in multiple types of mammalian cells. JAG1 and Hey1 are critical for TGF- β -induced epithelial–mesenchymal transformation (EMT) in cells derived from several organs [183]. In addition, JAG1 upregulation also contributes to TGF- β effects on cell cycle by stimulating p21 expression and cytostasis in epithelial cells [184]. Second, TGF- β and Notch can synergistically regulate common target genes in many cell types, for example, Smad3, a downstream transcription factor of TGF- β and Notch1 NICD can directly interact and form a complex with CSL that binds to specific DNA sequences as those found in the promoter of *Hes-1* [185]. Notch1 NICD not only interacts with activated Smad3 and facilitates its nuclear translocation [186], but also remains bound with pSmad3 in the nucleus where they jointly upregulate the transcription factor Forkhead box P3 (Foxp3) that is involved in immune processes [187].

6.2.4. VEGF/VEGFR-2 signaling—Vascular endothelial growth factor (VEGF) is the major angiogenic factor in physiological and pathological angiogenesis [188,189]. The expression of the VEGF gene is enhanced in a variety of angiogenic tumors [188,189]. VEGFR-2, receptor type 2 (KDR or flk-1) is generally recognized to have a principal role in mediating VEGF-induced responses and is considered as the earliest marker for EC development [190]. Moreover, VEGFR-2 directly regulates tumor angiogenesis [189–191]. In addition to its angiogenic actions in ECs, the VEGF/VEGFR-2 signaling paracrine–autocrine loop functions as an important survival process in breast cancer cells [190,192].

VEGF was first shown to act upstream of Notch in determining arterial cell fate in vascular development [193]. VEGF was demonstrated to increase Dll-4 and Notch expression, in turn leading to the activation of Notch signaling and arterial specification (expression of a set of arterial genes). Further studies in several systems established that VEGF regulates the expression of Notch signaling components [194–196]. Blocking VEGF, by intravitreal injection of soluble VEGF receptors, results in decreased sprouting and reduced expression of Dll-4 in retinal vessels [197]. Similar interactions among VEGF signaling, growing vessels and Notch components expression were found in tumor vessels [195,198–200].

Providing a feedback mechanism, Notch signaling in turn can alter expression levels of all three VEGF receptors. For example, VEGFR-2 was down-regulated by either Notch1,4 or Hey1 in ECs [201]. Reciprocally, VEGFR-2 expression increased in vessels of Dll-4 heterozygous mice or as a result of Dll-4 blockade [197]. Thus, Notch signaling can provide negative feedback to reduce the activity of the VEGF/VEGFR-2 axis in ECs. Taken together, VEGF pathway acts as a potent upstream activating stimulus for angiogenesis, whereas Notch pathway helps to shape that action appropriately [202,203]. Thus, an important feature of angiogenesis is the manifold ways in which the VEGF and Notch pathways interact [203].

6.3. Inflammatory cytokines

6.3.1. IL-6—IL-6, a multifunctional cytokine, produced by various types of cells, including macrophages and cancer cells, is an important factor for immune responses, cell survival, apoptosis, proliferation and angiogenesis [204–206]. IL-6 signals via a heterodimeric IL-6R/gp130 receptor complex, whose engagement triggers the activation of Janus (JAK) kinases, and the downstream effectors STAT proteins [204]. A number of studies implicated IL-6 and STAT3 as pro-tumorigenic and pro-angiogenic agents in many cancers including breast cancer [207–211].

Sansone et al. first determined that Notch pathway was a critical downstream target of IL-6 [212]. IL-6 treatment triggered Notch3-dependent upregulation of the Notch ligand JAG1 and promotion of primary human mammospheres and MCF-7-derived spheroid growth. Moreover, autocrine IL-6 signaling relied upon Notch3 activity to sustain the aggressive features of MCF-7-derived hypoxia-selected cells. These data support the hypothesis that IL-6 induces malignant features in Notch3-expressing stem/progenitor cells from human ductal breast carcinoma and normal mammary gland. It was also shown that the hypoxia resistance gene carbonic anhydrase (CA-IX) was activated in breast cancer cells by IL-6/Notch/JAG action and provided survival advantages under hypoxic conditions. Very recently,

within the IL-6 gene promoter region, the signature binding motif of CSL was identified and found to overlap with a consensus of NF- κ B-binding site [213]. These authors demonstrated that Notch1 positively regulates IL-6 expression via NF- κ B in activated macrophages [213].

Lee et al. established a HeLa/rtTAA/TRE-N1-IC cell line capable of doxycycline-induced expression of human Notch1 NICD [214]. They found that the induction of Notch signaling activated HIF-1 α and its target gene expression in the above cells. Interestingly, HIF-1 α expression was required for Notch signaling enhanced STAT3 phosphorylation required under hypoxia conditions. Furthermore, Src (a proto-oncogenic tyrosine kinase) was also required for the enhanced STAT3 phosphorylation in response to Notch signaling. Notch signaling activated Src/STAT3 pathway was dependent on the Notch effector Hes1 transcription factor. However, the treatment of Trichostatin A (TSA) that interferes with Hes1 transcriptional regulation did not affect STAT3 phosphorylation, and dominant negative Hes1 failed to interfere with Hes1-dependent Src/STAT3 pathway and induction of HIF-1 α . These observations indicate that Hes1-dependent activation of Src/STAT3 pathway is independent of Hes1 transcription regulation. Therefore, Hes1-dependent Src/STAT3 pathway provides a functional link between Notch signaling and hypoxia pathway.

6.3.2. IL-1 signaling—IL-1 family belongs to pro-inflammatory/-angiogenesis cytokines that is represented by two ligands: IL-1 α , IL-1 β , an antagonist: interleukin-1 receptor antagonist (IL-1Ra) and two receptors: IL-1R tI (type I receptor) and IL-1R tII (type II receptor) [215]. IL-1 plays a key role in the onset and development of the host reaction to invasion, being an important factor in the initiation of the inflammatory response and immune functions. IL-1 is also abundant at tumor sites, where it may affect the process of carcinogenesis, tumor growth and invasiveness, the patterns of tumor–host interactions and tumor angiogenesis [216]. There is also convincing evidence that IL-1 family and leptin (the major adipocytokine) crosstalk represents a major link among obesity, inflammation, angiogenesis and cancer progression [217–220].

IL-1 activates Notch signaling pathway probably through the NF- κ B pathway [221–223], which is present as a latent, inactive, light polypeptide gene enhancer (I- κ B, inhibitor of NF- κ B)-bound complex in the cytoplasm in majority of cells. IL-1 activates NF- κ B via IL-1 receptor-associated kinase (IRAK) and mitogen-activated protein kinase (MAPK) dependent inhibition of I- κ B [224,225]. c-Rel (an NF- κ B subunit) can trigger Notch1 signaling pathway by inducing expression of JAG1 [226,227]. Results from our laboratory suggest that leptin is an important inducer of IL-1 system in breast cancer cells [218]. Moreover, IL-1, Notch and leptin-induced upregulation of their gene components and NF- κ B, HIF-1 α and VEGF/VEGFR-2 are interconnected [190,228].

6.3.3. Leptin signaling—Leptin, a pluripotent cytokine secreted primarily not only by adipocytes but also by breast cancer cells, plays key roles in regulating energy intake and energy expenditure, including appetite and metabolism [229]. In the past decade, accumulating evidence indicates that leptin actions are related not only to energy metabolism, but also to reproduction, proliferation, inflammation and angiogenesis [230,231]. More recently, leptin signaling was also demonstrated to associate with BCSCs [232]. Breast cancer cells express higher levels of leptin and leptin receptor, OB-R, than

normal mammary cells. Importantly, higher levels of leptin/OB-R levels correlated with metastasis and lower survival of breast cancer patients [233–235]. In vitro, leptin was demonstrated to stimulate the proliferation of breast cancer cell lines [233,236,237]. In vivo studies clearly demonstrated a role for leptin in mammary tumor initiation and development as evidenced by the fact that mutant mice deficient in leptin ($Lep^{ob}Lep^{ob}$), or with non-functioning leptin receptors ($Lepr^{db}Lepr^{db}$) do not develop transgene-induced mammary tumors [238,239]. The disruption of leptin signaling using pegylated leptin peptide receptor antagonist (PEG-LPrA2) markedly reduced the growth of tumors in mouse models of syngeneic and human breast cancer xenografts [240,241]. These effects were accompanied by a significant decrease in VEGF/VEGFR-2, IL-1 R α , cyclin D1 and PCNA levels. Moreover, tumor angiogenesis was also impaired [240,241].

Leptin and IL-1 are associated in several pathological situations [161,242], suggesting an interplay between them. Indeed, leptin regulates IL-1 family members in a diabetic context [243] and in endometrial cancer cells [244]. Leptin was found to increase protein and mRNA levels of all components of the IL-1 system in a mouse mammary cancer cell line. Leptin-induced canonical signaling pathways (JAK2/STAT3, MAPK/ERK 1/2 and PI-3K/Akt1) were mainly involved in IL-1 upregulation. In addition, leptin upregulation of IL-1 α promoter involved the activation of SP1 and NF- κ B transcription factors [218].

Little information on leptin–Notch interactions is available. An earlier report shows that leptin regulates the expression of JAG1 and Notch4 in human cord blood CD34⁺ cells and early differentiated ECs (HUVEC) where leptin promotes cell differentiation [245]. We and others recently observed that leptin was able to activate the Notch signaling pathway in breast cancer cells [220,246]. Moreover, leptin increased the expression of both Notch receptors and ligands [220]. In these cells leptin also up-regulated Notch-target genes Hey2 and Survivin [190,220]. Leptin-induced non-canonical signaling pathways (PKC, p38 and JNK) differentially impacted on CSL promoter activity and on the expression of IL-1 system in mouse 4T1 mammary cancer cell line [218]. Interestingly, effects of leptin upregulation on pro-angiogenic factors IL-1, VEGF/VEGFR-2 and Notch were significantly abrogated by a γ -secretase inhibitor, DAPT as well as siRNA against CSL in 4T1 cells [220].

6.4. Oncogenic kinases and transcription factors

6.4.1. Ras signaling pathway—Ras signaling plays an important role in transmitting signaling from RTKs to ser/threo kinases. Among the effector molecules connected with the group of cell surface receptors, Ras transduces extracellular signals to diverse intracellular events by controlling the activities of multiple signaling pathways [247]. The multifunctional signal transducer Ras is a proto-oncogene that is frequently mutated in human cancers, including angiosarcomas [248]. Because Ras signaling impacts many cellular functions, including cell cycle regulation, apoptosis, cell survival, EC function and angiogenesis, it is a major target for the development of novel cancer treatments [248,249]. The signaling networks regulated by Ras are very complex due to their multi-faceted functions and crosstalks [250].

Crosstalk between Ras and Notch pathways has been described in pancreatic ductal adenocarcinoma [251], colorectal tumors [252], astrocytic gliomas [253], leukemia

[254,255], as well as breast cancer [256,257]. In an early report [50], Weijzen et al. demonstrated that oncogenic Ras activates Notch signaling. Notch1 was necessary to maintain the neoplastic phenotype in Ras-transformed human cells in vitro and in vivo [50]. Ras increased the expression and activity of Notch1 NICD and upregulated Notch ligand Dll-1 and presenilin-1, a protein involved in Notch processing, through a p38-mediated pathway [50]. These observations established that Notch signals were among the key downstream effectors of oncogenic Ras. Gustafson et al. [256] observed that transformation of MCF-10A cells by Harvey-Ras (Ha-Ras) induced CCAAT/enhance binding protein beta (C/EBP β), a transcriptional factor, and activated the Notch signaling pathway to block SIM2s (a transcriptional factor) gene expression. High expression level of Notch receptors, ligands and their cooperation with the Ras/MAPK pathway in several breast cancers and early precursors place Notch signaling as a key player in breast cancer pathogenesis. This offers combined inhibition of the two pathways as a new modality for breast cancer treatment [257]. Given the regulation of Ras is important for EC function, angiogenesis and activated Ras signaling is critical for vascular malformations and angiosarcoma, crosstalk between Ras and Notch pathways might occur in ECs. However, the exact role and mechanism of these two pathways in ECs need to be determined.

6.4.2. PI-3K/Akt signaling pathway—The phosphatidylinositol 3-kinase (PI-3K/Akt) pathway is a central player in a variety of cellular processes including cell growth, proliferation, motility, survival, angiogenesis, as well as EMT in tumor cells [258–261]. PI-3K/Akt pathway acts upon tumor cells in both autocrine and paracrine manners [262–264]. Notch has been shown to regulate the Akt (ser/threo) or Protein kinase B (PKB) pathway. Liu et al. [265] reported that Notch1 activation enhanced melanoma cell survival via activation of the Akt pathway. Palomero et al. [266] found that Notch1 induced up-regulation of the PI-3K/Akt pathway via Hes1, which negatively controlled the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in T-cell acute lymphoblastic leukemia (T-ALL). Additional reports also demonstrated that Notch1 crosstalks with Akt pathway in T-ALL, melanoma as well as breast epithelial cells [264,267,268]. On one hand, activation of Akt was necessary for Notch-induced protection against apoptosis in MCF-10A. On the other hand, inhibiting Notch signaling in breast cancer cells induced a decrease in Akt activity and an increase in apoptotic sensitivity [264]. Down-regulation of Notch1 or JAG1 mediated the inhibition of cell growth, migration and invasion, and the induction of apoptosis in prostate cancer. These effects were in part due to inactivation of Akt, mTOR, and NF- κ B signaling pathways [269]. In an early report [270], activated Notch1 synergizes with papillomavirus oncogenes in the transformation of immortalized epithelial cells, leading to the generation of resistance to anoikis, an apoptotic response induced by matrix withdrawal. This resistance to anoikis by activated Notch1 is mediated through the activation of PKB/Akt. The cellular responsiveness to Notch signaling dependent PI-3K/Akt pathway has also been observed in other types of cells, such as Chinese hamster ovary (CHO) cells, primary T-cells and hippocampal neurons [271].

PI3-K/Akt pathway also regulates Notch1 and DLL4 in ECs. VEGF can induce gene expression of Notch1 and DLL4 in human arterial ECs. Furthermore, the VEGF-induced specific signaling is mediated through VEGFR-1 & 2 and is transmitted via the PI3-K/Akt

pathway [272]. Other reports confirmed that PI3-K/Akt pathway can regulate Notch signaling in ECs [273,274].

6.4.3. mTOR signaling—mTOR, a key protein kinase, controls signal transduction from various growth factors and upstream proteins to the level of mRNA translation and ribosome biogenesis. mTOR is a ser/threo kinase that is often a downstream effector of PI-3K/Akt signaling pathway in breast and many other types of cancer cells. However, MAPK pathway was identified as the preferential upstream regulator of mTOR in the induction of inflammatory/pro-angiogenic molecules in endometrial cancer cells [244]. mTOR can also phosphorylate Akt [275]. mTOR has been intensely studied for over a decade as a central regulator of cell growth, proliferation, differentiation, autophagy, angiogenesis and survival [276–278]. mTOR functions as two distinct multiprotein complexes, mTORC1 and mTORC2 [275,279]. mTORC1 phosphorylates p70 S6 kinase (S6K1), eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and integrates hormones, growth factors, nutrients, stressors and energy signals. In contrast, mTORC2 is insensitive to nutrients or energy conditions. However, in response to hormones or growth factors, mTORC2 phosphorylates Akt, and regulates actin cytoskeleton and cell survival [275]. Aberrant activation of mTOR pathway is frequently found in cancer and its role in breast cancer cell proliferation and anti-cancer drug resistance has been implicated [280–282]. mTOR signaling has been reported to crosstalk with the Notch signaling pathway in several malignant cell lines [143,283–285].

Inhibition of p53 by Notch1 NICD mainly occurs through mTOR linked to PI-3K/Akt pathway. Moreover, rapamycin treatment abrogated NICD inhibition of p53 and reversed the chemoresistance [285]. Chemoresistant MCF-7 and MOLT4 (T-cell acute lymphoblastic leukemia) cells have aberrant Notch1 that can be reversed by using both PI-3K and mTOR inhibitors [285]. Efferson et al. [284] used an ERbB2-transgenic mouse model of breast cancer (neuT) to show that Notch signaling plays a critical role in tumor maintenance. Inhibition of the Notch pathway with a γ -secretase inhibitor (GSI) decreased both the Notch and mTOR/Akt pathways. Antitumor activity resulting from GSI treatment was associated with decreased cell proliferation [285]. Since mTOR is closely linked to the PI-3K/Akt pathway in ECs [286–288], it is reasonable to speculate that mTOR should also be a regulator of Notch pathway in ECs.

6.4.4. NF- κ B signaling pathway—The family of NF- κ B transcription factors is involved in the expression of key genes for innate and adaptive immunity, cell proliferation and survival, and lymphoid organ development. NF- κ B is activated in a variety of cancers [289,290] linked to tumor angiogenesis [228]. NF- κ B family, RelA (p65), RelB, c-Rel, p105/p50 and p100/p52 are evolutionarily conserved molecules that form hetero- or homodimers. The p65/p50 heterodimer, the most abundant form of NF- κ B is regulated by the so-called canonical pathway [289,291].

Numerous reports have described the bidirectional regulation of Notch and NF- κ B through different context-dependent mechanisms. First, Oswald et al. [76] clearly demonstrated that Notch was able to transcriptionally regulate NF- κ B members. RBP-J κ is a strong transcriptional repressor of p100/p52 whose effects can be overcome by activated Notch1,

suggesting that p100/p52 is a Notch target gene. Cheng et al. [292] further observed that Notch1 upregulated the expression of p65, p50, RelB, and c-Rel subunits in hemopoietic progenitor cells using Notch1 antisense transgenic (Notch-AS-Tg) mice. Second, NF- κ B subunits are also able to transcriptionally regulate Notch family members. This is supported by the findings of Bash et al. [226] that demonstrated c-Rel can activate Notch signaling pathway by up-regulating JAG1 gene expression in lymphocytes. A role for JAG1 in B-cell activation, differentiation or function was also suggested [226]. Lastly, members from Notch and NF- κ B family could physically interact with each other. Wang et al. [293] demonstrated that the N-terminal portion of Notch1 NICD interacted specifically with p50 subunit and inhibited p50 DNA binding in human NTera-2 embryonal carcinoma cells. In contrast, in T-cells Notch1 NICD was found to activate NF- κ B by directly interacting with NF- κ B and competing with I κ B α . These processes lead to the retention of NF- κ B in the nucleus. It seems that in T-cells there are two ‘waves’ of NF- κ B activation: an initial, Notch-independent phase, and a later, sustained activation of NF- κ B, which is Notch dependent [294]. Two recent reports also confirmed that Notch activation was required for NF- κ B activation in ECs [54,77].

6.4.5. HIF signaling pathway—A critical aspect of tumor biology is the sensation of oxygen in the microenvironment. In response to hypoxia, a hallmark of most solid tumors, cells adapt by regulating metabolism, erythropoiesis, and angiogenesis and by modulating pathways that result in survival or cell death. HIF is a key molecule upregulated in response to oxygen deficiency, as it acts as a master regulator of genes involved in tissue reoxygenation [295]. Additionally, HIF has been known to facilitate cancer progression by promoting tumor neoangiogenesis, cell motility, and invasion [296]. HIF is a heterodimer consisting of a constitutively expressed HIF-1 β subunit and an oxygen-regulated, unstable HIF-1 α subunit. HIF interactions with DNA are mediated through hypoxia-responsive elements (HRE) [295]. Several studies have demonstrated that HIF-1 plays important roles in the development and progression of cancer through activation of various genes involved in crucial aspects of cancer biology, including energy metabolism, vasomotor function, erythropoiesis, cell survival and angiogenesis [297].

Gustafsson et al. [298] showed evidence that hypoxia promotes the undifferentiated cell state in various stem and precursor cell populations. In this process, hypoxia blocks neuronal and myogenic differentiation in a Notch-dependent manner. Upon Notch activation under hypoxic conditions, Notch1 NICD can interact with HIF-1 α , and the complex is recruited to Notch1-responsive promoters. Sahlgren et al. [299] further demonstrated that a hypoxia/Notch/EMT axis exists in tumor cells, where Notch serves as a critical intermediate in conveying the hypoxic response into EMT. Hypoxia-induced increased motility and invasiveness of the tumor cells require Notch signaling, and activated Notch mimicked hypoxia in the induction of EMT. In this process, Notch signaling acts in synergy to control the expression of Snail-1, a zinc-finger transcriptional factor repressor of E-cadherin and a critical regulator of EMT. First, NICD could interact with the *Snail-1* promoter, and second, Notch potentiated HIF-1 α recruitment to the *lysyl oxidase (LOX)*; a copper-dependent amine oxidase) promoter and elevated the hypoxia-induced up-regulation of LOX, which stabilizes the Snail-1 protein [299]. Hypoxia increased Notch1 mRNA and protein level as well as

Notch activity, measured as Hes1 and Hey1 expression and Hes1 promoter activity. This effect was dependent on HIF-1 α [267]. These results suggest that Notch1 is under the control of oncogenes and the tissue microenvironment. Therefore, HIF-1 α and Notch signaling pathways play a critical role in the regulation of EMT and open up perspectives for pharmacological intervention within hypoxia-induced EMT, cell invasiveness and angiogenesis in tumors.

6.5. Other crosstalk signaling

6.5.1. ER signaling—Estrogens, in particular 17 β -estradiol (E2), play a pivotal role in sexual development and reproduction and are also implicated in a large number of physiological processes, including the cardiovascular system. The recognized risk factors for breast cancer are ages at: menarche, first pregnancy, and menopause. This suggests that endogenous ovarian steroids may profoundly affect initiation, promotion, and progression of carcinogenesis through a cascade of reactions initiated by activation of the ER [300,301]. ERs are known to regulate a huge number of genes affecting cancer proliferation and vascular function [302,303].

Soares et al. [51] first demonstrated that a crosstalk between estrogen and Notch signaling occurs in breast cancer and EC. The authors observed that E2 promoted 8-fold and 6-fold increases in Notch1 and JAG1 expression, respectively, in MCF-7 breast cancer cells. A similar up-regulation of both Notch1 receptor and JAG1 ligand was also found in EC. Notch gene expression was required for tubule-like structure formation in EC. Moreover, Notch gene expression, together with HIF-1 α , was upregulated by E2. In another report, E2 and parathion (an organophosphate compound and potent insecticide) alone and in combination also led to the activation of Notch signaling in MCF-10F, in the process of malignant transformation as indicated by anchorage independency and in vitro invasive capabilities [304]. Notch and ER α crosstalk in breast cancer suggests that combinations of antiestrogens and Notch inhibitors maybe more effective in treating ER α (+) breast cancers [305]. Overall, the crosstalk between Notch and estrogen signaling pathway has a significant role in human breast carcinogenesis and angiogenesis.

6.5.2. miRNA actions—There are several reports on the crosstalk between miRNA and Notch signaling pathways [306–308]. Yoo and Greenwald first reported that Notch activation leads to miR-61 mediated down-regulation of Vav, a proto-oncogene in *Caenorhabditis elegans* [308]. Interestingly, miR-61 could control the expression of oncogene orthologues Ras and Vav, indicating miRNA capacity to act as tumor suppressors [309]. Therapeutic potential of let-7 in cancer (initially identified as a timing developmental regulator in *C. elegans*) was recently reviewed [310]. In various human cell lines, Notch activation up-regulates miRNA let-7 [307]. Let-7 regulates self renewal and tumorigenicity of breast cancer cells [311], as well as ER α signaling in ER positive breast cancer [312].

On the other hand, miRNAs can regulate Notch pathways. miR-34a down-regulated the expression of Notch1 and Notch2 proteins in glioma cells [306]. miR-34 down-regulated JAG1 and Notch1 in cervical carcinoma and choriocarcinoma cells [313]. miR-34 was required for a normal cellular response to DNA damage in vivo. Therefore, a potential

therapeutic use for anti-miR-34 as a radiosensitizing agent in p53-mutant breast cancer is predicted [314]. In addition, altered miRNA signatures including miR-34 may be associated with breast carcinogenesis and metastasis [315]. Loss of miR-8/200 has been commonly observed in advanced tumors [316] and correlates with their invasion [317–319] and acquisition of stem-like properties [320,321]. Recently, miR-8/200 was identified to have the ability to inhibit Jagged1, thus attenuating Notch signaling and impeding proliferation of human metastatic prostate cancer cells [322].

7. Notch signaling as a therapeutic drug target in breast cancer

The prevailing new strategy for rationally targeted cancer treatment is aimed at the development of target-selective “smart” drugs on the basis of characterized mechanisms of action. The connection between Notch signaling, carcinogenesis and angiogenesis, as well as its crosstalk with many oncogenic signaling pathways suggest that Notch signaling may be such a candidate for multi-target drugs. The major therapeutic targets in the Notch pathway are the Notch receptors, in which GSIs prevent the generation of the oncogenic NICD and suppress the Notch activity [323,324].

Gamma-secretase is a large membrane-integral multisubunit protease complex, which is essential for Notch receptor activation [325]. Rasul et al. [326] tested the effects of three different GSIs in breast cancer cells. One inhibitor (GSI1) was lethal to breast cancer cell lines including MCF-7, MDA-MB-231, ZR-75-1, T47D and CAL-51 cells (range of IC₅₀ values: 0.6–0.9 μ M). No effect on the non-tumorigenic 226-L-U19 and 226-L-TS4 cell lines was seen in the range 0.5–40 μ M, which showed IC₅₀ values around 50 μ M. GSI1 treatment resulted in a marked decrease in γ -secretase activity and down-regulation of the Notch signaling pathway with no effects on expression of the γ -secretase components or ligands. Differential responses between tumourigenic and non-tumourigenic cell lines may be explained by the differential expression of Numb, a negative regulator of the Notch pathway, and NICD [326,327]. Non-tumourigenic cells express Numb but not NICD and the Notch pathway is not activated, in contrast, cancer cells have Numb downregulated, NICD upregulated and the Notch pathway activated, thus are sensitive to the cytotoxic effect of GSI1 by its effect on the Notch pathway [48]. In a recent report [284], the authors observed that inhibition of the Notch pathway with a GSI decreased both the Notch and mTOR/Akt pathways. Antitumor activity resulting from GSI treatment was associated with decreased cell proliferation as measured by Ki67 and decreased expression of glucose transporter Glut1 [284]. GSI effects are much higher in HER2/*neu*-positive cell lines where HER2 is amplified and/or over-expressed (ZR-75-1 and MDA-MB-453) compared with HER2-negative cells (MCF-7 and MDA-MB-231) that lack ERBB2 amplification and show low HER2 expression [86,305,326]. Since HER2 can influence the activity of Notch [96] and inhibition of HER2 via trastuzumab can activate Notch signaling [328], it will be important to consider GSI as a monotherapy or in combination with trastuzumab or lapatinib in HER2 breast cancer patients.

Triple-negative breast cancer (TNBC) is characterized by the lack of expression of ER, PgR, and HER-2. This difficult-to-treat form of breast cancer shows an undesirable tendency to overcome drug effectiveness [329,330]. In TNBC there is higher intratumoural expression of

VEGF than non-TNBC [331]. BCSCs are thought to be responsible for the development of drug-resistance and relapse of TNBC [332]. Basal-like breast cancer (BLBC) frequently expresses a CD44⁺/CD24⁻ phenotype, which has been associated with a 'stem-cell' phenotype. These cells show resistance to conventional treatment and allow repopulation of the cancer. BCSC shows some specific molecular alterations including activation of the Notch pathway [333,334]. Therefore, there is strong evidence to suggest that the Notch pathway is a key event in TNBC etiology, and that targeting the Notch pathway may improve patient outcomes by targeting angiogenesis and the hormone-insensitive chemoresistant BCSCs. As expected, GSIs can reduce the growth and dissemination of MDA-MB-231TNBC xenografts [335]. However, a recent report shows that GSIs only reduced sphere formation and xenograft growth from TNBC CD44⁺/CD24^{low+} cells, but CD44⁺/CD24^{neg} were resistant to GSI treatment [336]. Thus, while GSIs hold promise for targeting BCSCs, stem cell heterogeneity could limit GSI efficacy.

Although several GSIs have been developed into clinical trials [324], GSIs fail to distinguish individual Notch receptors. In addition, GSIs inhibit other signaling pathways [337] and cause intestinal toxicity [338], probably attributable to dual inhibition of Notch1 and Notch2 [339]. Very recently, Wu et al. [340] utilized phage display technology to generate highly specialized antibodies that specifically antagonize each receptor paralogue, enabling the discrimination of Notch1 versus Notch2 function in rodent models as well as in humans. Their results showed that inhibition of either receptor alone reduces or avoids toxicity, demonstrating a clear advantage over pan-Notch inhibitors. The gastrointestinal toxicity and abnormalities in the thymus and spleen are major side-effects with GSI use [341], likely resulting from inhibition of Notch cleavage in regulating cell-fate decisions. Therefore, close attention needs be paid to the therapeutic window so that the minimally active dose needed to inhibit Notch is employed, thereby reducing adverse side effects. In addition, development of a practical combination therapy [133] should minimize problematic side-effects.

8. Conclusion and overall perspectives

Notch signaling and its crosstalk with many signaling pathways play an important role in breast cancer cell growth, migration, invasion, metastasis and angiogenesis, as well as CSC self-renewal (see Fig. 1). Therefore, increasing attention has been paid in recent years to the development of clinically useful antagonists of Notch signaling. Better understanding of the structure, function and regulation of Notch intracellular signaling pathways, as well as its complex crosstalk with other oncogenic signals in cancer cells will be essential to ensure rational use of treatment and development of new combinatory therapeutic possibilities. Emerging novel opportunities arise from the discovery of Notch crosstalk with inflammatory and angiogenic cytokines and their links to obesity-related cancers. Combination therapy with drugs designed to prevent Notch oncogenic signal crosstalk may be advantageous over GSIs alone.

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Glossary

4T1 cells	mouse mammary cancer cell line
ADAM	a disintegrin and metalloprotease
Akt	protein kinase B
ALDH	aldehyde dehydrogenase
ANK	ankyrin
Axin2	the Axin-related protein
Bcl-2	B-cell lymphoma 2
BCSCs	breast cancer stem cells
BMP	bone morphogenetic protein
CBF1	centromere-binding factor 1
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
c-myc	Myc proto-oncogene protein
Co-A	recruits coactivator
Co-R	co-repressor
CSC	cancer stem cell
CSL	CBF1/Su(H)/Lag-1
Cyclin D1	kinase and regulator of cell cycle D1
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DBZ	dibenzazepine
DLL-1	Delta-like 1
DOS	Delta and OSM-11-like proteins
DSL	Delta/Serrate/LAG-2
E2	17 β -estradiol

EC	endothelial cell
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial–mesenchymal transformation
ER	estrogen receptor
ERK 1/2	extracellular regulated kinase 1 and 2
GSI	a γ -secretase inhibitor
HD	heterodimerization
HDAC	histone deacetylases
HIF-1α	hypoxia regulated factor-1 α
HUVECs	human umbilical vein ECs
ICN	intracellular region of Notch
IL-1	interleukin-1
IL-1R tI	interleukin-1 type I receptor
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
JAK2	Janus kinase 2
MAPK	mitogen activated protein kinase
MCF-7	ER positive human breast cancer cell line
MDA-MB-231	ER negative human breast cancer cell line
MFE	Mammosphere-forming efficiency
miRNA	MicroRNA
mTOR	mammalian target of rapamycin
NECD	Notch extracellular domain
NEXT	Notch extracellular truncation
NF-κB	eukaryotic nuclear transcription factor kappa B
NICD	Notch intracellular domain
NRR	negative regulatory region
OB-R	leptin receptor

PDGF	platelet-derived growth factor
PEST	proline, glutamine, serine and threonine residue
PI-3K	phosphoinositide 3-kinase
RhoC	Ras homolog gene family, member C
Src	a proto-oncogenic tyrosine kinase
STAT3	signal transducer and activator of transcription 3
TACE	tumor necrosis factor- α -converting enzyme
TAD	transactivation domain
TAM	tamoxifen
T-ALL	T-cell acute lymphoblastic leukemia
TGF-β	transforming growth factor beta
TNBC	Triple-negative breast cancer
TNF-α	tumor necrosis factor alpha
TSA	Trichostatin A
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor 2 or KDR or Flk-1

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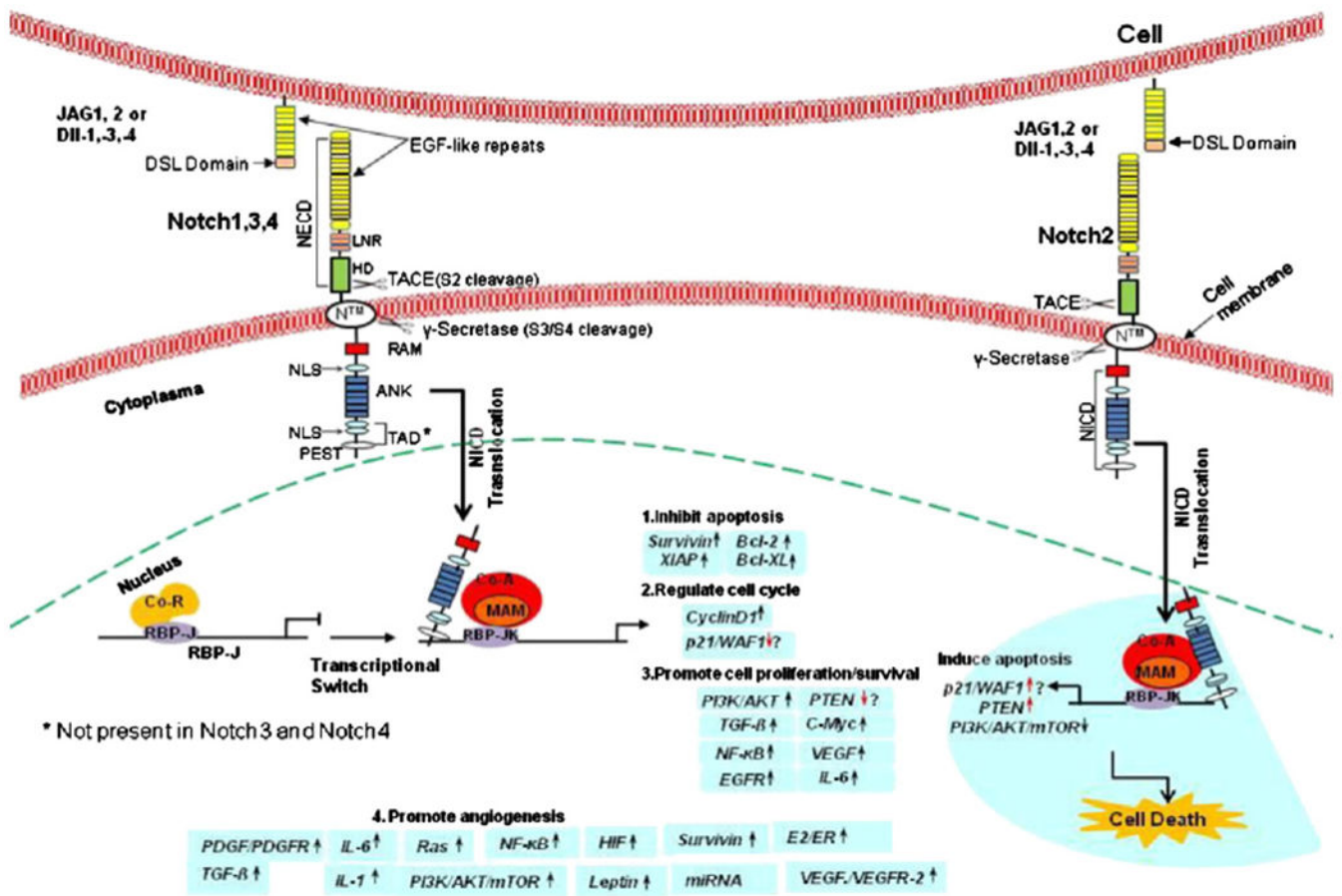


Fig. 1. Notch signaling and its possible downstream targets in human cancers and angiogenesis. Mammalian ligands of Notch are membrane-bound proteins containing an extracellular NH₂-terminal Delta/Serrate/LAG2 (DSL) motif followed by epidermal growth factor (EGF)-like repeats. Notch receptors are broadly expressed on the cell surface as heterodimers containing a Notch extracellular domain (NECD) composed by multiple extracellular EGF-like repeats and three Lin12/Notch repeats (LNR). Notch receptor cytoplasmic region or Notch intra-cellular domain (NICD) contains one nuclear localization signals (NLS) linking RAM domain to six ankyrin (ANK) repeats (ANK domain) followed by an additional bipartite NLS, a loosely defined transactivation domain (TAD), and a conserved proline/ glutamic acid/ser/threo-rich domain (PEST domain). In the absence of activated Notch signaling, the DNA binding protein RBP-Jk (CSL/CBF1/Su (H)/Lag1, a transcription factor) forms a complex with corepressor molecules that represses transcription of target genes. Ligand binding to NECD triggers successive proteolytic cleavages of Notch cytoplasmic region by ADAM and γ -secretase, proteases resulting in the release of NICD, which translocates into nucleus and removes corepressors from RBP-Jk. This allows RBP-Jk to recruit a coactivator complex composed of Mastermind (MAM) and several transcription factors to transcriptionally activate Notch target genes. Activation of Notch could impact on the following processes in human cancer: 1) inhibition of apoptosis through upregulation of Survivin [86,87] and Bcl-2 protein family [342]; 2) activation of the cell cycle through

upregulation of Cyclin D1 [95]; 3) promotion of cell proliferation/survival through upregulation of PI-3K/Akt [265], TGF- β [184], *c-myc* [80], NF- κ B [109], EGFR[165] and IL-6 [343] pathways; 4) stimulation of angiogenesis and VEGF/VEGFR-2 autocrine/paracrine loop by upregulation of IL-1 system and VEGF/VEGFR-2 [190,220]; 5) suppression of cancer growth in some cellular situations. For example, Notch2 signaling may function as a tumor suppressor through upregulation of PTEN or down-regulation of PI-3K/Akt/mTOR [344]. Coordinated actions of Notch affect cancer cell growth, migration, invasion, metastasis and angiogenesis, as well as CSC self-renewal.

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Table 1

Function of Notch signaling in cancer stem cells (CSCs).

Cancer types	CSC marker	Notch/ligand	Function or changes	References
Bladder cancer	K5, p63, BMI-1, OCT-4	Notch1	Activation	[110]
Brain medulloblastoma	CD133, CD15	DLL1	Cell proliferation	[111]
Brain glioblastoma	CD133	Jagged1, DLL1, DLL4	Cancer stem-like cell self-renewal	[112]
Brain glioblastoma	CD133	Notch1	Cell proliferation	[113]
Brain glioblastoma	CD133, NESTIN, BMI1	Notch2	Tumor growth	[114]
Brain medulloblastoma	CD133	HES1	Cell proliferation and anchorage	[115]
Brain medulloblastoma	CD133	Notch pathway	Stem-like cells self-renewal	[116]
Breast cancer	CD44Hi/CD24Low	Notch1	CSC self-renewal	[117]
Breast cancer	CD44Hi/CD24Low	Notch1	Brain metastases	[118]
Breast cancer	CD24(-) CD44(+)	Notch1	Cell proliferation	[119]
Breast cancer	ESA(+)/CD44(+)/CD24(low)	Notch4	Stem cell activity & tumor formation	[92]
Colon cancer	CD133, CD44, ESA, ALDH1	Notch	Prevention of apoptosis	[120]
Lung cancer	ALDH(+)	Notch3	CSC maintenance	[121]
Liver cancer	Oct3/4, OV6, CD133, EpCAM	Notch	Self-renewal, extensive proliferation	[122]
Liver cancer	EpCAM	Jagged1	Tumorigenesis	[123]
Ovary cancer	CD44 High/CD24 Low	Notch1	Proliferation/division and survival	[124]
Pancreatic cancer	CD44 and EpCAM	Notch1	Acquisition of EMT phenotype	[125]