

EGFR-Ligand Signaling in Breast Cancer Metastasis: Recurring Developmental Themes

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1. Introduction

1.1 ErbB receptors, ligands and signaling

Breast cancer affects nearly 1 out of 9 women worldwide. The quality of treatment for breast cancer has improved to the point that close to 80% of patients in countries with advanced healthcare delivery systems survive the disease (1). Yet over 20% of breast cancer patients succumb to the disease, and the majority of these have metastatic breast cancer cells that occupy and compromise the function of distal organs (1). There has been an intensive effort to improve treatments for metastatic breast cancer. Novel treatment strategies have arisen from the study of the molecular and cellular biology of breast cancer cell lines. These studies have produced a group of agents called targeted therapeutics because they are often directed at a single molecule rather than a general process such as DNA replication or cytoskeletal function. The ErbB family represents a target that is present in breast cancer. Therapeutics to ErbB2 have been used to treat aggressive breast cancer for over a decade with considerable success (2). However, therapeutics that primarily target the EGFR have not been used extensively in breast cancer, and there are some improved agents for the receptor that are just entering the clinic. Recent conclusions from studies of metastatic breast cancer suggest new possibilities for the use of EGFR therapeutics. This review will describe the members of the EGFR signaling family, discuss the cellular context in which they function in development, and correlate this with the biological role of these molecules in breast cancer metastasis.

1.2 ErbB family members

The ErbB family consists of 4 receptors: ErbB1 or more commonly called EGFR, ErbB2/Her2, ErbB3 and ErbB4 (3). Signaling is generated when EGFR and ErbB4 bind to their ligands. In contrast, the ErbB2 extracellular binding domain fails to bind any of the 15 agonists, and in ErbB3 the kinase domain is not functional. Upon ligand stimulation, EGFR and ErbB4 receptors can transduce their signals as homodimers or heterodimers; however,

the signal generation from ErbB2 or ErbB3 require heterodimerization with another ErbB family member (3).

The ErbB receptors are stimulated by 15 ligands but the situation is complicated because several of these agonists can bind more than one receptor. The EGFR exclusive agonists are epidermal growth factor (EGF), transforming growth factor alpha (TGF- α), amphiregulin (AREG) and Epigen (Epi) (3, 4). ErbB4 is specifically bound and activated by Neuregulins (NRG) 3, 4, 5 (3, 4). Heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EREG), and β -cellulin (BTC) bind and activate both the EGFR and ErbB4 (3, 4). NRG 1 and 2 binds both ErbB3 and ErbB4 and NRG 1 can bind the EGFR with low affinity (3, 4).

All of the ErbB agonists are synthesized as plasma membrane bound integral membrane proteins (5). In some cases, the transmembrane ligands stimulate ErbB signaling on adjacent cells through a juxtacrine mechanism which may mediate the stromal-epithelial interactions (6) (7). Most ErbB signaling requires proteolytic cleavage termed ectodomain shedding for the ligand to be released and available to bind receptors that may be on the same cell (autocrine signaling), or on neighboring cells (paracrine signaling) (5). The proteases that mediate the process are from the "a disintegrin and metalloproteinase " or (ADAM) family (8, 9). There are 40 members of the ADAM protein family that function in cell adhesion and ectodomain shedding. These ADAMs are integral membrane proteins in which the extracellular region contains a protease as well as a disintegrin domain that modulates integrin binding (9). ADAMs can be activated by a wide range of stimuli that signal through G-protein-coupled receptors and these signals are often transduced by Src (10). The shedding of AREG, EREG, HB-EGF, Epigen, TGF α and NRG 1&2 is typically catalyzed by the single family member ADAM 17, whereas BTC and EGF are cleaved by ADAM 10 (8). In addition, ADAM 17 cleaves many other cytokines, growth factors, receptors, adhesion molecules and extracellular matrix proteins, suggesting its activity may be a key determinate of cellular behavior (9). Nevertheless, emerging data suggest that EGFR ligands can be shed by other proteases such as the ADAM thrombospondin (ADAMTS) family that is structurally related to the ADAM family, but is secreted and the disintegrin domain is replaced by a thrombospondin domain that binds to matrix (11, 12). Also, it is likely that other metalloproteinases secreted from cells in a paracrine relationship are capable of releasing ligands (13).

Over the past two decades the expression of ErbB receptors, ligands, and their activating proteases in normal breast and breast cancers have been intensively studied. Various mRNA detection methods and immunohistochemistry studies have concluded that the entire ErbB family is expressed in various breast cancers. In fact, it appears the vast majority of the family is expressed in the mammary epithelia (14-16). Since newer therapeutics that target the EGFR are being considered for use in cases of advanced breast cancer, in the rest of this review we will focus on how this receptor is activated and describe its role in development and cancer progression.

1.3 EGFR homodimer signaling

EGF was the first ligand identified and due its abundance in the mouse salivary gland and relative ease of purification from this source (17). EGF has historically been used for receptor binding, signaling, trafficking, and cell fate studies resulting in a model of receptor signaling that is in many ways considered to be the prototype for receptor tyrosine kinases (18-20). The binding of EGF to the EGFR exposes the dimerization arm in the extracellular domain

that permits interaction with another EGFR receptor or heterodimerization with other ErbBs. Ligand binding also induces a conformational change in the receptor that activates the intracellular kinase domain, which in turn can phosphorylate tyrosine residues on the adjacent C-terminal tail of the dimerized ErbB receptor. The 10 phosphorylated tyrosine residues serve as docking sites for adapter proteins or other signal transduction components, resulting in activation of Ras, MAPK, src, STAT 3/5 and PLC γ /PKC and the PI3 kinase-AKT-pro survival pathway. Activation of these signaling pathways by ErbB dimers has profound impact on proliferation, resistance to apoptosis, differentiation, as well as motility/migration associated behaviors. Not all tyrosine phosphorylation of EGFR C-terminal tail results in stimulation of downstream signaling pathways. For example, phosphorylation of the 974 residue triggers endocytosis of the receptor, and phospho 1045 binds to Cbl, mediating ubiquitination of the receptor and subsequent proteosomal degradation (4, 21). Trafficking studies suggest that ~50% of EGF stimulated EGFR is degraded, whereas the remainder is recycled back to the plasma membrane (22). Thus, activation of the EGFR by EGF directly stimulates a broad group of cellular signaling pathways, many of which converge on elements of the ERK/MAPK pathway (3), but this signaling is dampened by receptor turnover. The rapid turnover of the EGF stimulated EGFR is believed to limit stimulation of cellular proliferation, permitting a balance with various differentiation-inducing stimuli present in a normal tissue (3, 22, 23). In cancers, autocrine EGFR homodimer signaling is substantially attenuated, shifting the cell fate balance towards proliferation and survival rather than differentiation, apoptosis and senescence.

1.4 Attenuating EGFR signaling with heterodimerization

Probably the most well understood attenuation of EGFR signaling occurs when the receptor heterodimerizes with the ErbB 2 receptor (24, 25). It is believed that EGFR heterodimerization with ErbB2 frequently occurs in a number of breast cancers (26, 27). Despite being unable to bind ligand, the ErbB2 dimerization arm is constitutively exposed, which allows this receptor to more efficiently dimerize with other liganded ErbB family members (4). The resulting ErbB2 containing heterodimers attenuate EGFR signal transduction in several ways (28-32). First, the affinity of this ErbB2 complex for ligands is enhanced. Second, the ErbB2 phosphotyrosine domains bind most adapter proteins with higher affinity than those of the ErbB homodimers, resulting in more efficient signal transduction. Third, ErbB2/EGFR heterodimers are slowly endocytosed, and are more frequently recycled to the plasma membrane than the EGF stimulated homodimers. By virtue of its strong interactions with adapter proteins and altered trafficking downstream of endocytosis, an EGFR/ErbB2 heterodimer can amplify and extend the duration of EGFR ligand signaling, leading to proliferation and survival at the expense of other cell fates (28-31).

In contrast to the fairly well established understanding of ErbB2 containing heterodimers, there have been few studies on the EGFR heterodimerized with ErbB4 or ErbB3. Co-immunoprecipitation experiments have confirmed the presence of the ErbB4/EGFR in a lung epithelial cells and type II pneumocytes; however, the specific function of this complex was not determined (33, 34). Co-expression of ErbB4 and EGFR plasmids in model NIH 3T3 fibroblasts or CHO lines, provided evidence of dimerization of these receptors, and suggested that this complex could induce cellular transformation in the presence of EGF or NRG1. Further analysis of the CHO system found that the ErbB4/EGFR heterodimer

specifically induced B-Raf kinase activity, which was speculated to induce transformation by increasing the activity of the ERK/MAPK pathway (35). Recently, ErbB3/EGFR heterodimers have been identified in pancreatic cancer cell lines (36, 37). It appeared that the ErbB3/EGFR complex may be a more effective stimulus of proliferation in pancreatic cancer cell lines than EGFR homodimers (36). Additionally, these studies suggest the ligand AREG is able to stimulate activity of the ErbB3/EGFR heterodimer (36, 37). Unfortunately, the comprehensive binding, signal transduction and trafficking studies completed for ErbB2 containing receptor complexes have not been completed for EGFR/ErbB4 or ErbB3 heterodimers. This information, coupled with the identification of the specific cell types and tumors that express heterodimers and the function of these complexes will be important considerations for expanded use of ErbB targeted therapeutics.

1.5 Other EGFR ligands

As studies of receptor binding, conformation, phosphorylation, and trafficking are completed for each ligand, it is becoming clear that each agonist induces signaling that can be viewed as a variation of the basic EGF-EGFR homodimer scenario. This attenuated signaling produced by each ligand has the potential to induce subtle differences in downstream signaling, which would be expected to result in altered gene expression and cellular behavior. In the preceding section, the emerging differences in signaling are detailed for each of the ligands that bind the EGFR.

1.5.1 TGF α

Next to EGF, the most intensively studied ligand has been TGF α . Similar to EGF, TGF α exclusively binds to and activates the EGFR. Binding studies suggest that TGF α binds to the receptor with similar affinity as EGF (32). However, conclusions from structural studies involving ligand-receptor complex data indicates there are subtle differences in the conformation of the extracellular ligand-binding domain (sub domain II) induced by TGF α as compared to EGF (4). It is unclear whether this conformational change induced by TGF α could generate alterations in EGFR kinase activity or accessibility of C-terminal tyrosines. Although not comprehensively studied at this point, some of our early studies with breast cancer cell lines suggest that TGF α does not induce the extensive receptor phosphorylation observed with mouse salivary gland derived EGF (Fig 1). Additionally, it has been long recognized that TGF α induces different trafficking of the receptor than EGF (38). Close to 100% of receptors internalized after TGF α treatment are recycled to the plasma membrane (22). At physiological pH of 7.4 in the extracellular environment, TGF α and EGF have similar binding affinities for the EGFR (22, 38). However, at pH close to 5 such as in the endosome, TGF α has decreased affinity for the EGFR (22, 38). It appears that dissociation of the ligand from the EGFR in the endosome permits the receptor to be recycled back to the plasma membrane where it can be reengaged by ligand. It is thought that the three additional histidines found in the receptor binding domain of TGF α provide a greater sensitivity to pH for agonist-receptor interactions (38). In fact, mutations that add histidines to this region of EGF decreased ligand-receptor binding at low pH (39, 40). Together, the altered ligand induced receptor conformation, phosphorylation and trafficking appear to result in TGF α being a more potent stimulator of proliferation of EGFR expressing cell lines than EGF.

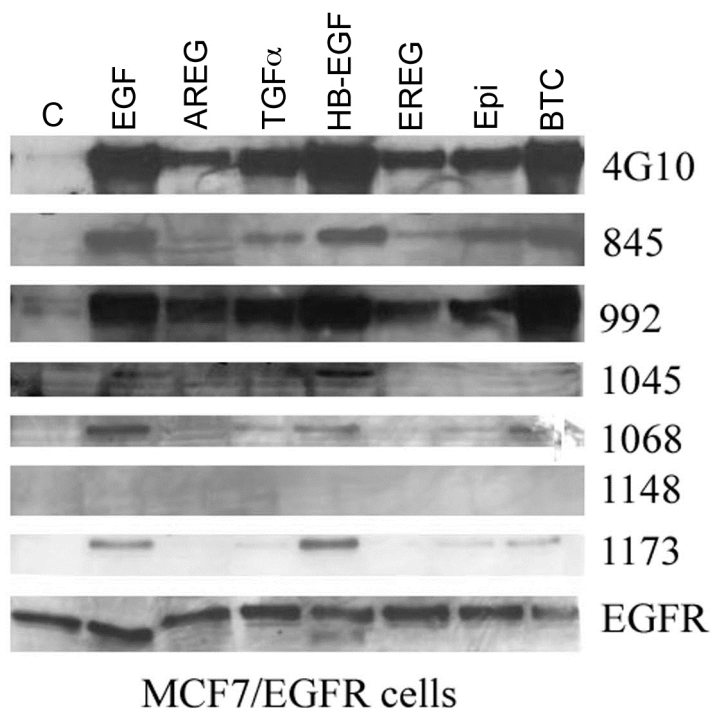


Fig. 1. EGFR phosphorylation after ligand stimulation.

The human breast cancer cell line MCF7 was engineered to overexpress high levels of the EGFR after retroviral transduction. Cells were grown to 80% confluence and placed on ice for 30 minutes. Ligands (R&D, Minneapolis) were applied for 10 minutes to the media and then the media was removed, cells washed and proteins extracted. The EGFR was concentrated with Concanavalin A beads and extracted with Laemmli sample and applied to gels and western blotted with specific antibodies to phosphorylated tyrosines listed on the left. The various ligands used are listed on the top of the figure (C) represents vehicle treated cells.

1.5.2 AREG

The differential impact on breast cancer cell behavior that AREG exhibits compared to EGF has drawn considerable attention to the concept that various EGFR ligands have discrete functions (41-43). Among the ErbB receptors, AREG appears to exclusively bind and activate the EGFR. In addition, the ligand contains a heparin-binding domain N-terminal to the receptor binding region (44, 45). It appears that interaction with heparin-sulfated proteoglycans on the plasma membrane enhances the ability of exogenous AREG to activate the EGFR (46). What has been a matter of controversy has been the relative strength of AREG binding to the EGFR as compared to other ligands. The initial identification of human AREG by Shoyab and colleagues, reported the fully processed ligand isolated from breast cancer cells had reduced affinity for the human EGFR, as compared to salivary gland derived mouse EGF (44). In contrast, subsequent studies with human recombinant ligands

found that AREG has similar affinity for the EGFR as EGF and TGF α (47, 48). Inducing further complexity, additional analyses of ligand receptor interactions have suggested that recombinant AREG does not induce efficient dimerization of the EGFR, as compared to recombinant EGF and TGF α (49). Interestingly, proteolytic processing of AREG in mammalian cells may eliminate the C-terminal portion of the ligand binding domain that is required for high affinity for the receptor (50). In addition, the terminal portion of the receptor binding domain in all other EGFR ligands contains a leucine, whereas a methionine is found in AREG, and this is speculated to reduce affinity for the receptor (50).

More recent studies have focused on the distinct downstream signaling and cellular behavior induced by AREG. Unlike exogenous EGF treatment, AREG stimulation of model cell lines and breast cancer cell lines is unable to induce efficient phosphorylation of many of the tyrosine residues in the C-terminal tail of the EGFR (22, 43, 51, 52) and (Fig. 1). Notably, the Cbl binding 1045 tyrosine residue is not efficiently phosphorylated by AREG and this ligand fails to induce rapid turnover of the EGFR. Trafficking studies indicate that AREG liganded EGFR is rapidly internalized, but then is recycled back to the plasma membrane. In addition, AREG binding to the EGFR is very resistant to acidic pH suggesting that the ligand does not disengage in the endosome as does TGF α (22). It appears that AREG may be unique among the ligands in that it induces EGFR trafficking through Rab 4 and Rab 11 containing endosomes (22, 43). AREG induces prolonged phosphorylation of ERK relative to EGF (41, 52). This altered signaling appears to be the basis of AREG stimulating the loss of cell-cell adhesion and increase motility/migration associated behaviors in breast and other epithelial cells (41, 53). AREG overexpression has also been found to selectively activate interleukin-1 induced NF κ B signaling in breast epithelial cells (41-43).

1.5.3 Epigen

This was the last ErbB family member identified in 2000, and it has not been as intensively studied as other ligands. The ligand activates the EGFR and does not activate ErbB3 or ErbB4 when these receptors are expressed in isolation (54, 55). However, epigen can activate ErbB4 and ErbB3 when these receptors are co-expressed with ErbB2 (54, 55). Epigen appears to have ~100 fold less affinity for the EGFR relative to recombinant human EGF. Not surprisingly, we found that epigen induced modest phosphorylation of breast cancer cells (Fig. 1). The binding of epigen to the EGFR appears to be sensitive to pH similar to TGF α . Modeling suggests that additional histidines in the receptor binding domain are responsible for the dissociation of the ligand from the EGFR at low pH (54, 55). In comparison with EGF, epigen induced significantly weaker ubiquitylation and degradation of EGFR, and once internalized, it appears that the receptor is efficiently recycled to the plasma membrane. As expected for lower affinity ligands, epigen is a more potent mitogen than EGF and displays prolonged MAPK signaling (54, 55).

1.5.4 HB-EGF

Exogenous HB-EGF is a high affinity ligand for the EGFR but it also binds and activates ErbB4 (32). Exogenous HB-EGF does not as robustly stimulate phosphorylation of ErbB4 as it does the EGFR (56). In addition, pro-HB-EGF serves as the diphtheria toxin receptor in human cells (6). Similar to EGF, exogenous HB-EGF induces extensive EGFR tyrosine phosphorylation in most cell types studied (22, 56), and we found this to be the case for breast cancer cells (Fig. 1). Upon binding to and activation by HB-EGF, the EGFR is rapidly

endocytosed and the majority of ligand engaged receptors are trafficked to lysosomes and degraded (22). The binding of HB-EGF to the EGFR was resistant to low pH. HB-EGF shows similar effects on cell proliferation and migration to those exhibited by EGF (57).

As its name implies, HB-EGF has a heparin-binding region N-terminal to the EGF domain. This domain has been shown to interact with heparin sulfated plasma membrane proteins such as the tetraspanin, CD9 and the extracellular matrix binding/cell differentiation marker protein CD44 (6, 57). In particular the heparin-mediated interaction between HB-EGF and CD9 appear to be crucial to juxtacrine signaling by the proligand (58). Finally, the associations between the heparin binding domain and cell membrane associated heparin sulfated proteoglycans appear to be crucial to localizing HB-EGF to regions of cell-cell contact. Furthermore, the interaction with these heparin-sulfated proteoglycans prevented proteolytic cleavage of the pro-ligand, whereas exogenous heparin increased shedding of HB-EGF (7, 58). In contrast to the impact of shed ligand, juxtacrine signaling by the pro HB-EGF appears to be antiproliferative (58).

1.5.5 β -cellulin

Exogenous β -cellulin is a high affinity ligand for the EGFR and ErbB4 (32). In general, exogenous β -cellulin phosphorylates the EGFR to a similar extent as EGF in model cell types, and this is what we observed with breast cancer cells (Fig. 1) (22, 59). This exogenous ligand also stimulates total ErbB4 phosphorylation to an extent similar to NRG1, but there may be differences in phosphorylation of specific tyrosine residues (60). Upon binding to and activation by β -cellulin, the EGFR is rapidly endocytosed and trafficked to lysosomes where the majority of it is degraded (22). The binding of β -cellulin to the EGFR was resistant to low pH. Exogenous β -cellulin was slightly less efficient than EGF at inducing proliferation in some specific cell types (60). There have been some reports that β -cellulin binds to heparin and may participate in juxtacrine signaling (61).

1.5.6 EREG

Unlike the other dual receptor ligands HB-EGF and BTC, EREG is a low affinity ligand for the EGFR (32). EREG typically induces much less phosphorylation of the EGFR than EGF in model cell lines (62-65). In breast cancer cells, we found that the epiregulin induced phosphorylation of the various tyrosines on the EGFR to an extent similar to AREG and the low affinity ligand epigen (Fig. 1). EREG is not as effective as NRG or BTC in stimulating ErbB4 phosphorylation (62-65). The ligand appears to preferentially activate heterodimers and efficiently induces EGFR heterodimers with all three other receptors (65). Upon binding to and activation by EREG, the EGFR is rapidly endocytosed, but then is recycled back to the plasma membrane, and its binding to the receptor was resistant to low pH (22). Exogenous EREG was more efficient than EGF at inducing proliferation in some cell types (63, 65). Also EREG does not induce as great of activation of the MAPK pathway as EGF, but the duration of MAPK phosphorylation was increased relative to the prototype ligand (63, 65).

Thus, the expression of specific EGFR ligands could influence the progression of breast cancer in several ways. First, three of the ligands (HB-EGF, BTC and EREG) could induce ErbB4 signaling on breast cancer cells themselves or their microenvironment, whereas the other 4 ligands EGF, TGF α , AREG and epigen would only induce EGFR signaling. Second, if juxtacrine EGFR signaling between breast cancer cells may require interaction with heparin sulfate proteoglycans, only HB-EGF and AREG would be likely mediators of this signaling.

Juxacrine EGFR signaling might be antiproliferative and also only occur in breast cancer that lacked active sheddases that released HB-EGF and AREG. Third, EGFR signaling induced by soluble ligands appears to be dependent on the relative ratio of receptor degradation versus recycling to the plasma membrane. For example, those ligands whose binding leads to rapid receptor degradation (EGF, HB-EGF and BTC) would activate high levels of downstream signal transduction, but this would likely be of short duration. In contrast, the ligands that induce recycling of the internalized receptor to the plasma membrane would produce longer duration EGFR signaling. In the context of autocrine signaling in breast cancer cells, the longer duration EGFR signaling is likely to more efficiently induce mitogenesis. What remains to be determined is whether the duration of EGFR signaling differentially impacts other cellular behaviors relevant to breast cancer progression such as resistance to apoptosis, and the stimulation of invasive/motile behaviors. Finally, in the context of paracrine signaling it is not clear how EGFR turnover influences the supportive functions of stromal and immune cells of the tumor microenvironment.

2. EGFR/ErbB signaling in development

2.1 ErbB signaling and mammary gland development

The majority of research on mammary gland development is performed in the mouse model due to its biologically and histologically similarity with humans, and the power of transgenic knockout murine models (66). Embryonic mammary gland development in the mouse begins around embryonic day 10.5 (E 10.5), where bilateral milk lines are formed from front to hind paws. Between E11.5 and E12.5, five placodes on each milk line develop with eventual epithelial bud formation at each placode. These epithelial buds remain quiescent until E15.5, where minor branching permits the migration of mammary epithelia into the fat pad, and mesenchymal differentiation forms the overlying nipple epidermis. This rudimentary mammary gland will remain quiescent until after birth (67). The majority of mammary gland growth and development occurs in postnatal life during puberty and pregnancy, and ErbB signaling impacts this phase.

The primary hormone that drives post-natal mammary gland development is the nuclear steroid hormone, estrogen. Estrogen stimulates proliferation of luminal cells within the mammary ducts, causing ductal elongation and branching (68). Progesterone also increases cellular proliferation of the mammary ducts, and acts synergistically with estrogen during periods of high hormone levels such as pregnancy (68). Prolactin, a non-steroid hormone released from the pituitary gland, is active in mammary gland development, late in pregnancy, stimulating alveolar development and triggering milk production during lactation (69).

The mouse mammary gland begins pubertal outgrowth between 3-4 weeks of age, and is complete at 8-12 weeks of age. The gland requires both longitudinal ductal growth, as well as ductal branching to fully infiltrate the mammary fat pad. Pubertal growth is directed by the cells within structures called terminal end buds (TEBs), which are a bulbous expansion of the epithelia. The TEBs are found at the distal end of each growing duct, and consist of 3 to 4 cell layers, including cap cells that make up the 'basal' layer and multiple layers of interior luminal-like body cells that line the duct. Signaling within the cells of the TEBs and the surrounding stroma will determine the extent of continued ductal branching (70) (71). From this point, the mammary gland will show minimal growth with each estrous cycle. Upon pregnancy, estrogen and progesterone drive another large spurt of growth resulting

in extensive ductal branching. Progesterone also works with prolactin to signal differentiation of the secretory or alveolar cells throughout the duct system, which produces the large volumes of milk post parturition (72, 73). Termination of lactation will eventually lead to involution, where large-scale apoptosis will eliminate the secretory alveoli and remodel the remaining ducts of the mammary gland, returning the structure to a state similar to that of the virgin gland.

2.1.1 Role of EGFR in mammary gland growth

In virgin mice, EGFR, ErbB2, and ErbB3 are present in the developing ductal structure while there is minimal ErbB4. During pregnancy, this pattern changes to greatly increase expression of ErbB4 in the mammary epithelium, while ErbB4 levels will again regress during lactation and involution (74). Even though three of the receptors are present during growth, there is minimal ErbB phosphorylation observed until ductal morphogenesis begins. During pubertal growth, phosphorylated EGFR and ErbB2 are detected, which suggested that these receptors may mediate the impact of estradiol (E2) on the gland (75).

The EGFR^{-/-} mice die within 8-days after birth and show a wide range of dysfunctional epithelia, but their mammary glands were similar to their wild-type littermates. Transplantation of pre-pubertal glands from EGFR^{-/-} mice into cleared fat pads of wild-type littermates failed to infiltrate the structure, but glands from wild-type mice produced normal ductal systems (76). In contrast, when a purified mammary epithelium from the EGFR^{-/-} mouse was implanted into cleared fat pads with mammary stroma from wild-type mice it produced a normal ductal tree, whereas the opposite combination of wild-type epithelium and EGFR^{-/-} stroma failed to penetrate the fat pad. Thus, postnatal mammary ductal growth is dependent on the presence of the EGFR in mammary stromal fibroblasts. It appears that signaling by the receptor triggers the production of stromal growth factors important to TEBs in ductal elongation (76).

The EGFR ligands EGF, TGF α , and AREG, are found in different locations within the TEB during ductal growth. Using immunohistochemical techniques, TGF α is found exclusively in the basal cap cell layer while the luminal cells express only EGF (70). AREG has been found in both the basal cap cells and the luminal cell layers of TEBs (77). Luekette and colleagues produced knockout mice for each of these ligands separately or as double and triple knockouts. While all three mice null for the individual ligands and their various crosses were fertile, not all had distinct mammary phenotypes. Double-knockout mice for EGF and TGF α , but which contained AREG, displayed normal ductal growth and TEB formation. Mice that were single-knockout for AREG or a triple-knockout for all ligands displayed almost a complete lack of ductal growth into the fat pad at 8-12 weeks (78). In the AREG^{-/-} mice, mammary epithelial failed to fill the fat pad even after multiple pregnancies, strongly suggesting that AREG-EGFR signaling mediated the impact of estrogen on mammary ductal growth. This suggests that EGF and TGF α are dispensable for mammary gland growth, while AREG plays a vital role in glandular development. Recombination grafts indicated that estrogen stimulated pubertal mammary gland growth will not occur without AREG signaling to the stroma (79). The AREG gene is regulated by estrogen receptor alpha (ER α), which apparently accounts for its requirement in postnatal mammary gland development (80, 81). Whether there are any ligand specific effects of the AREG ligand in mammary development have not been explored.

Studies performed with mice deficient for ADAM-17 have shown cardiac insufficiencies, a constellation of epithelial defects and die soon after birth similar to the phenotype of EGFR^{-/-} mice. The ADAM17^{-/-} mice have small, immature mammary glands with minimal branching or ductal growth (82). To verify the requirement of ADAM-17, the defective growth of ADAM-17-null mammary epithelia can be rescued in the presence of exogenous AREG, EGF, or TGF α (82). In conclusion, estrogen induced growth of the mammary pad requires mammary epithelial cell ADAM-17 to shed AREG which then, is necessary to stimulate EGFR signaling in the stromal fibroblasts.

2.1.2 Breast epithelial stem cell and ErbB signaling

Potential mammary gland stem and progenitor cells have been identified using a series of methodologies used to identify the hierarchy of cells that produces that mature hematopoietic system. A single human mammary gland stem cell has been shown to regenerate all the cellular components of the human mammary gland, as well as produce milk proteins in immunocompromised mice (83-86). This work, coupled with mouse work has given rise to an epithelial hierarchy illustrated in figure 2. In this hierarchy, the mammary stem cells give rise to “ the common or bipotent” progenitor; the bipotent progenitor gives rise to a luminal progenitor, as well as a cell type that gives rise to mature myoepithelial cells; and the luminal progenitor produces derivatives that ultimately differentiate into mature duct and alveolar cells (83-86).

The reproducible isolation of stem and progenitor cells from mammary epithelia has permitted profiling the various cell types for the expression of the receptors involved in post-natal mammary gland growth and breast cancer progression (85). The subpopulation containing putative mammary multipotent stem cells appear to lack expression of estrogen and progesterone receptors, whereas the EGFR is expressed in ~12% of this fraction. Nearly 50% of the luminal progenitor inclusive population expressed high levels of EGFR. The relatively small subset of the differentiated luminal cell segment (ductal and alveolar cells) express the EGFR (83-86). Of possible significance is that both the mammary stem cell and luminal progenitor population are routinely propagated in a media supplement containing EGF. Whether this implies that propagation of these stem and progenitor cells are dependent on EGFR signaling or simply that there is a requirement for generalized receptor tyrosine kinase activity remains to be determined.

Taken together, the post-natal development of the mammary gland is regulated in large part by the EGFR. EGFR signaling in stromal fibroblasts is required for the estrogen-stimulated invasion mammary epithelium into the stromal fat pad that establishes the adult virgin mammary gland. It appears that the stromal EGFR signaling is mediated primarily by the estrogen-controlled ligand, AREG. Interestingly, the EGFR and its ligands are expressed in the mammary epithelia, but the recombination experiments suggest that autocrine receptor activity in this compartment is dispensable for the establishment of the adult mammary gland. At this time, it is not clear if autocrine EGFR signaling in a mammary epithelial stem or progenitor cells might be required for maintenance of the organ throughout adult life.

2.2 EGFR signaling and cardiac development

Careful reexamination of the EGFR^{-/-} mice along with observations from the Waved-2 mice (these express a mutant form of the receptor with only 10% kinase activity) found defective

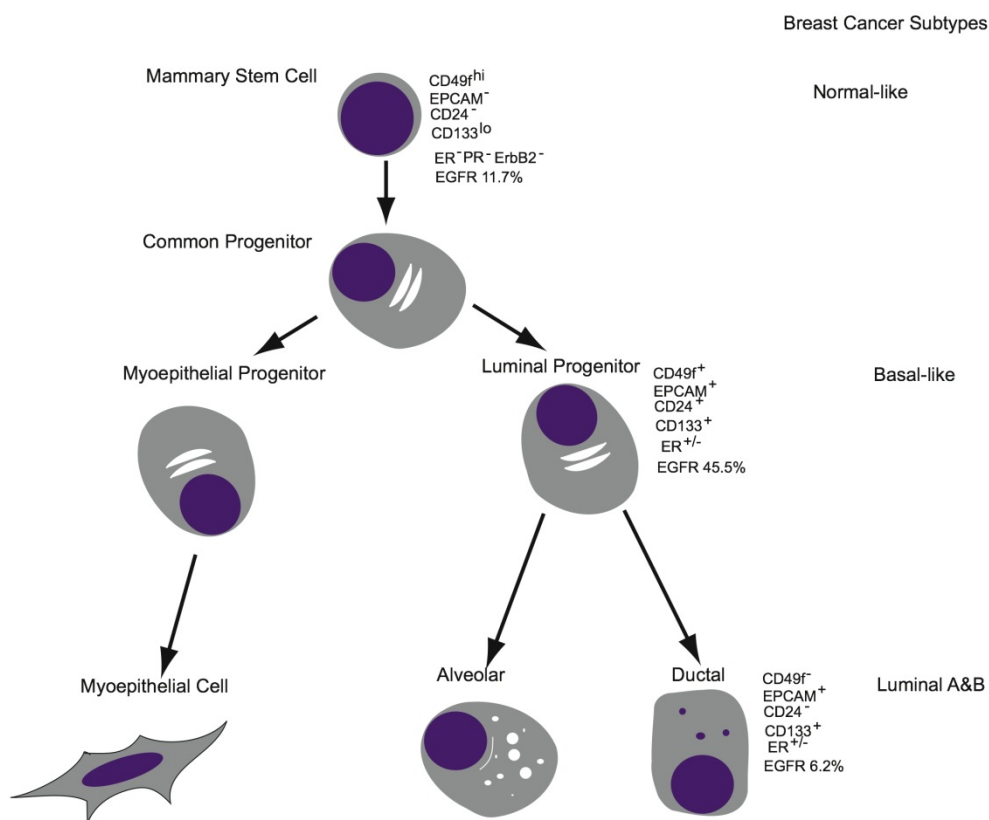


Fig. 2. Hierarchy of mammary epithelial cells.

The various cells of the mammary epithelium and their relationships are represented. To the right, is the expression pattern of the various cell surface markers. Below this is the ErbB and ER α expression along with breast cancer cell types the various cells are related to.

cardiac valve morphogenesis and maturation (94, 96). HB-EGF KO mice also exhibit defective maturation of cardiac valves, suggesting that this may be the relevant ligand that induces EGFR signaling in this process. Also, the HB-EGF^{-/-} cardiac defect was phenocopied in the ADAM-17, suggesting that this protease released the ligand during cardiac development (97).

In the context of cellular behaviors, it appears that the EGFR signaling system plays a role in differentiation. In the EGFR, HB-EGF and ADAM-17 KO mice which have hyperplastic valves it appears that the impact of this signaling is distinct from a proliferation and migration defect observed with the other ErbB knockouts. It is thought that HB-EGF-EGFR signaling decreases BMP expression, the factor which drives cardiac valve maturation, and hence is a differentiation factor (97).

2.3 EGFR signaling and nervous system

In the mouse, EGFR is highly expressed during brain development (E-7 to E-17) and is present on multipotent precursors of both neurons and glia, as well as developing astrocytes and some neurons (98, 99). The initial reports of the EGFR knockout did not identify a nervous system defect. However, reexamination of the KOs with extended post natal survival due to breeding of the knockout allele onto other mouse strains was able to identify brain defects (98). Among the defects identified were smaller or thinner forebrain regions, including the cerebral cortex, olfactory bulb and neocortex (98). Both HB-EGF and TGF α are expressed in portion of the fore brain during late embryonic and early postnatal life (98, 100). Modest histological defects were observed in the prefrontal cortex of mice with a conditional KO of HB-EGF in the forebrain, and these mice displayed behavior and defects in dopamine metabolism that have been observed in schizophrenia (98).

The defects observed in the forebrain of the EGFR-KO mice appear to result from disruption of the cellular interactions required to support neurons. There was substantial neuronal apoptosis in the early postnatal forebrain regions affected (98). However, this occurred in EGFR-expressing and non-receptor bearing neurons. In addition, there was a delay in the appearance of glial fibrillary acidic protein (GFAP) positive astrocytes in the glial limitans and white matter tracks of the fore brain. Although EGFR ligands can stimulate both the proliferation of astrocytes and recruitment of these cells from multipotent precursor cells, the major defect of the knockout mice appears to have a defect in the migration these glial cells from germinal centers. It is speculated that the delay in formation of contacts between neurons and astrocytes results in a deficiency of trophic support, resulting in neuronal cell death in the forebrain (98, 99).

EGFR expression is high in developing astrocytes, but the receptor is not present in mature astrocytes of the healthy adult brain. Upon injury or disease, EGFR expression is up regulated in reactive astrocytes (101). Reactive astrocytes lengthen processes produce plasma membrane pseudopodia and increase expression GFAP in response to all forms of CNS injury or disease (102). Stimulation of the EGFR on reactive astrocytes results in the upregulation of motility chemokines and extracellular matrix remodeling genes that are likely to contribute to glial scarring (101). Intriguingly, the use of EGFR tyrosine kinase inhibitors reduced nerve loss and lead to greater nerve fiber regeneration in optic nerve crush a model of a glial scarring (101). Thus, EGFR signaling in astrocytes facilitates neuronal survival during development, but receptor activity in reactive astrocytes actually contributes to neuron loss in pathologies.

2.4 EGFR signaling and bone

Bone phenotypes had not been reported in the original characterization ErbB receptor KO mice. However, work on the problem of malignancy-associated hypercalcemia had long established that TGF α increased the formation of bone resorbing osteoclasts in bone marrow

cultures and whole animals (103, 104). The EGFR is expressed on both chondrocytes and cells of the osteoblast lineage in animals and humans (105). However, the function of the receptor was not established until a human EGFR gene-knockin mouse was created (106). This human EGFR transgene had a limited expression in mouse tissues that normally express the receptor, probably due to the presence of the Neo gene in the first intron of the construct. The human EGFR was expressed in the heart and nervous system and provided a rescue of the murine EGFR KO, but the receptor was not expressed in epithelia and bone. The human EGFR knockin mice were growth retarded and the skeletal phenotype appeared to be largely due to premature hypertrophy of the growth plate cartilages. Although routine histology did not reveal defects in the bones, growth of the knockin osteoblasts *in vitro* resulted in the increased formation of calcified nodules, which represent the end point of differentiation for these cells. Thus, in both cartilage and bone, EGFR signaling inhibits differentiation and helps maintain chondrocytes and osteoblasts in a proliferative state. At this point, it is unknown if any other ErbB receptors play a functional role in bone development or physiology.

Further insight into the role of EGFR in bone resulted from a study of global changes in osteoblast gene expression induced by the main serum calcium regulator, PTH. Activation of the PTH receptor on osteoblasts rapidly upregulates AREG mRNA expression 10 to 20-fold, as well as increasing the TGF α and HB-EGF ligands (107, 108). In addition, PTH signaling induces shedding of ADAM-17 controlled ligands in the kidney (109). Further experiments indicated that addition of exogenous AREG to osteoblasts stimulated their proliferation. However ligand-EGFR signaling also inhibited osteoblast differentiation and dramatically decreased mineralization of osteoblast cell lines. Consistent with the role for AREG in stimulating the proliferation of osteoblasts, 4-week-old AREG-knockout mice exhibited less trabecular bone in the tibia than wild type littermates (107). These experiments suggested that EGFR signaling may mediate the impact of PTH on the recruitment and expansion of cells committed to the osteoblast lineage, but excessive signaling by this system could prevent these cells from undergoing terminal differentiation and forming mineralized bone. The inhibition of osteoblast differentiation and subsequent mineralized bone matrix deposition by-EGFR signaling may contribute to the uncoupling of bone formation from the accelerated bone resorption.

3. EGFR and breast cancer

3.1 ErbB and EGFR expression in primary tumors

The development of platforms capable of simultaneously evaluating gene expression from a large portion of the genome have led to identification of gene expression profiles that correlate with various established and some novel classes of breast cancer. These profiles have produced further insights into the impact of ErbB family members in breast cancer progression. Based on these studies, breast cancers are now divided into the following subclasses: ErbB2 amplified, luminal A, luminal B, normal breast-like, and basal (110-112). The ErbB2 amplified, basal and luminal B subtypes had substantially worse prognosis than the normal breast-like and luminal A.

3.1.1 ErbB2 amplified tumors

Among the molecular subclasses of breast cancer, the ErbB2 amplified, has the most well established functional role for an ErbB member in disease development and progression. The ErbB2 amplified tumors typically express ErbB3 and cell line experiments suggest the

ErbB2/ErbB3 heterodimers stimulate proliferation of these cells through the PI3 kinase-AKT pathway (113). Despite the identification of the ErbB2 co-receptor, the precise ligand activating the ErbB3 has not been established. In addition, the correlation between high ErbB2 expression and poor prognosis suggests that ErbB2 contributes to metastasis and how the receptor contributes to these processes, is still under investigation (110-112).

3.1.2 ER+ tumors: luminal A&B

Luminal A tumors express ER α along with GATA binding protein 3, X-box binding protein 1, trefoil factor 3, and other estrogen-regulated genes and high levels of the luminal keratins K8 and 18 (110-112). Luminal B tumors tend to express the above markers at slightly reduced levels, but have an upregulated cassette of genes, including proliferation related genes such as Myb and components involved in DNA replication. There is no specific ErbB family member included in luminal A or B signature. Further evaluations of ER α + tumors have indicated that the majority of these tumors lack ErbB2 and EGFR expression, but close examination of data from microarray and PCR studies suggest there are occasional luminal type tumors that express these receptors (16, 110-112). A large fraction of ER α + tumors also contain ErbB4 (110-112), and there is some indication this receptor may be involved in a reciprocal regulatory loop with ER α signaling (114). Surprisingly, AREG was not in the original gene set that defined ER α + luminal tumors. A follow up interrogation of data that was used to relate disease outcome to cancer subclasses identified a correlation between higher levels of AREG expression, ER α , and the luminal A subclass (115). Also, this analysis indicated that ADAM-17 levels were low in the luminal A class relative to other tumor subtypes. These observations suggest that although most ER α + luminal A breast cancers express AREG, they lack the EGFR; therefore, autocrine signaling by this ligand receptor system should not be present in most of these tumors. Because the tumor cells express low levels of ADAM-17 it is unclear whether AREG could even participate in paracrine signaling between luminal A breast cancer cells and the tumor stroma. There is a possibility that other proteases produced by the tumor cell or microenvironment lead to shedding of AREG by luminal A breast cancers but whether this signaling impacts progression is unclear.

3.1.3 Normal-like breast cancers

The gene expression signature of these tumors clustered with the normal breast samples. These tumors had a signature that was not associated with epithelial cell types. They instead express high levels of collagen receptors, lipoprotein lipase and glycerol-3-phosphate dehydrogenase normally found in stromal cells or adipocytes. High levels of AREG and moderate levels of ADAM-17 were observed in occasional samples from this group of tumors, but the EGFR was absent. At this time there is no evidence that ErbB signaling plays any role in the biology of these tumors (110-112).

3.1.4 Basal tumors

These tumors lack the expression of the estrogen, progesterone and ErbB2 receptor-for this reason they are often called triple receptor negative tumors-and these cancers express some markers consistent with the myoepithelial cells that are in contact with the basement membrane. These tumors express high levels of the epithelial markers keratin 5 and 14 (basal keratins), P cadherin as well as troponin (110-112). Basal breast cancers are correlated

with poor survival, high rates of distant metastasis and are generally high grade, large tumors. Once the category became established, antibody labeling studies indicated that 50 to 70% of the basal cancers expressed high levels of EGFR immunoreactivity (116). Low levels of EGFR expression is correlated with reduced numbers of distant metastasis (117). These tumors also frequently express elevated levels of $TGF\alpha$ and ADAM-17 (115).

Within basal breast cancers there could be a fraction that exhibits autocrine $TGF\alpha$ -EGFR signaling. The correlation of ADAM-17, $TGF\alpha$ and EGFR with poor prognosis implicates some role for this signaling system in metastasis. At this time, functional testing of $TGF\alpha$ in basal breast cancer models has not been completed. One major question that remains is: does $TGF\alpha$ participate in autocrine tumor cell signaling or paracrine tumor-stroma interactions. Future studies will identify which prometastatic cellular behaviors are activated by $TGF\alpha$ -EGFR signaling, providing insight into whether receptor-targeted therapeutics might provide benefit in a metastasis prevention paradigm.

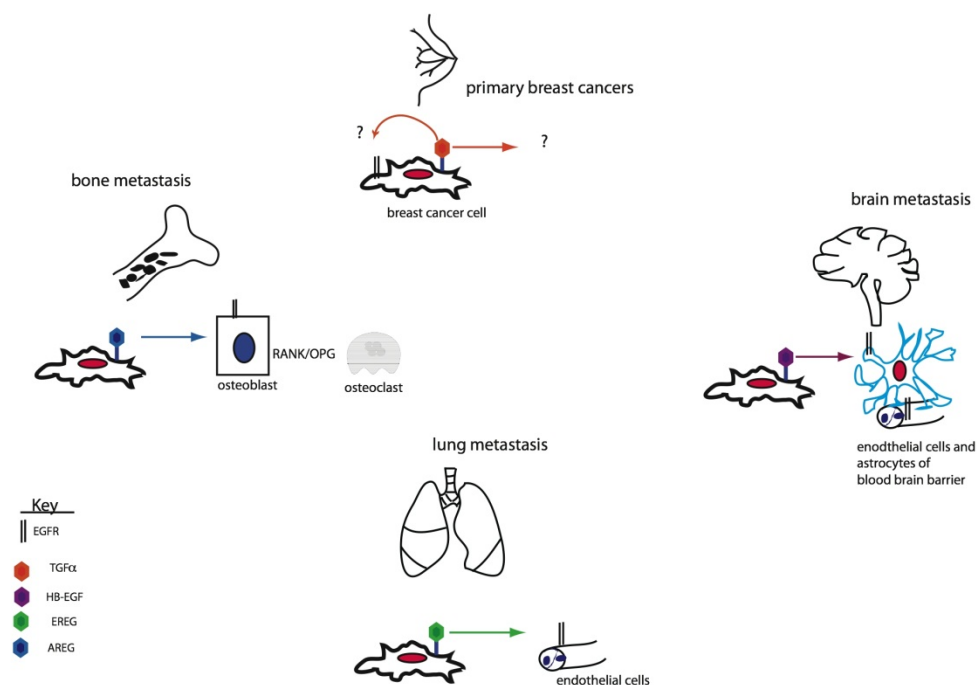


Fig. 3. Summary of EGFR-ligand signaling in models of basal breast cancer metastases. The specific ligand involved in primary tumors and metastatic site are indicated by the colored hexagons. The cell type that responds to the signal is also indicated. Paracrine signaling interactions appear to be important to metastases, whereas the relative role of autocrine versus paracrine signaling has not been explored in primary basal breast cancer.

3.2 ErbB signaling in breast cancer metastasis

Metastasis requires a set of cellular behaviors that are distinct from primary tumor formation. To spread from the site of the primary tumor to distant organs cancer cells must:

1) move out of the primary tumor and invade through local connective tissue; 2) enter capillaries (intravasation); 3) survive in the blood stream; 4) exit the blood stream (extravasation) and invade into a new organ; 5) survive in the new organ possibly as a micro metastasis; 6) adapt to the new organ and grow as a macrometastasis (virulence) (118, 119). These steps can be viewed as two generalized processes: 1) invasion, which is movement of cancer cells through normal tissue and entry into the blood stream, and 2) colonization, which is escape from the blood stream and growth in a distant organ. Substantial progress is being made in identifying metastasis genes that mediate these generalized steps of the process. Metastasis genes are thought dispensable for primary tumor initiation and growth, but are crucial to the novel processes involved in the spread of cancer (119). In general, genes that facilitate invasion for carcinomas such as breast cancer are associated with EMT. Some of the genes involved in invasion are expressed in primary tumors. Colonization is considered to be the most inefficient part of the metastatic process and the growth of a cancer cells in a novel organ is likely to require novel changes in gene expression. Thus, the expression of colonization/virulence-associated metastasis genes are thought to be limited tumors within the specific target organ, rather than being present in most primary tumors (118, 119). Due to less availability, comprehensive gene expression studies of metastases have lagged behind the studies of primary tumors. As a result, alternative strategies for identifying gene signatures that are functionally involved with metastasis have been developed. For breast cancer metastases, Massague and colleagues selected *in vitro* various subclones of the aggressive breast cancer cell line MDA-MB-231. They found that many of the subclones had differing capacities to colonize various organs after intracardiac injection into mice (12, 119-123). Gene expression profiles were generated from the subclones that colonized specific organs, and these were compared to signatures from subclones that colonized the other organs. These signatures were then compared to larger data bases generated from human primary tumors that had ultimately metastasized to the organ of interest producing a refined signature. Subsequent functional analysis of these gene signatures has resulted in the identification of specific EGFR ligands as breast cancer metastasis genes.

3.2.1 EREG and lung metastases

The identification of a lung metastatic signature was derived from subclones of the MDA-MB-231 (LM) breast cancer cells that produced lesions in the lung after intracardiac injection. Among the genes that emerged from this analysis was the ErbB ligand, EREG (121, 123). Knockdown of EREG alone failed to slow the growth of LM as primary tumors in the mammary fat pad or in lung. However, knockdown of EREG in conjunction with cyclooxygenase 2 (COX2), MMP1 and MMP2 (these genes had also been identified as upregulated in the LM signature) had a dramatic impact on both primary tumor growth and subsequent metastasis to lungs. The decreased primary tumor growth in the cells with reduced levels of EREG, COX2, MMP1 and MMP2 appeared to result from reduced angiogenesis. The LM cells recruited abundant dilated tortuous and leaky blood vessels, and the repression of these 4-genes resulted in reduced capillary branching, length and dye effusion. However, VEGF levels were not reduced in the quadruple knockdown tumors relative to controls. Also, no differences were observed in pericyte recruitment to the capillaries between the LM cells and the quadruple knockdowns, suggesting that capillary defects were the result of altered endothelial cell behavior. Also, these multiple knockdowns

exhibited reduced ability to colonize lungs after intravenous injection as compared to the parental LM cells. Close evaluation of the lungs of animals injected with the quadruple knockdown cells found abundant cells trapped in the vasculature, suggesting a failure in extravasation. The deficiencies in quadruple knockdown extravasation were also observed with an *in vitro* assay. Consistent with the knockdown studies, single therapeutic agents that targeted EGFR (such as cetuximab, an EGFR blocking antibody), COX2 (celecoxib) or MMPs (GM6001) also had modest impact on behavior of the LM cells grown as primary tumor and their spontaneous metastases to the lung. However, combinations of two agents slowed the growth of primary tumors and reduced subsequent lung colonization. The efficacy of cetuximab suggests that EREG is stimulating this the EGFR and not ErbB4. The fact that LM cells lacked upregulation of VEGF production, (an EGFR regulatory target in most cancer cells) was interpreted as evidence that EREG functioned in paracrine signaling with endothelial cells rather than being engaged in autocrine signaling.

Although microvasculature defects do not appear to be a major component of the various EGFR^{-/-} or other ErbB-knockout mice, studies of normal and tumor capillaries *in vitro* suggest signaling by family members has an influence on angiogenesis. Intriguingly, ErbB receptor expression is altered in tumor capillary endothelial cells as compared to those in normal vessels. ErbB 2, 3, 4 but minimal EGFR is found in most normal endothelial cells in culture (124). In contrast, tumor endothelial cells acquire the EGFR and down regulate ErbB3 expression both *in vitro* and *in vivo*. Endothelial cells in culture also express EGFR and ErbB4 ligands HB-EGF and NRG-1 (124-126). It is thought that HB-EGF signaling through both the EGFR and ErbB4 helps recruit pericytes to capillaries to stabilize the structures (125). The addition of exogenous EGF to tumor endothelial cells *in vitro* increases their proliferation (124). Shedding of HB-EGF and activation of the EGFR leads to down regulation of tight junction proteins and migration of normal endothelial cells (127). Also, NRG-1-ErbB4 signaling stimulates endothelial cell proliferation, migration and angiogenesis in animals; however, NRG-1 inhibits proliferation of tumor derived endothelial cells (124, 126). In light of the impact of EGFR signaling on tumor endothelial cells, breast cancer cell upregulation of EREG would contribute to the recruitment of a leaky vasculature that is common to aggressive tumors. In addition, EREG-EGFR/ErbB4 signaling might contribute to intravasation and extravasation by down regulation adhesion molecules between endothelial cells within capillaries. The question that remains is whether the low affinity ligand EREG might be more efficient at stimulating proliferation, migration and downregulation of junctional complexes than the high affinity ligand, HB-EGF normally present in endothelial cells.

3.2.2 HB-EGF and brain metastasis

A brain metastasis signature was derived using methods similar to those described for lung metastasis (120, 128). Among the genes that emerged as upregulated in brain metastatic cells was the ErbB ligand, HB-EGF (120). Treating mice cardiac-injected with brain seeking sublines MDA-MB-231 and CN34-BrM2C with cetuximab resulted in reduced numbers of brain metastases. Knockdown of both EREG and HB-EGF, or cetuximab treatment, reduced migration of MDA-MB-231 and CN34-BrM2C through consecutive monolayers of endothelial cells and astrocytes in a model of blood brain barrier extravasation. Brain endothelial cells and astrocytes both express the EGFR (100, 127). Activation of the EGFR has been shown to down regulate components of tight junctions in brain endothelial cells (127). This coupled with EGFR signaling induced upregulation of motility, chemokines and

extracellular matrix remodeling genes in astrocytes would likely aid in extravasation of breast cancer cells through the blood brain barrier into the parenchyma. Beyond this EGFR signaling produces reactive astrocytes that are components of the microenvironment of brain metastases. This raises the question of whether breast cancer generated EGFR ligands could play a role in breast cancer cell virulence in the brain by generating increased numbers of reactive astrocytes (129). HB-EGF is the EGFR ligand most abundantly expressed in the brain and endothelial cells, suggesting it may be the most well suited agonist to mediate in paracrine interactions among cancer cells, capillaries and the brain parenchyma.

3.2.3 AREG and bone metastasis

Tumor cell colonization of bone may be a less complex process than that of lung and brain because the capillaries in bone called sinusoids, have large openings in them to facilitate entrance of bone marrow derivatives into the circulation (119). It is thought that cancer cells may be able to exit through these openings dispensing with the intricacies of extravasation from continuous capillaries.

The growth of breast cancer macrometastases is described as a vicious cycle (130, 131). In this cycle, breast cancer cells exploit the natural renewal process based on the paracrine interactions between the bone forming osteoblast and the bone resorbing osteoclast. In normal bone, osteoblasts regulate osteoclast numbers and activity by releasing chemokines that recruit osteoclast precursors and then differentiate and activate them with a cell surface ligand for the receptor for activation of NF κ B (RANKL). Osteoblasts also produce a soluble decoy receptor osteoprotegerin (OPG) that prevents RANKL from engaging its receptor; therefore, the level of the ratio of OPG to RANKL controls osteoclastogenesis. Once activated, osteoclasts adhere to the bone surface and secrete proteases and acid that degrades mineralized matrix. Growth factors including TGF β and IGF-1 are released from the bone matrix, and this in turn stimulates new bone matrix formation by osteoblasts. This is a regenerating system that is in balance within the local environment; however, the rate of turnover and formation can be modulated by a series of endocrine hormones including PTH and calcitonin. Within the bone marrow, breast cancer cells produce a series of cytokines and growth factors including IL-11 and PTH-related protein (PTHrP) that increase the levels of RANKL relative to OPG leading to increased osteoclastogenesis. The increased bone resorption and resulting high level of growth factors enhance the survival of breast cancer cells, and also TGF β increases gene expression of IL-11 and PTHrP, which begets more osteoclasts.

Profiling bone metastasis subclones of MDA-MB-231 cells resulted in an 11-gene signature (122). Several of the genes identified were factors like IL-11 that directly altered the RANKL/OPG ratio or connective tissue factor that enhanced osteoblast proliferation. Ectopic expression of a single gene from the profile had very little impact on the ability of modestly osteolytic 231 subclones to grow within the bone of immunocompromised mice. However, the combination of 3 of the genes from the signature induced destructive growth in bone after intracardiac injection. Among the genes identified in the signature that produced increased osteolysis when overexpressed as part of a 3 gene cassette were the proteases, MMP1 and ADAMTS-1 (12). It was unclear what the role of these molecules would play in bone metastasis. Eventually, a careful evaluation of aggressively osteolytic MDA-MB-231 lines that were engineered to overexpress ADAMTS-1 and MMP1 were found to shed dramatically increased levels of AREG. Conditioned media from the ADAMTS-1

and MMP1 engineered MDA-MB-231 lines caused an increased RANKL/OPG ratio in primary murine bone cell cultures (12, 132). The conditioned media from the MDA-MB-231-ADAMTS-1 and MMP1 cells activated osteoclastogenesis in the primary bone cell cultures, and this could be inhibited by the EGFR inhibitors gefitinib or cetuximab. Remarkably, these agents (Gefitinib 100mg/kg daily or Cetuximab 100 mg/kg weekly) completely prevented the formation of osteolytic lesions by the MDA-MB-231 ADAMTS-1+MMP1 line delivered by either the intracardiac or intratibial injection method (12). These findings clearly support the notion that EGFR signaling on cells of the osteoblast is a major regulator of the RANKL/OPG ratio, but point out the requirement for appropriate protease expression to make EGFR ligands accessible to the bone microenvironment. These experiments provide an explanation of how bone resorption could be uncoupled from bone formation by breast cancer cells because stimulation of the EGFR should block osteoblast differentiation and matrix production.

To some extent, the identification of a role for AREG in bone metastasis is confounding given that its expression is associated with ER α + breast cancers that generally have good prognosis (115). The MDA-MB-231 cells are ER α - and have a phenotype that is similar to basal cancers. So we interrogated the breast cancer transcriptome datasets GSE2034(133), GSE2603 (123), and GSE12276 (120) from the NCBI Gene Expression Omnibus to evaluate AREG expression in ER α - tumors. We found that AREG expression was lower in the ER α -negative tumors that ultimately metastasized to bone as compared to those that did not (134). A similar pattern of expression has been reported for the classical bone metastasis virulence factor PTHrP. Low expression of the peptide is observed in the primary tumors that ultimately metastasize to bone (135-137). PTHrP gene expression is thought to be activated by TGF β when breast cancer cells enter the bone microenvironment (138). In contrast to PTHrP, the enhanced activity or expression of the proteases that cleave AREG would be sufficient to increase its expression in the bone microenvironment without activating gene expression. Consistent with this concept, high expression ADAMTS-1 and MMP1 protein have been observed in primary breast cancer tumors that ultimately metastasized to bone (12). Thus, the complex post-genomic regulation of EGFR ligand processing and receptor interactions provides mechanism beyond transcription where the amplitude of signaling of this system can be increased to contribute to colonization. Given that AREG appears to be the physiological mediator of EGFR signaling in the bone, it may be ideally suited to uncouple bone formation from bone resorption, which is a component of osteolytic predominant bone metastases that arise from breast cancers.

3.3 Conclusions: EGFR ligand function in breast cancer

EGFR ligands and receptors are frequently expressed together on epithelial cells and the cancers that are derived from these tissues, including the breast. Addition of exogenous EGFR ligands to breast epithelial or cancer cells typically has a profound impact on proliferation or migratory behavior, leading to the concept that autocrine receptor signaling would contribute to tumor progression. Despite this perspective, the unraveling of the developmental breast, heart and brain phenotypes of the various family member KOs suggest that EGFR, ligand and ADAM-17 function as part of a complex paracrine-signaling network. In addition, *in vitro* and *in vivo* models based on MDA-MB-231 cells where EGFR ligands have been found to function as metastasis genes, suggest they signal in a paracrine fashion to key elements of the microenvironment. On the basis of these conclusions, we

speculate that TGF α expression in primary basal breast cancers may also be engaged in paracrine signaling with cells in the microenvironment. This conclusion suggests that the efficacy of EGFR targeted therapeutics will depend upon their uses in combination with other compounds that target the tumor microenvironment in primary basal tumors, as well as those that have metastasized to the lung, brain and bone.

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