Amphiregulin Is a Critical Downstream Effector of Estrogen Signaling in ERα-Positive Breast Cancer

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Abstract

Estrogen stimulation promotes epithelial cell proliferation in estrogen receptor (ERα)-positive breast cancer. Many ERα target genes have been enumerated, but the identities of the key effectors mediating the estrogen signal remain obscure. During mouse mammary gland development, the estrogen growth factor receptor (EGFR) ligand amphiregulin acts as an important stage-specific effector of estrogen signaling. In this study, we investigated the role of amphiregulin in breast cancer cell proliferation using human tissue samples and tumor xenografts in mice. Amphiregulin was enriched in ERα-positive human breast tumor cells and required for estrogen-dependent growth of MCF7 tumor xenografts. Furthermore, amphiregulin levels were suppressed in patients treated with endocrine therapy. Suppression of EGFR receptor signaling appeared necessary for the therapeutic response in this setting. Our findings implicate amphiregulin as a critical mediator of the estrogen response in ERα-positive breast cancer, emphasizing the importance of EGFR receptor signaling in breast tumor pathogenesis and therapeutic response. Cancer Res; 75(22); 4830–8. ©2015 AACR.

Introduction

Estrogen is an essential hormone for mammary gland development and is a key driver of proliferation during the development of estrogen receptor–positive (ERα+) breast tumors. The actions of estrogen are primarily mediated by its receptor, the ERα transcription factor, which is required for mammary gland development (1). Microarray and chromatin immunoprecipitation experiments have identified several hundred estrogen-responsive genes in breast cancer cells (2, 3); however, among these targets, the identity of the key effectors of this proliferative signal in breast cancer remains unclear. A more detailed understanding of the mechanisms involved will provide insight into the processes driving ERα+ breast tumor initiation and progression.

Analysis of human mammary glands demonstrated that it is the epithelial cells adjacent to ERα+ cells (rather than the ERα+ cells themselves) which enter the cell cycle following estrogen stimulation (4), implicating an estrogen-responsive paracrine growth factor in proliferation control. In the mouse, mammary gland development from ERα-deficient cells can be rescued by cotransplanting with wild-type mammary epithelial cells, supporting a role for a paracrine factor (5). We and others have previously reported that amphiregulin (AREG), a ligand of the estrogen growth factor receptor (EGFR), is induced during the proliferative phase of mouse pubertal mammary growth, where it is a direct transcriptional target of ERα (6, 7). Mammary glands of amphiregulin knockout mice have a striking defect in pubertal epithelial outgrowth but retain the ability to undergo differentiation during pregnancy, indicating a stage-specific requirement (8). This phenotype is rescued by cotransplantation of wild-type and Areg−/− mammary epithelial cells (6). Thus, amphiregulin appears to be a key mediator of estrogen action during normal mammary gland development.

Studies of human breast cancer cell lines indicate that amphiregulin is induced by estrogen treatment (9), and that its experimental overexpression can confer EGFR signaling self-sufficiency (10), but whether endogenous amphiregulin plays an important role in the estrogen-dependent proliferation of human breast cancer cells remains unknown. In this study, we test the hypothesis that co-option of this key stage-specific mammary developmental pathway might be the primary driver of estrogen-dependent proliferation of ERα+ human breast cancer cells.

Materials and Methods

Cell culture

MCF-7, T47D, and ZR751 were obtained from ATCC, and independently validated by STR profiling at our institution. These cell lines were cultured in DMEM (Cellgro) with 10% FBS (Hyclone). Suppression of AREG expression was achieved by lentiviral infection with two independent pLKO.1 constructs with the following sequences: shAREG-1, cactgccaagtcatagccata; shAREG-2, gaacgaaagaaacttcgacaa; or the empty vector control. For three-dimensional (3D) culture and in vivo experiments, FACS sorting was used to enrich for cells from shRNA-transduced pools which lacked cell surface amphiregulin.

Quantitative reverse transcriptase PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. One microgram of RNA was used for cDNA synthesis using the ImProm-II Reverse
ELISA

The human amphiregulin DuoSet ELISA Development System (R&D Systems) was used to analyze amphiregulin levels according to the manufacturer’s instructions, as previously described (11).

Tumor xenografts

All xenografts were performed in athymic mice and were approved by the Institutional Animal Use and Care Committee of the Albert Einstein College of Medicine (New York, NY). Two series of AREG knockdown experiments were performed. In the first, 14 nulliparous 5-week-old athymic mice were implanted with 0.72-mg 17β-estradiol 60-day release pellets, and injected orthotopically with 1 × 10^6 MCF7 cells in a 1:1 mixture of DMEM and Matrigel in the right (AREG knockdown: shAREG-2) or left (empty vector control) fourth inguinal mammary fat pad of each mouse. Tumor growth was monitored for 51 days. The second series was performed identically, except 12 mice were used and monitored for 44 days.

Immunohistochemistry

Breast tumor tissue microarrays (TMA) were provided by The Ohio State University’s Human Genetics Sample Bank. Slides were dewaxed in histoclear and rehydrated by serial incubations in 100% to 70% ethanol. Slides were rinsed with water and then with TBS. Antigen retrieval was performed by incubation of slides in a steamer for 20 minutes in a preboiled solution of 10 mmol/L sodium citrate (pH 6.0). Slides were washed in TBS and incubated for 30 minutes in a solution of 2% hydrogen peroxide in 1:1 methanol/PBS. Slides were washed in PBS, blocked (5% rabbit serum in PBS), and immunostained with goat anti-AREG antibody (15 mg/mL, AF262, R&D Systems) overnight at 4 °C. Slides were washed five times in TBS, followed by incubation for 30 minutes at room temperature in a 1:300 dilution of biotinylated secondary antibody (Vector Laboratories, Inc.). Samples were incubated for 30 minutes at room temperature in Vectastain Elite ABC-HRP, washed twice in TBS and developed using 3, 3′-diaminobenzidine (Vector Laboratories, Inc.). Samples were washed with water and counterstained with hematoxylin, rinsed with water, dehydrated by serial ethanol washes to 100%, incubated in histoclear for 3 minutes, and mounted in Permount (Fisher Scientific). Amphiregulin staining intensity was assessed semiquantitatively using a three-point scale by two investigators working independently on blinded samples. Discordant scores were resolved by joint review. Proliferation was assessed using mouse anti-BrdU (Roche) at a 1:400 dilution.

3D culture proliferation assay

Three-dimensional laminin-rich extracellular matrix cultures were prepared by seeding of single cells on top of a thin layer of growth factor–reduced Matrigel (BD Biosciences) and the addition of a medium containing 5% Matrigel, as previously described (12, 13). The cell lines were seeded at a density of 1,000 cells/cm^2 for MCF7 and 625 cells/cm^2 for T47D and ZR751. Cells were seeded in DMEM supplemented with 1% charcoal/dextran-stripped FBS (Gemini Bioproducts), 0.292 mg/mL L-glutamine, 1× nonessential amino acids, 10.11 mg/mL sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL of streptomycin (Hyclone), and 6 ng/mL of human recombinant insulin (Calbiochem). Digital pictures of each well were taken and colony cross-sectional area was measured using ImageJ.

Microarray and clinical data

Gene expression profiles of breast cancer cell lines in 3D culture (12) are available from ArrayExpress (#E-TABM-244). Gene expression profiles (NCBI accession number: GSE5462) from a study of paired tumor core biopsies taken before and after 14 days of treatment with letrozole (14, 15) were examined for the expression of amphiregulin and other ERBB ligands and receptors. Of the 58 patients included in the study, response data were not available for 6 cases, resulting in a total of 52 paired samples included in our analysis.

Results

We examined the distribution of amphiregulin expression in 295 breast cancer patients (16), which revealed a striking enrichment of AREG mRNA expression in ErbB-2 tumors (Fig. 1A). We further evaluated the quantitative relationship between AREG and ErbB1 (ESR1) mRNA levels in 13 luminal human breast cancer cell lines grown in 3D culture (12). The highest levels of AREG are found in luminal cell lines with the highest levels of ErbB2 expression (Fig. 1B). We confirmed the association between AREG and ErbB2 expression in an independent cohort of 118 breast cancer patients by immunostaining for amphiregulin on tumor tissue microarrays (Fig. 1C). Analysis of AREG levels in 88 ErbB-2+ and 30 ErbB-2+ tumors showed that ErbB-2+ tumors most frequently express high levels of the amphiregulin protein (P = 0.0194). Representative examples of staining intensity are provided and additional sections can be seen in the Supplementary Materials and Methods.

As previously reported (9), we found that AREG mRNA is regulated by estrogen in MCF7 cells and extended this finding to T47D cells, an additional ErbB-2 breast cancer cell line. AREG mRNA was induced by estradiol and is suppressed by ErbB2 antagonists with distinct mechanisms of action such as 4-hydroxytamoxifen (OHT) and fulvestrant (ICI182,780) in MCF7 (Fig. 2A) and T47D (Fig. 2B) cells. Using ELISA, we found that production of soluble amphiregulin protein was increased upon estradiol treatment, and was suppressed by both ErbB2 antagonists in MCF7 (Fig. 2C) and T47D (Fig. 2D) cells.

These data are consistent with AREG being a transcriptional target of ErbB2 in both human breast tumors and breast cancer cell lines; however, the extent to which AREG, among hundreds of known ErbB2 target genes (2, 3), is a key effector of ErbB2 function remained unclear. To rigorously test the requirement for AREG in ErbB2-dependent proliferation, we used two shRNA constructs to establish pools of MCF7 cells with stable suppression of amphiregulin expression. Efficient knockdown of amphiregulin in these pools compared with the empty vector control (pLKO.1) was confirmed by both quantitative reverse transcription (qRT)-PCR (Fig. 3A) and ELISA (Fig. 3B) analysis. To evaluate the impact of amphiregulin depletion on the proliferative response to estrogen, we performed 3D culture experiments. The vector control (pLKO.1) MCF7 cell line exhibited a robust growth response to
estrogen, while neither shRNA-transduced subline responded (Fig. 3C). To test whether the requirement for amphiregulin for growth in 3D culture was a general feature of ER$^+$ breast cancer cell lines, we suppressed amphiregulin expression in two additional lines, ZR751 and T47D (Fig. 3D). In both cases, the vector control lines grew well in response to estrogen, while the shAREG-transduced pools responded weakly or not at all (Fig. 3E and F).

To ascertain the importance of estrogen-dependent induction of amphiregulin in vivo, control and knockdown MCF7 cells were injected into mammary fat pads of athymic mice implanted with slow-release estrogen pellets. The two shRNA lines were evaluated on different dates and the contemporaneous vector control is shown in each case (Fig. 4A). Tumors in which amphiregulin was knocked down grew significantly more slowly than control tumors ($P < 0.05$ at day 17 and beyond, black curves, and day 27 and beyond, gray curves). Sustained suppression of AREG expression was confirmed by ELISA analysis of tumor lysates at the endpoint of the experiment which included the shAREG#2 cell line (Fig. 4B). These data indicate that, among ER$^+$ target genes, amphiregulin expression is necessary for the robust growth of MCF7 tumors in vivo.

To determine the extent to which these in vitro and in vivo findings could be generalized to human breast cancer cases, we examined the change in expression of amphiregulin mRNA following a 2-week treatment with the aromatase inhibitor, letrozole, in 52 postmenopausal breast cancer cases previously described by Miller and colleagues [14, 15]. Response to treatment was assessed as a reduction in tumor volume of greater than 50%, as measured by ultrasonography, after 3 months of neoadjuvant letrozole. Figure 5A shows the fold change in amphiregulin from baseline following 2 weeks of letrozole treatment. The majority of cases (41/52) had a substantial reduction in amphiregulin expression levels, a finding observed in both responders and nonresponders. These data suggest that amphiregulin expression is regulated by ER activity in human breast tumors.

It was interesting to see that some of these tumors had a substantial reduction in amphiregulin levels yet failed to achieve a 50% reduction in tumor volume. To determine whether the ERBB pathways might remain active in the nonresponding tumors even when amphiregulin mRNA was suppressed, we examined differences in expression levels of all ERBB family receptors and ligands between responders and nonresponders in the post-letrozole treatment samples. Nonresponding tumors had consistently and significantly higher expression levels of the genes encoding the ERBB2 receptor and the ligands TGF$\alpha$, epiregulin, neuregulin 1, and neuregulin 2 (Fig. 5B). These data suggest that tumors in which the primary estrogen-responsive ERBB family ligand, amphiregulin, is suppressed by endocrine therapy but which express alternate ligands and/or receptors which can activate these same signaling pathways may escape the growth-suppressive effects of endocrine therapies. To determine whether some of these genes might be acting coordinately, we examined their expression in the individual tumors (Fig. 5C). Although statistically significant (Fig. 5B), the differences in epiregulin and TGF$\alpha$ expression between responders and nonresponders were not very striking. In contrast, the nonresponders were enriched for tumors with elevated levels of ERBB2, NRG1, and NRG2, suggesting that tumors in which elevated expressions of these ligands (whose
receptors heterodimerize with ERBB2) may be less likely to respond to endocrine therapy.

Discussion

In this study, we found that amphiregulin expression is frequently associated with estrogen receptor positivity in human breast tumors and cell lines, and that estrogen-dependent amphiregulin expression is necessary for the growth of MCF7 xenografts in vivo, and for the estrogen-responsive growth of several ER+ breast cancer cell lines in 3D culture. These data indicate that the co-option of this stage-specific mammary developmental pathway may be a key feature of ERα+ human breast cancer. The clinical relevance of these experimental findings is supported by the strong suppression of amphiregulin expression observed in a large cohort of breast cancer patients who received a neoadjuvant endocrine therapy, and by the demonstration that several tumors that did not respond to treatment had alternate, likely estrogen-independent, mechanisms of activating ERBB signaling pathways.

Several mechanisms have been proposed to explain the proliferative response to estrogen in ERα+ breast cancer cells, including the upregulation of c-Myc (17), Cyclin D1 (18), c-Myb (19), GREB1 (20), interaction with RSK (21), and activation of Cyclin E/cdk2 (22). Each of these potential mechanisms relies on cell-autonomous effects of the estrogen receptor, yet in the normal human breast, it is not the ERα+ cells, but the cells immediately adjacent to them that proliferate (4), suggesting that a paracrine effector is also involved. Similarly, in ERα+ breast tumors, significant proportions of the neoplastic cells can lack ERα expression, yet these tumors often respond well to endocrine therapy (23). Together, these findings indicate that estrogen has both cell-autonomous and non–cell autonomous effects during mammary gland development and in breast cancer, and that autocrine and paracrine mechanisms, which likely include amphiregulin, may play an important role in both settings.

Autocrine amphiregulin expression has been implicated in the growth of inflammatory and other ER− breast cancer cell lines in culture (24, 25); however, despite the reported importance of amphiregulin as an estrogen effector during mouse mammary gland development (6), and evidence that amphiregulin is also regulated by estrogen in human breast cancer cell lines (9), the actual contribution of amphiregulin/EGFR to estrogen-dependent human breast cancer initiation and progression has not received widespread attention. This lack of attention may reflect, in part, the disappointment that initial trials of EGFR inhibition in breast cancer were not very successful (summarized in ref. 26), although the understanding of the biology of EGFR was less advanced at that time and there was often little attempt at rational

Figure 2.
Amphiregulin expression is induced by estrogen and suppressed by ERα antagonists. A, quantitative RT-PCR analysis of AREG mRNA levels in MCF7 cells treated with estrogen (E2) alone, or supplemented with 4-hydroxytamoxifen (OHT) or fulvestrant (ICI) at the indicated concentrations. B, quantitative RT-PCR analysis of AREG mRNA levels in T47D cells treated as described in A. C, MCF7 and D, T47D, ELISA analysis of soluble AREG protein production by cell lines treated as indicated in A. Error bars represent SD. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001.
Figure 3.
shRNA-mediated knockdown of amphiregulin strongly attenuates ERα⁺ breast cancer cell line growth in vitro. A, qRT-PCR analysis of AREG knockdown using two independent shRNA constructs. B, decreased AREG soluble protein production in shRNA transductants, detected by ELISA. (Continued on the following page.)
tumors, randomized to anastrazole alone or breast tumors to getinib, which
may function indirectly as EGFR pathway inhibitors. If these anti-EGFR
and anti-estrogen therapies are indeed impinging on the same key
pathway, overly simplistic conclusions from combination clinical
trial data may merit closer examination.

For example, a phase II trial in the neoadjuvant setting of 206 women
with ER\textsuperscript{+} tumors, randomized to anastrozole alone or
anastrozole plus getinib (i.e., aromatase inhibitor \pm EGFR
inhibitor) showed that getinib added no additional benefit to
aromatase inhibition (27), which could suggest that either getinib
has no activity in breast cancer or that it targets the same
pathway as aromatase inhibitors. Studies with single-agent getinib
arms indicate that a substantial proportion of ER\textsuperscript{+} tumors exhibit either a molecular or clinical response to EGFR inhibition,
particularly among patients not heavily pretreated with other
agents. For example, Guix and colleagues treated 41 women
preoperatively with the EGFR inhibitor, erlotinib, and saw a
significant downregulation of Ki67 levels in ER\textsuperscript{+} but not
HER2\textsuperscript{+} or triple-negative (ER\textsuperscript{+}, PR\textsuperscript{−}, HER2\textsuperscript{−}) breast cancer
(28). Polychronis and colleagues randomized 54 women with
ER\textsuperscript{+} breast tumors to getinib with or without anastrozole and
clearly showed that getinib alone significantly downregulated
both tumor cell proliferation and tumor bulk when given for 4 to
6 weeks preoperatively (29). In ER\textsuperscript{+} metastatic breast cancer,
adding getinib to anastrozole significantly increased progression-
free survival (PFS) compared with anastrozole alone (median
PFS 22 vs. 14.7 months) with the benefit being particularly
pronounced in women who had not previously received endo-
crine therapy (median PFS 20.2 vs. 8.4 months; ref. 30).

In patients with ER\textsuperscript{+} tumors with acquired resistance to
tamoxifen, getinib was associated with a 53.6% clinical benefit rate,
significantly more than the 11.5% rate observed in ER\textsuperscript{+} tumors
in the same trial (31); however, a trial in which the ER\textsuperscript{+} tumors
had already acquired resistance to both tamoxifen and an
aromatase inhibitor showed no benefit of getinib (32). Similarly,
a study by Baselga and colleagues in advanced breast cancer patients
with 1 to 2 prior chemotherapy regimens did not show benefit for
getinib (33); however, this trial involved a smaller number of
patients (n = 31), less than half of whom actually expressed EGFR
in their tumor. Taken together, although EGFR inhibitors have not
proven to be a panacea for breast cancer, several lines of evidence
suggest that EGFR plays a role in ER\textsuperscript{+} breast cancer, at least up to
the stage of treatment resistance, and is likely functioning down-
stream of ER\textsubscript{a}. The ER\textsubscript{a} target gene and EGFR ligand, amphiregulin,
is an attractive candidate to link these pathways.

If amphiregulin/EGFR signaling contributes to a significant
proportion of ER\textsuperscript{+} breast cancer cases, the lack of enduring
responses to getinib and erlotinib requires some explanation.
We speculate that achieving complete and sustained inhibition of
wild-type EGFR using these single agents in vivo is difficult; thus,
many of these tumors may be dependent on the EGFR pathway,
yet at the same time insensitive to EGFR inhibitors. A key aspect
of this pathway is the extent to which ligand binding by a minor
fraction of receptors can yield a robust pathway activation due to
the stoichiometry between receptors and the large number of
downstream signaling intermediates, and the signaling amplifi-
cation that takes place at each step of the pathway. Chen
and colleagues have reported an advanced mathematical model (34)
describing the relationship between the various ERBB receptors
and downstream intermediates and computed the rates of signal
propagation via these intermediates at various levels of ERBB
activation (using either EGF or heregulin across a concentration
range of several logs). Importantly, the model equations were
validated against biologic experiments, providing a detailed
quantitative analysis of all of the key parameters in several cancer
cell lines. As one might expect, activation of the EGFR itself by EGF

(Continued)
was substantially governed by the concentration of the ligand and by the enzymology of the receptor. However, examining the propagation of signal through the network reveals a substantial departure from linearity. In one example, a 50-fold reduction in the EGF stimulus (5 nmol/L to 0.1 nmol/L) resulted in an approximately 95% reduction in pEGFR while only reducing pMAPK by half and leaving pAkt essentially unchanged. In a gefitinib or erlotinib-treated tumor under steady-state conditions, the sub-10-minute half-life of the receptor–inhibitor complex (35), the high local concentrations of ERBB ligands (36, 37), and

Figure 5.
Amphiregulin expression is strongly suppressed following neoadjuvant letrozole treatment of human breast cancer patients. A, amphiregulin mRNA analysis by gene expression microarray showing suppression of AREG levels following 2 weeks of letrozole treatment. The data represent the fold-change in AREG mRNA between the pretreatment and 14-day biopsy specimens. B, mRNA analysis of selected ERBB family receptors and ligands in 14-day biopsy specimens, stratified by ultrasonographically evaluated response after 90 days of neoadjuvant letrozole treatment. The between-group fold-difference in expression is indicated in each case, $P \leq 0.05$; **, $P \leq 0.01$. The following genes were also assessed and were not significantly different between the groups: AREG, BTC, HBEGF, NRG3, NRG4, EGFR, ERBB3, and ERBB4. C, heatmap of relative expression of each of the genes in B in each of the tumors. Levels of each gene were normalized to their average level across the population.
the capacity of the cellular signal transduction machinery to amplify small transient signals from occasionally uninhibited receptors at the cell surface, may mean that even 95% inhibition of the EGFR might not be sufficient to inhibit this pathway to an extent necessary to elicit a sustained tumor response in vivo. Experimental evidence indicates that the responsiveness of cancer cell lines to many inhibitors is highly regulated by the local concentration of receptor tyrosine kinase ligands (38). Thus, using higher-affinity EGFR inhibitors (35) or approaches to reduce ERBB family ligand bioavailability, such as using endocrine therapy to block amphiregulin induction or ADAM10/17 inhibitors to prevent ERBB ligand shedding (24), may have the potential to increase EGFR inhibitor effectiveness in this patient population.

In the clinic, almost half of patients with advanced ERα+ tumors fail to respond to tamoxifen in the first-line setting and, of the patients who respond initially, all will subsequently progress to endocrine resistance (39). Elevated ERBB signaling activity has been associated with endocrine resistance in the clinic. For example, ERα tumors that express high levels of the EGFR ligand, TGFβ, tend to be tamoxifen nonresponsive (40). Our observations on the downregulation of AREG expression following letrozole treatment (Fig. 5A), and on the frequency of expression of ERBB signaling pathway activators in tumors not responding to letrozole (Fig. 5B and C), are consistent with a role for ERBB signaling generally in the proliferation of ERα breast tumor cells and with our hypothesis that endocrine therapy-induced suppression of EGFR family ligand bioavailability, such as using endocrine therapy to block amphiregulin induction or ADAM10/17 inhibitors to prevent ERBB ligand shedding (24), may have the potential to increase EGFR inhibitor effectiveness in this patient population.

In conclusion, we have implicated amphiregulin as a key effector of estrogen receptor activity during breast cancer growth in vivo. In light of our experimental data, we believe that the issue of EGFR signaling in ERα+ breast tumors merits renewed attention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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