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Transforming Growth Factor Beta Signaling in Growth of Estrogen-Insensitive Metastatic Bone Lesions

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While many women with breast cancer will use estrogen receptor therapeutics such as fulvestrant, resistance is nearly inevitable. Recently, it has been noted that the epidermal growth factor receptor (EGFR) family plays a vital role in metastasis to bone; also cross signaling with the transforming growth factor receptor type II (TGFBR2) has been noted to increase levels of destructive environmental cytokines. Therefore we are examining the role of EGFR/TGFBR2 cross talk in bone metastatic breast cancer.

The study of these pathways are important to metastatic breast cancer, as they have been known to increase levels of the destructive cytokine parathyroid hormone related protein (PTHrP), which causes increased osteolysis within the bone environment. To elucidate the connection between these two vital receptors may led to further progression in therapeutics for those women with EGFR positive tumors.

Transforming Growth Factor Beta, Breast Cancer, Bone Metastasis, Epidermal Growth Factor Receptor, Parathyroid Hormone Related Protein
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>6</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>7</td>
</tr>
<tr>
<td>Conclusion</td>
<td>7</td>
</tr>
<tr>
<td>References</td>
<td>8</td>
</tr>
<tr>
<td>Appendices</td>
<td>8</td>
</tr>
</tbody>
</table>
INTRODUCTION

Nearly 80% of breast cancers will eventually metastasize to the bone, causing destructive and painful osteolytic lesions (1). Many of these women will have gone through a series of anti-estrogen treatments. General first line antiestrogen therapy is tamoxifen, which acts as an antagonist with partial agonist characteristics on the estrogen receptor α (ERα) (2, 3); though the majority of women will eventually become resistant to this therapy within 5 years time. Second line therapy thereafter is fulvestrant, which acts as a true ERα antagonist, though ultimately many women will develop resistance to this therapy as well (4-6). The majority of women resistant to fulvestrant will then eventually develop metastases to the bone, and currently there are no available pharmacological therapies for treatment of these bone lesions. We have developed a fulvestrant-resistant MCF7 breast cancer cell line (MCF7-F), which has decreased levels of the estrogen receptor α (ERα) as well as increased expression of the transforming growth factor beta type II receptor (TGFβR2) (7). We have recently adopted direct intratibial injections in our lab for delivery of cells to the bones of mice (8, 9). This method has allowed us to examine the growth of MCF7 cells within the bone microenvironment. Based upon this data and the increased expression of TGFβR2, a hypothesis was formed where the TGFβR2 pathway plays a critical role in metastasis of estrogen-insensitive breast cancer cells to the bone.

The TGFβ pathway is a dichotomy in its actions in the cellular environment. In normal cells, TGFβ regulates cellular homeostasis and acts to regulate proliferation. Neoplastic cells are able to overcome this TGFβ regulation, and frequently use the pathway instead for uncontrolled growth and invasion (10). Another family of receptors often involved in breast cancer metastasis to bone is the epidermal growth factor receptor (EGFR). The EGFR has been linked with increased growth, motility, and proliferation of cancer cells; increased EGFR expression has also been connected to antiestrogen resistance in breast cancers (7, 11). A major connection between the TGFβR2 and the EGFR is the parathyroid hormone related protein (PTHrP). PTHrP is a well-known player in breast cancer metastasis, as it stimulates activation of the destructive osteoclast cells within the bone environment (12). Considering the vicious osteolytic cycle that occurs within bone, metastatic cancer cells secrete large quantities of PTHrP and TGFβ to activate osteoclast destruction in the bone (13). TGFβ released from the bone will stimulate the TGFβR2 on the surface of the cancer cell, stimulating further proliferation. The cycle continues causing an accelerated breakdown of bone as well as a large increase in tumor size. For the EGFR, it is well known that downstream activation will turn on the PTHrP P3 promoter, further increasing levels of circulating PTHrP (12). We have recently found a possible connection between the EGFR and TGFβR2 signaling, which would lead to a new and interesting view on why many cancers are able to overcome the usual path of TGFβ inhibition.

BODY

Task 1 was to examine the ability of MCF7-F cells to grow in bone in vivo.

Task 1 was previously completed and reported in the 2009-2010 annual report. We examined the ability of MCF7-F cells to grow in bone simultaneously with human mesenchymal stromal cells (hMSC). We observed that the combination of MCF7-F with hMSC cells caused an increase in osteolytic lesion formation over either cell line injected alone (Figure 1). This finding that MCF7-F cells form destructive lesions with co-injection of hMSC cells suggests that surrounding stroma within the bone plays a vital role in lesion formation after cancer cell metastasis. Task 1 is complete.
Task 2 was to examine the ability of MCF7-F cells to form osteolytic lesions and influence osteoblast to osteoclast signaling in vivo. Upon completion of the MCF7-F/hMSC experiments from Task 1, mice were sacrificed and hind limbs removed for histological analysis. Histological sections were prepared and stained for tartrate resistant acid-phosphatase (TRAP) for the detection of active osteoclasts, and also stained for hematoxylin and eosin (H&E). With TRAP staining, an increase in osteoclast counts was seen in sections with both MCF7-F/hMSC cells in comparison to sections with MCF7-F or hMSC cells alone (Figure 2). To investigate if this increase in osteoclast activation is directly from the MCF7-F cells, co-culture experiments were performed with osteoclast precursor cells. Osteoclast precursor cells were primed for 3 days with receptor activator of nuclear factor kappa-B ligand (RANKL), a key factor found on osteoblast cells, and is also involved in osteoclast activation (14-16). The invasive MDA-MB-231 breast cancer cell line, MCF7 cells, or MCF7-F cells were then incubated with the primed osteoclast precursors, followed by TRAP staining in culture. MCF7-F cell co-culture increased the number of TRAP positive osteoclasts as seen in Figure 3. These data suggest the MCF7-F cells may have a more invasive phenotype than wild-type MCF7 cells. We were unable to obtain sufficient cells to perform Pit assays on dentin slices, and so this assay was not performed. The remainder of Task 2 is complete.

Task 3 was to examine the cooperation between EGFR and TGFβ signaling in breast cancer cell lines. In order to examine the cooperation between EGFR and TGFβ in the MCF7 cell lines, an initial stable MDA-MB-231 cell line was produced with a lentiviral shRNA to EGFR (shEGFR). The shEGFR line showed a 70% decrease in the EGFR compared to vector control cells (Figure 4A). Since parathyroid hormone related protein (PTHrP) is a vital player in the osteolytic cycle, we investigated if there was a change in PTHrP levels in the shEGFR line. shEGFR cells show a significant basal decrease in PTHrP levels compared to vector control. Also, after TGFβ treatment, shEGFR cells continue to show a decrease in PTHrP (Figure 4B). Collectively, these data suggest there is a connection between EGFR/TGFβ signaling and PTHrP expression in osteolytic destruction.

In order to proceed efficiently to complete tasks for this grant as well as complete the PhD in a timely manner, the MDA-MB-231 shEGFR line was used for continued work. Ongoing experiments include production of a shEGFR line in MCF7-F cells. Since both osteoblasts in the bone microenvironment as well as cancer cells harbor the EGFR, this suggests that EGFR ligands participate in both an autocrine and paracrine manner in the metastatic bone environment. Both paracrine and autocrine EGFR signaling were inhibited with a neutralizing amphiregulin antibody, PAR34, whereas shRNA to the EGFR was used to specifically block autocrine signaling in MDA-231 cells.

Breast cancer metastasis to bone was modeled in female athymic nude mice with intratibial inoculation of MDA-231 cells, and cancer cell-bone marrow co-cultures. EGFR knockdown, but not PAR34 treatment, decreased osteoclasts formed in vitro, reduced osteolytic lesion tumor volume, increased survivorship in vivo, and resulted in decreased MDA-231 growth in the fat pad. These results are currently in press with PLoS One, and may be found in the appendix, as well as publication 1 in Reportable Outcomes.

As the EGFR has a number of active ligands, we are investigating the role for a number of these specific ligands in the cooperation of EGFR and TGFβ signaling. We have created a number of stable shRNA MDA-231 cell lines using a lentiviral packaging system (17, 18) and

5
shRNAs to EGFR ligands Amphiregulin, HB-EGF, and TGFα. We have also created stable MDA-231 cell lines that overexpress the same ligands, Amphiregulin, HB-EGF, and TGFα. As expected, we observed decreased levels of PTHrP in the shRNA lines and an increase in PTHrP expression in the overexpression lines as seen in Figure 5. Interestingly, invasion was inhibited in both the AREG shRNA and AREG overexpressing line, while overexpression of TGFα caused a significant increase in invasion (Fig 6).

We then injected both the shRNA and overexpressing cell lines into the mammary fat pad of female athymic nude mice. As expected, the shRNA lines increased survival and created smaller tumors by both mass and volume (Fig XX). Simultaneously, the overexpressing lines caused significant decreases in survival as well as larger tumors (Fig XX). Interestingly, \textit{in vitro} it appears that TGFα stimulates more aggressive behavior. In contrast, AREG tumors grew more rapidly than other overexpressing lines.

Together, these results suggest TGFα leads to more invasive behavior, where as AREG leads to larger, more rapidly growing tumors. To defend these findings, we are creating cell lines using the shRNA to AREG or TGFα, and overexpressing the opposite ligand. By doing so, we hope to allow AREG and TGFα to be dominant without competition from the opposing ligand. We are also creating a double knockdown line of both AREG and TGFα inhibition, in hope to nearly completely inhibit signaling by either ligand. After characterization of these new lines, we will inject them into the mammary fat pads of athymic nude mice to observe their tumorigenic capabilities. From these experiments, we expect TGFα overexpression will cause more motile, invasive cells that will create fast crowing, large tumors. We expect that AREG overexpression will create modestly more invasive, motile cells, though not as drastic as the TGFα dominant line. We expect these results to be submitted for publication in May 2012.

Task 4 was to investigate involvement of miRNAs in metastasis and formation of osteolytic lesions. We were unable to initiate experiments for Task 4 due to time constraints.

**KEY RESEARCH AND TRAINING ACCOMPLISHMENTS**

- Provided evidence that MCF7-F cells are capable of growing osteolytic lesions within mouse bones with support of stromal hMSC cells.
- Demonstrated this combination of MCF7-F/hMSC cells stimulate an increase in osteoclast activation within the bone microenvironment relative to the parental MCF7 line.
- Training in production of Lentiviral constructs as well as subsequent generation of stable cell lines.
- Found that autocrine inhibition of the EGFR is more vital to reducing growth of metastatic breast cancer in the bone than inhibition of receptor signaling in the micro environment.
REPORTABLE OUTCOMES

Manuscripts


Degrees
Completion of a PhD in Pharmacology will occur in May 2012.

Poster Presentations
2011 Breast Cancer Cell Epidermal Growth Factor Signaling and Osteolysis (Poster presentation). Congressionally Directed Medical Research Programs, Era of Hope Conference, Orlando, FL.

2009 Involvement of Amphiregulin-EGFR Signaling in Breast Cancer Induced Osteolysis (Poster presentation). Gordon Conference: Bones & Teeth, Biddeford, ME.

Personnel Receiving pay from this effort: Nicole Nickerson

CONCLUSION
Greater than 50% of women with breast cancer will eventually develop metastases to the bone. There is a current lack of pharmacological treatments for these painful bone lesions, requiring ongoing research to produce new therapeutics. In the bone of these patients, a destructive cycle occurs whereby increased expression levels of PTHrP cause increases in the levels of bone destruction. We have noted a possible connection between the pro-oncogenic response of cancer cells to the TGFBR2 and the EGFR; whereby activation of the EGFR may be causing increased expression of the TGFBR2, which in turn may considerably increase available levels of PTHrP. We have also examined how MCF7-F cells interact with the bone microenvironment through co-injection with hMSC cells. These connections are of considerable importance to metastatic breast cancer patients, and may eventually offer a new insight to pharmacological intervention for the TGFBR2 as well as treatment involving the surrounding stromal cells.
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APPENDICES
Reprints:


Figure 1: Intratibial injection of MCF7-F and hMSC cells

Figure 1: 4-5 week old female athymic nude mice were intratibially injected with MCF7-F, hMSC, or MCF7-F with hMSC cells. Lesions were monitored weekly with X-ray.

Figure 2: TRAP staining of MCF7-F/hMSC injected mouse legs

Figure 2: Combination MCF7-F/hMSC cell injections increase osteoclast activation. Representative TRAP staining from MCF7-F/hMSC or MCF7-F injected animals. Arrows denote positive TRAP stained osteoclasts. B = Bone, T=Tumour.
Figure 3: Co-culture of osteoclast precursors with breast cancer cell lines

![Bar chart showing osteoclast number for different cell lines](image)

Figure 3: Breast cancer cells stimulate osteoclast maturation. MDA-MB-231, MCF7, or MCF7-F cells were co-cultured with osteoblast pre-cursor cells followed by TRAP staining. Both MCF7 and MCF7-F cells caused an increase in active osteoclasts in culture. * P<0.05, **P<0.001.

Figure 4: Knockdown of the EGFR results in a decrease in PTHrP levels

A) Representative western analysis of EGFR knockdown in the shEGFR cell line versus Vector control. B) Quantitative PCR analysis of PTHrP levels in Vehicle or TGFβ treated Vector or shEGFR cells.

Figure 4: MDA-MB-231 cells were induced with lentiviral shEGFR. A) Representative western analysis of EGFR knockdown in the shEGFR cell line versus Vector control. B) Quantitative PCR analysis of PTHrP levels in Vehicle or TGFβ treated Vector or shEGFR cells.
Figure 5: Basal mRNA levels of PTHrP were measured by qRT-PCR in all shRNA or overexpressing cell lines. Significant differences were observed in the shEGFR, AREG, and TGFα cell lines.

* p<0.05, ** p<0.01.

Figure 6: Modifications to AREG or TGFα cause changes in cellular invasion.

* p<0.05, ** p<0.01.

Figure 6: Cells were allowed to invade through matrigel coated BD Transwell invasion chambers for 24 hours before staining the invading cells. Significant changes were observed in both the shRNA and overexpression lines of AREG and TGFα.

* p<0.05, **p<0.01.
Figure 7: Injection of shRNA and overexpressing cell lines into the mammary fat pad. (A) Ligand overexpressing cell lines cause a decrease in survival. (B) shRNA of ligands causes an increase in overall survival. (C) Ligand overexpressing cell lines caused an increase in speed and size of tumor volume. (D) shRNA of ligands caused a decrease in speed of growth and tumor volume. (E) As expected, overexpression of ligands increased total tumor mass while shRNA of ligands decreased overall tumor mass.
Decreased Autocrine EGFR Signaling in Metastatic Breast Cancer Cells Inhibits Tumor Growth in Bone and Mammary Fat Pad

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Keywords: Breast Cancer; Metastasis; EGFR; Amphiregulin; Bone

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Abstract

Breast cancer metastasis to bone triggers a vicious cycle of tumor growth linked to osteolysis. Breast cancer cells and osteoblasts express the epidermal growth factor receptor (EGFR) and produce ErbB family ligands, suggesting participation of these growth factors in autocrine and paracrine signaling within the bone microenvironment. EGFR ligand expression was profiled in the bone metastatic MDA-MB-231 cells (MDA-231), and agonist-induced signaling was examined in both breast cancer and osteoblast-like cells. Both paracrine and autocrine EGFR signaling were inhibited with a neutralizing amphiregulin antibody, PAR34, whereas shRNA to the EGFR was used to specifically block autocrine signaling in MDA-231 cells. The impact of these was evaluated with proliferation, migration and gene expression assays. Breast cancer metastasis to bone was modeled in female athymic nude mice with intratibial inoculation of MDA-231 cells, and cancer cell-bone marrow co-cultures. EGFR knockdown, but not PAR34 treatment, decreased osteoclasts formed in vitro (p<0.01), reduced osteolytic lesion tumor volume (p<0.01), increased survivorship in vivo (p<0.001), and resulted in decreased MDA-231 growth in the fat pad (p<0.01). Fat pad shEGFR-MDA-231 tumors produced in nude mice had increased necrotic areas and decreased CD31-positive vasculature. shEGFR-MDA-231 cells also produced decreased levels of the proangiogenic molecules macrophage colony stimulating factor-1 (MCSF-1) and matrix metalloproteinase 9 (MMP9), both of which were decreased by EGFR inhibitors in a panel of EGFR-positive breast cancer cells. Thus, inhibiting autocrine EGFR signaling in breast cancer cells may provide a means for reducing paracrine factor production that facilitates microenvironment support in the bone and mammary gland.

Keywords: Breast Cancer; Metastasis; EGFR; Amphiregulin; Bone
Introduction

The epidermal growth factor (EGFR) has long been recognized as a therapeutic target in breast and other epithelial cancers due to its ability to potently stimulate cell proliferation, motility, and invasion. The EGFR is activated by a family of ligands that include epidermal growth factor (EGF), Amphiregulin (AREG), transforming growth factor α (TGFα), heparin-binding EGF (HB-EGF), betacellulin, epiregulin, epigen, and Neuregulin 2β [1]. These factors are synthesized as plasma membrane proteins tethered by a transmembrane domain, requiring proteolytic cleavage to be accessible to receptors [2]. These individual ligands may induce differential signaling pathways downstream of the EGFR, both from the plasma membrane and intracellular compartments, which can result in certain ligands being more efficient stimulators of proliferation [1,3,4,5,6]. Breast cancer cells frequently express the EGFR, one or more of its ligands and proteases that shed the ligands, resulting in autocrine signaling that may contribute to their rapid growth and invasive behavior.

The EGFR is frequently expressed in the basal subtype of breast cancer, which typically lack the expression of estrogen receptor α (ERα), progesterone receptor (PR) and Her2 receptor, accounting for only ~15-20% of the total disease [7,8,9]. However, 50-75% of basal breast cancers express EGFR and are more aggressive than similar tumors lacking the receptor [10,11]. Co-expression of the ADAM17 protease and the TGFα ligand in primary basal tumors has been associated with reduced survival [12]. These observations suggest that more aggressive basal-like breast cancers have the capacity to be stimulated by autocrine EGFR signaling, whereas the ligands produced by other subtypes of breast cancer (luminal, HER2 positive) may serve as paracrine signaling molecules [13].
Models of breast cancer metastasis to specific organs have provided evidence that EGFR ligands mediate paracrine signaling with cells of the tumor environment. Recent gene expression profiling of a bone-homing MDA-231 subline found that MMP-1 (matrix metalloproteinase 1) and ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motifs) were upregulated, leading to increased AREG shedding [14]. The increased AREG appeared to signal via the EGFR present on osteoblasts, leading to reduced production of osteoprotegrin, the decoy ligand to the major controller of osteoclast differentiation and activation, receptor for nuclear factor κβ ligand (RANKL) [14]. Increased osteoclast numbers and activity is a key element in the growth of breast cancer cells in the bone [15]. The metastatic growth of these MDA-231 sublines could be inhibited by the EGFR-targeted therapeutics cetuximab or gefitinib alone, or in combination with other targeted agents [14,16,17].

Autocrine activation of EGFR on breast cancer cells may also influence signaling with the bone microenvironment. Models of bone metastasis have provided evidence that cancer cell activation of EGFR often leads to the production of paracrine signaling molecules necessary for survival and rapid growth within the bone. Among the most well characterized factors that facilitate the growth of cancer cells in the bone is parathyroid hormone related protein (PTHrP), which signals through its receptor on osteoblasts, and leads to an increase of RANKL expression and increased osteoclast activity [18,19]. Autocrine activation of EGFR is a major regulator of PTHrP in both breast and lung cancers [20]. Intriguingly, the stimulation of the PTH receptor on osteoblasts stimulates the expression and shedding of AREG, thus potentially initiating a second autocrine loop of EGFR signaling in osteoblasts [21,22]. Taken together, autocrine EGFR-driven cytokine production, as well as paracrine interactions of the EGFR ligands themselves,
both appear to drive growth of bone-metastatic lesions suggesting various agents that disrupt this signaling could be effective treatments for breast cancer metastasis to bone.

In this study, we evaluated EGFR ligand expression by a bone-homing subline of the human breast cancer cell line MDA-231, with regard to their impact on specific malignant phenotypes and breast cancer cell signaling, as well as paracrine signaling to a mouse bone cell line. To specifically inhibit autocrine signaling in the MDA-231 cells we reduced EGFR expression by a lentiviral shRNA, and to inhibit both autocrine and paracrine EGFR signaling, an AREG neutralizing antibody was used. Finally, we evaluated the impact of altered autocrine and paracrine signaling on MDA-231 cell growth in vitro as well as in vivo, in the bone and mammary fat pad.
**Materials and Methods**

*Ethics Statement*

Animal care and experiments were approved by the Indiana University Animal Care and Use Committee (IACUC), OLAW assurance #94094-01, protocol #10-014.

*Cell lines and cell culture*

MDA-MB-231 cells were obtained from T. Guise [18] and MC3T3 cells were obtained from A. Robling [23], and were grown in DMEM (Sigma, St Louis, MO) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 10ng/mL insulin (Sigma). S1T3 and NS2T2A1 cells were both obtained from Z. Bouizar [24], and grown in a 50:50 mixture of RPMI and DMEM:F12 (Sigma) supplemented with 10% FBS. SUM149 cells were purchased from Asterand (Detroit, MI) and grown in F12 Hams (Sigma) supplemented with 10% FBS (Atlanta Biologicals).

Production of shEGFR-MDA-231 and shControl cells: MDA-MB-231 cells [18] were plated in 12-well dishes and grown to 50% confluence. 10L of either EGFR shRNA lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA) or Control shRNA lentiviral particles (Santa Cruz Biotechnology) with 6µg/mL polybrene (Santa Cruz Biotechnology) was added to the wells. Cells were grown for 24 hours before removal of lentiviral particles, then grown another 24 hours before 1.5µg/mL puromycin selection. Pooled colonies were tested for EGFR expression by western blotting, and cultures maintained in media with 1.5µg/mL puromycin.

*Animal injections and therapeutic dosing*

For intratibial inoculation, either 7.5 x 10³ MDA-MB-231, shEGFR-MDA-231, or shControl cells were inoculated into the left tibia of 3-4 week old female athymic nude mice (Harlan, Indianapolis, IN). Mice were anesthetized with 5% isoflurane and laid in a dorsal
position. Autoclaved 100μL Hamilton syringes with 27 gauge needles were used to puncture the skin at the proximal end of the left tibia. The syringe was gently pushed through the epiphysis to about 3mm deep to assure it passed the through metaphysis, and 10μL of cell suspension was inoculated slowly over 20 seconds. Mice were anesthetized with 5% Isoflurane, and lay in a prone position for weekly radiography to monitor lesion progression (35kV for 10 seconds; Faxitron, Lincolnshire, IL). Animals were x-rayed at 2x magnification, and lesions were first detected at 14 days post inoculation. End point for these studies was 4-6 weeks after tumor inoculation, or earlier if the size of the x-ray lesion reached 25% of the upper tibia area, swelling of knee region exceeded 2-fold the diameter of the non-injected limb, the limb could not be used for ambulation, or the animals displayed signs of excessive pain, per our veterinarian-guided animal protocol and pain-scale. Osteolytic area on x-ray was measured using ImageJ software (NIH).

Upon sacrifice, hind limbs were removed and fixed in 10% neutral buffered formalin for 48 hours, and then 70% EtOH for at least 24 hours. After microCt imaging, bones were decalcified (10% EDTA for 1 week), and embedded in paraffin. Tibiae were sectioned at 7μM in the sagittal plane and mid-sagittal sections were stained with hematoxylin and eosin (H&E) or tartrate resistant acid phosphatase (TRAP). TRAP staining for osteoclasts was performed using an azo-dye coupling method with fast red violet LB salt (F-3881, Sigma) as described [25]. After rehydration through graded alcohols, sections were incubated in freshly prepared TRAP stain at 37°C for 15 minutes, counterstained in hematoxylin, and mounted in glycerin jelly. Serial slides were stained with hematoxylin and eosin (H&E) as described [26]. For this study, PAR34 antibody was administered for 4 weeks through intraperitoneal injection once weekly at 10mg/kg in a sterile 0.9% saline solution [27].
For mammary fat pad tumors, shControl or shEGFR-MDA-231 cells were combined with 50% Matrigel and inoculated at 1x10^6 cells in 100μl total volume in the first mammary fat pad of female athymic nude mice, aged 3-4 weeks (Harlan). One group of shControl mice was administered PAR34 at 10mg/kg/week by intraperitoneal injection. Tumors were measured twice weekly for length (L) and width (W), and tumor volume (V) calculated as: 

$$V = (L \times W^2) \times 0.5.$$  

**Micro-CT**

Fixed tibiae were scanned using a SkyScan micro-CT (SkyScan 1172; SkyScan, Belgium) as previously described [28], with the following scanner settings: voltage, 60kV; resolution, 6μm; 0.5mm aluminum filter; stage rotation, 0.7; and frame-averaging, 2. Flat-field corrections were used to minimize background noise. NRecon software (SkyScan), was used to reconstruct the images, with post-alignment optimization performed for each separate tibia. CTan software (SkyScan), was used to analyze reconstructed images, separating bone from surrounding soft tissue with a threshold range of 100 to 255 (binarized 0-255 scale). Bone volume was reported from analysis of 700 sections per tibia. 3D images were obtained using MeshLab software (MeshLab, 3D-CoForm) with smoothing option.

Trabecular bone analysis regions were chosen in the secondary spongiosum, with a consistent total length of 1mm measured for each tibia. Region of interest was chosen as only the internal bone cavity containing trabecular bone with cortical bone excluded.

**Osteoclastogenesis assays**

Osteoclastogenesis assays were performed as in [29]. Briefly, 1-4 month old mice were euthanized, hind limbs dipped in 70% ethanol and removed at hip. Femur and tibia ends were cut to expose the bone marrow cavity, and each marrow cavity flushed with 5-10mL of DMEM cell culture medium. 50μL of cell suspension was mixed with 450μL of 2% acetic acid to lyse
red blood cells, and remaining cells counted. $4 \times 10^5$ cells were plated in each well of a 24-well dish with 60ng/mL RANKL (PeproTech) for 3 days. After 3 days, $2 \times 10^3$ MDA-231, shControl, or shEGFR-MDA-231 cells were plated with the bone marrow cells, with 60ng/mL RANKL and 10ng/mL MCSF (PeproTech) and grown for 3 days before TRAP staining. For TRAP staining, cells were washed with 1xPBS, fixed with ice cold methanol for 10 minutes, and stained in fresh TRAP solution for 15 minutes at 37°C. TRAP solution was replaced with 1xPBS for cell counting under the microscope.

**Statistical analysis**

Results of *in vitro* experiments are expressed as the mean ± SD of triplicate or quadruplicate measures of independent replicates for single experiments. Results of *in vivo* experiments are expressed as the mean ± SEM of three to six replicates of samples taken from ten individual animals. All statistical comparisons were based on two-tailed analysis of the Student's $t$ test. A $P$ value of $<0.05$ was considered to be significant.
Results

Amphiregulin is highly secreted by MDA-231 cells

Previously we determined a subline of the aggressive breast cancer cell line MDA-231 efficiently colonizes mouse bone after intracardiac inoculation, expresses high levels of EGFR protein and modest levels of the ErbB2 and ErbB3 receptors, and sheds AREG [20]. To more completely evaluate EGFR ligand production in these cells, we examined the expression of five EGFR ligands, including epidermal growth factor (EGF), AREG, betacellulin, heparin-binding EGF (HB-EGF), and transforming growth factor α (TGFα) using ELISA for both conditioned media and membrane extracts. MDA-231 cells release high levels of AREG (0.048pM), and maintain similar concentrations associated with the membrane fraction. Surprisingly, higher levels of HB-EGF also remained associated with the cell membrane (0.254pM), with lower levels (0.008pM) detectable in the media (Fig 1A). Low concentrations of TGFα were present on the membrane fraction (0.008pM) while higher levels were detected in the media (0.063pM). Betacellulin was present in low concentrations (0.004pM) and EGF protein was undetectable using this methodology (Fig 1A). In terms of autocrine signaling in vitro, AREG appears to be shed at the highest concentrations, while high levels of membrane-associated HB-EGF indicate that this could be the predominant ligand if it were cleaved from the membrane.

Amphiregulin activates EGFR phosphorylation on both MDA-231 and MC3T3 cells

To determine if the impact of EGFR signaling in the bone microenvironment is similar to that of breast cancer cells, we used a mouse preosteoblastic cell line MC3T3 as a model to compare receptor phosphorylation induced by exogenous ligand treatment. Here, we used 100 nM recombinant human ligands (AREG, TGFα, and HB-EGF), as well as recombinant human EGF (10 nM) to serve as the prototype ligand. MC3T3 or MDA-231 cells were treated with EGF,
AREG, TGFα, or HB-EGF, followed by western blotting with their respective phospho-specific antibodies. Modest levels of basal EGFR tyrosine phosphorylation could be detected in MDA-231 cells at Y992 and Y1086, whereas baseline EGFR levels could not be detected in the MC3T3 line with any of the phospho-specific antibodies (Fig 1B). Human EGF and HB-EGF were able to induce receptor phosphorylation on both MDA-231 and MC3T3 cells, as detected with 4G10, a pan phosphotyrosine antibody, as well as the other site-specific antibodies. Exogenous AREG induced modest phosphorylation of some residues in MDA-231 cells compared to EGF, but appeared to increase phosphorylation of all tested residues in MC3T3 cells. We noted that TGFα caused very little phosphorylation in the human cells and was not able to induce detectable changes in EGFR phosphorylation in mouse MC3T3 cells. Though both AREG and TGFα are shed and capable of inducing receptor phosphorylation in MDA-231 cells, AREG appears to be the highest cleaved ligand and it is able to potently activate the EGFR on mouse osteoblast-like MC3T3 cells providing the rationale to target this ligand as the main inducer of both autocrine breast cancer signaling and paracrine receptor signaling in mouse tissues.

**shRNA to the EGFR causes a decrease in migration and PTHrP expression in MDA-231 cells**

To inhibit breast cancer cell autocrine and paracrine signaling, we used shRNA to the EGFR as well as a monoclonal antibody (PAR34) (Figure S1). To reduce autocrine EGFR signaling in the MDA-231 line, cells were transduced with a lentiviral shRNA to the receptor (shEGFR-MDA-231 cells) or a shRNA scrambled control (shControl). As detected by western blot, there was a 64% knockdown of the EGFR as compared to MDA-231 or shControl cells, and this knockdown not affect levels of other EGFR family receptors (Fig 2A). Introduction of the shEGFR construct had no effect on production of AREG, TGFα, or HB-EGF mRNA production
We verified by ELISA that ligand protein levels were not disrupted by the shEGFR construct, as AREG, TGFα, and HB-EGF were present in the media or on cell membranes at the same levels in shEGFR-MDA-231 cells, as compared to MDA-231 and shControl cells (Fig 2B). Treatment of MDA-231 or shControl cells with PAR34 antibody or control IgG had no effect on ligand expression of AREG, TGFα, or HB-EGF (Fig 2B). As expected, a decrease (p<0.05) in PTHrP levels in the shEGFR-MDA-231 cells was observed as compared to control cells (Fig 2C), indicative of reduced autocrine EGFR signaling.

We then examined impact of PAR34 on breast cancer cells grown in vitro. PAR34 inhibited exogenous AREG-induced phosphorylation of tyrosines 992 and 1173 in MDA-231 cells, when compared to IgG control (Figure S2B), and this inhibition was AREG-specific, as PAR34 did not inhibit stimulation by EGF. Similar inhibition of exogenous phosphorylation was noted in the non-tumorigenic, epithelial breast cell line S1T3 (S1 cells).

To further test the impact of autocrine EGFR signaling inhibition by PAR34 antibody and shRNA knockown, cell proliferation and migration were examined in vitro. EGFR signaling has been reported to stimulate motility, but does not induce proliferation in MDA-231 cells [30,31]. Using the MTT assay we found that shEGFR-MDA-231 cells and controls treated with PAR34 proliferated at a similar rate to non-treated controls (Fig 2C). As shown in Figure 2D, PAR34 inhibited migration (p<0.001) of both MDA-231 and shControl cells by 20%, and migration was decreased (p<0.001) by 65% in shEGFR-MDA-231 cells relative to controls. Taken together, these in vitro assays confirm that inhibition of EGFR by PAR34 or shRNA decreases breast cancer cell motility.

PAR34 treatment modifies the trabecular patterning factor of bone
To examine the impact of inhibiting AREG signaling within the bone, we first evaluated PAR34 antibody treatment in non-tumor bearing animals. Female athymic nude mice aged 3-4 weeks received intraperitoneal injections of PAR34, at 10mg/kg/week, for 4 weeks. Upon sacrifice, tibiae were removed and prepared for microCT and histological sectioning. While PAR34 treatment did not affect the gross bone structure, as analyzed by both x-ray and microCT (data not shown), microCT showed a decrease (p<0.001) in trabecular pattern factor in PAR34 tibiae when compared to vehicle treated animals (Table S1 and Figure S2A). We also evaluated osteoclasts present in the newly deposited bone under the hypertrophic zone of growth plate chondrocytes, and observed an increase (p<0.01) in the number of these cells per bone surface area in PAR34 treated animals versus control animals (Figure S2B and Table S1). Overall, PAR34 treatment influenced bone growth, thus validating this dose and schedule as effective for targeting bone in vivo.

*shEGFR-MDA-231 cells produce smaller tumors in bone*

To examine global inhibition of AREG signaling, or to specifically reduce cancer cell EGFR signaling during osteolytic lesion growth within the bone, female athymic nude mice (aged 3-4 weeks) were inoculated in the left tibia with MDA-231, shControl, or shEGFR-MDA-231 cells. Intratibial inoculation was chosen to insure that differential motility of the shEGFR-MDA-231 did not inhibit colonization of the bone. Three days after inoculation, treatment of one group of MDA-231 inoculated mice was initiated with weekly intraperitoneal injection of PAR-34 antibody (10mg/kg). The MDA-231, shControl, and PAR34 treated groups had extensive osteolytic lesion destruction as detected by x-ray and microCT at the experimental end-point, while the majority of shEGFR-MDA-231 mice had smaller regions of distinct bone loss measured by x-ray (Fig 3A). All PAR34 treated animals required sacrifice after the 3-week time
point, as they displayed experimental end-point criterion including maximum x-ray lesion size, swelling of the injected limb, or ambulation difficulties. Survival was increased (p<0.001) in shEGFR-MDA-231 tumor-bearing mice as compared to those inoculated with MDA-231 or shControl (Fig 3B), and osteolytic lesion size was decreased (p<0.01) in shEGFR-MDA-231 animals (Fig 3C). Although large lesions were readily apparent in the reconstruction of microCT scans from the MDA-231, shControl, or PAR34 groups, total tibia head bone volume was not significantly different as compared to the shEGFR-MDA-231 group (Fig 3D).

Examination of H&E stained tibiae from all groups verified large, destructive tumors within the MDA-231, PAR34 treated, and shControl groups (Materials and Methods S1). Interestingly, shEGFR-MDA-231 animals had smaller tumors (p<0.01) that remained within the bone marrow cavity (Fig 4A). Surprisingly, the PAR34 treated animals had a larger tumor volume (p<0.05) when compared to controls (Fig 4B). Tartrate resistant acid phosphatase (TRAP) staining indicated the number of osteoclasts per tumor bone interface in shEGFR-MDA-231 bones trended toward a decrease in comparison to MDA-231 or shControl tumor-bearing tibiae (Fig 4C). Additionally, we observed an increase in osteoclasts per tumor bone interface, though this was not significant (Fig 4C).

Thus, it appears that decreased EGFR was sufficient to reduce the size of osteolytic lesions and tumor volume within bone. Conversely, PAR34 antibody enhanced MDA-231 growth within the bone.

Modulation of EGFR signaling impacts osteoclastogenesis in vitro

We also examined the effects of EGFR knockdown or PAR34 treatment using an in vitro osteoclastogenesis assay, whereby MDA-231 or shEGFR-MDA-231 cells were co-cultured with mouse bone marrow (BM) to determine if osteoclast formation could be increased. As seen in
Figure 5A, co-culture of BM with shEGFR-MDA-231 cells stimulated fewer (p<0.01) osteoclasts than control cell co-cultures, correlating with the decreased osteolytic lesion size in vivo.

Next we evaluated the impact of PAR-34 and exogenous AREG on various permutations of the co-culture assay. We also observed that PAR34 antibody caused an increase in osteoclasts in BM alone (Fig 5B, p<0.01) or co-cultures with MDA-231 (p<0.01) and the shEGFR-MDA-231 cells (p<0.001) (Fig 5C&5D). In contrast, exogenous AREG ligand failed to increase osteoclasts in BM, but stimulated the formation in co-cultures that contained MDA-231 cells (Fig 5C, p<0.01). Intriguingly exogenous ligand did not increase osteoclast number in the shEGFR-MDA-231 containing co-cultures (Fig 5D). The impact of PAR-34 on BM alone or cancer cell co-cultures generally corresponded with in vivo findings where the antibody treatment produced increased osteoclasts in non-tumor bearing bones and increased tumor size in cancer cell injected bones.

To further investigate the impact of EGFR signaling inhibitors on osteoclastogenesis, BM and MDA-231 co-cultures were treated with a range of concentrations of gefitinib, a small molecule EGFR inhibitor [32]. As shown in Figure S3, 1μM gefitinib also increased osteoclasts (p<0.001) in co-cultures, but showed a trend toward decreased formation in BM cultures alone. These findings coupled with those from PAR-34 treatments suggest that different EGFR inhibitors can have distinct impacts on osteoclastogenesis and in some cases they may enhance it.

shEGFR-MDA-231 cells produce smaller mammary fat pad tumors

To determine if the reduced growth of the shEGFR-MDA-231 cells in bone was specific to that microenvironment, we examined the in vivo growth rate of mammary fat pad tumors
produced by shControl or shEGFR-MDA-231 cells. A group of shControl-inoculated animals were treated with weekly intraperitoneal injections of PAR34 (10mg/kg). As shown in Figure 6, tumor volume measures and final masses were decreased (p<0.01) in shEGFR-MDA-231 tumors as compared to shControl (Fig 6A&6B). While PAR34 treatment trended towards reduced tumor volume and mass, these results were not significant compared to shControl tumors (Fig 6A&6B). Histological analysis revealed an increased (p<0.05) necrotic area in shEGFR-MDA-231 tumors, despite unchanged cell proliferation as detected by an anti-Ki67 antibody (Table 1). However, fewer vessels (p<0.001) were stained by anti-CD31 antibody in the shEGFR-MDA-231 tumors than shControl cells (Table 1 and Fig 6C). Thus, reduced growth in vivo of the shEGFR-MDA-231 cells was observed in the mammary fat pad, likely correlated with reduced vascularization of the tumor.

*Decreased EGFR signaling causes a reduction in proangiogenic factor expression*

To explore the molecular basis of the reduced vasculature of the mammary fat pad tumors produced by shEGFR-MDA-231 cells, we first examined changes in expression of vascular endothelial growth factor (VEGF). Previous work has reported VEGF is regulated in breast cancer cells by EGFR signaling [33], however we observed no differences in control versus shEGFR-MDA-231 cells (Fig 7A). Previous publications have noted that EGFR signaling regulates macrophage colony-stimulating factor-1 (MCSF-1) expression in murine cancer cells [34]. MCSF-1 could influence angiogenesis by recruiting macrophages or various progenitors from the bone marrow, which could produce VEGF or directly contribute to neoangiogenesis [35,36]. MCSF-1 levels were lower (p<0.05) in shEGFR-MDA-231 cells when compared to controls, as measured by ELISA (Fig 7B).
To verify this finding is due to EGFR inhibition and not off target effects of the shRNA construct, we examined MCSF-1 levels in a panel of breast cancer cell lines following treatment with the small molecule EGFR inhibitor PD153035 or PAR34. PD153035 reduced MCSF-1 secretion from the parental MDA-231 (p<0.01), shControl (p<0.01), SUM149 (p<0.05), and the tumorigenic epithelial breast cancer cell line NS2TA1 (p<0.01) (Fig 7C). PAR34 decreased MCSF-1 levels in MDA-231, shControl and NS2TA1 cells (p<0.05) in which AREG is the predominant ligand, but not in SUM149 cells. We further evaluated the shEGFR-MDA-231 and control cells for matrix metalloproteinase 9 (MMP-9), a protease that promotes angiogenesis by releasing VEGF that is bound to extracellular matrix [37]. As shown in Figure 7D, MMP-9 levels were markedly reduced in cell extracts of shEGFR-MDA-231, as compared to shControl and MDA-231. Also, PAR34 reduced expression of the protease in SUM149 and NS2TA1 cell lines. PD153035 inhibition (6-hrs) had no effect on MMP-9 levels. These findings suggest that autocrine EGFR signaling regulates at least two proangiogenic factors in breast cancer cell lines, and disruption of receptor signaling would be predicted to reduce vascularization and decreased growth of tumors.
Discussion

In this study, we found that reduced EGFR expression decreased MDA-231 cell growth within bone and the mammary gland. Previous studies suggested that EGFR signaling promotes growth \textit{in vivo} as part of paracrine relationships between breast epithelia-derived cells and the microenvironment. The mammary epithelium expresses both EGFR and its ligands EGF, TGF\(\alpha\), and AREG, suggesting a potential for autocrine signaling [38,39]; however, elegant recombination experiments established that mammary gland ductal outgrowth requires EGFR expression in the fat pad, and AREG expression in the epithelium [39,40,41]. In lung and brain metastasis models, epigen or HB-EGF expressed by MDA-231 cells signal to the EFGR on endothelial cells to facilitate colonization of these organs. In a model of bone metastasis, breast cancer cell derived AREG is thought to signal to the osteoblast, facilitating osteoclast formation and driving osteolytic destruction. In contrast, autocrine EGFR signaling is typically associated with proliferation of epithelial cancers [13]. Since the MDA-231 line bears a mutation in K-ras, which activates the MAPK cascade the major driver of mitogenesis downstream of the EGFR [30,31], this line represented an ideal system for modulating receptor levels without reducing cell proliferation. The shEGFR-MDA-231 cells did not exhibit alterations in the expression of ligands or other ErbB receptors, and had identical rates of proliferation as compared to control lines (Fig. 2). Decreased EGFR expression in the MDA-231 resulted in slower tumor growth in both the bone and the mammary gland (Figs. 3 and 6). The shEGFR-MDA-231 cells produced smaller osteolytic lesions \textit{in vivo} and induced the formation of fewer osteoclasts \textit{in vitro} relative to controls (Figs. 3 and 5). Consistent with a central role for the breast cancer cell EGFR in the
stimulation of osteoclastogenesis, the addition of exogenous AREG to co-cultures containing shEGFR-MDA-231 cell failed to induce increased numbers of the bone resorbing cells (Fig. 5). We conclude that autocrine EGFR signaling contributes to MDA-231 tumor growth in bone and the mammary gland independent of driving cancer cell proliferation.

There is a growing appreciation that EGFR signaling in epithelial cancer cells stimulates the expression of many chemokines, cytokines, growth factors, and receptors that facilitate paracrine interactions with non-cancer cells of the tumor microenvironment [20,42,43,44]. EGFR signaling controls the expression of VEGF isoforms, which are pivotal factors that control angiogenesis in many tumors [33,42,45]. We did not detect differences in VEGF levels in the shEGFR-MDA-231 cells, consistent with previous studies of MDA-231 sublines [46]. However, we did detect decreases in proangiogenic factors such as MCSF-1 and MMP-9 in the shEGFR-MDA-231 cells, as well as a panel of breast cancer cell lines treated with an EGFR tyrosine kinase inhibitor or PAR-34. Reduced expression of MCSF-1 and MMP-9 would likely influence the growth of the MDA-231 cells in both the mammary fat pad and the bone. Also, the decreased production of PTHrP would be expected to contribute to reduced growth of the MDA-231 line in bone. Previously, it has been shown that PTHrP antibody inhibition dramatically decreased the number of osteoclasts per tumor bone interface, coupled with decreased osteolytic lesion size [18]. Consistent with the reduction of PTHrP, we observed a trend in reduction of osteoclasts in shEGFR-MDA-231 inoculated tibiae; also, in vitro studies indicated that shEGFR-MDA-231 cells generate fewer osteoclasts than control MDA-231 cells (Fig. 5A). It is likely that the EGFR on breast cancer cells controls the expression of many additional cytokines and growth factors that mediate tumor cell-microenvironment interactions, both in primary tumors and sites of metastasis.
Our attempt to block both autocrine and paracrine EGFR signaling by antagonizing
AREG interaction with its receptor, using PAR34 antibody, produced surprising results. Given
that we had previously found that AREG was the major ligand controlling PTHrP expression in
MDA-231 cells [20], it was not surprising that PAR-34 decreased MCSF-1 and MMP-9
production. However, the antibody only modestly inhibited MDA-231 cell motility in
comparison to knockdown of the receptor (Fig. 2D). This raises questions as to whether the
various ligands might exhibit differential impacts on cell motility and growth factor production,
as previously established for some of these agonists in the stimulation of EGFR-dependent
cellular proliferation [3]. Also, the failure of the antibody to potently inhibit motility may reflect
its inability of block signaling of the EGFR from internal compartments such as the endosome
[47]. In vivo, we observed that PAR-34 treatment increased MDA-231 tumor growth within the
bone, while also increasing active osteoclasts at the tumor bone interface. Correspondingly,
PAR34 increased osteoclastogenesis in BM alone as well as BM cancer cell co-cultures, and
increased osteoclast numbers below the growth plate of non-tumor bearing. These later findings
suggest that PAR-34 may induce a higher baseline bone turnover, and this could contribute to the
increased tumor growth that we observed in our in vivo experiments. Although an impact on
baseline osteoclastogenesis of BM cultures was not observed with gefitinb, we found that this
EGFR inhibitor also increased osteoclastogenesis MDA-231 containing co-cultures. Together
these unanticipated findings lead us to speculate that AREG-EGFR signaling in the bone marrow
microenvironment may influence other processes besides osteoblast differentiation and
subsequent osteoclastogenesis. Recent reports indicate that EGFR signaling decreases
hematopoietic stem cell mobilization in response to G-CSF [48]. Derivatives of hematopoietic
stem cells include osteoclasts, monocytes, myeloid suppressor cells and megakaryocytes that all
could influence the growth of breast cancer cells in the bone [49]. Our unexpected findings with
AREG antibody treatments of cancer cells in the bone marrow encourage a more careful analysis
of the impact of various inhibitors on EGFR signaling on all cell types in the breast cancer bone
metastasis microenvironment.

In conclusion, EGFR knockdown in MDA-231 cells reduced their motility and
production of secreted factors that stimulate osteolytic lesion growth and angiogenesis in vitro. In vivo, EGFR knockdown in MDA-231 cells reduced tumor growth both in the mammary fat
pad and the bone. MDA-231 cells act as a model for triple negative breast cancers, so these
findings raise the possibility that interventions that could reduce EGFR expression in triple-
negative breast cancer cells might provide therapeutic benefit to patients with metastatic disease.

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References

Figure Legends

Figure 1. *EGFR ligand expression and shedding in MDA-231 cells.* (A) ELISA measurement of media or membrane extracts from MDA-231 cells. Measurements were taken from two independent cultures and performed in triplicate. (B) Western blots of anti-EGFR and anti-phosphorylated tyrosine resides in MDA-231 or MC3T3 cells treated with EGF, AREG, TGFα, or HB-EGF.

Figure 2. *Characterization of the shEGFR-MDA-231 cell line.* (A) Extracts from MDA-231, shControl, and shEGFR-MDA-231 cells, probed with anti-EGFR or anti-ErbB2, ErbB3, or ErbB4 antibodies and anti-β-Tubulin (loading control). Histogram notes relative pixel density of EGFR protein of shEGFR-MDA-231 cells versus shControl and MDA-231 cells. (B) AREG, TGFα, and HB-EGF ELISA measurements of MDA-231, shControl, and shEGFR-MDA-231 cells, to verify no changes in basal or PAR34 treated ligand expression. ELISA measurements were performed in triplicate from two separate cultures. (C) Relative PTHrP mRNA levels in the shControl and shEGFR-MDA-231 cell lines. PTHrP was measured by qRT-PCR analysis and relative ratios of PTHrP mRNA to GAPDH mRNA levels were shown (mean of triplicate measures from a single experiment; bars, SD). (D) MTT proliferation assays were performed on shEGFR-MDA-231, MDA-231, and shControl cells, as well as PAR34-treated MDA-231 or shControl cells. MTT measurements were performed in quadruplicate, p<0.05. (E) 24 hour migration assay of shEGFR-MDA-231, MDA-231, and shControl cells, with PAR34 treatment to the latter two lines, p<0.001. Migrated cells were obtained from two separate migration wells, with four random fields chosen for counts from each well.

Figure 3. *In vivo analysis of autocrine or paracrine inhibition of EGFR.* (A) Representative end point x-rays for each treatment group (top row), with arrows denoting osteolytic lesion areas.
Corresponding 3D micro-CT images (bottom row). n=10 animals per treatment group. (B) Kaplan-meyer survival curve demonstrating significant increased survival in the shEGFR-MDA-231 injected animals, p<0.001. n=10 animals per group. (C) Osteolytic lesion area was measured using ImageJ software from x-ray images. n=10 mice, p<0.01. PAR34-treated animals required sacrifice at the 3-week time point due to maximum allowable lesion areas and pain scale (per our animal protocol). (D) Micro-CT bone volume analysis of tibiae in all treatment groups. 700 sections were analyzed per tibia. p=not significant.

Figure 4. Histomorphometric analysis of tumor bearing bones. (A) Representative images of H&E stained tibiae from each treatment group. Tumor region outlined in white, BM = bone marrow, T=Tumor. (B) Histomorphometric tumor volume analysis on H&E stained tibia sections. Care was taken to measure the same size tissue volume on each section. *p<0.05 and **p<0.01. (C) Osteoclast counts of TRAP stained slides from each treatment group. p=not significant, n=10 mice per group.

Figure 5. Activated osteoclast measurement by bone marrow and cancer cell co-culture. (A) Co-cultures of mouse bone marrow with MDA-231, shControl, or shEGFR-MDA-231 cells were TRAP stained to identify active osteoclasts. Four random fields were counted from two separate wells for each co-culture. **p<0.01. (B-D) Co-cultures of mouse bone marrow with MDA-231 cells, (C) bone marrow only, or (D) bone marrow with shEGFR-MDA-231 cells were treated with AREG ligand, PAR34 antibody, Control IgG antibody, or a combination of ligand with antibody as noted. Wells were TRAP stained to identify active osteoclasts, and four random fields were counted form two separate wells for each treatment. **p<0.01, ***p<0.001. (E) bone marrow only or co-cultured with MDA-231 cells were treated with 1μM gefitinib or DMSO control for 3 days followed by TRAP staining for active osteoclasts. ***p<0.001
Figure 6. *shEGFR-MDA-231 cells produce smaller tumors in the mammary fat pad.* (A) Tumor volume measurement for mammary fat pad tumors grown from injection of shControl, PAR34 treated shControl cells, or shEGFR-MDA-231 cells. PAR34 treated animals were administered 10mg/kg/week of PAR34 by intraperitoneal injection. Tumor measurements were taken three times per week. **p<0.01, n=6 mice per group.** (B) Upon sacrifice, tumor masses were assessed. **p<0.01, n=6 mice per group.** (C) Paraffin-embedded tumors were stained with anti-CD31 antibody for vessel formation (top row), black arrows denote areas of vessel staining. Ki67 staining (middle row) was examined for cellular proliferation. shEGFR-MDA-231 tumors contained large regions of necrosis, as seen in Necrosis in the bottom row. T=tumor, N=necrotic region. No necrosis was observed in shControl or PAR34 treated tumors. Vessel and proliferation counts, as well as percent changes of necrotic regions are noted in Table 1. n=6 animals per treatment group. Magnification bars, CD31 and Ki67 = 100μm. Necrosis = 1mm.

Figure 7. *MCSF-1 and MMP-9 decrease with EGFR inhibition.* (A) anti-VEGF probed western blot for MDA-231, shControl, and shEGFR-MDA-231 extracts, β-tubulin used for loading control. (B) Media was harvested from shControl or shEGFR-MDA-231 cells and analyzed for MCSF-1 by ELISA, *p<0.05. Measurements were obtained from two separate cultures, and performed in triplicate. (C) MDA-231, shControl, SUM149, or NS2TA1 cells were treated with the tyrosine kinase inhibitor PD153035 (10μg/mL) compound for 6 hours or PAR34 (10μg/mL) for 24 hours before media harvest for MCSF-1 ELISA, *p<0.05 and **p<0.01. Measurements were obtained from two separate cultures, and performed in triplicate. (D) anti-MMP9 antibody probed western blots for shControl, MDA-231, SUM149, or NS2TA1 cell extracts treated with PD153035 (10μg/mL) compound for 6 hours or PAR34 (10μg/mL) for 24 hours. shEGFR-MDA-231 cells were untreated. anti-β-tubulin used as loading control.
Supporting Information Legends

Figure S1. Model of inhibition of autocrine and paracrine EGFR signaling within the bone environment. (A) In an uninhibited situation, cancer cells produce and cleave AREG to act in autocrine or paracrine signaling. Autocrine EGFR signaling can activate the expression of paracrine factors such as PTHrP that can directly stimulate the PTH receptor on osteoblasts and this increases RANKL production and osteoclastogenesis. In addition, stimulation of the PTH receptor induces AREG-EGFR signaling on the osteoblast, leading to increased RANKL accessibility and osteoclastogenesis. Finally cancer cell derived AREG can stimulate the EGFR on the osteoblast in a paracrine manner resulting in increased RANKL accessibility and osteoclastogenesis. (B) shEGFR knockdown in cancer cells will decrease autocrine signaling and AREG-EGFR signaling in the endosome, in turn decreasing PTHrP levels. Decreased PTHrP secretion will lead to decreased osteoblast RANKL production, and a decrease in osteolysis. However this should not prevent cancer cell derived AREG from stimulating the EGFR on osteoblasts. (C) PAR34 inhibition of AREG binding the EGFR on cancer cells will decrease PTHrP secretion, and thus decrease RANKL production by the osteoblast. PAR34 may also inhibit cancer cell and autocrine AREG from stimulating the osteoblast EGFR thus reducing RANKL accessibility and osteolysis.

Figure S2. PAR34 inhibition on bone environment. (A) Female athymic nude mice aged 3-4 weeks were treated with weekly intraperitoneal injection of PAR34 antibody at 10mg/kg or an equal volume of sterile 0.9% saline as vehicle. Left column, paraffin-embedded tibiae were TRAP stained for active osteoclasts. Active osteoclasts were counted in the primary spongiosum directly under the growth plate. Arrows denote positively stained osteoclasts. Right column, microCT images were reconstructed from the secondary spongiosum, and denote changes in
trabecular bone. For both TRAP staining and microCT analysis, n=10 mice per group. Magnification bar = 170μm. (B) MDA-231 or S1 cells were treated with AREG ligand with or without PAR34 antibody, and compared to PAR34 inhibition with EGF ligand treatment. Cell lysates were resolved on 8% SDS-PAGE gels before membrane transfer and probed with the corresponding tyrosine phosphorylated antibodies.

**Figure S3. Gefitinib treatment of bone marrow co-cultures.** Mouse bone marrow (BM) was cultured alone or co-cultured with MDA-231 cells followed by treatment with 0.5μM, 1.0μM, or 5μM of EGFR kinase inhibitor gefitinib for three days. Osteoclasts were counted after TRAP staining from three random fields from two separate wells. *** p<0.001. & denotes all cultured cells in wells were dead after 5μM gefitinib treatment.

**Table S1.** MicroCT and histomorphometry measurements of PAR34 treated, non-tumor bearing tibiae.

**Materials and Methods S1.** Supporting Materials and Methods.
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Materials and Methods S1

Pharmacologic reagents

Mouse EGF was purchased from Sigma. Recombinant human ligands for TGFβ, HB-EGF, EGF, and AREG, as well as Normal Goat IgG, were purchased from R&D Systems (Minneapolis, MN). PAR34 clone was a gift from PDL BioPharma, and was harvested from mouse hybridomas, purified over a protein A column, and dialyzed into sterile 0.9% saline for injection. PD153035 was purchased from Tocris (United Kingdom). VEGF antibody was purchased from AbCam (Cambridge, MA). MMP-9 antibody was purchased from Santa Cruz Biotechnology. PTHrP (1-34) ligand was purchased from Bachem. EGFR, ErbB2, ErbB3, and Erbb4 antibodies were purchased from Santa Cruz Biotechnology. Gefitinib was purchased from Tocris Bioscience (Ellisville, MO).

Ligand and MCSF-1 ELISA assays

MDA-231 cells were grown to confluence in a 12-well dish and serum starved overnight. Conditioned media was collected and cleared by centrifugation for 10 minutes at 4°C. To measure concentration of ligand attached to cell membrane, cells were harvested in ligand extract solution (1M tris-HCl, 0.5M EDTA, 10% TritonX-100, protease/phosphatase cocktail) and cleared through centrifugation for 10 minutes at 4°C. Ligand concentrations (EGF, AREG, HB-EGF, Betacellulin, TGFβ) were measured using the manufacturer’s instructions for the respective ligand DuoSet ELISA kit (R&D Systems). For MCSF-1 ELISAs, cells were treated for 6 hours with PD153035 or 24 hours with PAR34. After various time points, media was harvested from the wells and assayed per the manufacturer’s instructions. Samples were assayed in triplicate.

Analyses of EGFR phosphorylation and expression
We adapted previously published procedures for assaying EGFR ligand stimulation by assessing tyrosine phosphorylation [1]. Briefly, cells were plated on a 100mm dish and grown to confluence. Cells were incubated on ice for 30 minutes, washed with ice cold PBS and treated with ligand for 7 minutes. Cells were lysed and incubated on ice for 20 minutes. Lysates were cleared by centrifugation at 13k rpm for 10 minutes at 4°C, and transferred to a fresh tube.

Concanavalin A-sepharose beads were used to precipitate glycoproteins (which include ErbB receptors) from cleared cell lysates. Precipitates were resolved by SDS-PAGE on a 7.5 % polyacrylamide gel and electrotransferred to PVDF membrane (BioRad). Blots were probed using an anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology) or an anti-phosphospecific rabbit monoclonal antibodies (Cell Signaling Technology). Primary antibody binding was detected using a goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (Kirkegarrd and Perry Labs Inc) and enhanced chemiluminescence (Santa Cruz). Immunoblots were then stripped and reprobed with an anti-EGFR rabbit polyclonal antibody (Santa Cruz) and detected as described above.

RNA isolation and quantitative real-time reverse transcription PCR (Q-RT-PCR)

Total RNA was prepared using the mini RNA isolation II kit from Zymo Research Corporation according to the manufacturer’s instructions. Reverse transcription (RT) and quantitative real-time PCR (QRT-PCR) was performed as previously described [2]. Quantitative real-time PCR (QRT-PCR) was performed using DyNAmo HS SYBR Green qPCR master mix (New England Biolabs) according to the manufacturer’s
PCR reactions were performed in a DNA Engine Opticon System (MJ Research Inc), primers and temperatures are listed in [3].

**Immunohistochemistry**

Histological tumor sections were rehydrated through alcohol gradation, followed by antigen retrieval for 20 minutes in boiling Tris/EDTA pH 9.0. Sections were blocked in 10% serum for 2 hours, followed by primary antibody for CD31 or Ki67 at 4°C for 24 hours. Sections were then incubated in peroxidase blocking solutions (3% H2O2 in TBS) for 15 minutes. Secondary biotinylated-antibody was added for 1 hour at room temperature (Vector Laboratories). Primary antibody was detected using HRP-Streptavidin and DAB peroxidase (both Vector Laboratories). CD31 stained sections were counterstained with hematoxylin, Ki67 slides were not counterstained, and all slides were dehydrated and cleared through xylene before mounting.

**Histomorphometry**

Bones: Histomorphometry was performed using a Leica DM2500 microscope, fitted with Q-imaging Micropublisher Camera (W. Nuhsbaum Inc., McHenry, IL). Histomorphometric analysis was performed with Bioquant OsteoII 2010 software (Bioquant image analysis corporation, Nashville, TN). Tumor volume was measured on H&E stained bone sections. Osteoclasts that were at the tumor-bone interface were counted at 20X on the tumor-bearing bones, and reported as osteoclasts per tumor bone interface. Osteoclasts in non-injected mice given therapeutics only, were counted in the trabecular region directly underneath the growth plate and reported as osteoclasts per bone surface [4,5]. Care was taken in measuring the same size region for each bone.
Tumors: Ki67 tumor counts: Four 10X images were randomly captured from the cortex of each tumor avoiding the necrotic centers. From each of these images all the labeled cells in 0.1 mm² area were counted and a mean number of positive cells for each tumor generated. Counts from each of the shEGFR-MDA-231, shControl, and PAR-34 treated tumors were averaged. Differences in counts among the tumors were not significant.

Necrosis: 10X images were obtained for each H&E stained tumor. Using ImageJ (NIH) the entire tumor area was measured and compared to only the necrotic areas of each tumor. Percent necrosis was calculated.

**MTT Assay**

500 cells per well were plated in a 96-well dish, in quadruplicate wells per cell line, and allowed to sit overnight in a 37°C/5% CO₂ incubator. PAR34 or control IgG was plated at 10μg/mL when specified. MTT measurements were taken on days 1, 3, 5, and 7 after plating. MTT working solution was used at 1mg/mL MTT in cell culture medium. On days of measurement, 50μL of MTT working solution was added to each well and incubated for 4 hours at 37°C/5% CO₂. Medium was removed and 150μL DMSO added to each well and read on a 96-well plate reader at 600nM.

**Migration Assays**

BD BioCoat™Control Inserts were purchased from Becton Dickinson, and used per manufacturers instructions. For each insert, 1x10⁵ cells per well were plated and allowed to migrate or invade for 24 hours as in [6]. After 24 hours, each insert was fixed and stained using the Hema3 Stat Pack (Fisher Scientific) per manufacturer’s instructions, and allowed to dry overnight. The next day, each insert was carefully cut from the plastic insert using a scalpel blade, and placed on clean microscope slides sealing with
microscope oil. Assays were performed in duplicate migration chambers, with four random images taken per chamber.

References

Figure 1

(A) Graph showing the pMoles/1x10^6 cells for different treatments: Media and Membranes. The treatments include Areg, EGF, Betacellulin, HB-EGF, and TGFα.

(B) Western blots comparing MDA-231 and MC3T3 cells under different conditions: Basal, EGF, AREG, TGFα, HB-EGF. Proteins analyzed include EGFR, β-tubulin, 4G10, Y845, Y992, Y1086, Y1173, and β-tubulin.
Figure 4

A

Vehicle  MDA-231  PAR-34  shControl  shEGFR-MDA-231

H&E

B

Tumor Volume (mm³)

MDA-231  PAR34  shControl  shEGFR

C

Osteoclasts per tumor bone interface (mm²)

MDA-231  PAR34  shControl  shEGFR
Figure 5

A

B

C

D

TRAP stained osteoclasts

TRAP stained osteoclasts

TRAP stained osteoclasts

TRAP stained osteoclasts

Basal  PTHrP  MDA-231  shControl  shEGFR
BM only
BM + cancer cells

Basal  Ctl  AREG  PAR34  AREG+ PAR34  AREG+ IgG
BM only

Basal  MDA-231  Ctl  AREG  PAR34  AREG+ PAR34  AREG+ IgG
BM + MDA-231

Basal  shEGFR  Ctl  AREG  PAR34  AREG+ PAR34  AREG+ IgG
BM + shEGFR-MDA-231
Figure A: Tumor Volume (mm^3) over Weeks

- shControl
- PAR34
- shEGFR-MDA-231

Figure B: Average Tumor Mass (mg)

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<tr>
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<td>PAR34</td>
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<td>shEGFR-MDA-231</td>
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Figure C: Immunohistochemistry Images

- CD31
- Ki67
- Necrosis

- shControl
- PAR34
- shEGFR-MDA-231
Figure 7

A

MDA-231  shControl  shEGFR-MDA-231

VEGF  
β-Tubulin

B

M-CSF (pg/mL)

shControl  shEGFR-MDA-231

C

M-CSF (pg/mL)

MDA-231  shControl  SUM149  A1

D

shControl  MDA-231  shEGFR-MDA-231

MMP-9  β-Tubulin

SUM149  A1

MMP-9  β-Tubulin
Supplementary Figure 1

A: Uninhibited

Cancer Cells → PTHrP → RANKL → Osteolysis

B: shEGFR knockdown

Cancer Cells → PTHrP → RANKL → Osteolysis

C: PAR34 inhibition

Cancer Cells → PTHrP → RANKL → Osteolysis
Supplementary Figure 2

A

TRAP stain

MicroCT

PBS Control

PAR34 Ab

B

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Supplementary Table 1: Impact of PAR34 treatment on bone

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<td>Trabecular pattern factor^ (1/mm)</td>
<td>25.77 ± 3.04</td>
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<td>Osteoclasts per bone surface^ (1/mm)</td>
<td>3.14 ± 1.23</td>
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** p<0.01
*** p<0.001
^ obtained by microCT
^ obtained by TRAP staining
EGFR-Ligand Signaling in Breast Cancer Metastasis: Recurring Developmental Themes

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²Harrison School of Pharmacy, Auburn University, Auburn, AL
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United States of America

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 juxtracrine 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, TGFα 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 disintgrin 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 PLCγ/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-immuno precipitation 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 TGFα
Next to EGF, the most intensively studied ligand has been TGFα. Similar to EGF, TGFα exclusively binds to and activates the EGFR. Binding studies suggest that TGFα 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 TGFα as compared to EGF (4). It is unclear whether this conformational change induced by TGFα 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 TGFα does not induce the extensive receptor phosphorylation observed with mouse salivary gland derived EGF (Fig 1). Additionally, it has been long recognized that TGFα induces different trafficking of the receptor than EGF (38). Close to 100% of receptors internalized after TGFα treatment are recycled to the plasma membrane (22). At physiological pH of 7.4 in the extracellular environment, TGFα and EGF have similar binding affinities for the EGFR (22, 38). However, at pH close to 5 such as in the endosome, TGFα 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 TGFα 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 TGFα 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 TGFα (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 TGFα (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 TGFα (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 interleuken-1 induced NFκβ 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 TGFα. 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 diptheria 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, TGFα, 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, TGFα, and AREG, are found in different locations within the TEB during ductal growth. Using immunohistochemical techniques, TGFα 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 TGFα, 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 TGFα 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 (ERα), 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 TGFα (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 hematopoetic 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$\alpha$ 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 postnatal 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 TGFα are expressed in portion of the forebrain 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 forebrain. 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 upregulated 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 TGFα 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 TGFα 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 lead 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 ERα 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 ERα+ 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 ERα+ tumors also contain ErbB4 (110-112), and there is some indication this receptor may be involved in a reciprocal regulatory loop with ERα signaling (114). Surprisingly, AREG was not in the original gene set that defined ERα+ 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, ERα, 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 ERα+ 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 kertatin 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 TGFα and ADAM-17 (115).

Within basal breast cancers there could be a fraction that exhibits autocrine TGFα-EGFR signaling. The correlation of ADAM-17, TGFα and EGFR with poor prognosis implicates some role for this signaling system in metastasis. At this time, functional testing of TGFα in basal breast cancer models has not been completed. One major question that remains is: does TGFα participate in autocrine tumor cell signaling or paracrine tumor-stroma interactions. Future studies will identify which prometastatic cellular behaviors are activated by TGFα-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 intrasavation and extrasavation 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 extrasavation 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 NFκβ (RANKL). Osteoblasts also produce a soluble decoy receptor osteoprotegrin (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 TGFβ 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 TGFβ 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 in 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 ERα+ breast cancers that generally have good prognosis (115). The MDA-MB-231 cells are ERα- 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 ERα- tumors. We found that AREG expression was lower in the ERα-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 TGFβ 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\(\alpha\) 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.

4. References


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EGFR signaling in breast cancer: Bad to the bone

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The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. This family includes EGFR/ErbB1/HER1, ErbB2/HER2/Neu ErbB3/HER3, and ErbB4/HER4. For many years it was believed that EGFR plays a minor role in the development and progression of breast malignancies. However, recent findings have led investigators to revisit these beliefs. Here we will review these findings and propose roles that EGFR may play in breast malignancies. In particular, we will discuss the potential roles that EGFR may play in triple-negative tumors, resistance to endocrine therapies, maintenance of stem-like tumor cells, and bone metastasis. Thus, we will propose the contexts in which EGFR may be a therapeutic target.

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin motif; AREG, amphiregulin; CSF-1, colony-stimulating factor-1; DTC, disseminated tumor cell; EGFR, epidermal growth factor receptor; ER, estrogen receptor; OPG, osteoprotegrin; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; RANK, receptor activator of nuclear factor κB-ligand; RANKL, RANK ligand; TACE, tumor necrosis factor alpha converting enzyme.

1. Introduction
1.1. EGFR ligands and signaling
1.2. EGFR signaling specificity
2. Manuscript body
2.1. EGFR and primary breast tumors
2.1.1. Triple-negative, basal breast tumors
2.1.2. Resistance to antiestrogens
2.1.3. Breast cancer stem cells
2.2. EGFR and bone metastasis
2.2.1. Latent bone colonization by breast tumor cells
2.2.2. Bone metastasis: a vicious cycle
2.2.3. EGFR and osteolysis
2.2.4. EGFR and osteoblast function
2.2.5. EGFR and PTHrP
2.2.6. EGFR ligands and activating proteases as bone metastasis virulence factors

Acknowledgements
References
1. Introduction

The study of breast cancer has provided opportunities to test concepts emerging from basic studies of cell proliferation, signal transduction and developmental biology. One subject of these basic studies is the epidermal growth factor receptor (EGFR) or ErbB family of receptor tyrosine kinases. This family includes EGFR/ErbB1/HER1, ErbB2/HER2 Neu/ErbB3/HER3, and ErbB4/HER4. These receptors play distinct roles in breast malignancies [1–15]. ErbB2 is a therapeutic target in breast tumors that overexpress the receptor. In contrast, the roles that ErbB4 plays in breast malignancies remain a subject of opposing views. For many years it was believed that EGFR plays a minor role in the development and progression of breast malignancies. However, recent findings have led investigators to revisit these beliefs. Here we will review these findings and propose roles that EGFR may play in breast malignancies. Thus, we will propose the contexts in which EGFR may be a therapeutic target.

1.1. EGFR ligands and signaling

EGFR signaling is stimulated by members of the epidermal growth factor (EGF) family of peptide growth factors, whose roles in stimulating ErbB receptor signaling and coupling to biological responses have been intensively studied [2,12,16–17]. EGFR agonists include the epidermal growth factor (EGF), transforming growth factor alpha (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AREG), epiregulin (EPI), epigen (EPC), betacellulin (BTC) and neuregulin (NRG) 2B. These agonists are expressed as integral membrane proteins and are cleaved by metalloproteinases to release soluble, mature ligands. These metalloproteinases are typically members of the ADAM (a disintegrin and metalloproteinase) family of membrane proteases. For example, ADAM17 (tumor necrosis factor α converting enzyme – TACE) cleaves AREG, EPR, HB-EGF and TGFα [18–22]. Because cleavage of the ligand precursors is required for release of soluble, mature ligands, ligand cleavage represents a potential point in which agonist-induced EGFR signaling can be regulated. However, the transmembrane ligands stimulate EGFR signaling on adjacent cells, apparently through a juxtacrine signaling mechanism that may mediate the stromal–epithelial interactions characteristic of the breast [23–25].

The mechanisms by which EGFR signaling is stimulated by agonist binding have been extensively studied [16,17,26,27]. To summarize, EGFR consists of an extracellular domain, a hydrophobic transmembrane domain, an intracellular catalytic tyrosine kinase domain, and several intracellular tyrosine residues whose phosphorylation is responsible for coupling to downstream effectors. Ligand binding to the extracellular domain stabilizes the EGFR in an extended conformation that is competent for receptor dimerization. Dimerization then enables the cytoplasmic domain of one receptor monomer (the regulatory monomer) to stabilize the tyrosine kinase domain of another monomer (the catalytic monomer) in the active conformation and presents the tyrosine residues of the regulatory monomer to the catalytic site of the catalytic monomer. In this manner EGFR dimerization enables its tyrosine phosphorylation (Fig. 1).

Approximately 10 EGFR tyrosine residues are phosphorylated following ligand engagement and receptor dimerization [17,28]. These phosphorylation sites bind adapter proteins and other signaling molecules that possess SH2 (Src-homology domain 2) or PTB (phospho-tyrosine binding) motifs. Several of the phosphorylated tyrosine residues can bind unique effectors and each EGFR agonist is likely to stimulate EGFR phosphorylation at a unique subset of tyrosine residues. Thus, EGFR agonists typically stimulate EGFR coupling to multiple effectors, including Ras, MAPK, Src, STAT 3/5, PLCγ, PKC, and PI3 kinase [17,29]. These effectors are typically coupled to increased survival, proliferation, motility and invasiveness displayed by malignant tumor cells.

In contrast, some EGFR agonists also stimulate coupling to downstream molecules that negatively regulate the receptor. For instance, phosphorylation of EGFR Tyr974 triggers EGFR endocytosis and phosphorylation of EGFR Tyr1045 triggers Cbl-dependent EGFR ubiquitination and proteosomal degradation [17,30]. EGFR phosphorylation also triggers EGFR binding to SHPTP protein tyrosine phosphatases, in which in turn dephosphorylates EGFR [17,31,32]. Thus, EGFR agonists also stimulate pathways that negatively regulate EGFR coupling to malignant phenotypes and the balance between these positive and negative regulators of EGFR coupling to malignant phenotypes may be altered in tumor cells.

1.2. EGFR signaling specificity

Several factors contribute to EGFR signaling specificity. One is the presence of other ErbB family receptors. For example, ErbB2 can stabilize EGFR in a conformation that is competent for dimerization and tyrosine phosphorylation even in the absence of ligand binding, thereby contributing to ligand-independent EGFR signaling and increased ligand affinity for the EGFR [16,33,34]. Furthermore, ErbB2 and ErbB4 heterodimerize with EGFR upon agonist binding to EGFR. This results in phosphorylation of the heterodimerization partner (ErbB2 or ErbB4) and may result in phosphorylation of a different set of EGFR tyrosine residues [16,33]. The latter mechanism may account for the observation that heterodimerization of ErbB2 with EGFR alters EGFR endocytosis and intracellular trafficking [35–37]. In any event, agonist-induced heterodimerization of EGFR with a partner ErbB receptor alters the consequences of stimulation with a given EGFR ligand by coupling to different signaling pathways and biological responses than EGFR homodimers.

Numerous studies indicate that different EGFR ligands induce distinct biological responses and patterns of EGFR coupling to signaling pathways. For example, TGFr and AREG are more effective stimuli of EGFR coupling to biological responses associated with tumor cell metastasis (motility, invasiveness, etc.) than is EGF. These biological differences appear to be due to differences in the sites of agonist-induced EGFR tyrosine phosphorylation. EGFr stimulates greater phosphorylation of EGFR Tyr1045 than does AREG. Thus, EGF stimulates greater EGFR ubiquitination and turnover than does AREG, presumably because of increased EGFR coupling to the ubiquitin ligase c-Cbl. Moreover, the duration of EGFR coupling to MAPK and PLC signaling is greater following stimulation with AREG than with EGF [38–43].

The mechanism by which different ligands cause phosphorylation of distinct sets of EGFR tyrosine residues is unclear. However, the crystal structure of the EGFR extracellular domain dimer when bound with EGF is distinct from the crystal structure of the EGFR extracellular domain when bound with TGFr. Thus, ligand-specific differences in the juxtapositioning of the receptor monomers within the receptor dimer may lead to differences in receptor tyrosine residue availability to the receptor kinase domain for phosphorylation [17].

2. Manuscript body

2.1. EGFR and primary breast tumors

The roles that EGFR and its ligands play in breast cancer have been a subject of intensive study and controversy. Some retrospective immunohistochemical studies have indicated that EGFR overexpression in primary tumors is an indicator of poor prognosis [44–47], whereas other similar studies have failed to establish
such a link [10,48]. Collectively, these studies suggest that EGFR is expressed in 18–35% of breast cancers but is not overexpressed relative to the normal breast epithelia [49]. Of course, because increased EGFR signaling is commonly associated with increased EGFR turnover, immunohistochemical analyses of EGFR protein expression may not be ideal for evaluating the role that EGFR may be playing in breast malignancies.

Initial studies have suggested that expression of EGF, TGFβ, or AREG is associated with larger and more aggressive tumors [9,50,51]. However, more extensive studies have failed to link ligand expression to prognosis [49,52]. This apparent dichotomy may be explained by the fact that immunohistochemical analyses of ligand expression in tumor samples primarily detects the immature, transmembrane form of the ligand, whereas signaling might be driven largely by the mature soluble form of the ligand.

2.1.1. Triple-negative, basal breast tumors

The development of platforms capable of simultaneously evaluating gene expression from a large portion of the genome has led to the identification of gene expression profiles that classify breast cancers. This has yielded further insights into the roles that EGFR and EGFR ligands may play in breast cancer. Basal-type breast cancers express markers frequently found in cells that are in contact with the basement membrane. Such markers include keratin 5 and 17 (basal keratins), P-cadherin, and troponin [53–56]. Basal-type breast cancers are associated with large size, high tumor grade, poor survival, and increased frequency of distant metastases [56]. These tumors typically lack expression of the estrogen receptor-alpha (ERα), progesterone receptor, and ErbB2. Thus, basal tumors are frequently referred to as “triple-negative” breast tumors [57]. Given the relative aggressiveness of these tumors and the absence of targeted therapeutics for treating these tumors, the identification of targets for treating these tumors is a priority.

Gene expression profiling and immunohistochemical studies have indicated that 50–70% of basal breast tumors exhibit EGFR expression [58]. Moreover, our preliminary analyses of breast cancer transcriptome datasets GSE2034 [59], GSE2603 [60], and GSE12276 [61] from the NCBI Gene Expression Omnibus reveal that the EGFR ligand TGFβ and the EGFR/ErbB3/ErbB4 ligand NRG2 are expressed at significantly higher levels in ERα-negative tumors than in ERα-positive tumors. Likewise, the expression of ADAMs and MMPs responsible for maturation (cleavage) of EGFR ligands is higher in ERα-negative tumors than in ERα-positive tumors. A low level of EGFR expression in basal tumors correlates with a reduced incidence of metastases [62]. Similarly, EGFR expression in basal tumors correlates with TGFβ and ADAM-17 expression [63]. Thus, a sizable fraction of basal breast cancers appear to exhibit autocrine TGFβ-EGFR signaling and this may account for the poor prognosis associated with these tumors [63].

In contrast, ERα-positive tumors tend to exhibit elevated AREG expression but no increase in EGFR expression [59–61]. This pattern of expression is similar to that exhibited by the normal mammary epithelia, in which ERα-positive cells exhibit little EGFR expression but do express AREG [64]. The AREG findings are consistent with previous reports from breast cancer cell lines that indicate this ligand is an estrogen regulated gene, but can be activated by several other pathways present in both ERα-positive and negative cancers [65,66]. One possible interpretation of the molecular profile data is that ERα-positive breast cancer would lack high levels of autocrine EGFR signaling, but could engage in paracrine EGFR sig-
naling with fibroblast-like cells in the microenvironment through AREG.

### 2.1.2. Resistance to antiestrogens

The estrogen receptor partial agonist tamoxifen (Tam) is commonly used to treat ERα-positive breast cancer in both pre- and post-menopausal women. However, a significant fraction of ERα-positive tumors exhibit intrinsic resistance to Tam and in many patients responsiveness of ERα-positive tumors to Tam is of limited duration due to acquired resistance [67,68]. Indeed, many ERα-positive tumors acquire complete resistance to Tam, resulting in a restoration of tumor growth and metastasis [67,68].

Tam resistance may arise through overexpression or phosphorylation of the ERα co-activator AIB1/SRC-3 (amplified in breast cancer 1/steroid hormone receptor co-activator 3) [67,68]. This alters the effects of Tam on ERα-mediated gene expression, leading to Tam stimulation of mitogenic signaling pathways [67,68]. Signaling pathways downstream of several different tyrosine kinases induce phosphorylation of AIB1, suggesting that EGFR signaling may cause Tam resistance via this mechanism [67–69].

Tyrosine phosphorylation of ERα causes tamoxifen resistance by enabling estrogen-independent ERα-mediated gene expression [69]. A number of different tyrosine kinases may catalyze ER tyrosine phosphorylation, including ErbB2 [68,69]. Because ErbB2 is a common heterodimerization partner of EGFR, ligand-induced EGFR signaling may contribute to ER tyrosine phosphorylation and tamoxifen resistance.

Fulvestrant (Faslodex®; ICI 182,780) triggers rapid ERα degradation via the proteasome and is frequently used to treat receptor positive, tamoxifen-resistant tumors [68]. However, acquired resistance frequently arises, limiting the utility of this approach [68]. Chronic treatment of ERα-positive breast tumor cell lines with fulvestrant leads to clones that display resistance to fulvestrant. These models of acquired resistance typically display a loss of ERα-expression and elevated EGFR or ErbB2 expression and receptor tyrosine phosphorylation [70,71]. These cell lines also display elevated TGFα expression and retain AREG expression [70,71]. These data suggest that enhanced autocrine EGFR/ErbB2 signaling may compensate for the loss of ER expression and signaling in fulvestrant-resistant breast tumors. However, this hypothesis has yet to be tested in breast cancer patient samples.

#### 2.1.3. Breast cancer stem cells

Solid tumors typically consist of a heterogeneous mix of cellular phenotypes that include poorly differentiated cells that undergo rapid cell division, differentiated cells that are incapable of cell division, and quiescent cells that possess the capacity for self-renewal and can give rise to the other types of tumor cells. This self-renewal and pluripotency have led this category of cells to be called cancer stem cells or stem-like cancer cells (Fig. 2) [72,73].

Breast cancer cells that have been isolated from pleural effusions exhibit a high level of CD44 expression and a low level of CD24 expression [74]. While these cells display a homogenous phenotype, they are extraordinarily efficient at forming phenotypically heterogeneous tumors in immunocompromised mice. Moreover, these cells readily form colonies in suspension cultures and exhibit very aggressive behaviors in metastasis and invasion assays [74]. Thus, these CD44+/CD24− breast tumor cells exhibit characteristics of tumor stem cells. ALDH1 has also emerged as a marker of tumor cells that exhibit stem-like characteristics [75,76].

There is no direct evidence indicating that EGFR and its ligands are involved in the establishment or maintenance of breast tumor stem cells. However, stem-like tumor cells are much more rare in ERα-positive breast tumors and breast cell lines (which typically have little EGFR and ErbB2 expression) than in triple-negative breast tumors and breast cell lines (which typically exhibit elevated EGFR expression) [76]. Ligand-induced EGFR signaling is required for stem-like breast tumor cells (including those derived from DCIS tumors) to form colonies in semi-solid medium [77]. Overexpression of ErbB2 in mammary epithelial cells and breast cancer cell lines increases the fraction of cells that display stem-like properties [78]. Finally, a preliminary report from a small clinical trial indicates that the dual specificity EGFR/ErbB2 tyrosine kinase inhibitor lapatinib reduces the number of CD44+/CD24− cells found in breast tumor specimens [79]. These reports provide intriguing hints that the ligand-induced EGFR/ErbB2 signaling may play a substantial role in establishing and maintaining breast cancer stem-like cells. Nonetheless, additional direct experimentation is necessary to evaluate this hypothesis.

#### 2.2. EGFR and bone metastasis

The most common metastasis site of breast cancer is the bone [80]. Nearly 70% of invasive breast cancer cases result in metastasis to the bone and generate severe pain and disability in the patient [80]. Destruction of bone matrix is responsible for the fractures and bone pain associated with advanced breast cancer [81]. The majority of tumors that metastasize to bone are ERα-positive [82], but there are a fraction of ERα-negative tumors that also metastasize to this location [83]. Bone metastases were largely refractory to the traditional systemic approaches (radiation therapy and chemotherapy) used to treat advanced breast cancer [80,81,84]. Recently, the integration of the fields of basic bone cell biology and cancer biology has produced insights that have generated new and partially effective therapeutic approaches to this devastating form of metastasis. Agents such as bisphosphonates reduce bone destruction and tumor growth by targeting the bone microenvironment rather than the tumor [84]. Recently, EGFR signaling has come into focus as a potential microenvironment target that could be exploited to reduce the morbidity associated with this form of metastasis.

Metastasis to any organ features invasion of cancer cells through normal tissue into the blood stream (initiation), extravasation and infiltration of a distant tissue (progression), and growth of a destructive colony within the new context (virulence) [85]. The genes that mediate these events are likely to be dispensable for primary tumor initiation and growth and may or may not be part of gene expression profiles exhibited by the primary tumor [85]. We have analyzed breast cancer transcriptome datasets from the NCBI Gene Expression Omnibus to compare the patterns of ErbB receptor and ligand in primary tumors that ultimately produced bone metastasis to the patterns found in tumors that failed to metastasize or produced metastases to other sites [59–61]. We have also compared ErbB receptor expression in a small set of bone metastasis samples with ErbB receptor expression in breast cancer samples removed from the lung, brain and liver [83]. ErbB2 expression was lower in those ErBα-negative tumors that produced bone metastases than in tumors that did not metastasize to bone, which suggests that tumors that overexpress ErbB2 typically metastasize to visceral sites [86]. Surprisingly, AREG expression was significantly lower in ERα-negative tumors that ultimately metastasized to bone than in other ERα-negative tumors. However, we found little additional evidence for differential expression of ErbB family receptors. These findings suggest that EGFR signaling may be dysregulated in bone metastases through post-transcriptional events. As indicated below, several emerging lines of evidence involving ligand-activating proteases support a role for the EGFR signaling in bone metastasis.

#### 2.2.1. Latent bone colonization by breast tumor cells

Frequently, bone metastasis arises in breast cancer patients years after the identification and treatment of the primary tumor [87]. This implies that breast cancer cells remain dormant and indolent...
Fig. 2. Complex interactions of tumor and bone cells regulate bone biosynthesis and breakdown. Breast cancer cells express PTHrP, IL-11, and CTGF, which stimulate RANK ligand (RANKL) expression by cells of the osteoblast lineage. RANKL binding to RANK on monocytes stimulates their differentiation to active osteoclasts and consequent bone breakdown. Breast cancer cells also express IL-8, which directly stimulates monocyte production and leads to increased osteoclast formation. Breakdown of the bone matrix by osteoclasts releases TGFβ and IGFs, which stimulate tumor cell survival, proliferation, and release of osteolytic factors. Both breast cancer cells and cells of the osteoblast lineage express EGFR and the EGFR ligand AREG.

within the body. Over the past two decades methodology has been developed to identify dormant/latent tumor cells within patients. Individual or small groups of tumor cells found in the bone marrow of patients who lack discernable bone metastases are termed disseminated tumor cells (DTCs) [87]. The presence of DTCs in the bone marrow is predictive of metastatic disease both in the bone and at other sites [87–89]. The vast majority of DTCs present in the bone marrow of breast cancer patients are CD44+/CD24−, making them reminiscent of stem-like breast cancer cells [90]. However, elevated EGFR and ErbB2 are also markers for DTCs [91,92]. This suggests that ErbB receptors play a role in the establishment or maintenance of stem-like breast cancer cells, but there is no further information regarding potential function of ErbB receptors in the infiltration of breast cancer cells into bone, or regarding their possible impact on latency/indolence [93].

2.2.2. Bone metastasis: a vicious cycle

Much of the advances in the understanding of breast cancer colonization of bone has stemmed from studies of the MDA-MB-231 ERα-negative breast cancer cell line in bone xenografts. MDA-MB-231 cells possess a basal phenotype [94] and various bone-seeking sublines have been developed to dissect the molecular and cellular regulators of osteolytic growth of this cell line [95,96]. On the basis of these studies, the concept of “the vicious cycle” of tumor cell growth linked to bone destruction has been developed [96]. This model holds that breast cancer cells direct the resident cells of bone to uncouple the physiological linkage between bone matrix destruction and new bone formation [96]. The MDA-MB-231 cells produce cytokines and growth factors that engage in paracrine signaling with osteoclasts, cells that dissolve bone matrix, and osteoblasts, which are responsible for bone formation [96,97]. Osteoclast formation is mediated mainly through RANK (receptor activator of nuclear factor-κB-ligand) and its agonist RANKL (RANK ligand), the latter of which is produced by osteoblasts and bone marrow stromal cells [93,96]. Osteoblastic cells also produce a soluble RANKL sink called osteoprotegrin (OPG) [80,93]. Thus, osteoclast formation is influenced by the balance between RANKL and OPG in the bone microenvironment [96]. In addition, osteoblasts produce colony-stimulating factor (CSF-1), which recruits monocytes from bone marrow progenitors that ultimately can be differentiated into osteoclasts in the presence of high levels of RANKL [96,98,99]. In the MDA-MB-231 xenograft models, the breast cancer cells produce several growth factors and cytokines that perturb the RANKL/OPG ratio and increase the number of monocytes that can be differentiated to osteoclasts in the presence of high levels of RANKL [96,98,99]. In the MDA-MB-231 xenograft models, the breast cancer cells produce several growth factors and cytokines that that perturb the RANKL/OPG ratio and increase the number of monocytes that can be differentiated to osteoclasts [95,97,99]. The osteoclast-mediated destruction of bone releases growth factors embedded in the bone matrix. These stimulate their cognate receptors on the cancer cells, resulting in increased tumor cell proliferation and production of cytokines that skew the RANKL/OPG ratio toward increased osteoclastogenesis, thereby propagating a vicious cycle of tumor cell proliferation and bone destruction [97,100]. It should be noted that this model is based on the activi-
ties of the ERα-negative MDA-MB-231 breast tumor cell line, and it is unclear whether all of the specific molecules and cellular interactions apply to the more common form of disease progression that arise from ERα-positive breast tumors.

2.2.3. **EGFR and osteolysis**

There is growing evidence suggesting that EGFR signaling in osteoblasts directs osteolysis or bone resorption. EGFR is expressed by cultured osteoblasts, but not osteoclasts or monocytes [101,102]. Furthermore, EGF, TGFα, and MDA-MB-231 cells (which express various ErbB ligands) stimulate bone turnover and osteoclastogenesis in various model systems [103–106]. This osteolysis is accompanied by decreased OPG expression and minimal change in RANKL expression by the bone cells [106]. EGFR TKIs inhibit CSF-1 and RANKL production from human bone marrow stromal cells and osteoclast formation in vitro [107]. These studies clearly support the concept that EGFR signaling within the osteoblast promotes osteoclastogenesis through perturbation of the RANKL/OPG balance.

2.2.4. **EGFR and osteoblast function**

Studies of bone biology suggest additional roles for EGFR ligands in the pathogenesis of osteolytic lesions. Parathyroid hormone (PTH), the main serum calcium regulator, stimulates AREG gene transcription 10–20-fold and stimulates more modest increases in transcription of the TGFα and HB-EGF genes [108,109]. The PTH receptor, like other serpentine G-protein-coupled receptors (GPCRs), appears to be coupled to proteases (such as ADAM-17) that cleave ErbB receptor ligand precursors and enable the release of the mature, soluble ligands [110].

Exogenous EGFR ligands stimulate the proliferation of osteoblasts, inhibit their differentiation, and decrease their mineralization [109]. Moreover, 4-week-old transgenic mice lacking AREG expression exhibit less trabecular bone in the tibia than do wild-type littermates [109]. Thus, EGFR signaling may mediate the impact of PTH on the recruitment and expansion of cells committed to the osteoblast lineage, whereas excessive ligand signaling could prevent these cells from undergoing terminal differentiation and forming mineralized bone [109]. The uncoupling of bone formation from the accelerated bone resorption would be a key feature of disease states like breast cancer-induced osteolysis (Fig. 3).

2.2.5. **EGFR and PTHrP**

In the MDA-MB-231 model, PTH receptor signaling is one of the key events in regulating the vicious cycle of breast cancer osteolysis and colonization [111]. MDA-MB-231 cells express parathyroid hormone-related peptide (PTHrP), another PTH receptor agonist that stimulates RANKL expression and inhibits OPG expression in cells of the osteoblast lineage [111]. The pattern of PTHrP expression by breast cancers at various stages of progression resembles that displayed by metastasis virulence factors [85]. PTHrP expression is lower in primary breast cancers that ultimately metastasize to bone than in other primary breast tumors; however, PTHrP expression is very high among metastatic tumor cells within the bone microenvironment [112–115]. PTHrP gene expression in these metastatic tumor cells appears to be stimulated by TGFβ released from the bone matrix via osteoclast activity [96,100]. Nonetheless, the signaling between the PTHrP and the EGFR system is not simply directed from cancer cell to the microenvironment. In many epithelial cells EGFR is coupled to PTHrP gene expression [116–118]. In fact, an autocrine loop of AREG-EGFR signaling activates PTHrP transcription in the MDA-MB-231 line in vitro [119]. Thus, autocrine EGFR stimulation in breast cancer cells may contribute to the release of cytokines, such as PTHrP, that directly perturb the RANK/OPG balance and indirectly stimulate EGFR signaling within cells of the osteoblast lineage.

2.2.6. **EGFR ligands and activating proteases as bone metastasis virulence factors**

Analysis of MDA-MB-231 subclones identified 11 genes whose overexpression is specific to clones that readily colonize the bone and form aggressive osteolytic lesions [95]. Moreover, combinations of 3 of these genes are sufficient to induce osteolytic growth by parental MDA-MB-231 cells. Thus, these 11 genes appear to influence distinct events in the process of bone metastasis. These 11 genes include IL-11, which alters the RANKL/OPG balance, and connective tissue factor, which stimulates osteoblast proliferation. These 11 genes also include the proteases MMP1 and ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motifs), whose roles in bone metastasis were not readily apparent [95].

Overexpression of MMP1 and ADAMTS-1 in MDA-MB-231 cells dramatically increased AREG shedding and resulted in a cell line that formed more aggressive osteolytic lesions in the bone. Conditioned medium from the MDA-MB-231/ADAMTS-1/MMP1 cells altered the RANKL/OPG balance in a primary mouse bone cell culture and enhanced osteoclastogenesis. This enhanced osteoclastogenesis could be inhibited by the EGFR TKI gefitinib or by the anti-EGFR antibody cetuximab. Moreover, these agents (gefitinib 100 mg/kg daily or cetuximab 100 mg/kg weekly) prevented MDA-MB-231/ADAMTS-1/MMP1 cells from stimulating the formation of osteolytic lesions in the bone of immunocompromised mice injected with these cells [120]. These findings suggest that EGFR ligands or the proteases that regulate their availability can serve as breast cancer metastasis virulence factors and that metastasis could be blocked by EGFR antagonists that have no apparent direct effect on the breast tumor cells themselves.

This finding that AREG expression is necessary but not sufficient for MDA-MB-231 cells to colonize the bone is consistent with the observation that AREG expression is lower in ERα-negative breast tumors that ultimately metastasized to bone than in ERα-negative breast tumor that failed to metastasize to bone. Presumably, differences in the ability of breast tumor cells to colonize bone is regulated by proteases cleave AREG and enable it to stimulate EGFR signaling. Indeed, elevated expression of ADAMTS-1 and MMP1 is observed in primary breast cancer tumors that ultimately metastasize to bone [63]. Furthermore, given that various GPCRs are coupled to increased activity of MMPs and ADAMs, we speculate that increased signaling by GPCRs on tumor cells in the bone microenvironment may contribute to bone colonization by coupling to increased activity MMPs and ADAMs [121,122].

To summarize, the complex post-transcriptional regulation of EGFR ligand processing and receptor interactions provides mechanisms through which EGFR coupling to bone colonization may be enhanced. Thus, numerous gene products that contribute to EGFR signaling in breast tumor cells or osteoblasts may function as bone metastasis virulence factors. (1) The combination of an EGFR ligand (such as AREG) and an active shedding protease (such as MMP1 or ADAMTS–1) in breast tumor cells could activate paracrine EGFR signaling in osteoblasts, resulting in reduced OPG expression, increased osteoclastogenesis and decreased bone mineralization. (2) Autocrine EGFR signaling in the tumor cell could couple to PTHrP expression and release by tumor cells, leading to increased RANKL and decreased OPG expression in osteoblasts. (3) PTHrP released by tumor cells could also stimulate AREG expression and ADAM17 activity in osteoblasts, leading to increased EGFR signaling in the osteoblasts. Thus, PTHrP could play a central role in two pathways that independently lead to a robust alteration of the RANKL/OPG balance to favor osteoclast formation and osteolytic activity.

The multiple mechanisms by which MDA-MB-231 cells can stimulate EGFR coupling to osteolytic effects in the bone microen-
Fig. 3. EGFR may play multiple roles in breast cancer-induced osteolysis. (A) In normal bone RANKL stimulation of osteoclast-mediated bone turnover and is balanced by the OPG antagonist of RANKL. (B) An EGFR ligand (light blue hexagon) expressed and shed by tumor cells may stimulate paracrine signaling by EGFR (double black bars) expressed by osteoblasts. This would inhibit OPG expression by osteoblasts, leading to increased RANKL stimulation of RANK expressed by osteoclasts and increased osteoclast-mediated bone turnover. (C) An EGFR ligand expressed and shed by tumor cells may stimulate autocrine signaling by EGFR expressed by the tumor cells, leading to PTHrP expression by these tumor cells. This stimulates RANKL expression and inhibits OPG expression by osteoblasts, again leading to increased RANKL stimulation of RANK expressed by osteoclasts and increased osteoclast-mediated bone turnover. (D) PTHrP expressed by tumor cells can also stimulate expression of an EGFR ligand by osteoblasts, leading to autocrine EGFR signaling and coupling to increased RANKL expression and decreased OPG expression in osteoblasts. Again, this leads to increased RANKL stimulation of RANK expressed by osteoclasts and increased osteoclast-mediated bone turnover.

Environment indicate that this pathway may be a major component of the pathogenesis of osteolytic lesions triggered by this ER-negative breast cancer line. Moreover, AREG transcription is positively regulated by ERα in the mouse mammary gland and breast cancer cells [64,66]. Thus, deregulated signaling through the AREG-EGFR pathway may be a general mechanism by which multiple types of breast cancer form osteolytic bone metastases.

Small-molecule EGFR tyrosine kinase inhibitors and antagonistic anti-EGFR antibodies have exhibited little effect on primary tumor growth or patient outcome in breast cancer monotherapy clinical trials. One possibility is that anti-EGFR agents will be effective against bone metastases, but will have little effect on the primary tumor [97,120,123–125]. The other possibility is that these agents may be effective only as part of combination therapy regimens. Indeed, emerging data appear to support this possibility, particularly in advanced ERα-positive breast cancers [126–128].

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