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Autocrine-Derived Epidermal Growth Factor Receptor Ligands Contribute to Recruitment of Tumor-Associated Macrophage and Growth of Basal Breast Cancer Cells In Vivo

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Abstract

Epidermal growth factor receptor (EGFR) expression has been linked to progression of basal breast cancers. Many breast cancer cells harbor the EGFR and produce its family of ligands, suggesting they may participate in autocrine and paracrine signaling with cells of the tumor microenvironment. EGFR ligand expression was profiled in the basal breast cancer cell line MDA-231 where AREG, TGF- α , and HBEGF were the three ligands most highly expressed. Autocrine signaling was modulated through silencing or overexpression of these three ligands using lentiviral constructs and the impact measured using motility, proliferation, and cytokine expression assays. Changes in receptor phosphorylation and receptor turnover were examined. Knockdown of AREG or TGF- α in vitro resulted in decreased motility ($p < 0.05$) and decreased expression of macrophage chemoattractants. Overexpression of TGF- α increased motility and chemoattractant expression, whereas AREG did not. HBEGF modulation had no effect on any cellular behaviors. All the cells with altered ligand production were inoculated into female athymic nude mice to form mammary fat pad tumors, followed by immunohistochemical analysis for necrosis, angiogenesis, and macrophage recruitment. In vivo, knockdown of AREG or TGF- α increased survival ($p < 0.001$) while decreasing angiogenesis ($p < 0.001$), tumor growth ($p < 0.001$), and macrophage attraction ($p < 0.001$). Overexpression of AREG appeared to elicit a greater effect than TGF- α on mammary fat pad tumor growth by increasing angiogenesis ($p < 0.001$) and macrophage attraction to the tumor ($p < 0.01$). We propose these changes in mammary tumor growth were the result of increased recruitment of macrophages to the tumor by cells with altered autocrine EGFR signaling. We conclude that AREG and TGF- α were somewhat interchangeable in their effects on EGFR signaling; however, TGF- α had a greater effect in vitro and AREG had a greater effect in vivo.

Keywords

Epidermal growth factor receptor (EGFR); Amphiregulin; Breast cancer; Transforming growth factor- α (TGF- α); Macrophage colony-stimulating factor-1 (MCSF-1)

INTRODUCTION

The epidermal growth factor receptor (EGFR) and its ligands are frequently expressed on breast cancer cells, suggesting that their binding could stimulate cancer cell proliferation and motility, as well as facilitate signaling with the microenvironment to promote invasion and metastasis (1,2). This receptor requires ligand binding to initiate homo- or heterodimerization and downstream signaling. Ligands include epidermal growth factor (EGF) (3), amphiregulin (AREG) (4), transforming growth factor- α (TGF- α) (5), heparin-binding epidermal growth factor (HBEGF) (6), β -cellulin (7), epiregulin (EREG) (8), and epigen (EPGN). The ligands are synthesized as transmembrane proteins and are released from the cell membrane by the ADAM (a disintegrin and metalloproteinase) family of proteases (typically ADAM10 or ADAM17) to participate in autocrine or paracrine signaling within the microenvironment (9). The downstream effectors of the EGFR couple to distinct cell behaviors and biochemical events: phospholipase-C to motility/migration, mitogen-activated protein kinase to proliferation/invasiveness, STATs and AKT to angiogenesis/survival, and Cbl to receptor degradation (10–13).

Some of the ligands induce differences in the intensity of receptor phosphorylation as well as eventual receptor fate. Ligands EGF, HBEGF, and β -cellulin cause high levels of EGFR phosphorylation, including phosphorylation of the tyrosine residue that mediates Cbl interaction, and their high affinity for EGFR in the endosome leads the receptor to be shuttled to the lysosome for degradation (11,13,14). In contrast, AREG, epigen, EREG, and TGF- α cause modest to low levels of receptor phosphorylation, and generally the dissociate from the EGFR in the endosome, allowing for receptor recycling back to the plasma membrane for further signaling (11,15–18). Therefore, ligands that have high affinity for the receptor within the endosome induce a full spectrum of downstream signaling that is rapidly terminated, whereas ligands with low affinity appear to induce limited downstream signaling of prolonged duration. As a consequence, ligands that fail to induce rapid EGFR degradation are thought to be more effective mitogens (13,18–21).

Of the five molecular subclasses of breast cancers (luminal A & B, normal-like, ErbB2 overexpressing, and basal), basal tumors most frequently express the EGFR (22). In addition, basal tumors frequently produce TGF- α and ADAM17, indicating a potential for autocrine signaling in this set of tumors (23). Basal tumors are linked to poor survival, high rates of metastasis and lack therapeutic interventions. This suggests that EGFR signaling participates in progression of these aggressive basal tumors and is a target for potential therapeutics (24).

The precise role of EGFR and its ligands in breast cancer progression is still being defined. In some breast cancer cell lines, the EGFR appears to be central to the production of various macrophage chemoattractants. Tumor-associated macrophages are associated with poor prognosis of many tumor types and they facilitate tumor growth by releasing factors that enhance angiogenesis (25–27). Macrophage chemoattractants produced by breast cancer cells bind their cognate receptors on macrophages, which in turn secrete EGF, thereby establishing a paracrine loop between the two cell types (25,26). In addition, the macrophage-derived EGF stimulates motility, invasiveness, and intravasation of breast

cancer cells in vivo (28–30); however, this is thought to occur independently of EGFR ligand production by breast cancer cells themselves.

We had previously determined that shRNA to the EGFR in the basal human breast cancer cell line MDA-MB-231 (MDA-231) reduced migration and in vitro expression of cytokines including parathyroid hormone-related protein (PTHrP) and macrophage colony-stimulating factor-1 (MCSF-1). Silencing EGFR expression in vivo caused decreased tumor growth in both the bone and mammary fat pad (31). Our attempt to inhibit AREG signaling with a monoclonal antibody, PAR34, was modestly effective in reducing EGFR-driven effects. This finding led us to consider that other EGFR ligands produced by this breast cancer cell line might contribute to the growth of the cells in vivo.

In this study, we examined the affects of knockdown or overexpression of HBEGF, AREG, or TGF- α , the EGFR ligands that are expressed at high levels in the MDA-231 cell line. Modulation of autocrine ligand production influenced motility and cytokine production in vitro as well as tumor growth in vivo. These findings suggest that EGFR ligand production and autocrine receptor signaling contribute to recruitment of macrophages by a basal breast cancer cell line in vivo.

MATERIALS AND METHODS

Pharmacologic Reagents

The anti-human amphiregulin antibody (#262-AR) and recombinant human TGF- α , HBEGF, EGF, and AREG ligands were purchased from R&D Systems (Minneapolis, MN). Phorbol 12-myristate 13-acetate (PMA) was purchased from Acros Organics. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO). Sheep EGFR antibody was purchased from Capralogics (P00367, Hardwick, MA). The β -tubulin antibody (sc-55529) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The EGFR phospho- Tyr992 (E1780) antibody was from Sigma, the EGFR phospho-Tyr1045 antibody (#2237) was from Cell Signaling (Danvers, MA), and the generic anti-phosphotyrosine antibody (clone 4G10) was from Millipore (Billerica, MA). Secondary HRP-labeled antibodies for Western analysis were purchased from KPL (Gaithersburg, MD). The Human Inflammatory Cytokines & Receptors RT² Profiler PCR Array was purchased from SA Biosciences (Valencia, CA) and was used according to the manufacturer's protocol.

Cell Lines and Cell Culture

MDA-231 cells were grown in DMEM (Sigma) and supplemented with 10% FBS and 10 ng/ml insulin (Sigma). The shEGFR cells (31) were maintained in 1.5 μ g/ml puromycin. The shAREG, shTGF- α , shHBEGF, and control lentiviruses (PKO-1) were purchased from Sigma. MDA-231 cells were seeded at 1.6×10^4 cells per well in a 96-well dish and incubated in a 37°C/5% CO₂ incubator overnight. The following day, to each well we added 8 μ g/ml polybrene (Sigma) and the appropriate lentivirus stock at an MOI of 0.5, 2, or 5. Following overnight incubation, the medium was replaced with 10% FBS growth media for another 24 h, after which we began selection with 1.5 μ g/ml puromycin (Invitrogen, Grand Island, NY). The cell line that overexpresses HBEGF was produced by seeding MDA-231 cells in a six-well dish and growing them to 70% confluence. The TransIT-LT1 (Mirus Bio LLC, Madison, WI) transfection reagent was mixed with Opti-MEM medium (Invitrogen) and 2.5 μ g/ μ l of the pIRESHy2-HBEGF plasmid (a kind gift from R. Adams). The mixture was added drop-wise to each well of the six-well dish, after which the cells were incubated for 24 h in a 37°C/5% CO₂ incubator. The growth medium was changed and cells were incubated for 24 h, after which we began selection using 600 μ g/ml hygromycin B (Thermo

Fisher, Pittsburgh, PA). Recombinant lentiviruses encoding AREG, TGF- α , and vector control (pLenti6/TR) were produced as described previously (21). Briefly, MDA-231 cells were seeded in 60-mm dishes and grown to 50% confluence. Cells were infected by incubation overnight with the respective lentivirus and 6 μ g/ml polybrene (Sigma). Each 60-mm dish of cells was passaged to three 100-mm dishes, after which we began selection using 600 μ g/ml blasticidin (Invitrogen). We rescued the silenced expression of AREG or TGF- α by infecting the shAREG or shTGF- α cell lines with AREG or TGF- α lentiviruses essentially as described above. The proper amounts of EGFR and/or ligand expression were confirmed by qPCR.

S1T3 cells were used as positive control for EGFR phosphorylation and turnover assays (32,33). These cells are SV-40 immortalized human breast epithelial cells that are not tumorigenic when grown on the back of nude mice. We have used these cells as a model for breast epithelial cells and they exhibited EGF-stimulated receptor turnover similar to other model cell lines (16).

ELISA Assays

MDA-231 cells were grown to confluence in a 12-well dish and starved of serum for 24 h. The conditioned medium was collected and cleared by centrifugation for 10 min at 4°C. To measure the concentration of ligand associated with the cell membrane, cells were harvested in the following buffer (1 M Tris-HCl, 0.5 M EDTA, 10% Triton X-100, protease/phosphatase inhibitor cocktail). Lysate was cleared by centrifugation for 10 min at 4°C. AREG, HBEGF, and TGF- α concentrations in the conditioned medium and lysate were measured using the respective DuoSet ELISA kit (R&D Systems) and manufacturer's instructions. M-CSF and CCL2 concentrations in the conditioned medium were measured using the respective ELISAs (R&D Systems) and manufacturer's instructions.

RNA Isolation and Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

RNA isolation and qRT-PCR were performed as previously described (31,34). EPGN primers were 5'-F-TGA CAGCACTGACCGAAGAG and 5'-R-CTCATGGTGG AATGCACAAG (35). EREG primers were 5'-F-TCCA GTGTCAGAGGGACACA and 5'-R-GGTTGGTGGG CCGTTAAAAA (36). HaCat and SUM 149 cells were used as positive controls for EPGN and EREG mRNA detection, respectively. DNA from the amplified bands was isolated and sequenced to confirm the specificity of the primer sets.

MTT Proliferation Assay

Cells were seeded at 500 cells per well in a 96-well dish and were incubated overnight at 37°C/5% CO₂. MTT measurements were taken at 1, 3, 5, and 7 days after seeding. The MTT working solution (50 μ l of 1 mg/ml) was added to each well and incubated for 4 h at 37°C/5% CO₂. The medium was replaced with 150 μ l DMSO and the plate was incubated on an orbital shaker for 10 min. The optical density of each sample was determined using a 96-well plate reader at 600 nm absorption. Data points based on quadruplicate measures.

Migration and Invasion Assays

BD BioCoat™ migration chambers (Becton Dickinson, Franklin Lakes, NJ) were used according to the manufacturer's instructions and as previously reported (37). Briefly, cells were seeded at a density of 1×10^5 cells per well and were allowed to migrate for 24 h. The inserts were fixed and stained using the Hema3 Stat Pack (Thermo Fisher) and allowed to dry overnight. The next day, the number of migratory cells was determined by microscopy. Relative migration for each experimental condition was calculated by dividing the number of migratory experimental cells by the number of migratory control cells.

EGFR Turnover

Cells were grown in 10-cm dishes to near confluence and starved of serum overnight. Basal EGFR turnover was determined in the presence of 10 μ M cycloheximide to block protein synthesis (Sigma). The effect of ligand treatment on EGFR turnover on cycloheximide-treated cells was determined using 1,000 nM AREG or 100 nM EGF, TGF- α , or HBEGF. Cells were harvested 0, 10, 20, 40, 60, 120, or 240 min after ligand treatment; cells were incubated in Laemmli buffer (63 mM Tris-HCl, 10% glycerol, 2% SDS, pH 6.8) and sonicated for three 20-s pulses. Samples were cleared by centrifugation for 10 min at 4°C, and 5% 2-mercaptoethanol and 0.0025% bromophenol blue were added to each. Samples were resolved by SDS-PAGE and electro-transferred to PVDF. EGFR was detected using anti-EGFR and secondary antibodies as described elsewhere (31). EGFR expression was quantified using ImageJ software (NIH).

Animals

Animal care and experiments were approved by the Indiana University Institutional Animal Care and Use Committee (IACUC). For mammary fat pad tumors, all stable cell lines were combined with 50% Matrigel and inoculated at 1×10^6 cells in 100 μ l total volume in the first mammary fat pad of female athymic nude mice, aged 3–4 weeks (Charles River, Wilmington, MA). Tumors were measured twice weekly for length (L) and width (W), and tumor volume (V) was calculated as: $V = (L \times W^2) \times 0.5$. Upon sacrifice of the mice, tumors were dissected and weighed.

Immunohistochemistry

Tumors were fixed in formalin for at least 24 h and in two changes of 70% ethanol for 48 h prior to paraffin embedding. Sections (7 μ m thickness) were placed on glass slides. To stain for vessels, sections were rehydrated through graded alcohols, boiled for 20 min in citrate buffer (10 mM, pH 6.0), and rinsed in water. Sections were blocked in 10% serum and incubated in the primary rabbit anti-CD31 antibody (Abcam, Cambridge, MA) overnight. Primary antibody binding was detected using a secondary biotinylated antibody, HRP-streptavidin, and DAB peroxidase (all from Vector Laboratories).

To stain for macrophages, sections were rehydrated through graded alcohols and were subjected to antigen retrieval using trypsin solution (38). Sections were blocked in 10% serum and incubated in a primary rat anti-macrophage antibody (Abcam) overnight. Primary antibody binding was detected using a secondary biotinylated antibody, HRP-streptavidin, and DAB peroxidase (all from Vector Laboratories). Sections stained for CD31 or macrophages were not counterstained.

Separate tissue sections were stained with H&E for measurement of necrotic regions. For necrosis measurements, ImageJ (NIH) was used to measure the entire tumor area as well as the necrotic regions (both at 4 $\times$), and the size of the necrotic area was reported as a fraction of the entire tumor area. Sections stained for CD31 and macrophage were inspected by microscopy using a 20 $\times$ objective, with measurements and counts obtained from four randomly selected regions of each tumor (six tumors per type of sample).

Statistical Analysis

Results of in vitro experiments are expressed as the mean $\pm$ SD of triplicate or quadruplicate measures of independent replicates for single experiments. Results of in vivo experiments are expressed as the mean $\pm$ SEM of six animals per group. All statistical comparisons were based on two-tailed analysis of the Student's t test. A value of $p < 0.05$ was considered to be significant.

RESULTS

AREG, TGF- α , and HBEGF Are Available for Signaling in MDA-231 Cells

We previously observed that silencing EGFR transcription in MDA-231 cells (shEGFR cells) inhibited cytokine expression, cell migration, and tumor growth in the bone and mammary fat pad (31). Next, we wanted to determine if alterations to autocrine EGFR ligand signaling would induce similar changes in cancer cell behavior in vitro and in vivo. We had previously determined that the MDA-231 cell subline expressed substantial AREG, TGF- α , and HBEGF proteins, but not β -cellulin or EGF (31). To investigate whether epiregulin EREG or EPGN were expressed by this line, we used qRT-PCR to assess transcription of EREG and EPGN, to compare them with AREG, TGF- α , and HBEGF levels. Cell lines HaCaT (EPGN) and SUM149 (EREG) were used as positive controls for each transcript to be sure our primers were sufficiently sensitive (Fig. S1, available at: https://docs.google.com/file/d/0B201FJdFr_iPUTRmUDV5a25nVDg/edit?pli=1). We observed substantial transcription of AREG, TGF- α , and HBEGF transcripts, but EREG and EPGN levels were more than three orders of magnitude below the other ligands (Fig. 1A). We next examined if exogenous AREG, TGF- α , HBEGF, or the prototype ligand EGF causes EGFR turnover in MDA-231 cells. The control cell line S1T3, immortalized breast epithelial cells (39), displayed EGFR turnover within 60 min of treatment with EGF (Fig. 1B). MDA-231 cells did not demonstrate basal receptor turnover, whereas treatment with the prototype ligand EGF caused some EGFR turnover after 240 min (Fig. 1C). As expected, AREG and TGF- α did not induce rapid EGFR turnover; moreover, HBEGF, which induces rapid EGFR turnover in other cell types (11), failed to do so in MDA-231 cells (Fig. 1C). Unlike most cell types investigated to date, the MDA-231 cell line does not display rapid EGFR turnover in response to stimulation by EGF or HBEGF (16,40).

Characterization of MDA-231 Cells That Reduce or Overexpress Individual Ligands

To further explore differences among AREG, TGF- α and HBEGF signaling, we created stable MDA-231 cells in which endogenous AREG, TGF- α , or HBEGF are silenced (shAREG, shTGF α , shHBEGF, respectively), or engineered to overexpress AREG, TGF- α , or HBEGF (AREG-OE, TGF-OE, HB-OE, respectively). Using qRT-PCR, we observed that ligand gene transcription was specifically reduced in the shAREG (98%; $p < 0.001$), shTGF- α (60%; $p < 0.01$), and shHBEGF (65%; $p < 0.01$) cells (Fig. S2A, available at: https://docs.google.com/file/d/0B201FJdFr_iPUTRmUDV5a25nVDg/edit?pli=1). Ligand gene transcription was specifically elevated in the AREG-OE (76-fold; $p < 0.001$), TGF-OE (10-fold; $p < 0.001$), and HB-OE (70-fold; $p < 0.001$) cells (Fig. S2B, available at: https://docs.google.com/file/d/0B201FJdFr_iPUTRmUDV5a25nVDg/edit?pli=1).

Compared to vector control cells, the shAREG cells shed 60% less AREG into the medium ($p < 0.01$) and the AREG-OE cells shed 1.5-fold more AREG into the medium ($p < 0.05$) (Fig. 2A). However, compared to vector control cells, the shAREG and AREG-OE cells did not exhibit any difference in the amount of AREG attached to the membrane (Fig. 2A).

Compared to vector control cells, the shTGF- α cells secreted 75% less TGF- α into the medium ($p < 0.01$) and the TGF-OE cells secreted 4.5-fold more TGF- α into the medium ($p < 0.001$) (Fig. 2B). Compared to vector control cells, the shTGF- α cells did not display a significant decrease in the amount of TGF- α attached to the membrane, whereas TGF-OE cells exhibited a fourfold increased ligand ($p < 0.05$) in this fraction (Fig. 2B).

Compared to vector control cells, the shHBEGF cells did not exhibit a significant decrease in the amount of HBEGF secreted into the medium, although they did display a 70% decrease ($p < 0.01$) in HBEGF attached to the membrane (Fig. 2C). Compared to vector

control cells, the HB-OE cells did not exhibit a significant increase in the amount of HBEGF secreted into the medium, although they did exhibit a greater than twofold increase ($p < 0.001$) in HBEGF attached to the membrane (Fig. 2C).

Finally, we noted that altering the transcription of one ligand did not elicit a compensatory change in the expression of the other two ligands (Fig. 2A—C). Together, these data suggest that the molecular alterations to AREG or TGF- α expression produce greater changes in ligand available for autocrine EGFR signaling, while the modulations in HBEGF expression only influenced the membrane-bound population of agonist in the MDA-231 line.

To determine if the changes in AREG, TGF- α , or HBEGF expression displayed by our MDA-231 cells resulted in altered basal EGFR expression or signaling we used immunoblotting to detect basal levels of the receptor and its tyrosine phosphorylation. As expected, EGFR expression and tyrosine phosphorylation were reduced in the shEGFR cell line (Fig. 2D). A significant reduction in tyrosine phosphorylation was not detected in the shAREG, shHBEGF, or shTGF- α cells, while the AREG-OE and TGF-OE cells exhibited a twofold increase ($p < 0.001$) in tyrosine phosphorylation (Fig. 2D). Total EGFR expression was not changed in any of the cells (Fig. 2D).

To verify that the AREG-OE, TGF-OE, and HB-OE cells expressed ligand capable of inducing EGFR phosphorylation, these cells were treated with PMA to stimulate ligand cleavage by ADAM17 (41,42). The medium conditioned by these cells stimulates EGFR tyrosine phosphorylation ($p < 0.001$) in wild-type MDA-231 cells (Fig. 2D). As expected, overexpression or knockdown of AREG, TGF- α , or HBEGF failed to alter EGFR turnover in MDA-231 cells (not shown).

Changing AREG or TGF- α Expression Alters Cellular Migration

To examine the impact of changing the expression of EGFR ligands on cellular functions, we measured changes in cell proliferation and migration. Neither ligand silencing nor ligand overexpression caused a change in the proliferation of MDA-231 cells (Fig. S3, available at: https://docs.google.com/file/d/0B201FJdFr_iPUTRmUDV5a25nVDg/edit?pli=1). This is not surprising since EGFR signaling in this line has previously been demonstrated to affect motility but not proliferation (43, 44). In contrast, silencing the transcription of AREG or TGF- α caused decreased migration ($p < 0.001$ and $p < 0.05$, respectively) of MDA-231 cells (Fig. 3A). Although silencing EGFR transcription caused decreased migration ($p < 0.05$) of MDA-231 cells, the effect of ligand silencing was greater than the effect of silencing EGFR transcription (Fig. 3A). We were surprised to note that AREG overexpression caused decreased migration ($p < 0.01$), while TGF- α overexpression caused increased migration ($p < 0.01$) (Fig. 3B). Cells in which HBEGF was silenced or overexpressed failed to exhibit any change in migration (Fig. 3A, B).

Altered Ligand Expression Impacts Cytokine and Growth Factor Expression

EGFR signaling regulates PTHrP expression in breast cancers (39). As expected, PTHrP transcription was decreased 70–90% in cells in which EGFR, AREG, or TGF- α was silenced ($p < 0.001$ for each) (Fig. 4A). Overexpression of AREG caused a 2.5-fold increase in PTHrP transcription ($p < 0.001$), whereas overexpression of TGF- α caused a ninefold increase in PTHrP transcription ($p < 0.001$) (Fig. 4B). Neither silencing endogenous HBEGF expression nor HBEGF overexpression caused a significant change in PTHrP (Fig. 4A, B).

Autocrine and paracrine EGFR signaling has been shown to regulate the production of MCSF-1 in breast cancer cell lines, and the expression of MCSF-1 recruits macrophage and other bone marrow progenitors to tumors, promoting tumor growth and angiogenesis

(25,26,28,31). MCSF-1 was reduced in MDA-231 cells in which EGFR transcription was silenced ($p < 0.001$) and in MDA-231 cells in which AREG transcription was silenced ($p < 0.05$) (Fig. 4C). Furthermore, TGF- α and HBEGF overexpression each caused a modest increase in MCSF-1 secretion ($p < 0.05$ for each) (Fig. 4D). We were surprised to note that AREG overexpression caused a modest decrease in MCSF-1 secretion ($p < 0.05$) (Fig. 4D).

We employed a gene expression PCR array specific for inflammatory cytokines and receptors (SA Biosciences) to profile changes in the expression of 84 inflammatory cytokines and their cognate receptors that result from altering the expression of EGFR ligands. We focused on transcripts whose expression *increased* in at least one cell line in which an EGFR ligand was overexpressed and whose expression *decreased* in both the shEGFR cell line and at least one cell line in which the expression of ligand was silenced.

The cytokine CCL2 has been implicated in tumor macrophage attraction in prostate tumors as well as stimulation of osteoclastogenesis in breast cancer metastasis to bone (45,46). Our PCR array analyses revealed that CCL2 transcription is decreased in cells in which either EGFR or TGF- α is silenced, whereas CCL2 transcription is increased in the cell line in which TGF- α is overexpressed (data not shown). Analyses of CCL2 expression by ELISA yielded similar results in these cell lines. CCL2 expression was 30% lower ($p < 0.001$) in cells in which EGFR was silenced and 50% lower ($p < 0.001$) in cells in which TGF- α was silenced. In contrast, CCL2 expression was unchanged in cells in which AREG was silenced (Fig. 4E). Overexpression of TGF- α cells caused a fourfold increase in CCL2 expression ($p < 0.001$), but AREG overexpression caused a decrease in CCL2 expression (Fig. 4F). Neither HBEGF silencing nor HBEGF overexpression caused a change in CCL2 expression (Fig. 4E, F).

Characterization of Combination Knockdown/ Overexpression Cells

A surprising set of observations from the characterization of the ligand-overexpressing cells was that AREG overproduction reduced motility and cytokine production relative to controls. This suggested there might possibly be some sort of antagonism of EGFR signaling caused by AREG overexpression. To address this we created combination transduced cells that knocked down either TGF- α or AREG and then overexpressed the other ligand (shTGF- α /AREG-OE and shAREG/TGF-OE). Messenger RNA levels for these cells were determined by qRT-PCR (not shown). A ninefold increase in AREG was secreted in the shTGF- α /AREG-OE line, with 18-fold more AREG present on the membrane when compared to MDA-231 cells ($p < 0.001$) (Fig. S4A, available at: https://docs.google.com/file/d/0B201FJdFr_iPUTRmUDV5a25nVDg/edit?pli=1). There was a 19-fold increase in TGF- α secretion in the shAREG/TGF-OE line, with sevenfold more TGF- α present on the membrane ($p < 0.001$) (Fig. S4B, available at: https://docs.google.com/file/d/0B201FJdFr_iPUTRmUDV5a25nVDg/edit?pli=1). No changes to EGFR or ligand expression were observed in these transduced cells (data not shown). We also confirmed the overexpressed ligand was functional with PMA shedding, which induced an increase ($p < 0.001$) in pan-tyrosine phosphorylation similar to the other ligand-overexpressing cells (data not shown).

To determine if overexpression of AREG or TGF- α on the opposing knockdown background would recover decreases in migration and cytokine expression, we performed migration assays and used ELISA to measure ligand expression. Overexpression of either AREG or TGF- α ligands increased cellular migration compared to single AREG or TGF- α ligand knockdown cell lines ($p < 0.05$, $p < 0.001$) (Fig. 5A). As shown in Figure 5B, PTHrP mRNA levels were increased in the ligand-overexpressing cells ($p < 0.001$). Overexpression of either AREG or TGF- α rescued reduced MCSF-1 levels from the individual knockdowns (Fig. 5C). Lastly, CCL2 levels were modestly recovered by AREG overexpression in the

shTGF- α /AREG-OE cell line ($p < 0.001$), but TGF- α overexpression in the shAREG/TGF-OE line increased CCL2 levels to the extent observed previously in the TGF- α -OE line ($p < 0.001$) (Fig. 5D). With the exception of CCL2 production, overexpression of AREG or TGF- α in the opposite ligand knockdown cells rescued most cellular behaviors.

Tumor Growth in Cells With Altered Ligand Expression

To compare how ligand modifications influenced tumor growth, transduced cells that exhibited differential behavior in vitro (cells with altered AREG and TGF- α expression) were inoculated into mammary fat pads of female athymic nude mice. The tumor growth rate of the cells with modulated ligand expression was compared to various controls and EGFR knockdown cells. Silencing of the receptor, AREG, or TGF- α increased survival and produced slower growing tumors compared to controls (Fig. 6A, C), whereas overexpression of AREG and TGF- α whether alone or in combination with knockdown decreased survival and increased tumor volume (Fig. 6B, D). Histological analysis revealed an increased ($p < 0.05$) necrotic area in tumors produced from cells where the receptor, AREG, and TGF- α were silenced, whereas this parameter was decreased relative to controls in the ligand overexpression and combination cells (Table 1). CD31 antibody labeling of the tumors revealed fewer vessels in tumors from cells with reduced receptor or ligand levels ($p < 0.05$) (Fig. 7A). Overexpression of AREG alone or in combination with reduced TGF- α produced tumors that contained increased vessel count compared to controls (Fig. 7A, B). MCSF-1 and CCL2 have been implicated in attracting macrophage and other bone marrow progenitors to tumors, which may contribute to tumor cell growth and angiogenesis (31,47). The number of macrophages recruited to the tumor was decreased in tumors produced from EGFR silenced cells but not the knockdown of individual ligands (Fig. 7A, C). All ligand-overexpressing cells produced tumors with elevated numbers of macrophages ($p < 0.01$) (Fig. 7A, C). Taken together, reduction of AREG or TGF- α cells impacted tumor growth similar to silencing the EGFR, while overexpression of ligands tended to increase tumor volume and these neoplasms exhibited increased vessels and macrophages. However, AREG overexpression appeared to induce markedly more aggressive tumors than excess production of TGF- α .

DISCUSSION

This study builds on our previous work exploring the role of autocrine EGFR signaling in orthotopic and metastatic growth of the basal breast cancer cell line MDA-231. Among the benefits of the MDA-231 system for the study of EGFR ligand function is that knockdown or over-expression of AREG, TGF- α , or HBEGF did not cause a compensatory change in other EGFR ligands (48). Also, EGFR is not rapidly turned over after exposure to exogenous EGF or HBEGF (which are high-affinity ligands that stimulate receptor degradation in many cell types) (11,40,49). Finally, modulation of EGFR signaling does not influence proliferation of the line due to the presence of an activated K-ras gene (43). These attributes provided a cellular system where changes in a specific ligand had a proportional effect on the level of EGFR signaling, and the various assays used were not impacted by increased proliferation rates of engineered cells. In the future, it will be important to more comprehensively characterize EGFR signaling in additional basal breast cancer lines to determine if the receptor is generally resistant to turnover in this cell type, and whether the various ligands will have differential effects on proliferation.

Previously, we observed that decreased EGFR expression reduced MDA-231 cell growth in the mammary fat pad (31). We found that reduction of AREG and TGF- α levels by knockdown also reduced the growth rate of tumors in the mammary fat pad. Similar to EGFR knockdown cells, the reduced tumor growth of AREG and TGF- α silenced cells was

accompanied by increased necrotic area within the tumor and reduced tumor vasculature. Tumors produced by EGFR knockdown cells had reduced numbers of macrophages; however, this parameter was not significantly reduced in the ligand knockdowns. In the various ligand-overexpressing cells used, those with increased AREG produced markedly faster growing tumors as compared to controls and those that had increased levels of TGF- α (Fig. 6). Measures of tumor necrosis, vasculature, and macrophage counts were similar to tumors derived from cells that overexpress AREG or TGF- α ; however, tumors from AREG-OE cells were harvested a full week earlier than controls, which likely kept these parameters low. Thus, it appears that reduction of AREG or TGF- α has an impact on tumor growth, generally consistent with reduced autocrine EGFR signaling. On the other hand, the substantial impact of AREG overexpression on tumor growth compared with that of TGF- α raises the possibility that the ligands may influence tumor growth in a manner independent of autocrine EGFR-driven signaling. It is possible that cancer cell-derived EGFR ligands target the EGFR in the tumor microenvironment on fibroblasts or endothelial cells (50,51). The heparin-binding domain of AREG could enhance EGFR signaling through interaction with heparin residues on extracellular matrix proteins or plasma membrane proteins on cells in the microenvironment (52).

Also our work revealed subtle differences in autocrine ligand signaling between TGF- α and AREG in vitro. For example, cellular migration and MCSF-1 and CCL2 production were reduced in the AREG-overexpressing cells relative to controls, whereas the parameters were increased in the TGF- α overexpressing cells (Figs. 3 and 4). In the combination knockdown/OE cells, the increased AREG expression rescued decreased motility, and MCSF-1 and PTHrP mRNA expression observed in the TGF- α knockdown, but failed to reverse decreased CCL2 expression (Fig. 5). This finding suggested that AREG overexpression might antagonize some forms of EGFR signaling in the MDA-231 line. Both AREG and TGF- α induce similar levels of phosphorylation at various EGFR tyrosine residues in the MDA-231 line (31), so it is likely that the differential cytokine production stimulated by overexpression of AREG and TGF- α may stem from other aspects of ligand receptor interactions. TGF- α , similar to EGF, may stimulate prolonged EGFR signaling in the endosome, whereas AREG causes increased receptor localization to cell-cell junctions and does not appear to trigger signaling from the endosomal compartment (15). These differences could result in subtly distinct impacts on the expression of specific genes. Additional sources of interference from endogenous overexpression of AREG in the MDA-231 cells could include production of cleaved fragments of the ligand that eliminate the C-terminal binding domain required for high receptor affinity, resulting in partial antagonists (53). Additionally, increased AREG-EGFR binding may produce a physical receptor alteration that antagonizes endogenous TGF- α binding (13). Nevertheless, the substantially more aggressive growth of the AREG-OE tumor in vivo suggests the subtle differences between autocrine EGFR signaling induced by ligands in vitro are overridden by other mechanisms.

The tumor growth studies using the ligand knockdown cells provide some insight into the role of autocrine ligand production in growth of EGFR-positive basal breast cancer cells in the environment of the mammary fat pad. The established role of the EGFR in the growth of breast cancer tumors is to produce monocyte recruitment and enhanced production of macrophage chemokines (25,27,54). Macrophages in turn provide EGF at regions of close contact with the cancer cell and stimulation of the EGFR on breast cancer cells further activates gene expression for chemokines and cytokines, as well as triggering invasiveness (25,27,54). Our results suggest that autocrine-derived ligands can also stimulate the EGFR on breast cancer cells, essentially priming the system to facilitate the recruitment of macrophages to the tumor microenvironment, which will enhance tumor growth by promoting angiogenesis (Fig. 8).

Overall, it appears that multiple EGFR ligands expressed by a basal breast cancer cell line have both overlapping and distinct impacts on autocrine receptor signaling. HBEGF is not shed from the plasma membrane and does not participate in autocrine receptor signaling in MDA-231 cells, while shed TGF- α and AREG activate the EGFR and stimulate the production of cytokines that attract and differentiate monocytes. However, AREG overexpression appears to stimulate much more rapid MDA-231 tumor growth in the mammary fat pad than TGF- α .

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Figure 1.

AREG, TGF- α , and HBEGF are highly expressed in MDA-231 cells but do not induce receptor turnover. (A) Relative mRNA levels of AREG, TGF- α , HBEGF, EREG, and EPGN in MDA-231 cells. Ligands were measured by qRT-PCR analysis and relative ratios of ligand mRNA to GAPDH mRNA levels are shown (mean of triplicate measure from a single experiment; error bars, SD). (B) EGFR turnover in S1T3 cells. Western blots probed for EGFR or β -tubulin from basal S1T3 cells or S1T3 cells treated with EGF for the noted time points. (C) EGFR turnover in MDA-231 cells. Western blots probed for EGFR or β -tubulin from cells harvested after EGF, AREG, TGF- α , or HBEGF treatment for the noted time points.

Figure 2.

Characterization of reduced and overexpressing ligand cell lines. ELISA measurements to verify changes in EGFR ligands in overexpression and knockdown cells (A) AREG, (B) TGF- α , and (C) HBEGF levels in all cells. ELISA measurements were performed from triplicate wells derived from two independent cultures. shCtl refers to the PKO vector and the levels of various ligands was similar to the Plenti6TR overexpression vector and untransduced MDA-231 cells. These later controls are not shown for reasons of clarity. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Extracts were created from MDA-231, shEGFR, shAREG, shTGF- α , shHBEGF, AREG-OE, TGF-OE, and HB-OE cells. Basal S1T3- and EGF-treated S1T3 cells are included on the left of the panel as controls for phosphotyrosine labeling. Extracts were probed with antibodies for EGFR, pan-tyrosine 4G10, and β -tubulin as loading control. (E) AREG-OE, TGF-OE, and HB-OE cells were treated with PMA for 1 h, media harvested, and applied to MDA-231 cells for 20 min. Basal S1T3- and EGF-treated S1T3 cells are included on the left of the panel as controls for phosphotyrosine labeling. Western blots were probed for EGFR, 4G10, and β -tubulin as loading control.

Figure 3.

Migration of ligand knockdown and overexpression cell lines. Twenty-four-hour migration assay for (A) ligand knockdown and (B) ligand overexpression cell lines. Cells that migrated through the chamber membrane after 24 h were obtained from separate migration wells, with four random fields chosen for counts from each well. Each assay was performed at least twice, with counts obtained from at least four wells for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.

Characterization of cytokine expression in knockdown and overexpression cell lines. Ligand knockdown and overexpression cells were analyzed for cytokine expression. (A, B) PTHrP was measured by qRT-PCR analysis and relative ratios of PTHrP mRNA to GAPDH mRNA levels are shown (mean of triplicate measure from a single experiment; error bars, SD). *** $p < 0.001$. (C) Ligand knockdown and (D) ligand overexpression cells were serum starved for 24 h; media was harvested from all cell lines and analyzed for MCSF-1 by ELISA. Measurements were obtained from two separate cultures and performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (E, F) Twenty-four-hour serum-starved media was harvested from all groups and analyzed for CCL2 by ELISA. Measurements were obtained from two independent cultures and performed from triplicate wells. *** $p < 0.001$.

Figure 5.

Cytokine expression profile of shTGF- α /AREG-OE and shAREG/TGF-OE cell lines. shTGF- α /AREG-OE and shAREG/TGF-OE cells were analyzed for changes in migration and cytokine expression versus vector control and MDA-231 cells. (A) Cells that migrated through the chamber membrane after 24 h were obtained from at least four separate migration wells, with four random fields chosen for counts from each well. The assay was performed at least twice. $*p < 0.05$, $***p < 0.001$. (B) PTHrP was measured by qRT-PCR analysis and relative ratios of PTHrP mRNA to GAPDH mRNA levels are shown (mean of triplicate measure from a single experiment; error bars, SD). $***p < 0.001$. (C) Cells were serum starved for 24 h; media was harvested from all cell lines and analyzed for MCSF-1 by ELISA. Measurements were obtained from two separate cultures and performed in triplicate. (D) Twenty-four-hour serum-starved media was harvested from all groups and analyzed for CCL2 by ELISA. Measurements were obtained from two independent cultures and performed from triplicate wells. $***p < 0.001$.

Figure 6.

Tumor growth of ligand knockdown and overexpression cell lines. Noted cell lines were injected into the first mammary fat pad of female athymic nude mice. (A) Curve demonstrating a significant increase in survival in shEGFR, shAREG, and shTGF-α cell line tumors versus control lines. (B) Curve demonstrating decreased survival with AREG-OE cell lines tumor versus control lines. $n = 6$ animals per group, $**p < 0.01$, $***p < 0.001$. (C, D) Tumor volume measurements from all cell line injections; measurements obtained twice per week. $n = 6$ animals per group, $**p < 0.01$, $***p < 0.001$.

Figure 7.

Immunohistochemical staining for blood vessels and macrophages in tumors. (A) Immunohistochemical staining was performed using an anti-CD31 antibody for vasculature or anti-macrophage antibody for macrophages within the tumors grown from MDA-231 in the reduced ligand and ligand overexpression cells. Scale bar: 100 μ m in right corner of panel. (B) Vessel counts were performed on four random sections for each tumor, $n = 6$ animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C) Macrophage counts were performed on four random sections on each tumor, $n = 6$ animals per group. * $p < 0.05$, ** $p < 0.01$.

Figure 8.

Impact of paracrine and autocrine EGFR signaling and macrophage recruitment and tumor growth. (A) In a model of paracrine signaling between breast cancer cells and tumor-associated macrophage, production of EGF results in increased expression of macrophage chemoattractant MCSF-1. Resultant increased macrophage infiltration allows for chemoattractant binding on their cognate receptors on macrophage, stimulating EGF secretion and greater invasiveness. (B) Our work suggests increased autocrine EGFR signaling increases multiple macrophage chemoattractants including MCSF-1 and CCL2. Increased macrophages in the tumor, release of angiogenic factors that ultimately lead to greater vasculature support of the tumor promoting tumor cell survival and growth.

Table 1

Necrotic Tumor Area

	Necrotic Area (mm ²)	Fold Increase	Percent Decrease
MDA-231	19%	N/A	
shCTL	20%	N/A	
CTL-OE	18%	N/A	
shEGFR	39%	2.05 ^{**}	
shAREG	20%	1.05	
shTGF- α	21%	1.11 [*]	
AREG-OE	17%		11%
TGF-OE	14%		26%
shTGF- α /AREG-OE	16%		11%
shAREG/TGF-OE	14%		22%

*
 $p < 0.05$,

**
 $p < 0.001$.