Notch-EGFR/HER2 bidirectional crosstalk in breast cancer

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INTRODUCTION
Current research has revealed many interesting features of cell signaling networks implicated in the development of breast cancer and continues to define methods by which cancer cells become able to exploit these networks to promote their growth, survival, and invasiveness. Communication between cell signaling pathways is critical to a cell’s response to an ever changing environment, and is harnessed by cancer cells to progress their disease state to obtain a growth advantage over healthy cells. Such pathways include the Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase (HER or HER) and Notch pathways, which have been found to communicate with one another to overcome treatment as well as promote Breast Cancer Stem Cell (BCSC) cell fate. This review presents current perspectives on research of HER–Notch crosstalk in breast cancer and culminates recent publications to give an up-to-date view of the intricate mechanisms that describe how communication between these two pathways is affecting the development of malignancies, drug resistance, recurrence, and metastatic progression.

BREAST CANCER AT A GLANCE
1. Breast cancer is the second leading cause of cancer-related death among women worldwide. Women have a one in eight lifetime risk of being diagnosed with breast cancer in the US alone (1). Breast cancer is divided into four major subtypes based on receptor overexpression during tumorigenesis as well as a pre-invasive subtype known as ductal carcinoma in situ (DCIS).

The Notch pathway is a well-established mediator of cell–cell communication that plays a critical role in stem cell survival, self-renewal, cell fate decisions, tumorigenesis, invasion, metastasis, and drug resistance in a variety of cancers. An interesting form of crosstalk exists between the Notch receptor and the Epidermal Growth Factor Receptor Tyrosine Kinase family, which consists of HER-1, -2, -3, and -4. Overexpression of HER and/or Notch occurs in several human cancers including brain, lung, breast, ovary, and skin making them potent oncogenes capable of advancing malignant disease. Continued assessment of interplay between these two critical signaling networks uncovers new insight into mechanisms used by HER-driven cancer cells to exploit Notch as a compensatory pathway. The compensatory Notch pathway maintains HER-induced downstream signals transmitted to pathways such as Mitogen Activated Protein Kinase and Phosphatidylinositol 3-Kinase (PI3K), thereby allowing cancer cells to survive molecular targeted therapies, undergo epithelial to mesenchymal transitioning, and increase cellular invasion. Uncovering the critical crosstalk between the HER and Notch pathways can lead to improved screening for the expression of these oncogenes enabling patients to optimize their personal treatment options and predict potential treatment resistance. This review will focus on the current state of crosstalk between the HER and Notch receptors and the effectiveness of current therapies targeting HER-driven cancers.

Keywords: Notch, EGFR, HER2, crosstalk, breast cancer

2. The luminal A and B breast cancer subtypes comprise 60–70% of all breast cancers and are derived from the luminal epithelium of breast ducts with cancer cells overexpressing the ER and/or PR. First line therapy for the Luminal A and B breast cancer subtypes are anti-hormonal therapies that target estrogen-mediated activation of the ERα isoform. Aromatase inhibitors (AI), tamoxifen, and fulvestrant are examples of anti-estrogen therapies, with the luminal A subtype being more sensitive to such inhibitors than the highly proliferative and inherently more resistant luminal B breast cancer subtype. The HER2/Luminal A subtype has an amplification of the HER2 gene. The HER2 gene is a proto-oncogene and if mutated or overexpressed is a bonafide oncogene. HER2 amplification results in overexpression of the HER2 receptor on the surface of breast cancer cell. The HER2+ subtype occurs in 20% of breast cancers and is sensitive to HER2-based-targeted therapies such as the humanized, monoclonal antibody trastuzumab, or the small molecule tyrosine kinase inhibitor, lapatinib. Mechanisms of action of these anti-HER2
therapies will be expounded later in this review. Among others, the TNBC/BLBC subtype does not express therapeutically targetable ER, PR, or HER2 receptors making this aggressive subtype difficult to treat. Since TNBC lacks expression of targetable receptors, treatment options for TNBC tumors are limited to cytotoxic chemotherapy such as tubulin-destabilizing taxanes (docetaxel, paclitaxel), DNA damaging alkylating/methylating agents (Cyclophosphamide, Chlorambucil, Temozolomide), or DNA untangling (topoisomerase II) stabilizers (doxorubicin, camptothecin) aimed at killing highly proliferative TNBC cells.

3. Early stage Ductal Carcinoma In situ (DCIS) is a non-invasive form of breast cancer in which luminal cells that line mammary gland ducts are morphologically and physiologically changed to resemble cancer cells, to some extent. If left untreated, DCIS can proceed to a metastatic disease in three steps. First, a population of cells in the lesion begins to fill in the hollow luminal space of the mammary duct. Second, these tumorigenic cells can invade the surrounding breast tissue to become invasive ductal carcinoma. Third, the lesion becomes metastatic as the cancer cells begin to access blood or lymph circulation to invade distal parts of the body to form new tumors. If undiagnosed or left untreated, DCIS has a two in three chance of progressing to malignant disease (13). Molecular markers are similar between DCIS and invasive breast cancer and these include ER+ and HER2+. The expression of such markers can aid in determining the inter-connected signaling pathways that cancer cells exploit for continued survival and proliferation has led to many interesting findings that may help improve breast cancer treatment. This review delves into recent research exploring the role of the Notch and HER/HER pathways in breast cancer and how these potentially complementary pathways are able to communicate with one another to promote breast cancer and/or tumor growth.

HER/HER PATHWAY
The HER/HER family is made up of four structurally related receptor tyrosine kinases (RTKs) with the EGFR as the founding member of the family. In humans, these include: HER1 (EGFR), HER1 (HER1), HER2 (Neu, HER2), HER3 (HER3), and HER4 (HER4). HER2, the gene symbol, is derived from a homologous viral oncogene, Erythroblast Leukemia Viral Oncogene, and has the official name: V-Erb-B2 Avian Erythroblast Leukemia Viral Oncogene Homolog or HER2 accordingly (16).

Activation of the HER/HER family of receptors requires binding of a soluble, growth factor-ligand located in the intracellular cavity of the receptor that triggers receptor dimerization and phosphorylation, and activation of downstream pathways to elicit an appropriate response inside of the cell to the environmental stimuli outside of the cell. EGFR is activated by growth factor-ligands such as epidermal growth factor (EGF), Heparin binding EGF (HB-EGF), amphiregulin (AREG), or transforming growth factor alpha (TGF-α). In contrast, HER3 and HER4 are activated by the heregulin (neuregulin) family of growth factors (Hrg/Nrg 1, 2, 3, 4) (Figure 1A). Each HER/HER receptor, upon activation by growth factor binding, initiates hetero-dimerization or homo-dimerization of HER/HER receptors (Figure 1B). HER/HER receptor(s) dimerization stimulates auto-phosphorylation, followed by trans-phosphorylation of the receptor partner. HER/HER phosphorylation occurs at specific tyrosine residues within the intracellular domain of the HER/HER receptors. The phosphorylation of HER/HER tyrosine residues

Table 1 | Breast cancer subtypes and therapies.

| Subtype       | Molecular markers | First line therapy                                                                 | Second line therapy                                      | Approximate occurrence (%) | Reviewed in reference |
|---------------|-------------------|----------------------------------------------------------------------------------|----------------------------------------------------------|----------------------------|-----------------------|
| DCIS          | ER±, HER2±        | Surgery, radiotherapy, chemotherapy, none                                         | Adjuvant to chemo/radiotherapy such as tamoxifen (ER+) or | 20                         | Narod (4), Leonard (5), Wiechnmann (6) |
| Luminal A     | ER+/PR+           | Tamoxifen (pre or post menopausal)                                               | Aromatase inhibitor (AI) (post menopausal)               | 40                         | Ignatiadis (7), Davison (9) |
| Luminal B     | ER+/PR+/HER2+/Ki67| Tamoxifen (pre or post menopausal)                                               | Aromatase inhibitor (AI) (post menopausal)               | 20                         | Ades (9)              |
| HER2+         | ±ER+/HER2-        | Trastuzumab, pertuzumab                                                         | Lapatinib                                                | 10                         | Baselga (10), Zhang (11) |
| Triple negative (TNBC) | EGFR/Notch-1/Pi3K-PTEN/claudins/Hsp90/Ras | Molecular targeted therapies depending on subtype combined with chemotherapy, surgery, radiotherapy | Chemothrapy, surgery, radiotherapy                          | 10-20                     | Lehmann (12)          |
FIGURE 1 | The HER receptor and pathway: (A) there are four members of the HER family of RTKs: EGFR/HER1, HER2, 3, and 4. Each HER receptor is composed of four functional extracellular domains (domain I, II, III, and IV) as well as a tyrosine rich (Tyr) intracellular domain. Epidermal growth factor (EGF), amphiregulin (AREG), heparin binding EGF (HB-EGF), and heregulin 1, 2, 3, 4 growth factor-ligands bind to domains II and III of EGFR/HER1 and HER3, HER4, respectively. Growth factor-ligand binding initiates HER conformational rearrangement causing exposure of the dimerization domain (DD), which is sandwiched between domains II and IV, as well as allowing the closed conformation of the HER receptor (portrayed by EGFR/HER1, HER3, 4) to assume an open conformation (displayed by HER2). It is important to note the truncated intracellular domain of HER3, which lacks kinase activity therefore, the HER3 receptor is the kinase dead member of the HER family of RTKs. (B) Ligand-mediated exposure of the dimerization domain enables the binding of two like HER receptors to form a homo-dimer (left, EGFR-EGFR) or two different HER receptors to form a hetero-dimer (right, HER2-HER3). It is important to note that the HER2 receptor is in a fixed, open conformation and does not require ligand binding to dimerize with a ligand bound HER receptor. (C) Upon HER dimerization, the HER receptors undergo auto-phosphorylation of their intracellular tyrosine residues. Auto-phosphorylation of the HER receptors primes the receptors to transphosphorylate the tyrosine residues of their binding partners (D) in preparation to initiate activation of downstream targets (E) activated HER receptors initiate the AKT (left) and mitogen activated protein kinase (MAPK) (right) pathways through a series of residue targeted phosphorylations, or a phosphorylation cascade. The Src homology domain 2 (SH2) of p85 (regulatory domain of PI3K) docks to the phosphotyrosine residue of the activated HER receptor (EGFR). p85 docking to EGFR enables Src homology domain 3 (SH3) of p110 (catalytic domain of PI3K) to join the proline rich region (Pro) of p85 to initiate PI3K 3’ phosphorylation of phosphatidylinositol (4, 5)-bisphosphate (PIP) transforming PIP2 into phosphatidylinositol 3, 4, 5-triphosphate (PIP3). PIP3 recruitment is aided by the Plekstrin homology domain (PHD) within the catalytic domain of phosphoinositide dependent kinase-1 (PDK1). The lipid binding PH domain recruits AKT to the plasma membrane where it docks to PIP3, as the physical interactions and activations of PI3K, PDK1, as well as AKT occurs at the surface of the cell. PDK1 is able to phosphorylate AKT at the Thr 308 residue as well as aid in PKCa activation. AKT phosphorylation disrupts the formation of the tuberous sclerosis 1/2 (TSC 1/2) dimer, inhibits hydrolyzation of the GTP binding protein, Ras homolog enriched in brain (Rheb), to activate the mammalian target of rapamycin complex 1 (mTORC1) complex as well as the mTORC2 complex. The mTORC1 complex is composed of: G protein beta subunit-like (GβL), the metabolically sensitive regulatory-associated protein of mTOR (Rptor), DEP domain-containing mTOR-interacting protein (DEPTOR), and the serine/threonine kinase mammalian target of rapamycin (mTOR). The fully formed mTORC1 complex is able to promote cell growth, proliferation, and autophagy by directing protein translation by mediating the ability of Rptor to recruit the formation of the eukaryotic initiation factor 4F complex (eIF4F (eIF4E, G, etc.)) and the ribosomal protein S6 kinase beta-1 (S6K1). The mTORC2 complex is also involved in cellular metabolism but mainly facilitates fluctuations in cytoskeletal formation and degradation throughout the cell via serum (Continued)
categorizes the HER/HER family RTKs (Figures 1C,D). These phosphorylated tyrosine residues recruit adaptor proteins such as Grb2 and the p85 subunit of the PI3K complex, which elicit the activation of several downstream pathways such as Protein Kinase B (AKT/PKB) and the mitogen activated protein kinase (MAPK) pathways (Figure 1E). HER/HER-mediated activation of downstream pathways enables a growth factor to elicit proliferation, survival, or migration of the cancer cell (Reviewed in Bubil and Kopan) (17, 18).

A significant number of ligand–HER receptor combinations are engaged to maintain normal cell physiology during development and throughout adult life. The mechanism of HER activation is controlled by ectodomain shedding in which proteolysis is mediated by a matrix metalloproteinase (MMP9 or ADAM12) that cleaves the ligand growth factors from the surface of the cell, near the transmembrane domain, to release the soluble ligand into the extracellular environment (19). All four members of the HER family of RTKs share structural similarities, but they have specific features that confer unique regulatory characteristics. Unlike EGFR, the extracellular region of HER2 resembles a ligand activated or fixed state, making HER2 an orphan receptor, thus not needing to bind ligands for its activation (20, 21). HER3 does not possess kinase activity and can only potentiate downstream pathways (Reviewed in Altomore) (41). Many components of the AKT pathway have been implicated in tumorigenesis events such as the oncogenic activity of PI3K and the loss of the tumor suppressor gene deleted on chromosome TEN (PTEN)” . These mutations have been studied.

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FIGURE 1 | Continued
and guccorticoid-regulated kinase 1 (SGK1), for instance. In a similar fashion, the SH2 domain of growth factor receptor-bound-2 (Grb2) protein docks to the phosphotyrosine residue of the activated HER receptor (HER2). SH2 interaction with the Grb2 scaffold protein initiates the binding of two SH3 domains to the proline rich region of the son of sevenless (SOS) protein. SOS is a guanine-nucleotide exchange factor (GEP) that acts on Ras-GTPases (Ras-GDP) (rat sarcoma): SOS facilitates the unbinding of guanosine diphosphate (GDP) from Ras, there by destabilizing Ras and enabling guanosine triphosphate (GTP) binding to form Ras-GTP, and initiate the MAPK phosphorylation cascade. A GTPase activating protein (GAP) hydrolyzes Ras bound GTP (Ras-GTP), forming GDP and halting downstream phosphorylation of the MAPK pathway. Ras activation begins the MAPK phosphorylation cascade, which is a series of serine/threonine specific protein kinases occurring on: rapidly accelerating fibrosarcoma/mitogen activated protein kinase kinase kinase (raf/MAPKKK), mitogen/extracellular signal-regulated kinase/mitogen activated protein kinase kinase (MEK/MAPKK), and extracellular signal-regulated kinase 1/2/mitogen activated protein kinase (Erk1/2/MApK).

Activation of the MAPK pathway initiates the transcription of a host of genes including: c-Myc, c-Fos, and cyclins to orchestrate cell motility, invasiveness, and proliferation.
(42). Similar to the AKT pathway, several components of the MAPK pathway are involved in promoting tumorigenesis making these two pathways central nodes where activating mutations are known to trigger tumorigenesis and metastasis. Several inhibitors were designed to target components of the MAPK and mTOR pathways such as: U0126 and rapamycin, which inhibit Mitogen/Extracellular signal-related Kinase/Mitogen Activated Protein Kinase Kinase (MEK/MAPKK) and mTOR, respectively. EGFR or HER2-mediated stimulation of cell proliferation and survival pathways can cause cancer cells to become dependent on these pathways, or “addicted,” to EGFR or HER2 expression to maintain tumor growth and survival (43).

Unfortunately, cancers become resistant to many types of therapies that specifically target EGFR, HER2, or components of their downstream pathways. Resistance to these targeted treatments leads to tumor recurrence and in some cases a more aggressive cancer, ultimately ending in death of the patient. Cancer cells can adapt to treatments using a variety of mutations that enable the cancer cell to overcome targeted therapies and enable them to propagate under such conditions. It has come to light that cancer cells are able to become resistant to therapies by recruiting the use of a similar, compensatory pathway that continue downstream activation of protein synthesis, i.e., the AKT pathway, and/or transcriptional up-regulation of survival genes mediated by the MAPK pathway. Using compensatory pathways to divert dependence from one receptor to another is referred to as crosstalk, or bidirectional communication, between two pathways to allow continued cancer cell growth and survival. Extensive research has been done in G-Protein Coupled Receptor (GPCR) crosstalk with EGFR/HER2 as some GPCR agonists such as Lysophosphatidic acid (LPA), carbachol (muscarini acetylcholine inhibitor), and thrombin are known to be able to increase HER activation in two different ways (reviewed in Bray) (44). The first consists of a GPCR-dependent increase in the ectodomain shedding of HER ligands. The second phenomenon includes GPCR activation of c-Src to mediate phosphorylation of HER tyrosine residues. HER activation via GPCR may be required for GPCR-mediated mitogenic activity via the MAPK pathway (45). Both HER–MAPK pathway initiation, as well as activation of number of steroid hormones, can trigger the transcription of HER ligands and evidently initiate a positive feedback loop. Mechanisms of crosstalk between the HER family of RTKs and several other pathways including the: Wnt/β-catenin (46), TNFa/IKK/NK-xB (47, 48), and Notch pathway (49, 50) have been under thorough investigation for several years. Interesting findings have been made, which describe the novel mechanisms of HER–Notch bidirectional crosstalk. Recent data illustrate how these new mechanisms of crosstalk have been implicated in treatment resistance, mechanisms of Epithelial to Mesenchymal Transitioning (EMT), as well as the propagation and survival of BCSCs.

NOTCH PATHWAY

The Notch receptor is a single-pass, type I, transmembrane receptor (Reviewed by Kopan et al.) (Figure 2A) (51). A mature Notch receptor is formed as a hetero-dimer, composed of two domains bound by a non-covalent bond, typically mediated by calcium (Ca^{2+}). Upon maturation, the full length Notch polypeptide is cleaved at the S1 site, within the Golgi-apparatus by a furin-like convertase, into two domains: the Notch extracellular (NECD) and transmembrane/intracellular (NTMICD). The extracellular portion of the Notch receptor contains a series of EGF-like repeats that mediate ligand interactions from a neighboring cell. The EGF repeats are followed by the Negative Regulatory Domain (NRR) that prevents ligand-independent activation of the Notch receptor. There are five known Notch-ligands: Jagged-1 and -2 (Jag-1, -2), Delta-Like-1, -3, and -4 (DLL-1, -3, -4) that bind to and activate the Notch receptor when two neighboring cells are in close proximity to each other (Figure 2C). The affinity of a Notch-ligand to its Notch receptor can be attenuated by Fringe glycosyltransferases (Lunatic, Manic, or Radical) by adding N-acetylglucosamine moieties to the NECD region. The intracellular domain of the Notch receptor contains the active portion of the Notch receptor, the Notch Intracellular Domain (NICD), which is composed of the: RBP-Jκ associating module (RMA) domain, ankyrin domain, Cysteine Response region (NCR), Transactivation Domain (TAD), proline/glutamic acid/serine/threonine-rich motifs (PEST domain), and a pair of Nuclear Localization Sequences (NLS) that sandwich the Ankyrin domain to direct cleaved NICD to the nucleus enabling NICD to initiate transcriptional activation of target genes. Activation of the Notch pathway requires a series of events, which begins with the Notch receptor on a signal receiving cell binding to a Notch-ligand on the neighboring signal sending cell. Consequently, this ligation of ligand to receptor is required for removal of the inhibitory NECD from the NTMICD causing two subsequent cleavages at S2 mediated by ADAM10/17 and S3/4 mediated by the γ-secretase complex. The ADAM and γ-secretase targeted cleavage sites are buried within the transmembrane domain of the Notch Extracellular Truncation (NEXT) fragment. Cleavage by the γ-secretase complex releases the NICD portion of the Notch receptor (Figure 2A). NICD shuttles to the nucleus where it acts as a Recombining Binding Protein-Jκ/Core Binding Factor-1 (RBP-Jκ/CBF-1) mediated transcriptional activator of target genes. NICD binds to CBF-1, which is already bound to DNA, enabling the release of negative co-regulatory proteins such as C-terminal-Binding Protein 1 (CtBP1) and SMRT/HDAC-1-Associated Repressor Protein (SHARP) and the recruitment of co-activating proteins such as Mammal Mastermind-like-1 (MAML-1) and the Histone Acetyltransferase (HAT), p300, to CBF-1, to form the Notch transcriptional activating complex.Canonical Notch target genes are primarily basic helix-loop-helix transcriptional repressors including, Hairy/Enhancer of Split-1 (HES-1-5) and Hairy/Enhancer of split with a unique YRPW motif-1 (HEY-1, HEY-L) (Figure 2C). There are four Notch paralogs (Notch-1, -2, -3, -4) each with quite different structures and possibly synergistic and/or counteracting functions (Figure 2B).

The Notch pathway is most commonly associated with lateral inhibition in which the Notch-ligand, Delta, if expressed in one cell can inhibit Delta expression by a neighboring cell. In a cluster of undifferentiated cells, when one cell begins to develop into an epithelial cell, it signals its neighbors to do the same. Initially, an undifferentiated cell expresses both the Notch receptor and the Delta ligand. Activation of the Notch receptor inhibits Delta production in the same cell. Through juxtaacrine signaling, neighboring cells compete to produce Delta, resulting in a feedback
FIGURE 2 | The Notch receptor and pathway. (A) The Notch receptor is composed of several domains that can be divided between the extracellular domain (NECD) and the transmembrane and intracellular domain (NTMICD). The NECD begins at the N-terminus (NH$_3$) end of the Notch receptor and contains ligand binding epidermal growth factor-like (EGF) repeats and the negative regulatory region (NRR). Fringe proteins target the EGF repeats for glycosylation, which can augment their ligand binding ability to the Notch receptor. The NRR is composed of Lin12-Notch repeats (L) and the heterodimerization domain (HD), which aid in maintaining a closed conformation of the Notch receptor until ligand binding. The NTMICD contains: the transmembrane domain (T), the RBP-$\kappa$ associating module (R) or RAM domain, one of two nuclear localization signals (N), the ankyrin domain, a second nuclear localization signal, the cysteine response region (INCR), the transactivating domain (TAD), and a proline/glutamic acid/serine/threonine-rich motif (PEST) at the C-terminal (COOH) end of the Notch receptor. Ligand bound Notch is cleaved at a series of scissile sites (S1, 2, 3, 4), which are represented just outside of (S1) and inside the transmembrane domain (S2, 3, 4) (TMB enlargement). Furin-like convertase cleaves immature Notch to create the mature, heterodimeric Notch receptor, which is stabilized by a non-covalently bound calcium ion (Ca). The RAM domain facilitates core binding factor-1 (CBF-1) binding, the ankyrin domain binds mammal-like mastermind-1 (MAML-1) and p300 (p300), which are necessary components of the Notch transcriptional activating complex. The PEST domain is responsible for degradation of the Notch intracellular domain (NICD). (B) There are four Notch receptor paralogs, each similar in overall composition but with slight structural differences that facilitate their diverse attributes. From top to bottom are Notch-1, -2, -3, and -4. Notch-1 is the most studied Notch receptor containing all of the above mentioned domains with 36 EGF-like repeats. Notch-2 is similar to Notch-1 except for differences in its EGF-like repeats as well as the ability of Notch-2 to bind to Jagged-1 upon fringe modification [118]. Notch-3 has a slightly truncated EGF-like domain with 34 repeats and lacks a transactivating domain. Notch-4 is the smallest of the Notch receptor paralogs with 29 EGF-like repeats, a shorter NICD due to a lack of a transactivating domain and cysteine response region, as well as the addition of a PDZ Domain (Post-synaptic Density Protein (PSD)-95) (PDZ) that plays a critical role in proper neuronal development [119]. (C) The Notch pathway is activated by a Notch-ligand (jagged-1, -2, DLL-1, 3, 4) expressed on a signal sending cell binding to a Notch receptor (Notch 1, 2, 3, 4) on a signal receiving cell. Ligand bound Notch undergoes a series of cleavages, first by a disintegrin and metalloproteinase/tumor necrosis factor alpha converting enzyme (ADAM/TACE) at the S2 cleavage site to form the Notch extracellular truncation (NEXT) fragment. The NEXT fragment is targeted by the $\gamma$-Secretase complex for cleavage. The $\gamma$-secretase complex is composed of nicastrin, anterior PHarynx-defective 1 (APH-1), presenilin ENhancer 2 (PEN-2), and the active component of the complex, presenilin-1. $\gamma$-Secretase is inhibited by $\gamma$-secretase inhibitors (GSIs), which prevent Notch cleavage and transcriptional activation. Proper cleavage of the Notch receptor releases NICD, allowing NICD to travel to and enter the nucleus where NICD binds to promoter bound CBF-1, allowing CBF-1 to release negative co-regulators (NCoR) and recruit transcriptional co-activators. NICD-mediated transcription cannot occur without the recruitment of mammalian mastermind-like-1 (MAML-1) and the histone acetyltransferase, p300, to unwind the compressed DNA allowing transcription of the Notch targeted genes. Notch activates the transcription of canonical target genes such as: hairy enhancer of split-1 (HES-1), hairy/enhancer of split with an YRPW motif-1 (HEY-1), and non-canonical Notch target genes such as survivin.
loop that drives the two neighboring cells to assume different cell fates based on the number of Notch receptors or Delta ligands expressed by each cell. Notch signaling is used to establish a border between two different populations of cells such as stromal and progenitor cells. Boundary formation by oscillating Notch activity is most notable in somite formation in which the constant activation and inactivation of Notch-mediated transcriptional activity causes the segmented formation of somites in vertebrates (52–54).

Notch is able to dictate cell lineage by asymmetrical inheritance of Notch regulators between two dividing, daughter cells, and is most evident during neurogenesis (55). Protein encoded by the gene NUMB (Numb Drosophila Homolog), is an inhibitor/negative regulator of Notch activity. Asymmetric distribution of Notch and Numb between two dividing cells determines if one daughter cell is a signal sending cell and if the other is a signal receiving cell. This uneven distribution of Notch and Notch regulators, Numb, is passed down through multiple cell divisions to maintain stem cell populations as well as influence cell lineage decisions (56). The culmination of the Notch receptor studies resulted in determining the Notch pathway and its ability to act as a form of short range communication between cells. Notch juxtacrine signaling has been found to be involved in a variety of processes, most notably in stem cell differentiation, self-renewal, and cell fate determination as well as cell proliferation, growth, and survival (Reviewed by Artavanis-Tsakonas) (57).

The Notch pathway has been referred to as the Notch-survival pathway due to promotion of cell growth and proliferation. One of the Notch gene targets responsible for regulation of cell longevity is called survivin. Survivin is an Inhibitor of Apoptosis Protein (IAP) and functions to down-regulate caspase 3, 7, and 9 apoptotic activity as well as regulate the cell cycle by interacting with spindle microtubules during mitosis (58). Cyclin Dependent Kinase-1 (CDK1) phosphorylates the threonine 34 residue of survivin to stabilize its protein structure (59, 60) and enabling survivin to escape degradation mediated by the X-linked IAP-X-linked Associating Factor-1 complex (XIAP-XAf1). The XIAP-XAf1 complex induces E3 ligase activity that targets survivin for ubiquitination and subsequent proteosomal degradation. Prolonged survivin expression has been associated with breast cancer cell survival via the MAPK/ERK and AKT/PI3K pathways (61). A correlation has been made between poor breast cancer prognosis and an increase in the expression of both Notch-1 and survivin in ER-breast cancer and has been described as the Notch-Survivin signaling axis (62).

There is a growing body of evidence that Notch up-regulation or mutation results in several events that enable breast cancer cells to: become resistant to targeted treatments, undergo EMT, or mutation results in several events that enable breast cancer disease progression in various carcinomas. Notch-1-EGFR crosstalk promoting drug resistance, EMT, and non-small cell lung cancer by Xie et al. as a route of acquired resistance to EGFR targeted TKIs and development of the EMT phenotype via Notch-1 mediated down-regulation of E-cadherin (epithelial marker), as well as up-regulation of vimentin (mesenchymal marker), and Snail (mesenchymal marker) expression (86). Notch–EGFR crosstalk has been shown to increase MUC5AC expression (87) causing increased goblet cell secretion of mucin, a feature attributed to chronic airway inflammatory disease as well as a potential activator of inflammatory induced lung cancer. EGFR has been shown to inhibit expression of the Notch-1 gene in a squamous-cell carcinoma model by up-regulating c-Jun, which in turn down-regulates p53, causing repression of Notch-1 transcription, and this form of Notch-1-EGFR crosstalk occurs via the MAPK/MEK/ERK pathway (84). These are just a few examples of Notch–EGFR crosstalk promoting drug resistance, EMT, and disease progression in various carcinomas.

Activation of the PI3K pathway by deletion or inactivation of PTEN or oncogenic mutation of PIK3CA has been observed in many types of cancer as well as being implicated in drug resistance. Down-regulation of PTEN and oncogenic mutation of PIK3CA compounded with EGFR over expression has been correlated with poor response to trastuzumab treatment as well as a poor clinical outcome in women with HER2+ breast cancer (88, 89). While PI3K is a downstream target of EGFR signaling, growing evidence suggests that Notch activates the PI3K pathway to promote survival. The regulation of the PI3K pathway by Notch has been shown...
by Palomero et al. Specifically, the transcriptional target of Notch-1, HES-1 has been shown to repress PTEN expression resulting in increased PI3K signaling in T-ALL (90). Furthermore, Jagged-1-mediated activation of Notch signaling was shown to increase the phosphorylation and thus the activation status of AKT1 in cervical cancer cell lines to promote EMT and increase cell motility (91). Previous studies have shown that inhibition of the PI3K pathway, using small molecule inhibitors that target AKT or the mTOR signal transduction pathway, are able to restore sensitivity to trastuzumab treatment (92). Similarly, Eichhorn et al. showed that the use of a dual PI3K and mTOR inhibitor, NVP-BEZ235, can reverse anti-EGFR/HER2 resistance in HER2+ breast cancers that express low PTEN or contain PIK3CA activating mutations (93). These results strongly indicate that both Notch and EGFR activate the PI3K pathway suggesting that the two pathways converge to promote proliferation and survival.

Hyperactivation of both the Notch and EGFR pathways were observed in TNBC cells by Dong et al. leading them to hypothesize that Notch–EGFR crosstalk occurs in this aggressive breast cancer subtype (94). Inhibition of EGFR or Notch alone was insufficient to reduce TNBC tumor burden, leading to the possibility that these complementary pathways are capable of interacting with one another to confer resistance to EGFR or Notch targeted therapies, hence bidirectional crosstalk between the EGFR and Notch pathway. Therefore, combined Notch (DAPT, Compound E) and EGFR (gefitinib) inhibition caused a decrease in TNBC cell proliferation and an increase in TNBC cell death both in vitro and in vivo indicating that a synthetic lethal relationship exists between the EGFR and Notch pathways. Furthermore, the AKT pathway has been shown to promote resistance to Notch inhibition (95, 96) as well as resistance to EGFR inhibition (97–99). Inhibition of EGFR or Notch receptors alone has little effect on the activity of AKT, but dual inhibition showed dramatically reduced level of serine-473 phosphorylation of AKT in vitro. Overexpression of NICD1 in the EGFR expressing TNBC cell line, HCC1806, caused resistance to combined DAPT and gefitinib treatment indicating that the Notch–AKT pathway confers resistance to combined EGFR and γ-secretase inhibition in this TNBC model. Unfortunately, all TNBCs do not have the same mutation profiles and some lack overexpression of EGFR. Importantly, a large group of TNBC malignancies with low EGFR expression are not affected by anti-EGFR or anti-Notch treatments, as seen by in vitro studies performed in MDA-MB-231 with a K-Ras mutation (100, 101) (Figure 3). These results indicate a bidirectional crosstalk between

**FIGURE 3 | Notch–EGFR crosstalk in TNBC.** Dong et al. ascertained a form of crosstalk between EGFR and Notch receptors that enabled resistance to EGFR targeted therapy, gefitinib, in TNBC cells. It is proposed that EGFR targeted resistance occurs by Notch-1-mediated activation of the AKT pathway. Notch–EGFR crosstalk facilitates an increase in serine-473 residue phosphorylation of AKT, which allows TNBC cells to survive EGFR targeted treatments. Dual inhibition of EGFR via the tyrosine kinase inhibitor (TKI) gefitinib and the Notch pathway by GSIs, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) in vitro, and compound E in vivo, are synergistically lethal to TNBC tumorigenesis.
Notch and EGFR occurs in breast cancer. Clear evidence provided shows that both Notch and EGFR pathways are used by TNBC cells to escape targeted inhibition by compensatory activation of the Notch pathway. Compensatory Notch pathway activation maintains MAPK and/or AKT downstream signaling pathways making the breast cancer cell resistant to EGFR targeted treatment. Preclinical studies have combined EGFR and HER2 inhibitors with MAPK/MEK or AKT/Pi3K/mTOR inhibitors to reduce resistance to EGFR/HER2 TKIs (92, 93, 102). One mechanism that has been proposed is that EGFR regulates the Notch pathway through the repression of the global transcriptional co-repressor, Goucho/TLE (Transducer like Enhancer of split) (103) to attenuate Notch-mediated gene activation and promote growth of cancer cells. EGFR targeted inhibition alone has been an ineffective treatment strategy for TNBC and understanding the mechanisms of EGFR resistance as well as improving therapeutic strategies for TNBC are critical areas of research to improve survival rates for these women. Dual targeting of the EGFR and Notch pathways may become a viable treatment option for women diagnosed with EGFR over expressing TNBC.

**NOTCH–HER2 CROSSTALK**

Notch–HER2 crosstalk was first identified in breast cancer cells by Osipo et al. who observed that HER2+ breast cancer cells had low Notch-1 expression and activation. Upon treatment with an anti-HER2 therapy (trastuzumab), or observing HER2+ cells with an acquired resistance to an anti-HER2 therapy, it was noted that Notch-1 expression and Notch transcription of canonical target genes increased (50). This important research described siRNA knockdown or GSI-mediated inhibition of Notch-1 enhanced trastuzumab sensitivity and reversed resistance to trastuzumab treatment *in vitro*. These findings led to the conclusion that HER2 inhibition increased Notch-1 activity in a compensatory manner to promote survival and resistance of HER2+ breast cancer cells. Combinatorial targeted treatment of Notch and HER2 signaling pathways not only inhibited HER2+ tumor formation but more importantly recurrence (104).

One mechanism by which HER2 restricts Notch signaling was identified by Ju et al. These investigators showed that HER2 overexpressing breast cancer cells activate the ERK pathway to block the activity of the γ-secretase complex thus resulting in reduced levels of NICD1. Interestingly, these investigators also showed that Notch-1 is a transcriptional repressor of the *Survivin* gene, a potent anti-apoptotic gene that is regulated at the transcription level by the E3 ligase complex, XIAP1:XIAP. Specifically, Ju et al. showed that the HER2–ERK axis represses XIAP1, which is required to ubiquitylate the survivin protein. This resulted in increased survivin protein stability to promote survival of HER2+ breast cancer cells. Therefore, these results suggest that HER2 signaling via ERK inhibits Notch-1 cleavage thus limiting the formation of NICD1 and stabilizes the survivin protein (105) (Figure 4).

Conversely, crosstalk between HER2 and Notch could be stimulatory rather than inhibitory. One of the first studies to implicate crosstalk between Notch-1 and HER2 was published by Chen et al. where they showed that the HER2 promoter contained a putative CBF-1 binding site (106). Specifically, these investigators demonstrated that overexpression of NICD1 induced transcription of HER2 suggesting that Notch-1 positively regulates HER2 expression. Moreover, Notch has been shown to regulate HER2 expression in BCSCs thereby controlling the survival and differentiation of BCSCs (107), which have been implicated in promoting breast cancer cell drug resistance, metastasis, EMT, as well as tumor recurrence and growth. Furthermore, Pradeep et al. demonstrated that HER2 overexpression in MCF-10A-DCIS cells increased Notch-3-mediated transcription of Notch target genes to promote a more malignant phenotype (108). In the HER2+/DCIS cell model, Notch-3 nuclear localization was increased along with ADAM17, presenilin-1, Jag-1, DLL-1, and Notch-1, all components of the Notch pathway. Knock down of Notch-3 reduced HER2+ DCIS cell proliferation, spheroid formation, and luminal spheroid filling indicating that HER2-mediated up-regulation of Notch-3 promoted HER2+ DCIS cell proliferation and survival. Findings by Pradeep et al. imply that HER2-mediated activation of Notch-3 is necessary during the early steps of mammary tumorigenesis and targeted inhibition of these pathways can reduce early tumor progression in DCIS-HER2+ cancers with Notch-3 overexpression.

Combined inhibition of Notch and HER pathways, EGFR or HER2 via gefitinib or lapatinib, respectively has been investigated in a DCIS model by Farnie et al. to deduce the effects of dual inhibition of the Notch–EGFR/HER2 pathways on DCIS stem cells (109). Using a 3D culture model to assess luminal filling of DCIS acini as well as DCIS Cancer Stem Cell (CSC) activity, two DCIS cell lines, SUM225 (HER2−), and MCF10DCIS HER2 (HER2+), as well as human primary DCIS samples (HER2±) were studied. These two cell lines were treated with GSI (DAPT) and/or anti-HER1/2 (gefitinib or lapatinib) therapies to deduce the role of Notch–EGFR/HER2 crosstalk in HER± DCIS breast cancer. Anti-Notch treatment reduced mammosphere formation and acini size in the HER2+ cells yet had no effect on the HER2− cells. Anti-EGFR/HER2 treatment had the opposite effect by reducing mammosphere formation and acini size on HER2− cells with no effect on the HER2+ cells. Interestingly, combined inhibition of the Notch and EGFR/HER2 receptors reduced both acini size and mammosphere formation regardless of HER2 expression indicating that the crosstalk between Notch and EGFR/HER2 receptors maintains BCSC survival and self-renewal (Figure 5). In addition to these studies, others have shown that HER2 may facilitate increased expression of components of the Notch pathway such as Notch-ligands (Jag-1, Jag-2, DLL-1) or Notch activating metalloproteinases (ADAM, presenilin-1).

Notch–HER crosstalk can involve upstream and/or downstream components of either pathway. Recent data have shown Notch-dependent up-regulation of the ADAM12 metalloproteinase causes an increase in ectodomain shedding of HB-EGF, an EGFR growth factor, in head and neck squamous-cell carcinoma cells under hypoxic conditions. Notch-mediated release of HB-EGF causes the formation of invadopodia to aid in cancer cell invasion (110). Notch–EGFR crosstalk has been implicated as a paracrine mediator of estrogen to promote ER+/ER− low BCSC survival and proliferation within ER+ breast cancer cell lines and patient samples (111). Recent data have shown that estrogen increases ER+ bulk cell (Non-BCSC) production of EGFR,
Ju et al. determined an intricate form of crosstalk between HER2 and Notch-1 in HER2+ breast cancer. HER2+ breast cancer cells exhibit an increase in survivin protein expression concomitantly with a decrease in survivin mRNA expression. HER2 promotes activation of the AKT pathway, which threonine 308 phosphorylated AKT can inhibit p21-mediated inhibition of cyclin dependent kinase-1 (CDK1) dimerization to cyclin B1. Concomitantly, HER2-mediated activation of the MAPK pathway enables Erk-mediated CDK1-Cyclin B1 dimerization as well as inhibition of ubiquitination and proteasomal degradation of survivin by restricting the formation of the X-linked Inhibitor of apoptosis protein-X-linked associating factor-1(XIAP-XAF-1) complex. Together, AKT and Erk promote dimerization of CDK1 to Cyclin B1, which stabilizes survivin by phosphorylating the threonine 34 residue (Thr34) of the survivin protein. Increased survivin stabilization causes an increase in survivin expression. Survivin mRNA is reduced by Erk-mediated inhibition of γ-secretase, which in turn inhibits Notch receptor-mediated transcriptional activation. Pradeep et al. established an interesting form of crosstalk between the HER2 and Notch-3 receptors in HER2+ DCIS. HER2 up-regulates the transcription of the Notch pathway components: jagged-1, -2 (Jag-1/2), DLL-1, ADAM17, presenilin-1 (Pres-1), as well as Notch-3 and Notch-1 (Notch-3/1) through the MAPK pathway. Increased transcription of the Notch pathway components causes Notch-mediated up-regulation of c-Myc, Cyclin D1, phosphorylation of Serine-473 of Atk (+P-Ser 473 AKT), and down-regulation of the tumor suppressor, PTEN. Combined GSI and trastuzumab (Trast) treatment reduce HER2+ DCIS cell survival and invasiveness.

FGFR, and Notch-ligands, which are able to regulate ER−/ER low BCSC differentiation and expansion via the EGFR/MAPK/ERK, FGF/FGFR/Tbx3, and DLL-Jag/Notch/Pea3 pathways. Estrogen-driven BCSC regulation can be inhibited by blocking estrogen using tamoxifen, EGFR using gefitinib, or Notch using a GSI to attenuate BCSC survival and early progenitor cell differentiation, and possibly de-differentiation, respectively. Crosstalk between EGFR, FGFR, and Notch pathways are responsible for estrogen induced changes in the ER−/ER low BCSC population and may play a role in endocrine resistance as well as offer suitable targets for the treatment of ER+ breast tumors. Combinatorial drug treatments simultaneously targeting Notch and EGFR receptors continue to be assessed to reduce the occurrence of chemoresistance in cancers such as glioblastoma. Sooman et al. treated six glioblastoma cell lines with drugs such as imatinib (TKI), camptothecin, or temozolomide, and measured gene expression using microarray to identify molecular pathways associated with drug resistance, then combined the cytotoxic treatments with targeted inhibitors to determine optimal combinatorial treatments for glioblastoma (112). Synergistic effects of camptothecin with...
Elevated p63 increased Jagged-1-mediated Notch signaling caused an increase in STAT3, which in turn increased p63 expression and signaling (96). Activated Notch-1 signaling was found to inhibit p53 activation by blocking nuclear localization as well as phosphorylation at Ser15, Ser20, and Ser932 residues. Blocking mTOR signaling using rapamycin treatment was shown to sensitize cancer cells to chemotherapy by preventing Notch-1-mediated down-regulation of p53. Conversely, up-regulation of the mTOR downstream target eukaryotic initiation factor 4E (eIF4E) caused chemo-resistance as well as inhibition of p53-mediated apoptosis. These findings also support the critical role of the PI3K/mTOR pathway on Notch-1-mediated regulation of apoptosis and drug resistance.

An intriguing mechanism of communication between the Notch and MAPK pathways has been elucidated by Izrailit et al. (114). Active Notch-1 expression in the TNBC cell line MDA-MB-231 was found to use the MAPK/ERK pathway to facilitate Jagged-1 up-regulation and subsequent Notch activation. The TGFB pathway has also been implicated in regulating Jagged-1 expression to promote bone metastasis in breast cancer (115). Using high throughput screening, Izrailit et al. found that the pseudokinase Tribble-3 (TRB3) regulates activation of both the MAPK/ERK and TGFB pathways. Knock down of TRB3 reduced MDA-MB-231 cell proliferation while Jagged-1 over expression in the TRB3 silenced cells rescued cell proliferation. Similar studies were done in vivo in which the MDA-MB-231 cells with TRB3 knock down had significantly smaller tumors than mice with without TRB3 knock down. These results indicate that TRB3 increases Jag-1 expression through the MAPK/ERK pathway and this is critical to MDA-MB-231 cell proliferation and tumorigenesis.

The studies in this review reveal communication between the Notch and HER pathways in breast cancer cells that enable the cell to compensate for the loss of one pathway with the potential compensatory increase in the other. This research is continuing to unveil multiple interactions between multiple pathways to discern the in depth complexity by which breast cancer cells are able to thrive under a variety of targeted or cytotoxic cancer treatments. Continued work in this area may shed light onto the intricacies of pathway to pathway communication that could be utilized in other forms of cancer and diseases. The Notch and HER pathways have overlapping functions that justify their compensatory relationship as both are able to promote cell survival, growth, and motility, but nuances of either pathway may be able to be teased out to further assess effects of compiling multiple pathways.
targeted cancer treatments. For instance, the Notch pathway has also been associated with increased angiogenesis via DLL4, IL-1, or Leptin-mediated up-regulation of VEGF/VEGFR (116) as well as AKT/mTOR-mediated up-regulation of glucose metabolism via up-regulation of Glut 1 (117).

This review has summarized the communication mechanisms between the Notch and HER receptors with a particular focus on PI3K and MAPK signaling pathways. More studies are needed to uncover novel interactions, downstream pathway mutations, microRNA dysregulation, or epigenetic changes that may be critical to understanding how cancer cells move between pathways to create their own signaling circuit for survival. Further assessment of Notch–HER crosstalk in BCSC and stromal-cell communication could reveal differences in autocrine signaling to promote BCSC growth and survival. Continuing to determine mechanisms of action between critical oncogenic pathways such as Notch and HER may enable scientists to better diagnose and treat breast and other solid tumors using an increasingly specialized treatment plan that may preemptively avoid development of treatment resistance, CSC growth, and tumor recurrence.

CONCLUSION

Notch–HER crosstalk continues to yield insight into the compensatory mechanisms used by breast and other cancer cells to propagate cell survival, growth, metastasis, as well as drug resistance. Combinatorial treatments targeting the HER and Notch pathway simultaneously have yielded positive results in attempts to combat acquired resistance to molecular targeted and cytotoxic therapies. The goal is ultimately, to prevent or reverse drug resistance, as well as providing gene signatures that could preemptively reveal potential resistance and sensitivity to combinatorial treatment plans, in women diagnosed with HER+ or ER+ breast cancer. Continued research will unravel mechanisms of Notch–HER crosstalk to further improve clinical response to emerging therapies as well as revitalize current therapies in hopes of continued improvement of treatment strategies for women with breast cancer and other solid tumors.

Each breast cancer subtype is represented in Table 1 as well as the molecular markers that each breast cancer subtype tends to over express. The most common first and second line treatment therapies for each subtype are included and it is important to note that these therapies change based on the patient’s family history, age, race, as well as tumor stage (invasiveness) and histological grade. The rates of breast cancer subtype occurrence are approximated in this table (1). Advances in genomic driven classification of breast cancer continue to evolve the subtypes, particularly in TNBC, which can be further divided into: basal-like 1, basal-like 2, immunomodulatory, mesenchymal-like, mesenchymal stem-like, and luminal androgen receptor (12). Typical treatment options are neoadjuvant, or adjuvant, meaning they include variations of chemotherapy and/or radiotherapy before or after surgery, respectively, in an effort to remove/eradicate as many tumor/cancer cells as possible.

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