Trends in Developmental Biology
Vol. 6, 2012

Review

Neuregulin signaling in mammary morphogenesis and function

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ABSTRACT

The ErbB family of receptor tyrosine kinases has four members, EGFR, ErbB2, ErbB3 and ErbB4. Although the causal roles of EGFR and ErbB2 overexpression in breast cancer have been intensely studied, less attention has been paid to ErbB3 and ErbB4. However, several recent studies demonstrate an increasing research interest in ErbB3 and ErbB4 as potential therapeutic targets in breast cancers. The signaling pathways at play in breast cancers are often the same pathways that drive cell growth and survival in the untransformed breast epithelium. The roles of ErbB3 and ErbB4 in the normal mammary epithelium have been studied in depth using genetically engineered mouse models. This review will discuss ErbB3 and ErbB4 signaling pathways in the context of epithelial diversity in the breast throughout the many developmental phases that characterize the breast, and we will relate their physiological functions to potential parallels in breast tumorigenesis and malignant progression.

KEYWORDS: ErbB3, ErbB4, neuregulin, heregulin, mammary gland epithelium

INTRODUCTION

Neuregulins (NRGs) are a family of secreted low molecular weight ligands that bind to and activate cell surface receptors to induce a wide variety of cellular responses ranging from migration to adhesion, from growth to apoptosis and terminal differentiation. Four known *NRG* genes encode unique NRG ligands, NRG-1 (also termed heregulin), NRG-2, NRG-3, and NRG-4. Multiple splice variants for each NRG ligand have been described. Many of the reported NRG ligand splice variations affect intracellular signaling in distinct ways, and therefore may impact the biological consequences of ligand-receptor interactions. The NRGs are capable of binding to only two receptors encoded in the mammalian genome, ErbB3/HER3 and ErbB4/HER4. These two receptor tyrosine kinases (RTKs) are members of the ErbB RTK family, which also includes epidermal growth factor receptor (EGFR) and ErbB2/HER2. In the absence of ligand, ErbB3 and ErbB4 exist as catalytically inactive monomers. Upon NRG binding, the heterodimerization interface of the receptors are exposed, allowing for homo- or hetero-dimerization within the ErbB family, activation of the tyrosine kinase domain, and trans-phosphorylation of tyrosine residues in the C-terminal domains of heterodimeric partners [1-3]. Phospho-tyrosine residues recruit SH2-domain containing signaling molecules to ErbB3 and ErbB4, thus initiating NRG-induced signal transduction through intracellular pathways. It should be noted that, while NRGs do not bind to EGFR or ErbB2, NRG binding to ErbB3 and ErbB4 promotes heterodimerization between all ErbB family members, which can result in catalytic activation of EGFR and ErbB2. Similarly, the EGF-like ligands that directly activate EGFR can induce heterodimeric signaling through ErbB3

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and ErbB4, thus lending to the complexity of ErbB family signaling. NRGs are the only ligands known to bind to ErbB3. However, ErbB4 can also be ligand-activated by several EGF-like ligands, including heparin binding (HB)-EGF, epiregulin, and betacellulin [4-6].

Each ErbB family member is expressed in unique but partially-overlapping patterns during specification, patterning, and growth of the breast epithelium [7]. Genetically engineered mouse models lacking EGFR, ErbB2, ErbB3, or ErbB4 in the mammary epithelium confirmed that each receptor is required in unique spatiotemporal patterns for development and homeostasis in the breast [8, 9]. The NRG receptors ErbB3 and ErbB4 are primarily expressed in the luminal mammary epithelium, but their expression is not uniform throughout this epithelial compartment, reflecting perhaps transient intracellular expression dynamics, or heterogeneity within luminal compartments.

Although ErbB signaling is highly studied, predicting biological outcomes of ErbB signaling is difficult in the breast, considering the complexity of expression patterns, potential ErbB receptor-ligand and heterodimeric combinations, heterogeneity within the mammary epithelial population, cyclical changes in the mammary gland that occur in response to steroid hormones, and downstream signaling pathways that are differentially tapped in response to ligand-receptor interactions. These signaling pathways include the phosphatidyl inositol-3 kinase (PI3K), mitogen activated protein kinase (MAPK), Src/focal adhesion kinase (FAK) and signal transducer and activator of transcription (STAT) pathways, to name a few. It is important to understand and organize the complexity of ErbB-directed biological outcomes within the normal mammary epithelium, because ErbB dysregulation contributes profoundly to breast cancer formation, progression, and therapeutic response, and to numerous human pathologies, including cancers, neurodegenerative disorders, mental health diseases, and aging. This review will focus on NRG-induced signaling pathways that are critical for development of the mammary gland, and how these signaling pathways are often used pathologically by breast cancers.

NRG-ErbB3 signaling pathways

Although ErbB3 was originally identified as being devoid of tyrosine kinase activity, recent evidence demonstrates that ErbB3 possesses catalytic activity, albeit weak compared to the other ErbB family members [10]. Therefore, tyrosine phosphorylation of ErbB3 occurs most efficiently upon heterodimerization with other ErbB family members, but occurs nonetheless within ErbB3 homodimers. ErbB3 harbors six consensus YxxM motifs that, when tyrosine phosphorylated, activate signaling through the phosphatidyl inositol 3-kinase (PI3K pathway) by interacting with SH2 domains within p85, the regulatory subunit of PI3K [11, 12]. This relieves p85-mediated inhibition of the PI3K catalytic subunit p110. PI3K produces phosphatidyl inositol triphosphate (PIP_3), a second messenger that nucleates pleckstrin homology (PH) domain-containing proteins (e.g. Akt) at cell membrane sites to initiate phosphorylation cascades promoting cell survival, cell cycle progression, protein translation, cellular metabolism, and motility [13-15]. It is important to note that PI3K pathway mutations, such as RTK amplifications, activating mutations in p110, or amplification of Akt, are the most frequently occurring pathway mutations in breast cancers.

The affinity of the PI3K regulatory subunit p85 for any one interaction motif within ErbB3 is higher than the affinity between p85 and any other ErbB receptor [16]. Because ErbB3 has six high affinity p85 interaction motifs [16], more than any other RTK in the genome, phosphorylated ErbB3 has the potential to amplify PI3K signaling output in response to NRG. This observation underlies the intense research focus on the role of ErbB3 in the ontogeny of PI3K-dependent cancers, including many breast cancers. Recent findings also suggest that upregulation of ErbB3 expression and tyrosine phosphorylation in response to therapeutic targeting of other PI3K pathway components can circumvent the therapeutic benefit of the inhibitor by efficiently reactivating PI3K signaling. Furthermore, it is becoming increasingly apparent that heterologous RTKs are capable of ErbB3 tyrosine phosphorylation. For example, gene amplification of the hepatocyte growth factor (HGF) receptor

MET in lung cancer cells results in MET-dependent ErbB3 tyrosine phosphorylation. Similarly, gastric cancer cells with gene amplification of fibroblast growth factor (FGF) receptor-2 (FGFR2) display FGFR2-induced ErbB3 tyrosine phosphorylation. These findings support the idea that despite the weak intrinsic tyrosine kinase activity of ErbB3, cellular mechanisms are in place to maximize signaling from ErbB3 to the PI3K pathway.

NRG-induced ErbB3 signaling is also reported to activate the mitogen-activated protein kinase (MAPK) pathway, consistent with the observations that phosphorylated ErbB3 can recruit Shc1, Shc3, Grb2, and Grb7. ErbB3 was also reported to interact strongly with Nck1, Nck2, Crk and CrkL, although the biological significance of these interactions remains to be investigated.

NRG-ErbB4 signaling pathways

Sequence analysis of human and mouse *ERBB4* cDNAs revealed alternative *ERBB4* splicing in two areas - the extracellular juxtamembrane (JM) region (thus producing JMa or JMb isoforms) [17, 18] and a small cytoplasmic (Cyt) domain (thus producing Cyt1 and Cyt2 isoforms) [19, 20]. While the ErbB4-JMa isoform contains an extracellular proteolytic site which is subject to cleavage by the metalloprotease tumor necrosis factor converting enzyme (TACE), the JMb isoform lacks the TACE cleavage site [21]. ErbB4-JMa cleavage by TACE occurs upon NRG-mediated activation of ErbB4-JMa in a manner dependent on the ErbB4 kinase [22]. Once cleaved by TACE, ErbB4-JMa is cleaved a second time by the intra-membrane enzyme γ-secretase [23, 24], thus releasing the intracellular domain of ErbB4 into the cytoplasm. This ErbB4 intracellular domain is referred to herein as ErbB4-ICD. Once liberated into the cytoplasm, ErbB4-ICD exhibits nuclear localization, thus explaining the nuclear ErbB4 immuno-localization frequently seen in human breast cancer specimens, normal human and mouse mammary tissue, endometrial cancers and normal auditory sensory epithelium. Nuclear-cytoplasmic translocation of ErbB4-ICD is dependent on its acidic nuclear localization sequences [23, 25, 26]. There are also three putative nuclear export sequences [23], and a D-box motif often found in nuclear proteins that are targeted for proteosomal destruction by the anaphase-promoting complex, a nuclear E3-ubiquitin ligase [27].

While the precise physiological function of ErbB4-ICD is unclear at this point, we do know that ErbB4 kinase activity is required in the mammary epithelium for phosphorylation and nuclear translocation of the transcription factor Signal Transducer and Activator of Transcription 5 (STAT5), because ErbB4-deficient mammary epithelial cells fail to demonstrate activation of STAT5a, even in the context of other known pathways of STAT5a phosphorylation, including prolactin-induced activation of the intracellular tyrosine kinase Jak2, the canonical activator of STAT5a in the mammary gland. Proteomic studies identified more phosphorylated tyrosine residues in NRG-stimulated ErbB4 than have previously been observed on any other RTK [28], suggesting that ErbB4 has a strong potential to transduce signals to intracellular targets. Despite the number of phosphorylated tyrosine residues in ligand-activated ErbB4, high resolution proteomic mapping of ErbB4-interacting proteins revealed very few binding proteins, whereas similar experiments identified numerous proteins capable of binding to EGFR, ErbB2, and ErbB3. Therefore, ErbB4 may potently activate a select few signaling pathways with great intensity.

Of the proteins identified in complex with ErbB4, the p85 regulatory subunit of PI3K and Shc1, an upstream activator of MAPK signaling, were found. These data are consistent with the known ability of NRG-induced ErbB4 to activate PI3K and MAPK signaling in HEK293 cells. It is interesting to note that the p85 interaction motif at ErbB4 Y1056 is located within the 16 amino acid region that is included in ErbB4-Cyt1 but excluded from ErbB4-Cyt2 [19], which lends support to the idea that these two ErbB4 splice variants might have profoundly distinct biological properties. Consistent with its known role in STAT5 activation, a high affinity interaction between phosphorylated ErbB4 and STAT5 was found using an unbiased proteomics approach [29]. Investigators have also identified STAT1 interactions with ErbB4 [28], although the biological consequences of the ErbB4-STAT1 interaction are currently not known, but may relate to the ability of both ErbB4 and STAT1 to induce apoptosis in several cell types.

NRG signaling drives cellular behaviors important in the normal breast epithelium

Based on the identified interactions that occur in response to NRG-induced ErbB3 and ErbB4 activation, it is expected that ErbB3 would drive PI3K and MAPK signaling in mammary epithelial cells. This has been confirmed using mouse models of ErbB3 ablation in the developing mammary epithelium. Loss of ErbB3 resulted in impaired signaling through the PI3K pathway to Akt, a serine-threonine kinase that lies at the apex of several growth-promoting and cell survival-inducing cascades. Loss of ErbB3 also decreased MAPK signaling in the normal mammary epithelium. Mammary glands lacking ErbB3 displayed decreased growth during puberty, when the ductal epithelium is actively proliferating and lengthening to fill the mammary fat pad with an arborized epithelial network. Interestingly, loss of ErbB3 from the mammary gland during periods of epithelial quiescence profoundly disrupted epithelial homeostasis by causing cell death in the luminal compartment of the mammary gland [30]. These observations suggest that ErbB3 signaling is differentially required at distinct phases of breast development/homeostasis, although the mechanisms by which the cell funnels ErbB3 signaling to the correct output remains under investigation.

In addition to PI3K and MAPK signaling, ErbB4 is capable of activating the transcription factor STAT5a. This is of critical importance to the biological function of the mammary gland as a milk-producing organ. STAT5a expression and activity are required to induce growth of the milk-producing alveolar epithelium during pregnancy, for transactivation of the genes encoding milk proteins during late pregnancy and lactation, and for survival of the alveolar epithelium for the duration of lactation. Mice lacking ErbB4 within the mammary epithelium are incapable of nursing offspring due to a failure of STAT5a activation, suggesting that ErbB4 is a critical upstream activator for STAT5-mediated lactogenesis. Unlike ErbB3-deficient mammary glands, loss of ErbB4 from the ductal epithelium did not impair growth or survival in this epithelial population at any time point observed. Therefore, ErbB3 functions primarily in the ductal epithelium of the breast, while ErbB4 functions primarily in the alveolar epithelium. In support of this hypothesis, the ductal epithelium expresses ErbB3 more abundantly than it does ErbB4, and conversely the alveolar epithelium harbors more ErbB4 than ErbB3.

NRG signaling in mammary gland development

The mammary gland is a dynamic organ that must accommodate drastic morphological changes in response to hormonal cues at puberty, pregnancy, and post-lactational involution, and profound differentiation at lactation. The mammary epithelium is also subjected to a lifetime of cyclical expansion and retraction in response to rising and falling hormonal phases of the estrus/menstrual cycle. Although changes in the mammary epithelium related to the estrus/menstrual cycle are often overlooked due to their subtle nature, the frequency with which they occur throughout the duration of a woman's reproductive lifespan make even these subtle changes capable of large accumulated effects, and it is therefore important to understand the events governing both the profound and the subtle changes to the mammary epithelium during its life-long developmental transitions.

Embryonic mammary development

Much of what is known regarding embryonic and pubertal mammary organogenesis has been determined in rodent model systems. The mammary epithelium is specified from the embryonic ectoderm at mid-gestation. In mice this occurs at embryonic day 11.5 (E11.5). The ventral ectoderm thickens in two symmetric lines that traverse from the forelimb bud to the hindlimb bud at locations lateral to the midline. These are referred to as the mammary ridges. Signals from the underlying mesoderm at E12 encourage growth and consolidation of the mammary ridge epithelium into mammary placodes, which demarcate the presumptive nipple and will give rise to the entire epithelial network of the future mammary gland [31]. Mesoderm-derived signals include fibroblast growth factor-10 (FGF-10) and NRG-3. Decreased NRG-3 expression in *Scaramanga* (*Sca*) mice impairs the formation of mammary buds during embryogenesis and complete loss of mammary epithelial structures in the adult mouse. Conversely,

ectopic placement of NRG-3-soaked beads along the mammary ridge of E11.5 embryos caused the formation of supernumery mammary structures [32]. In humans, the gene encoding NRG-3 is located at chromosome 10q22-q23, a genomic site associated with developmental delay, mild facial dysmorphism, cerebellar anomalies, cardiac defects and congenital breast aplasia, again pointing to NRG-3-induced signaling pathways in early breast specification [33].

Based on the central role of NRG-3 in mammary epithelial specification, NRG receptors must also be involved in mammary specification. Consistent with this idea, ErbB3 expression is seen in mammary placodes at E11.5 [34]. ErbB3 expression in the mammary epithelial placode precedes that of ErbB4, which is initiated at E12.0. ErbB3 and ErbB4 are confined to the mammary bud and adjacent epithelium through E14.5. However, loss of either ErbB3 or ErbB4 in the mammary epithelium in genetically-engineered mice did not impair mammary bud formation. While it is possible that ErbB3 may compensate for loss of ErbB4 under these conditions, or *vice versa*, rigorous testing of this hypothesis would require the combined loss of ErbB3 and ErbB4 in the embryonic ectoderm preceding mammary bud formation in order to eliminate all NRG receptor signaling within the epithelial cell population from which the mammary bud derives.

Continued signaling from the underlying mesoderm induces epithelial invagination from the placode into the underlying mesoderm and continued proliferation, forming a rudimentary mammary bud. Continued growth from E12-E18 lengthens the mammary bud to form a solid epithelial cord that bifurcates repeatedly, forming the nascent mammary ductal network. Apoptosis within the primary duct canalizes the lumen of what will become the lactiferous sinus, while the remaining 9-11 branches of the mammary epithelial tree remain filled [31].

Development of the luminal epithelium and myoepithelium

Post-natal stereotactic growth of the rudimentary ductal tree maintains the relative size of the mammary ductal network in relation to the growing mammary fatpad in which the mammary epithelium is embedded. Upon puberty (occurring at 3 weeks of age in female mice) systemic hormonal signals (primarily estrogen and progesterone) and locally produced growth factors (insulin-like growth factor-1, or IGF-1) increase proliferation of the mammary ductal epithelium, specifically within a structure known as the terminal end bud (TEB). This is a club-shaped structure located at the distal-most aspect of the mammary epithelium. Growth at the distal tip results in distal ductal lengthening throughout the mammary fat pad. The TEB consists of a single layer of cap cells that surround a multi-layered structure comprised of body cells. Cap cells are highly proliferative, and are thought to harbor a mammary stem cell (MaSC) population, bipotential mammary progenitor cells (BPCs, those cells capable of developing along the luminal or myoepithelial lineages) and committed myoepithelial cells, the contractile cells that contact the basement membrane of the duct. The multilayered body cells are also highly proliferative, and generally stain positive for E-cadherin. The body layer harbors committed luminal progenitor cells (capable of giving rise to either ductal luminal cells or alveolar luminal cells) and mature ductal luminal cells. Apoptosis within the solid club-shaped TEB is critical to generate a patent lumen within the mammary ducts. Therefore, proliferation, apoptosis, specification, differentiation, and cell migration are actively occurring within the confined space of a TEB, making the TEB a highly dynamic structure comprised of heterogeneous cells with heterogeneous behaviors that are tightly regulated in order to produce a lengthening, canalized ductal epithelium. As puberty draws to a close and the ductal epithelium reaches the distal edge of the mammary fat pad, TEBs regress and proliferation within the mammary epithelium is substantially reduced as compared to what is seen during puberty.

Genetically-engineered mice lacking each of the ErbB family RTKs demonstrate the requirement of ErbB3, but not ErbB4, for ductal growth and patterning of the ductal mammary epithelium during puberty. This is consistent with expression analyses of phosphorylated ErbB3 and ErbB4 in mouse mammary glands, which demonstrated high levels of total and phospho-ErbB3 in the lengthening ductal mammary epithelium during

puberty, while total and phospho-ErbB4 levels remained low. Loss of ErbB3 decreased proliferation and cell survival in the body cell population of the terminal end buds, correlating with a loss of Akt phosphorylation and MAPK signaling. Cellular loss within the TEB was accompanied by structural disorganization of the TEB, and a pathological expansion of the TEB cap layer. Loss of ErbB3 from the keratin 14-expressing cap layer did not recapitulate the expansion of the cap layer, suggesting that cap layer expansion was an indirect effect of ErbB3 loss within the body layer of the TEB, and is required to maintain a homeostatic balance of differentiated epithelial cell types within the mammary gland.

Development of the alveolar epithelium

The luminal lineage of the mammary epithelium is comprised of two distinct populations that arise from a common precursor, the ductal luminal cells and the alveolar luminal cells. Ductal luminal cells are critical for mammary gland homeostasis, as they perform a critical barrier function supported by cell-cell junctions. These cells also serve as the conduit for delivery of milk from the site of milk production to the offspring. The alveolar cells develop within lobular structures as opposed to ductal structures, and their primary function is to synthesize and secrete milk. Therefore, while the ductal epithelium is established at puberty, the majority of the alveolar epithelium is not established until pregnancy and lactation. In humans, but not rodents, some development of the alveolar epithelium occurs at puberty in terminal structures called terminal ductal lobular units (TDLUs), which serve as the precursors for the milk-producing alveolar epithelium that will develop during pregnancy.

Although the majority of alveolar development occurs during pregnancy, alveolar buds are generated through proliferation of alveolar progenitor cells during the estrus cycle in rodents and menstrual cycle in humans. This occurs in response to rising systemic steroid hormones. In the event of pregnancy, continued hormonally-driven alveolar proliferation allows for rapid expansion of this population, followed by secretory differentiation of the alveolar cells upon birth of offspring [35]. While the ductal epithelium sparsely populates the mammary fatpad prior to pregnancy, the alveolar epithelium completely fills the mammary gland during lactation, and can increase the mass (wet weight) of the mammary gland by nearly 10-fold. The extensive alveolar mammary epithelium is maintained until offspring are weaned.

In the absence of pregnancy-derived signals, falling hormonal levels at the end of the estrus/menstrual cycles result in apoptosis of alveolar progenitor cells and regression of alveolar buds. Similarly, post-lactational alveolar cells undergo apoptosis upon the cessation of nursing in a process termed post-lactational involution. While both processes involve apoptosis of the alveolar lineage, the extent of the apoptotic burden is much more profound during post-lactational involution than at any other point in the natural history of the mammary gland. Upto 90% of the entire mammary epithelial population can be culled within a single week in rodents, or within a month in humans. Cell death and clearance of the apoptotic load in the mammary gland is followed by extensive mammary gland remodeling, ultimately returning the mammary gland to a state in which it comprised primarily of a ductal epithelial network without alveolar cells, and the mammary gland can again respond to hormonal cues [36, 37].

Early studies demonstrated that NRG signaling is important for lactogenic development of the mammary epithelium. NRG-1-releasing pellets implanted into mammary fatpads of mice induced alveolar expansion and secretion of milk proteins into lumens [38]. Conversely, disruption of NRG-1 expression in the mouse mammary gland impaired alveolar growth and reduced β-casein expression at late pregnancy [39]. While NRG-1 appears to be critical for lactogenesis, loss of ErbB3 did not appear to inhibit alveolar growth or survival during pregnancy. It should be noted, however, that lactational success was not measured in this ErbB3-deficient model, as these analyses were performed by transplantation of ErbB3-deficient mammary tissue into cleared mammary fat pads, a technique that unfortunately severs the mammary epithelium from the nipple, therefore eliminating lactation. Nonetheless, the results suggest that ErbB3 is not required for alveolar expansion during pregnancy.

In contrast to ErbB3, expression and phosphorylation of ErbB4 increases in the mammary gland during pregnancy, primarily in the expanding alveolar population. Mice lacking ErbB4 fail to fully expand the alveolar epithelium during late pregnancy, fail to activate STAT5 and transcription of STAT5a target genes, and produce inadequate milk for nursing offspring. Conversely, expression of constitutively active ErbB4-ICD in mammary glands of transgenic mice resulted in precocious STAT5 phosphorylation and expression of β-casein in virgin mice. Taken together, these observations suggest that alveolar growth is driven by NRG-ErbB4 signaling.

Aberrant signaling of neuregulins

As with all developmental processes, perturbations of these developmental signals during adulthood can lead to pathological consequences, including tumorigenesis. Because ErbB3 and ErbB4 receptors and their NRG ligands are each involved in phases of luminal expansion in the mammary epithelium, it is possible that the NRG signaling pathway may be commandeered by transformed cells to promote aberrant growth and survival. ErbB4 overexpression in breast tumor cells in some cases resulted in increased cell growth [40-42], while in other cases resulted in decreased cell growth. Examination of ErbB4 expression in clinical breast cancer datasets has also proven inconclusive, as many studies claim that ErbB4 expression predicts lower tumor grade and increased overall survival [43, 44], while others show that ErbB4 expression correlates with reduced overall survival. Clear evidence suggests that *ERBB4* mRNA expression is highest in luminal breast cancers as compared to other breast cancer molecular subtypes (including HER2-enriched, normal-like, basal-like, and claudin-low). Using mRNA expression in 158 breast cancers clustered according to their histological type of cancer, it was found ErbB4 protein expression was typically upregulated in estrogen receptor (ER)-positive HER2-negative lobular breast cancer [41]. These observations are consistent with the known role of ErbB4 within the luminal epithelium of the untransformed breast, specifically in lobular-alveolar structures. Because luminal breast cancers have a more favorable prognosis than other breast cancer subtypes, it will be important to take into account molecular subtype of all breast cancers used to assess the prognostic significance of ErbB4. Earlier studies examining ErbB4 expression in breast cancers would not have routinely clustered datasets according to molecular subtype, which may therefore skew many of the interpretations resulting from these studies.

Similar to *ERBB4*, expression levels of *ERBB3* mRNA are highest in luminal breast cancers as compared to other molecular subtypes. Interestingly, *ERBB3* overexpression, which occurs in up to 50% of luminal breast cancers, correlates with increased metastases, increased risk of recurrence, and decreased overall survival [44-49]. The increased expression of ErbB3 may increase PI3K activity, therefore driving cellular proliferation, survival, and motility [50]. Increasing evidence suggests that ErbB3 and ErbB4 signaling in ER-positive breast cancers may promote resistance to tamoxifen. Both receptors are upregulated in response to tamoxifen treatment in human breast cancer cells, as *ERBB3* and *ERBB4* are transcriptionally repressed by estrogen signaling [51, 52]. There is some evidence of mutually exclusive regulation of the ER and ErbB pathways. In fact, tamoxifen-induced upregulation of ErbB3 and ErbB4 sensitizes ER-positive breast cancer cells to the growth promoting effects of NRG-1, suggesting that compensatory signaling through ErbB3 and ErbB4 might be therapeutically relevant.

Based on these observations, it can be predicted that NRG ligands are expressed and contribute to the pathogenesis of breast cancers. This hypothesis is supported by studies demonstrating that transgenic NRG-1 overexpression induced mammary tumor formation in mice [53]. NRG ligand upregulation is reported to increase therapeutic resistance in a number of breast cancer cell lines, including luminal and HER2-amplified cells. Despite the known role of NRG-3 in fundamental mammary epithelial specification, very little is known about NRG-3 in breast cancers. A single study of 363 breast cancers reported that NRG-3 expression was detected in 43% of all tumors. In this study, NRG-3 (but not NRG-2) expression positively correlated with ErbB3 and ErbB4 expression [54]. Interestingly, this study demonstrated that NRG-2 correlated with a poor

prognosis, confirming a previous report suggesting that NRG-2 protein levels correlated strongly with positive lymph node status in patients with breast cancer [55]. A recent report demonstrated that ErbB3, ErbB4, NRG-3 and NRG-4 each correlated positively with ER+/progesterone receptor (PR)+ breast cancers, while NRG-2 and HB-EGF (a ligand that activates ErbB4 and EGFR, but not ErbB3) correlated with PR-negative tumors [56], which generally carry a worse prognosis than those expressing PR. NRG-4, in combination with epigen, was a powerful predictor of overall survival in 100 breast cancers surveyed by immunohistochemistry [57].

CONCLUSION

Taken together, these studies support further investigations into neuregulin signaling in breast tumor formation, therapeutic response, and overall outcome of patients with breast cancer. Increasing evidence supports ErbB3 as a potential therapeutic target in *HER2*-amplified and perhaps Luminal A/B breast cancers. However, less is known regarding the role of ErbB4 in breast cancer, how different splice variations in ErbB4 may contribute to neuregulin-driven biological outcomes, or how nuclear translocation of ErbB4 may affect critical signaling networks at play in breast cancers. It is critical, then, that we continue pursuing neuregulins and their receptors in breast cancers as well as in the normal breast. These studies will begin to bridge the knowledge gap between events occurring in normal breast development and those at the earliest steps in breast epithelial transformation, when breast cancers can be effectively prevented. A clear understanding of the molecular events that drive proliferation of specific epithelial compartments of the mammary epithelium will further allow us to increase the specificity of therapeutic drugs to the precise epithelial cell type within a breast tumor, thus improving the outcome and quality of life for patients with breast cancers.

ACKNOWLEDGMENTS

This work was supported by NCI R01 grant CA143126 (RSC) and Susan G. Komen for the Cure grant KG100677 (RSC).

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