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Role of Notch and its oncogenic signaling crosstalk in breast cancer

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Abstract

The Notch signaling plays a key role in cell differentiation, survival, and proliferation through diverse mechanisms. Notch signaling is also involved in vasculogenesis and angiogenesis. Moreover, Notch expression is regulated by hypoxia and inflammatory cytokines (IL-1, IL-6 and leptin). Entangled crosstalk between Notch and other developmental signaling (Hedgehog and Wnt), and signaling triggered by growth factors, estrogens and oncogenic kinases, could impact on Notch targeted genes. Thus, alterations of the Notch signaling can lead to a variety of disorders, including human malignancies. Notch signaling is activated by ligand binding, followed by ADAM/Tumor necrosis factor-α-converting enzyme (TACE) metalloprotease and γ-secretase cleavages that produce the Notch intracellular domain (NICD). Translocation of NICD into the nucleus induces the transcriptional activation of Notch target genes. The relationships between Notch deregulated signaling, cancer stem cells and the carcinogenesis process reinforced by Notch crosstalk with many oncogenic signaling pathways suggest that Notch signaling may be a critical drug target for breast and other cancers. Since current status of knowledge in this field changes quickly, our insight should be continuously revised. In this review, we will focus on recent advancements in identification of aberrant Notch signaling in breast cancer and the possible underlying mechanisms, including potential role of Notch in breast cancer stem cells, tumor angiogenesis, as well as its crosstalk with other oncogenic signaling pathways in breast cancer. We will also discuss the prognostic value of Notch proteins and therapeutic potential of targeting Notch signaling for cancer treatment.

Keywords

Notch; Breast cancer; Leptin; Tumor angiogenesis; Carcinogenesis; Cancer stem cell; NILCO; IL-1

1. Introduction

Breast cancer is a major cancer leading in both incidence and mortality in women. The American Cancer Society estimated that in the United States approximately 192,370 new cases of invasive breast cancer, 62,280 in situ cases, and 40,170 deaths were occured among US women in 2009 [1]. Even though breast cancer recurrence rates have been significantly

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reduced by adjuvant therapies such as chemotherapy, hormonal and biological therapies, recurrence still occurs in a substantial proportion of breast cancer patients after these treatments. Recently, Notch has been described as a major culprit (one of the three members of the so-called axis of evil) in breast cancer; the other two being HER2 and cancer stem cells [2]. This is probably true since Notch was involved in a number of drug resistance incidents, including anti-tamoxifen and anti-trastuzumab resistance in breast cancer [3].

Although several valuable reviews have been focused on the role of Notch signaling in several types of cancer, including breast cancer [4–7] in this review we will focus on recent advancements in identification of aberrant Notch signaling in breast cancer. Further, we will review the possible underlying mechanisms of Notch in breast cancer, including its potential role in breast cancer stem cells and tumor angiogenesis as well as its crosstalk with other oncogenic signaling pathways.

2. Notch signaling--an overview

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 [8–9]. Such signals are mainly transmitted through direct contact between adjacent cells expressing Notch receptors and their ligands. Notch receptors act in response to the ligands expressed by adjacent cells to regulate cell fate specification, differentiation, proliferation, and survival [10].

Notch signaling pathway is frequently deregulated in several human malignancies. Up-regulated expression of Notch receptors and their ligands have been found in cervical, colon, head and neck, lung, renal carcinoma, pancreatic cancer, acute myeloid, Hodgkin and Large-cell lymphomas, as well as breast cancer [11–13]. Activation of Notch has been shown to cause mammary carcinomas in mice [14–16]. Notch is suggested to transform MCF-10 cells and to protect transformed cells from p53 mediated induction of pre-apoptotic protein NOXA [17]. Furthermore, Notch signaling inhibitors, NUMB and NUMB-like (NUMBL) were found to be lost in approximately 50% of human mammary carcinomas due to specific Numb ubiquitination and proteasomal degradation [18]. Low expression of NUMB and higher levels of Notch1 and BIRC5 (encoding survivin) have been linked to basal-like phenotype and cancer stem cell markers in primary breast cancer [19]. NUMB and NUMBL display a complex pattern of functions such as the control of asymmetric cell division and cell fate choice, endocytosis, cell adhesion, cell migration, ubiquitination of specific substrates and a number of signaling pathways [20–21].

Signals exchanged between neighboring cells through the Notch receptor can amplify and consolidate molecular differences, which eventually dictate cell fates. Thus, Notch signals control how cells respond to intrinsic or extrinsic developmental cues that are necessary to unfold specific developmental programs [22]. This concept is also applicable to activities elicited by Notch signals in breast and other cancer types. Indeed, Notch signaling has the potential to affect the path of differentiation, proliferation, and apoptosis in both normal developmental and abnormal cellular growth programs. Remarkably, the same signaling pathways within different contexts can trigger a variety of cellular activities. Therefore, cancer progression activities triggered by Notch and its crosstalks are context dependent. This represents an important point in understanding the variety of outcomes associated with Notch signaling.

3. 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 [12–13,22]. 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 composed by specialized tandem EGF repeats] and EGF motifs. The other subtypes of DSL canonical ligands include Dll-3 and Dll-4 that lack DOS motif [23–25]. Both the DSL and DOS domains are crucial for physical binding with Notch receptor [11]. 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* studies [24,26–31], which may be an explanation for the odd issues of the diverse and frequent effects of Notch signaling with the small number of canonical DSL ligands and receptors in vertebrate genomes [24].

Notch receptors belong to a large single-pass type 1 transmembrane protein. Notch extracellular domain consists of 29–36 tandem array of EGF (epidermal growth factor)-like repeats, followed by a conserved negative regulatory region (NRR or LNR) consisting of three cystein-rich Notch Lin12 repeats (N/Lin 12) and a heterodimerization (HD) domain [32]. Notch family members differ in the number of EGF-like repeats; however, they share many similarities in structure [11,33]. EGF-like repeats mediate ligand binding, whereas NRR functions to prevent both ligand-dependent and -independent signaling [33]. 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 (PEST) that controls receptor half life [11,34–35] (Figure 1).

S1 cleavage of precursor of the Notch receptor occurs constitutively in the Golgi network by a furin-like convertase and the two fragments are re-assembled as a non-covalently linked heterodimeric receptor at the cell surface [10]. Mature Notch receptors are heterodimers made up of an extracellular subunit, a transmembrane subunit (N™) and a cytoplasmatic subunit. Binding of Notch heterodimer to ligand triggers S2 cleavage. This process takes places at the cell surface. N™ 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) and then by γ-secretase_t Site 3 and 4 (S3 cleavage) [36]. S2 cleavage releases the Notch extracelluar domain (NECD) from the heterodimer and creates a membrane-tethered Notch extracellular truncation (NEXT), which becomes a substrate for γ-secretase. γ-secretase mediated S3 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 [10]. 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 [12–13]. In the absence of NICD, CSL may interplay with ubiquitous corepressor (Co-R) proteins and histone deacetylases (HDACs) to repress transcription of some target genes [37–38].

4. Notch signaling target gene

The best-characterized Notch targets are transcriptional repressors of the Hes (Hes1–7) and Hey subfamilies (Hey1, Hey2, HeyL, HesL/HelT, Dec1/BHLHB2, Dec2/BHLHB3) [39–41]. 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. Their biological function in breast cancer remains relatively unclear and may

be totally different. Hes1 is a tumor suppressor in epithelial cells and acts as a mediator of 17β-estradiol (E2) proliferative effects on breast cancer cells [42–43]. In contrast, Hes6 is a novel estrogen-regulated gene and a potential oncogene overexpressed in breast cancer with tumor-promoting and proliferative functions [44]. Other Notch target genes include proteins and factors involved in the control of the cell cycle and survival processes like: p21WAF1/Cip1 (a cyclin-dependent kinase inhibitor that acts as both a sensor and an effector of multiple anti-proliferative signals), *Deltex* (a positive regulator of Notch activity), nuclear factor-kappa B (NF-κB) (a transcriptional factor), *cyclin D1* (a mitogenic sensor and allosteric activator of cyclin dependent kinase CDK4/6) and *c-myc* (an oncogene and cell cycle regulator). c-myc deregulation is one of the hallmarks of many cancers [12–13,22,45–46].

Notch signaling was earlier identified as a modulator of apoptosis. NICD interacts and mediates p53 inactivation through phosphorylation [47]. Recently, survivin, a member of the inhibitor of apoptosis (IAP) family of proteins and an inducer of cell proliferation, was identified as a novel Notch target gene [48–49]. Notch stimulation resulted in direct activation of survivin gene transcription through at least one RPB-Jκ site in the survivin promoter [50]. Dr. Altieri's group uncovered a novel Notch-survivin signaling axis. They found that activation of Notch resulted in direct transcriptional up-regulation of survivin in ER∓breast cancer cells [51–52]. This Notch-survivin signaling axis may contribute to worse clinical outcome of basal breast cancer (triple negative) patients and may open new prospects for individualized therapy of these recurrence-prone patients.

5. Notch signaling in normal breast development

Mammary gland development is governed by a variety of signaling pathways involved in cell fate and cell differentiation decisions. The Notch4 gene has been shown to be involved in normal mammary development [53–54]. *In vitro*, overexpression of the constitutively active form of Notch4 (MW: 250kDa and NICD MW: 80kDa) inhibits differentiation of normal breast epithelial cells [54]. *In vivo*, transgenic mice expressing a constitutively active form of Notch4 in the mammary gland fail to develop secretory lobules during gestation, and subsequently develop mammary tumors [53]. This supports a role for Notch in normal breast development, and suggests that alterations in Notch4 might play a significant role in breast-cancer development. Indeed, Notch family members have been detected in mammospheres and Notch ligands may affect the self-renewal and differentiation of normal mammary epithelial cells [55–56].

Notch3 may also play a role in normal breast development [57–58]. Notch3 signaling was blocked in bipotent colony forming cells (CFCs). It promoted a substantial decrease in formation of luminal-type colonies and an increase in myoepithelial-type colonies. These data suggest Notch3 is one of the key genes for the luminal cell commitment. It seems likely that the bipotent CFCs correspond to stem and/or multilineage progenitor cells whereas the luminal CFCs correspond to a lineage-restricted progenitor population. The role of other Notch signaling members in normal breast development are not clear and need further studies [58].

6. Activation of Notch signaling in breast cancer

Among Notch receptors with potential roles in breast carcinogenesis, Notch1 (MW: 272kDa and NICD MW: 110–120kDa) is relatively the best studied [15–17]. The importance of Notch1 in carcinogenesis was first seen in transgenic mice generated by the mouse mammary tumor virus (MMTVD)/myc which can spontaneously develop oligoclonal $CD4^+CD8^+$ T-cell tumors [59]. Notch1, a putative novel collaborator of *c-myc*, was mutated in a high proportion (52%) of these tumors [59]. These mutations led to high expression of

truncated Notch1 proteins. The role of Notch1 in the development of mouse mammary tumors was further established by using MMTV/neu Tg mice infected with the MMTV. The Notch1 gene was identified as a novel target for MMTV provirus insertional activation. MMTV insertion in the Notch1 gene induced the overexpression of 5' truncated ~7 kb RNA (coding a 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* [15]. Notch1 was further found to be a mediator of oncogenic Ras (retrovirus-associated DNA sequence kinase, small cytoplasmic GTP-binding proteins) [60]. Mutated Ras occurs in early breast cancer and plays a central role in breast carcinogenesis [60]. Moreover, Notch1 and JAG1 were co-upregulated upon estrogen treatment not only in breast cancer MCF-7 cells, but also in endothelial cells, suggesting a role of Notch1-JAG1 in angiogenesis [61]. A synergistic effect of high levels of co-expression of Notch1 and JAG1 on overall survival of breast cancer patients has also been shown [62].

Activated Notch2 (MW: 205 and NICD MW: 110kDa) in normal mammary epithelial cells *in vivo* has not been reported. However, unlike other Notch members, Notch2 expression was demonstrated to be related with better survival in patients with breast cancer with high expression associated with well-differentiated tumors [63]. These data support the intriguing model that Notch2 activation corresponds to decreased tumor aggressiveness. O'Neill et al [64] found that constitutively activated Notch2 NICD in the human adenocarcinoma line MDA-MB-231 increased apoptosis *in vitro*. Notch2 NICD suppressed tumor take and growth, leading to 60% decrease in number of tumors that were significantly smaller and necrotic. These results demonstrated that Notch2 signaling is a potent inhibitory signal in human breast cancer carcinogenesis.

Upregulation of Notch3 NICD (MW: 244 and NICD MW: 86kDa) in mammary glands led to the development of mammary tumor, suggesting Notch3 has transforming potentials *in vivo* [65]. Yamaguchi et al [66] further found Notch1 and Notch3 NICD proteins in most breast cancer cell lines. Notch3 signaling activated CSL-mediated transcription in these cells. In a recent report [67], stable short hairpin RNA interference of Notch3 knockdown in breast cancer cells decreased osteoblast- and transforming growth factor (TGF)-β1-stimulated colony formations. Moreover, osteolytic lesions were significantly reduced following inoculation of cells with constitutively reduced Notch3 expression. These results suggest Notch3 knockdown may stand as a novel mechanism for decreasing breast cancer derived bone metastasis.

Genetic alterations of Notch4 that lead to deregulated levels of the Notch4 NICD represent gain-of-function mutations associated with mammary carcinogenesis [68–69]. 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 suggested Notch4 can transform MCF-10A cells [70]. 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 [14,53].

7. Notch signaling and breast cancer stem cells

A new theory about the initiation and progression of solid tumors, including breast cancer, is emerging from the idea that tumors, like normal adult tissues, contain stem cells (called cancer stem cells, CSC) and thus, what could arise from them [71]. 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. In particular, Notch activity is increasingly

investigated in breast CSC (BCSC) subpopulation [55–56,72], since Notch signaling pathway has key functions in normal breast development as well as in the development and progression of breast cancer as was shown in section 6. The data from Farnie et al consistently show that up-regulated Notch expression is found in BSCS and initiating cell populations identified by phenotypic markers $CD44^+/CD24^-$ [4]. $CD44^+/CD24^-$ phenotype in breast cancer cells have been linked to CSC-like cells showing tumor-initiating properties and invasive features. However, it is unclear whether their presence within a tumor has clinical implications [4].

Jolicoeur's group studied the function of Notch1 in mammary tumor development using transgenic mice expressing Notch1 NICD [73]. Their data suggest that Notch1 NICD impairs mammary stem cell ($CD24^+CD29^{high}$) self-renewal and facilitates their transformation through a cyclin D1- dependent pathway [73]. Moreover, Notch1 is related to BCSC self-renewal. ErbB2 (HER2) promoter contains Notch-RBPJκ binding sequences [74] that can be activated by Notch1 signaling to increase HER2 transcription in both mammary stem/progenitor cells [5,55] and BCSC. These Notch1 effects could affect self-renewal properties of BCSC [75]. Moreover, Notch1 interacts with erythropoietin (Epo) to maintain the self-renewing capacity of BCSC. Expression of erythropoietin receptor (EpoR) was found on the surface of BCSC [76]. In addition, Recombinant human Epo (rhEpo) increased the numbers of BCSC and self-renewing activity in a Notch-dependent manner through induction of JAG1 [76]. These observations may explain the negative impact of recombinant Epo on survival and/or tumor control of breast cancer patients with EpoR-positive tumors [76].

Notch1 mRNA is primarily expressed in luminal cells of normal breast epithelium [77]. In contrast, Notch4 is mainly present in the basal cell population and in the BCSC-enriched population [72]. These data suggest that Notch1 and Notch4 may impact on different subpopulation cells and have different roles in BCSC. Harrison et al found that 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 [72]. Based on these observations, they proposed that Notch4 signaling regulates the route from BCSC into progenitor populations. In contrast, Notch1 activity regulates the progenitor proliferation and luminal differentiation. Therefore, the single activation of Notch1 receptor gene is not sufficient to generate mammary carcinogenesis in mice. Conversely, activation of the Notch4 receptor inhibits mammary epithelial cell differentiation and is sufficient to generate mammary carcinogenesis in mice [78].

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 and strongly associated with ER+ versus ER− cancer [79]. Notch2 expression was particularly enhanced in carriers of the risk genotypes (AG/GG) of rs11249433 that may favor development of ER+ luminal tumors and affects tumor-initiating cells [79]. Notch3 is a poor activator of Hes-1 and Hes-5 in contrast to that of Notch1 [80]. The Notch3 intracellular domain represses notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters. Notch3 is critical for the differentiation of human progenitor cells to luminal lineage *in vitro* [77]. 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 differentiation [22,81–82].

In recent years, several oncogenes, such as HER2 [2,83], Akt [84] as well as transcriptional factors, such as signal transducer and activator of transcription 3 (STAT3) [85], NF-κB [86] were associated with BCSC. Since Notch signaling crosstalk with these oncogenic pathways (will be discussed below) it could impact on BCSC and breast cancer development.

8. Crosstalk among Notch signaling and other oncogenic pathways in breast cancer

In human cancers, 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 [12,87]. Therefore, it is not surprising that Notch signaling crosstalks with many oncogenic signaling pathways, such as developmental signals, i.e., Wnt and Hegdehog signaling, growth factors, cytokines, oncogenic kinases as well as transcriptional factors, etc.

8.1. Developmental signaling

8.1.1 Hedgehog signaling—Hedgehog is a developmental signaling pathway that plays key roles in a variety of processes, such as embryogenesis, maintenance of adult tissue homeostasis, tissue repair during chronic persistent inflammation, and carcinogenesis [88–90]. Hedgehog family ligands, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh), undergo autoprocessing and lipid modification to generate mature peptides [91–93]. Genetic evidence in mice as well as molecular biological studies in human cells clearly indicate that deregulated Hedgehog signaling can lead to mammary hyperplasia and tumor formation [94]. Notch ligand, JAG2 is induced by Hedgehog signaling during carcinogenesis [95]. However, the exact role(s) activated Hedgehog signaling plays in the development or progression of breast cancer remain unclear.

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 [96]. Hedgehog signals could induce Hes1 in both C3H/10T1/2 mesodermal and MNS70 neural cells [97]. In human breast cancer, deregulated Hedgehog, together with Notch, Wnt signals, could also regulate self-renewal and differentiation ability of BCSC [98].

8.1.2 Wnt signaling—Wnt signaling is a developmental signaling pathway that plays a critical role in regulating the normal development of the mammary gland. Remarkably, deregulation of Wnt signaling causes breast cancer [99–100]. Wnt and Notch signaling are well established oncogenic factors in the mouse mammary gland. Wnt1 expression led to subsequent activation of Notch signaling in human mammary epithelial cells (HMEC) [101]. Concomitant upregulation of the Wnt target genes *Lef1* and *Axin2* along with with Notch ligand Dll-3 and Dll-4 was found in a panel of 34 breast carcinomas, suggesting that the same process takes place in tumors [101]. Moreover, the blockade of expression Notch ligands abrogated HMEC transformation by Wnt1, demonstrating that there is a need for Notch-Wnt crosstalk during mammary tumorigenesis [101]. However, Wnt and Notch signals do not always cooperate in tumor initiation and progression. Both pathways are involved in skin stem cell regulation and differentiation. However, Notch1 is a tumor suppressor in the epidermis [102], whereas Wnt signaling is oncogenic [103–104]. Moreover, Wnt and Notch signals have opposing effects on the fate of stem cells, with Notch promoting differentiation and Wnt promoting stem cell self renewal and proliferation [105].

8.2. Growth factors

8.2.1. HER/ErbB—HER/ErbB genes (HER1/EGFR, HER3 and HER4) encode for receptor tyrosine kinase (RTK)-transmembrane proteins that induce signaling upon the binding of several ligands (epidermal growth factor, EGF; amphiregulin; heregulin or *neu*-mouse and transforming growth factor alpha, TGF-α) and are involved in the regulation of cell proliferation, differentiation and survival [106–107]. Deregulation of EGF receptor (EGFR) by over-expression or constitutive activation can promote tumor processes including angiogenesis and metastasis. Moreover, EGFR over-expression is associated with poor prognosis in many human malignancies including breast cancer [108–109].

ERbB2 or HER2/*neu* (HER2 in humans and *neu* in mice) is an orphan receptor without known ligands. The HER2/*neu* gene has been implicated in cancer with special emphasis in breast cancer [107,110]. Amplification of HER2 occurs in 20%–25% of breast cancers and is associated with an aggressive tumor phenotype and poor prognosis [111].

Notch signaling could regulate HER2 activity since the HER2 promoter contains Notch-binding sequences [74]. Yamaguchi et al [66] observed that down-regulation of Notch1 by RNA interference had little or no suppressive effects on the proliferation of either ErbB2-positive or ErbB2-negative cell lines. In contrast, down-regulation of Notch3 significantly suppressed proliferation and promoted apoptosis of the ErbB2-negative tumor cell lines. Their findings indicate that Notch3 rather than Notch1-mediated signaling plays an important role in the proliferation of ErbB2-negative breast tumor cells. Furthermore, targeted suppression of Notch3 signaling pathway may be a promising strategy for the treatment of ErbB2-negative breast cancer [66]. Magnifico, et al demonstrated that HER2-overexpressing cells display activated Notch1 signaling [75]. Their results show that the inhibition of Notch1 signaling by small interfering RNA or γ-secretase inhibitor resulted in down-regulation of HER2 expression and decrease of sphere formation [75]. These studies show important interactions between the Notch1 and HER2 pathways, both of which are involved in the progression of breast cancer and regulation of cancer stem cells. However, these authors did not investigate the effects of Notch3 silencing.

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 [112–113]. A crosstalk between EGFR and Notch pathways occurs in gliomas [114], lung [115], skin cancers [116] and breast cancers [117–118]. Dai et al found that forced overexpression of Notch1 by transfection increased EGFR expression in human breast cancer cells [117]. 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 [118] further observed that inhibition of either EGFR or Notch signaling alone was insufficient to suppress basal-like breast tumor cell (defined by its specific pattern of gene expression that is similar to normal breast basal cells) survival and proliferation. However, simultaneous inhibition of EGFR and Notch signaling uncovered a lethal relationship between these two oncogenic pathways. These results show that Notch pathway activation could contribute to the resistance to EGFR inhibition in triple negative cancer cells, and might provide a novel treatment strategy for basal-like breast tumor.

8.2.2. PDGF/PDGFR signaling—Platelet-derived growth factor (PDGF) is a potent angiogenic family of molecules comprised of four polypeptide chains encoded by different genes. Two of them have already been identified: PDGF-A and PDGF-B, while PDGF-C and PDGF-D were recently discovered [119–121]. 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 [122]. Therefore, the expression of PDGF correlates with advanced tumor stages and unfavorable prognosis in human breast carcinomas [123]. PDGF produced in carcinomas is generally thought to act on the non-epithelial tumor stroma promoting angiogenesis [124].

The growing body of literature strongly suggests that a crosstalk between PDGF-D and Notch signaling occurs in cancer [125]. Dr. Sarkar's group demonstrated that down-regulation of PDGF-D leads to the inactivation of Notch1 and NF-κB DNA-binding activity as well as down-regulation of its target genes, such as VEGF and MMP-9 in pancreatic cancer cells [126]. Therefore, the inactivation of PDGF-D-mediated cell invasion and angiogenesis could in part be due to inactivation of Notch1 [126]. Additionally, down-regulation of PDGF-D also inhibited the Notch1 expression in breast cancer cells [127]. Interestingly, mRNA and protein expression 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 a prostate cancer PC3 expressing PDGF-D cells, indicating PDGF-D was correlated to Notch signaling [125].

8.2.3. TGF-β signaling—Genes encoding components of TGF-β signaling pathway, including ligands TGF-β1 and TGF-β2 as well as receptor TGFBRI, are functionally polymorphic in humans [128–130]. TGF-β can regulate such diverse processes as cell proliferation, differentiation, motility, adhesion, organization, and programmed cell death. 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 [131–132].

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 [133]. In addition, JAG1 upregulation also contributes to TGF-β effects on cell cycle by stimulating p21 expression and cytostasis in epithelial cells [134]. Second, TGF-β and Notch can synergistically regulate same 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* [135]. Notch1 NICD not only interacts with activated Smad3 and facilitates its nuclear translocation [136], 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 [137].

8.2.4. VEGF/VEGFR-2 signaling—Vascular endothelial growth factor (VEGF) is the major angiogenic factor in physiological and pathological angiogenesis [138–139]. The expression of the VEGF gene is enhanced in a variety of angiogenic tumors including breast cancer [138–139]. 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 endothelial cell development (Review see [140]). Moreover, VEGFR-2 directly regulates tumor angiogenesis [139–141]. In addition to its angiogenic actions in endothelial cells the VEGF/VEGFR-2 signaling paracrine-autocrine loop functions as an important survival process in cancer cells [140].

VEGF was first shown to act upstream of Notch in determining arterial cell fate in vascular development [142]. 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 [143–145]. Blocking VEGF, by intravitreal

injection of soluble VEGF receptors, results in decreased sprouting and reduced expression of Dll-4 in retinal vessels [146]. Similar interactions among VEGF signaling, growing vessels and Notch components expression were found in tumor vessels [144,147–149].

Providing a feedback mechanism, Notch signaling in turn can alter expression levels of all three VEGF receptors. For example, VEGFR-2 was downregulated by either Notch1,4 or Hey1 in endothelial cells [150]. Reciprocally, VEGFR-2 expression increased in vessels of Dll-4 heterozygous mice or as a result of Dll-4 blockade [146]. Thus, Notch signaling can provide negative feedback to reduce the activity of the VEGF/VEGFR-2 axis in endothelial cells. A similar scenario might occur in breast cancer where VEGF/VEGFR-2 and Notch play prominent roles. However, this needs to be investigated.

The emerging picture is that VEGF pathway acts as a potent upstream activating stimulus for angiogenesis, whereas Notch pathway helps to shape that action appropriately [151–152]. Thus, an important feature of angiogenesis is the manifold ways in which the VEGF and Notch pathways interact [152].

8.3. Inflammatory cytokines

8.3.1. IL-6—IL-6, a multifunctional cytokine, is an important factor for immune responses, cell survival, apoptosis, angiogenesis and proliferation [153]. 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 [153]. A number of studies implicated IL-6 and STAT3 as pro-tumorigenic agents in many cancers, including breast cancer. IL-6 levels are significantly elevated in breast cancer patients, associated with poor prognosis [154–155].

Sansone et al first determined that Notch pathway is a critical downstream target of IL-6 [156]. 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. Thus, 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. These authors also pointed out that the hypoxia resistance gene carbonic anhydrase (CA-IX) is activated in breast cancer cells by IL-6/Notch/JAG action and provides survival advantages under hypoxic conditions.

Lee et al established HeLa/rtTAA/TRE-N1-IC cell line which was capable of doxycycline-induced expression of human Notch1 NICD [157]. They found that the induction of Notch signaling activates hypoxia inducible factor-1 alpha (HIF-1α) and its target gene expression in the above cells. Interestingly, Notch signaling enhanced STAT3 phosphorylation required for HIF-1α expression under hypoxia conditions. Furthermore, Src (a proto-oncogenic tyrosine kinase) was 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-1α. 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.

8.3.2. IL-1 signaling—IL-1 family belongs to pro-inflammatory/-angiogenesis cytokines and is represented by two ligands: IL-1α, IL-1β, an antagonist: interleukin-1 receptor

antagonist (IL-1Ra) and two receptors: IL-1R tI (type I receptor) and IL-1R tII (type II receptor) [158]. 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 [159]. There is also convincing evidence that IL-1 family and leptin (the major adipocytokine) crosstalk represents a major link among obesity, inflammation, angiogenesis and breast cancer progression ([160–161] and unpublished data).

IL-1 activates Notch signaling pathway probably through NF-κB pathway [162–164]. NF-κB is present as a latent, inactive, light polypeptide gene enhancer (I-κB, inhibitor of NF-κB)-bound complex in the cytoplasm in majority of cells. IL-1 activates NF-κB via IL-1 receptor-associated kinase (IRAK) and mitogen-activated protein kinase (MAPK) dependent inhibition of I-κB [165–166]. c-Rel (a NF-κB subunit) can trigger Notch1 signaling pathway by inducing expression of JAG1 [167–168]. Results from our laboratory suggest that leptin is an important inducer of IL-1 system in breast cancer cells [161]. Moreover, IL-1, Notch and leptin-induced upregulation of their gene components and NF-κB, HIF-1α and VEGF/VEGFR-2 are interconnected [140,169].

8.3.3. Leptin signaling—Leptin, a pluripotent cytokine, secreted primarily by adipocytes but also by breast cancer cells, plays key roles in regulating energy intake and energy expenditure, including appetite and metabolism [170]. In the past decade, accumulating evidence indicates that leptin actions are not only related to energy metabolism, but also related to reproduction, angiogenesis, proliferation and inflammation [171–172]. Moreover, elevated levels of leptin and its deregulated signals have been strongly associated to obesity-related cancers including breast cancer [173]. Breast cancer cells express higher levels of leptin and leptin receptor, OB-R, than normal mammary cells. More importantly, higher levels of leptin/OB-R levels correlated with metastasis and lower survival of breast cancer patients [174–176]. *In vitro*, leptin was demonstrated to stimulate the proliferation of breast cancer cell lines [174,177–178]. *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 [179–180]. In our previous reports, 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 [181–182]. These effects were accompanied by a significant decrease in VEGF/VEGFR-2, IL-1 R tI, cyclin D1 and PCNA levels. Moreover, tumor angiogenesis was also impaired [181–182].

Leptin and IL-1 have been shown to be associated in several pathological situations [183–184], suggesting an interplay between them. Indeed, leptin regulates IL-1 family members in a diabetic context [185] and in endometrial cancer cells [186]. We also found that leptin increased 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 up-regulation. In addition, leptin upregulation of IL-1α promoter involved the activation of SP1 and NF-κB transcription factors [161].

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 endothelial cells (HUVEC) and also promotes cell differentiation [187]. We recently observed that leptin was able to activate Notch signaling pathway (Notch1,3,4 NICD and CSL) in mouse (4T1, EMT6 and MMT) and human breast cancer cell lines

(MCF-7 and MDA-MB-231) under normoxic conditions. Moreover, leptin increased the expression of both Notch receptors and ligands. In these cells leptin also up-regulated Notch-target genes Hey2 and surviving ([140] and unpublished data). Leptin-induced non-canonical signaling pathways (PKC, p38 and JNK) differentially impacted on CSL promoter activity and on the expression of IL-1 system [161]. Interestingly, leptin upregulatory effects 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 (unpublished data).

We speculate that complex molecular mechanisms are involved in leptin interactions with Notch and IL-1 in breast cancer cells. Notch, IL-1 and leptin outcome crosstalk (NILCO) is required for leptin regulation of VEGF/VEGFR-2. As discussed above, Notch could crosstalk and also be regulated by STAT3, NF-κB, HIF-1α and HER2. These factors are also regulated/activated by leptin and IL-1. Then NILCO could impact on Notch signaling regulation as follows: (1) STAT3 activation: leptin binding to OB-Rb (long and full-functional isoform) activates JAK2 that is recruited/autophosphorylated- and phosphorylates OB-R and STAT3 [188–189]. Leptin–induced activation of STAT3 was required for the induction of CSL, IL-1 (unpublished data) and VEGF [169]. (2) Leptin activation of NF-κB is involved in the upregulation of IL-1 [161]. NF-κB and HIF-1α are essential factors for leptin regulation of VEGF in breast cancer cells [169]. Moreover, the blockade of leptin signaling markedly reduced the growth of tumors and the expression of VEGF in mouse models of syngeneic and human breast cancer xenografts [181–182]. Leptin increased VEGF/VEGFR-2 expression in endometrial cancer cells *in vitro* [186] and in breast cancer cells *in vitro* and *in vivo* [181–182]. (3) Leptin/HER2 crosstalk: Leptin treatment activates HER2 through trans-phosphorylation on Tyr-1248 in the HER2 intracytoplasmatic tail [190]. HER2 physically interacts with OB-R and, thus the leptin/OB-R signaling might contribute to enhanced HER2 activity in MCF-7 cells [191]. (4) Leptin increases estrogen levels and ERα activity: ERα and Notch have an active crosstalk (see section 8.5.1) [61,192]. Aromatase, a product of the *CYP19* gene and a member of the cytochrome P450 (CYP) enzyme family, catalyzes a rate-limiting step for the conversion of androstenedione to estrone and testosterone to E2 [193]. Leptin can enhance aromatase activity and expression either in stromal cells or breast cancer cell lines, thus it can increase estrogen production [194–195]. In addition, leptin transactivates ERα in MCF-7 cells [196]. Obesity has become a pandemic particularly in western countries. Increased leptin signaling is associated with obesity and breast cancer incidence. Combinatory therapy targeting NILCO would enhance the disruption of specific signaling pathways involved in breast cancer progression. This might help to design new strategies aimed at controlling growth, angiogenesis and metastasis in breast cancer.

8.4. Oncogenic kinases and Transcription factors

8.4.1. Ras signaling pathway—Ras signaling plays an important role transmitting signaling from RTKs to ser/threo kinases. Among the effector molecules connected with the group of cell surface receptors, Ras has an essential role in transducing extracellular signals to diverse intracellular events by controlling the activities of multiple signaling pathways [197]. Ras oncogene is mutated in a large number of cancers including breast cancer. Deregulated Ras signaling impacts on many cellular functions, including cell cycle regulation, apoptosis and cell survival, therefore, Ras is a major target for the development of novel cancer treatments [198]. The signaling networks Ras regulates are very complex due to their multiple functions and crosstalk [199].

Crosstalk between Ras and Notch pathways has been described in pancreatic ductal adenocarcinoma [200], colorectal tumors [201], astrocytic gliomas [202], leukemia [203–204], as well as breast cancer [205–206]. In an early report [60], 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* [60]. Oncogenic Ras increased levels and activity of Notch1 NICD and upregulated Notch ligand Dll-1 and also presenilin-1, a protein involved in Notch processing, through p38-mediated pathway [60]. These observations established that Notch signals are among the key downstream effectors of oncogenic Ras. Gustafson et al [205] observed that transformation of MCF-10A cells by Harvey-Ras (Ha-Ras) induces CCAAT/enhance binding protein beta (C/EBPβ), a transcriptional factor, and activates the Notch signaling pathway to block SIM2s (a transcriptional factor) gene expression. High level expression of Notch receptors, ligands and its cooperation with the Ras/MAPK pathway in several breast cancers and early precursors places 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 [206].

8.4. 2. PI-3K/Akt signaling pathway—The phosphatidylinositol 3-kinase (PI-3K/Akt) pathway plays a central role in a variety of cellular processes, including cell growth, proliferation, motility, survival and angiogenesis in tumor cells including breast cancer [207–208]. The PI-3K/Akt pathway is also instrumental in EMT during carcinogenesis [209]. Many of the transforming events in breast cancer are a result of enhanced signaling of the PI3-K/Akt pathway [208,210].

Notch has been shown to regulate the Akt (ser/threo) or Protein kinase B (PKB) pathway. Liu et al. [211] reported that Notch1 activation enhanced melanoma cell survival and the effects of Notch signaling were mediated via activation of the Akt pathway. Palomero et al [212] 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 [213–215]. On the 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 [215]. 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-κB signaling pathways [216].

In an early report [217], activated Notch1 synergizes with papillomavirus oncogenes in transformation of immortalized epithelial cells and leads 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 signals depending PI-3K/Akt pathway also occurs in other types of cells, such as Chinese hamster ovary (CHO) cells, primary T-cells and hippocampal neurons [218].

Akt is also a regulator of Notch signaling. Hyperactivated PI3-K/Akt signaling led to upregulation of Notch1 through NF-κB activity, while the low oxygen content normally found in skin increased mRNA and protein levels of Notch1 via stabilization of HIF-1α [213]. Taken together, these findings demonstrate that Notch1 is a key effector of both Akt and hypoxia in melanoma development. To the best of our knowledge, there no reports about whether Akt is also a regulator of Notch signaling in breast cancer.

8.4.3. 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 breasts and many 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 [186]. mTOR can also phosphorylate Akt [219]. mTOR has been intensely studied for over a decade as a central regulator of cell growth, proliferation, differentiation, autophagy, angiogenesis and survival [220–222]. mTOR functions as two distinct multiprotein complexes, mTORC1 and mTORC2 [219,223]. 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 [219]. Aberrant activation of mTOR pathway is found in many types of cancer and thus plays a major role in breast cancer cell proliferation and anti-cancer drug resistance [224–226]. mTOR signaling has been reported to crosstalk with the Notch signaling pathway in several malignant cells [96,227–229].

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 [229]. 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 [229]. Efferson et al [228] 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 [229].

8.4.4. NF-κB signaling pathway—The family of NF-κB transcription factors is involved in expression of genes involved in key genes for innate and adaptive immunity, cell proliferation and survival, lymphoid organ development. NF-κB is activated in a variety of cancers, including breast cancer [230–231] also linked to tumor angiogenesis [169]. NF-κB 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-κB is regulated by the so-called canonical pathway [230,232].

Numerous reports have described the bidirectional regulation of Notch and NF-κB through different context-dependent mechanisms. First, Oswald et al. [233] clearly demonstrated that Notch is able to transcriptionally regulate NF-κB members. RBP-Jk 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 [234] further observed that Notch1 upregulates the expression of p65, p50, RelB, and c-Rel subunits in hemopoietic progenitor cells using Notch1 antisense transgenic (Notch-AS-Tg) mice. Additionally, Notch1 regulated NF-κB in cervical cancer cells in part via cytoplasmic and nuclear IKK-mediated pathways [235]. Second, NF-κB subunits are also able to transcriptionally regulate Notch family members. This is supported by the findings of Bash J, et al. [167] 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 [167]. Lastly, members from Notch and NF-κB family could physically interact with each other. Wang J, et al. [236] demonstrated that 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-κB by directly interacting with NF-κB and competing with IκBα. These processes lead to the retention of NF-κB in the nucleus. It seems that in T-cells there are two 'waves' of NF-κB activation: an initial, Notch-independent phase, and a later sustained activation of NF-κB, which is Notch dependent [237]. In breast cancer, both Notch and NF-

κB are prominent therapeutic targets. If murine and *in vitro* data are confirmed in human cancer, the treatment of cancers dependent on Notch activity may benefit from combinations of agents targeting both pathways [168].

8.4.5. 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 try to adapt by regulating metabolism, erythropoiesis, and angiogenesis and by modulating pathways that result in survival or cell death. HIF is a key molecule unregulated in response to oxygen deficiency, as it acts as a master regulator of genes involved in tissue reoxygenation [238]. Additionally, HIF has been known to facilitate cancer progression by promoting tumor neoangiogenesis, cell motility, and invasion [239]. HIF is a heterodimer consisting of a constitutively expressed HIF-1β subunit and an oxygen-regulated, unstable HIF-1α subunit. HIF interactions with DNA are mediated through Hypoxia-Responsive Elements (HRE) [238]. 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 angiogenesis, energy metabolism, vasomotor function, erythropoiesis, and cell survival [240].

Gustafsson et al. [241] 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-1α, and then the complex is recruited to Notch1-responsive promoters. Sahlgren et al [242] 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-1α 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 [242]. Hypoxia increased Notch1 mRNA and protein as well as Notch activity, measured as Hes1 and Hey1 expression and Hes1 promoter activity. This effect was dependent on HIF-1α [213]. These results suggest that Notch1 is under the control of oncogenes and the tissue microenvironment. Therefore, HIF-1α and Notch signaling pathways play a critical role in regulation of EMT and open up perspectives for pharmacological intervention within hypoxia-induced EMT and cell invasiveness in tumors.

8.5. Other crosstalk signaling

8.5.1. ER signaling—Estrogens are important regulators of growth and differentiation in normal breast tissue, and they also play an important role in the development and progression of ER positive breast carcinoma [243–244]. The recognized risk factors for breast cancer are age at: menarche, first pregnancy, and menopause. This suggests endogenous ovarian steroids may profoundly affect initiation, promotion, and progression of carcinogenesis through a cascade reaction initiated by activation of the ER [243,245]. ERs are known to regulate a huge number of genes affecting cancer proliferation and vascular function [246–247].

Soares et al [61] first demonstrated that a crosstalk between estrogen and Notch signaling occurs in breast cancer and endothelial cells (EC). The authors observed that E2 promoted 8-fold and 6-fold increase in Notch1 and JAG1 expression, respectively, in breast cancer MCF-7 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-1α, was upregulated by E2. In another report, E2 and parathion (an organophosphate compound and potent insecticide) alone and in combination also led to an activation of Notch signaling in MCF-I0F, in the process of malignant transformation, indicated by anchorage independency and *in vitro* invasive of capabilities [248]. Notch and ERα crosstalk in breast cancer suggest that combinations of antiestrogens and Notch inhibitors may be more effective in ERα (+) breast cancers [192]. Overall, the crosstalk between Notch and estrogen signaling pathway has a significant role in human breast carcinogenesis and angiogenesis.

8.5.2. miRNA actions—MicroRNAs (miRNAs) are short non-coding RNAs that bind to the 3' untranslated region (UTR) of cognate messenger RNAs (mRNAs) through fully complementary or imperfect base-pairing repressing the translation or decreasing the stability of the bound mRNAs [249]. miRNA are involved in biological and pathologic processes, including cell differentiation, proliferation, apoptosis and metabolism, and are emerging as highly tissue-specific biomarkers with potential clinical applicability for defining cancer types and origins [250–251]. These RNAs can function as oncogenes or tumor suppressors depending upon the cell type in which they are expressed [252–253].

There are several reports on the crosstalk between miRNA and Notch signaling pathway [254–256]. Voo et al first reported that Notch activation leads to miR-61 mediated down-regulation of Vav, proto-oncogene in C. *elegans* [256]. Interestingly, miR-61, could control the expression of oncogene orthologues Ras and Vav indicating miRNA capacity to act as tumor suppressors [257]. Therapeutic potential of let-7s in cancer (initially identified as a timing developmental regulator in C. *elegans*) was recently reviewed [258]. In various human cell lines, Notch activation up-regulates miRNA let-7 [255]. Let-7 regulates self renewal and tumorigenicity of breast cancer cells [259] as well as ERα signaling in ER positive breast cancer [260].

On the other hand, miRNAs can regulate Notch pathways. miR-34a down-regulated the expression of Notch1 and Notch2 protein in glioma cells [254]. miR-34 down-regulated JAG1 and Notch1 in cervical carcinoma and choriocarcinoma cells [261]. 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 [262]. In addition, altered miRNA signatures including miR-34 may be associated to breast carcinogenesis and metastasis [263].

9. Clinical Significance of Notch signaling in human breast cancer

9.1. Tumor marker and prognostic value of Notch signaling in breast cancer

Farr et al earlier observed that high levels of Notch1 may be associated with a poorer prognosis in breast cancer patients, while high levels of Notch2 correlated to a higher chance of survival [63]. Therefore, Notch1 may possess tumor-promoting functions, while Notch2 could play a tumor-suppressive role in human breast cancer. Further data confirmed that Notch1 might be a novel prognostic marker for breast cancer [62].

Dickson et al [264] examined JAG1 protein expression in breast cancer using the Allred score (percent staining score + intensity score). Their data suggest that patients with high levels of JAG1 protein had a worse outcome than those with tumors expressing low levels. Patients with tumors expressing either high levels of JAG1 protein, mRNA or both had reduced 10-year survival as well as median survival. These results show that the Allred score for JAG1 mRNA and protein levels can be used to rapidly identify patients with significant survival disadvantage, which may benefit from anti-Notch therapies (such as

GSI). Another study examined 887 samples from a prospectively accrued lymph node-negative breast cancer cohort with a median follow-up greater than 8 years. In these patients, JAG1 protein and mRNA expression was associated with basal phenotype (triple negative cancer: ER−, PR− and HER2−) and higher recurrence, as well as reduced disease free survival [265]. Overall, these data suggest JAG1 might be a good prognostic marker for human breast cancer patients.

9.2. Notch signaling as therapeutic drug target

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 and carcinogenesis, as well as its crosstalk with many oncogenic signaling pathways suggest that Notch signaling may be such a candidate for multi-target drugs' candidate. 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 [266–267].

Gamma-secretase is a large membrane-integral multisubunit protease complex, which is essential for Notch receptor activation [268]. Effects of GSI treatments in solid tumors are shown in Table 1. Rasul et al [269] tested the effects of three different GSIs in breast cancer cells. One inhibitor (GSI1) was lethal to breast cancer cell lines, but had a minimal effect on the non-malignant breast lines. GSI1 treatment resulted in a marked decrease in γ-secretase activity and downregulation of the Notch signaling pathway with no effects on expression of the γ-secretase components or ligands. In a recent report [228], 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 [228]. GSI effects are much higher in HER2/*neu*-positive cell lines where HER2 is amplified and/or overexpressed (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 [51,192,269]. Since HER2 can influence the activity of Notch [74] and inhibition of HER2 via trastuzumab can activate Notch signaling [270], it will be important in considering GSI as a monotherapy or in combination with trastuzumab or lapatinib in HER2 breast cancer patients.

Although several GSIs have already developed into the clinic trial [267], GSIs fail to distinguish individual Notch receptors, inhibit other signaling pathways [271] and cause intestinal toxicity [272], probably attributed to dual inhibition between Notch1 and Notch2 [273]. Very recently, Wu et al [274] utilized phage display technology to generate highly specialized antibodies that specifically antagonize each receptor paralogue, enabling the discrimination of Notch1 versus Notch2 function in human patients and rodent models. Their results showed that inhibition of either receptor alone reduces or avoids toxicity, demonstrating a clear advantage over pan-Notch inhibitors.

10. Conclusion and overall perspectives

Notch signaling and its crosstalks with many signaling pathways play an important role in breast cancer cell growth, migration, invasion, angiogenesis and metastasis (see Fig2). Therefore, significant attention has been paid in recent years toward the development of clinically useful antagonists of Notch signaling.

There are still many gaps to uncover in the field of Notch signaling in breast cancer. There is an urgent need to build up studies to better understand the heterogeneity and complexity in the molecular biology of breast cancers and to develop tools to more accurately predict their

prognosis and design their customized treatment strategies. 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 ensuring 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 (i.e., NILCO) and their links to obesity-related cancers. Combinatory treatments 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
BALB/c	an albino, laboratory-bred strain of the house mouse
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
BCSC	breast cancer stem cells
BMP	bone morphogenetic protein
Br4	brain metastatic cell line
CaSki	cervical carcinoma cell
CBF1	Centromere-Binding Factor 1
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
C/EBPβ	CCAAT/enhance binding protein β
CFCs	bipotent colony forming cells
c-myc	Myc proto-oncogene protein
Co-A	recruites coactivator
Co-R	co-repressor
CpdE	compound E
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	dibenzazipene
DDP	cisplatin
DLL-1	Delta-like 1
DOS	Delta and OSM-11-like proteins
DSL	Delta/Serrate/LAG-2
E2	17β-estradiol
EC	endothelial cells
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMD	company name
EMT	epithelial-mesenchymal transformation
EMT6	a mouse mammary adenocarcinoma cell line
ER	estrogen receptor
ERK 1/2	extracellular regulated kinase 1 and 2
GBM	Glioblastoma
GSI	a γ-secretase inhibitor
HD	heterodimerization
HDAC	histone deacetylases
HIF-1α	hypoxia regulated factor-1 α
HT-29	colon cancer cell line
HUVECs	human umbilical vein endothelial cells
ICN	intracellular region of Notch
IGF-1	insulin like growth factor-1
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-MB231	ER negative human breast cancer cell line
MFE	Mammosphere-forming efficiency
miRNA	MicroRNA
mTOR	mammalian target of rapamycin
NECD	Notch extracelluare domain

NEXT	Notch extracelluar truncation
NF-κB	eukaryotic nuclear transcription factor kappa B
NICD	Notch intracellular domain
NRR	negative regulatory region
OB-R	leptin receptor
PDAC	pancreatic ductal adenocarcinoma
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
SiHa	human cervical tumor cell
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
TNF-α	tumor necrosis factor alpha
TSA	Trichostatin A
U87MG	a glioma cell line
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2 or KDR or Flk-1

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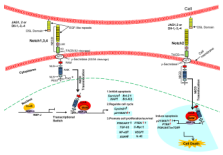

Fig. 1.

Notch signaling and its possible downstream targets in breast cancer. 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, three Lin12/Notch repeats (LNR or NRR) and a heterodimerization (HD) domain followed by a transmembrane subunit (N™). Notch receptor cytoplasmatic region or Notch intracellular 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 cytoplasmatic region; S2: by TACE (ADAM) and S3/S4: by 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 breast cancer: 1) inhibition of apoptosis through upregulation of survivin [51–52] and Bcl-2 protein family [294]; 2) activation of the cell cycle through upregulation of Cyclin D1 [73]; 3) promotion of cell proliferation/survival through upregulation of PI-3K/Akt [211], TGF-β [134], c-Myc [46], NF-κB [86], EGFR [117] and IL-6 [154] pathways; 4) stimulation of angiogenesis and VEGF/VEGFR-2 autocrine/paracrine loop by upregulation of IL-1 system and VEGF/VEGFR-2 ([140,161] and unpublished results); 5) suppression of cancer growth in some cellular situations. For example, Notch2 signaling may function as a tumor suppressor through upregulation of PTEN or downregulation of PI-3K/Akt/mTOR [295]. *Note*: (*): TAD is not present in Notch3 and 4.

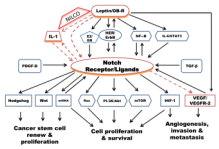

Fig. 2.

Potential Notch signaling crosstalk with other pathways in breast cancer. OB-R: leptin receptor ; IL-1: pro-inflammatory/-angiogenic cytokine interleukin-1; NILCO: Notch-IL-1-Leptin crosstalk outcome; E2: 17β-estradiol; HER/ErbB: HER1/EGFR, HER2, HER3 and HER4, encode for RTK-transmembrane proteins; NF-κB: a transcription factor family, nuclear factor kappa-light-chain-enhancer of activated B cells; IL-6: pro-inflammatory/-angiogenic cytokine, interleukin-6; STAT3: Signal transducer and activator of transcription 3; PDGF-D: Platelet-derived growth factor D; TGF-β: transforming growth factor β; miRNA: MicroRNA; PI-3K: phosphatidylinositol 3-kinase; mTOR: mammalian target of rapamycin; HIF-1: hypoxia-inducible transcription factor 1; VEGF: vascular endothelial growth factor; VEGFR-2: vascular endothelial growth factor receptor-2.

Table 1

Gamma-secretase inhibitors effects in cancer.

Compound	Type of cancer cells tested	Type of studies	Biological effect reported	Reference
Experimental models				
GSI-I(Z-LLNle,Calbiochem)	ER− Breast cancer cell lines	*In vitro*	Apoptosis; growth suppression Suppressed proliferation and metastasis,	[51]
	Xenograft	*In vivo*		
	Breast cancer cell lines	*In vitro*	GSI1 induces a G2/M arrest resulting in apoptosis through downregulation of Bcl-2, Bcl-XL	[269]
	ER+ Breast cancer cell lines	*In vitro*	Inhibition of the transcription of ERα-target genes via IKKα-dependent	[275]
GSIs IX (DAPT) CalBiochem, EMD Biosciences, Sigma)	Neuroblastoma cell lines.	*In vitro*	Induced complete cell growth arrest, promoted neuronal differentiation, and significantly reduced cell motility.	[276]
	U87MG human glioblastoma Human lung adenocarcinoma cell line A549	Tumor xenografts in nude mice	Inhibited the growth of tumor xenografts in nude mice	[277]
	Breast cancer	*In vitro* *In vitro*	Reversal of breast cancer-induced osteoclastogenesis and enhancement of cancer cell attachment	[278]
	Breast cancer stem cells	*in vitro* culture and *in vivo* transplant models	Reduce ductal carcinoma in situ (DCIS) mammosphere formation	[4]
	Mouse breast cancer cells 4T1	*In vitro*	Inhibition of leptin-induced VEGF/VEGF-R2 and IL-1/IL-1R tI	[279]
	Ovarian cancer cells A2780	*In vitro*	Inhibit cell growth, induce G1 cell cycle arrest and induce apoptosis	[280]
	CaSki and SiHa cells (cervical carcinoma cells lines	*In vitro*	Decreased RhoC activity and metastasis	[281]
	Br4 brain metastatic cells	*In vitro*	Inhibit the migration and invasion of Br4 cells	[282]
	Colon cancer cell lines HT-29	*In vitro*	Inhibition of proliferation and apoptosis	[283]
	Glioblastoma (GBM) stem cells	*In vitro*	Dispersion of the neurospheres leading to cell death	[284]
	Normal breast tissue, pure DCIS tissue of varying grades, and DCIS tissue surrounding an invasive cancer	*In vitro*	Reduce mammosphere-forming efficiency (MFE)	[285]
GSIs XXI (compound E [CpdE]) CalBiochem, EMD	Breast cancer	*In vitro* *In vivo*	Reversal of breast cancer-induced osteoclastogenesis and enhancement of cancer cell attachment	[278]
	Neuroblastoma cell lines.	*In vitro*	Induced complete cell growth arrest, promoted neuronal differentiation, and significantly reduced cell motility.	[276]
GSI18	Human pancreatic cancer cell lines	*In vitro*	Depletion in the proportion of tumor-initiating aldehyde dehydrogenase (ALDH)-expressing subpopulation	[286]
MRK-003(Merck)	BALB/c- ERBB2-transgenic mouse model of breast cancer (neuT) mice.	*In vivo*	Inhibition of tumor growth progression and increase of overall survival of transgenic mice (ERBB2- breast cancer ; neuT)	[228]

Compound	Type of cancer cells tested	Type of studies	Biological effect reported	Reference
Experimental models				
	Inducible Her2-driven invasive mammary adenocarcinoma models	*In vivo*	Inhibition of cell growth	[287]
	pancreatic ductal adenocarcinoma (PDAC)	*In vivo*	Inhibits PDAC cell growth	[288]
	Human lung cancers	*In vivo* xenograft model	Reduced tumor cell proliferation, inhibited serum independence and induced apoptosis	[289]
		In vitro		
Clinical trials				
PF-03084014(Pfizer)	Advanced cancer and leukemia	Phase I clinical trials	Cell growth inhibition via cell cycle arrest and induction of apoptosis	[290]
DAPT (Sigma)	Head and neck squamous cell carcinoma	Phase I clinical trails	Enhances cisplatin (DDP)-sensitivity	[291]
MK-0752 (Merck)	T-cell acute lymphoblastic leukemia/lymphoma	Phase I clinical trials	All doses were sufficient to inhibit gamma secretase	[292]
	Advanced or Metastatic Breast Cancer	Phase I/II study	Can target stem cells and prevent tumor recurrences	*
RO4929097 (Roche)	Advanced solid tumors	Phase I study	Antitumor activity and prolonged stable disease	[293]

* http://clinicaltrials.gov/ct2/show/NCT00645333