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Knock-down of Amphiregulin Inhibits Cellular Invasion in Inflammatory Breast Cancer

Andrea Baillo^{1,2},

Wayne State University Department of Oncology, Karmanos cancer Institute

Craig Giroux, PhD^{1,2}, and

Wayne State University Department of Oncology, Karmanos cancer Institute

Stephen P. Ethier, PhD^{1,2,*}

Wayne State University Department of Oncology, Karmanos cancer Institute

Abstract

We have previously shown that SUM-149 human breast cancer cells require an AREG/EGFR autocrine loop for cell proliferation. We also demonstrated that AREG can increase EGFR stability and promote EGFR localization to the plasma membrane. In the present studies we successfully knocked-down AREG expression in SUM-149 cells by lenti-viral infection of AREG shRNA. In the absence of AREG expression, SUM-149 cell growth was slowed, but not completely inhibited. Furthermore, cells infected with AREG shRNA constructs showed an increase in EGFR protein expression by western blot. Immunofluorescence and confocal microscopy showed that following AREG knock-down, EGFR continued to localize to the cell surface. Soft agar assays demonstrated that AREG knock-down cells retain anchorage-independent growth capacity. Additionally mammosphere forming assays and Adefluor staining analysis showed that knock-down of AREG expression did not affect the expression of stem cell phenotypes. However, following AREG knock-down, SUM-149 cells demonstrated a dramatic decrease in their ability to invade a Matrigel matrix. Consistent with this observation, microarray analysis comparing cells infected with a non-silencing vector to the AREG knock-down cells, identified genes associated with the invasive phenotype such as RHOB and DKK1, and networks associated with cell motility such as integrin-linked kinase signaling, and focal adhesion kinase signaling. AREG was also found to modulate WNT and Notch signaling in these cells. Thus, AREG functions in regulating the invasive phenotype, and we propose that this regulation may be through altered signaling that occurs when AREG activates plasma membrane localized EGFR.

Introduction

The epidermal growth factor receptor (EGFR) is transmembrane protein belonging to the ErbB tyrosine kinase family. EGFR is activated following binding of one of a number of EGFR ligands which include, epidermal growth factor (EGF), amphiregulin (AREG), betacellulin (BTC), heparin-binding EGF (HB-EGF), transforming growth factor alpha (TGF- α), epiregulin (EREG), and epigen (EPGN) (Olayioye et al., 2000). Ligand binding to the extracellular domain of EGFR initiates activation of receptor dimers resulting in phosphorylation of the C-terminal tail, and subsequent down stream signaling. De-regulation

Corresponding Author: Stephen P Ethier, 4100 John R HWCRC Rm840.1, Detroit, Mi, 48224, Phone: 313-576-8612, Fax: 313-576-8029, ethier@karmanos.org.

¹Wayne State University, Detroit, Mi

²Karmanos cancer Institute, Detroit, Mi

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of EGFR expression or signaling has been implicated in cancer progression. In fact, approximately 30% of breast cancers over express EGFR, which correlates with poor prognosis (Nicholson et al., 2001; Tsutsui et al., 2002).

AREG is a heparin binding growth factor that binds EGFR (Cook et al., 1991). It was first isolated from the conditioned medium of MCF-7 breast cancer cells following treatment with a tumor promoter, phorbol 12-myristate 13-acetate (PMA), by Shoyab et al. (Shoyab et al., 1988). AREG was named for its ability to stimulate the proliferation of human fibroblasts and keratinocytes as well as tumor cells, and its ability to inhibit the proliferation of some carcinoma cell lines in culture (Shoyab et al., 1988). Later studies showed that AREG is synthesized as a 252 amino acid transmembrane precursor that requires proteolytic cleavage for secretion. Cleavage results in two mature soluble protein forms consisting of either 78 or 84 amino acids and ranging from 19–21-kDa in molecular weight (Plowman et al., 1990; Shoyab et al., 1989). Post-translational modifications of pro-AREG produces a major soluble 43-kDa form, 28-, 26-, 16-kDa membrane anchored forms, and soluble 21-, 19-, and 9-kDa forms (Brown et al., 1998).

AREG acts as an autocrine growth factor in human urothelial cells, normal human keratinocytes, and human lung bronchial epithelial cells (Kansra et al., 2004; Tsao et al., 1996; Varley et al., 2005; Willmarth and Ethier, 2006). During development in the mouse mammary gland, AREG has been shown to play an important role in terminal end bud formation and ductal elongation (Ciarloni et al., 2007; Luetke et al., 1999). In addition, expression of AREG mRNA has been observed in a variety of cancers including colon, breast, liver, prostate, pancreatic, lung, bladder, ovarian, skin, myeloma, and squamous cell carcinoma (D'Antonio et al., 2002; Ebert et al., 1994; Fontanini et al., 1998; Mahtouk et al., 2005; Salomon et al., 1995b; Sehgal et al., 1994; Tsai et al., 2006).

SUM-149 breast cancer cells were isolated from a patient with triple negative, inflammatory breast cancer whose disease progressed through chemotherapy. Our lab found that SUM-149 cells over express constitutively active EGFR, are EGF independent for growth, and over express AREG mRNA and protein. The EGF-independent growth of SUM-149 cells is regulated predominately by AREG (Willmarth and Ethier, 2006).

Previously, our lab has shown that in SUM-149 cells AREG functions through a self-sustaining AREG/EGFR autocrine loop. In this loop, AREG stimulation of EGFR results in AREG transcription and secretion allowing for AREG to signal EGFR continuously. More recently we have shown that AREG activation of EGFR results in an increase in the steady state levels of EGFR and accumulation of EGFR at the cell surface (Willmarth et al., 2009; Willmarth and Ethier, 2006). Consistent with our previous findings, Baldys et al. (Baldys et al., 2009) showed that AREG promotes the recycling of EGFR to the plasma membrane. Still the role of AREG in cancer has not been fully elucidated. In this report we describe knock-down of AREG expression using shRNA techniques to analyze the cancer-associated phenotypes and to examine the genes regulated by AREG that contribute to these phenotypes.

MATERIALS AND METHODS

Reagents and Antibodies

The antibodies used were mouse monoclonal, anti-EGFR antibody MAB108 from a mouse hybridoma (gift from Julie Boerner, Karmanos Cancer Institute) for detecting EGFR in immunofluorescence experiments and, mouse monoclonal anti-EGFR antibody clone 31G7 (Zymed Laboratories, Inc., San Francisco, CA, USA) to detect EGFR, anti-EGFR human (mouse) antibody clone 225 (C225) (EMD Chemicals, Gibbstown, NJ, USA) to block ligand

binding to EGFR, goat polyclonal anti-AREG antibody (R&D Systems, Minneapolis, MN, USA) to detect AREG and neutralize AREG, mouse monoclonal antibody PY-20 (EMD Chemicals, Gibbstown, NJ, USA) to detect EGFR tyrosine phosphorylation, B-Actin (Sigma-Aldrich, St. Louis, MS) was used as a loading control, rabbit polyclonal anti-RhoB antibody (Cell Signaling Technologies, Danvers, MA, USA) to detect RhoB, goat polyclonal anti-EGF and mouse monoclonal anti-EPGN antibodies (R&D systems, Minneapolis, MN, USA) to neutralize EGF and EPGN, Phospho-specific antibodies Y845, 992, 1068, 1086, and 1148 (Cell Signaling Technologies, Danvers, MA, USA) to detect EGFR tyrosine phosphorylation, IgG mouse and goat (R&D systems, Minneapolis, MN, USA) to detect non-specific effects on cell growth. 0.5 $\mu\text{mol/L}$ Gefitinib/Iressa (AstraZeneca Pharmaceuticals, Wilmington, DE, USA).

Cell Culture

SUM149 cells were maintained in Ham's F-12 medium with 5% fetal bovine serum, 5 $\mu\text{g/ml}$ insulin, 2 $\mu\text{g/ml}$ hydrocortisone, 5 $\mu\text{g/ml}$ gentamicin, and 2.5 $\mu\text{g/ml}$ fungizone (5%IH). SUM-149sh4, SUM-149shPld, and SUM-149shNS cells were maintained in 5%IH plus 1 $\mu\text{g/ml}$ puromycin. The serum-free base medium for MCF10A and MCF10A+AREG cells was SFIH (Ham's F-12 with 1 $\mu\text{g/ml}$ hydrocortisone, 1 mg/ml bovine serum albumin, 10 mM Hepes, 5 mM ethanolamine, 5 $\mu\text{g/ml}$ transferrin, 10 nM triiodothyronine, 50 nM sodium selenate, 5 $\mu\text{g/ml}$ gentamicin, 2.5 $\mu\text{g/ml}$ fungizone, and 5 $\mu\text{g/ml}$ insulin), and MCF10A cells required 10 ng/ml EGF (SFIHE). MCF10A + AREG cells were MCF10A cells grown in SFIH medium with 20 ng/ml exogenous AREG (SFIHA), which was found previously to be the biologically equivalent concentration to EGF for these cells (Berquin et al., 2005). All cells were maintained in a humidified incubator at 37 °C and 10% CO₂.

Lenti-AREG shRNA infection

Knock-down of AREG expression was performed using Mission TRC human shRNA clone sets (OpenBiosystems, Huntsville, AL, USA). Lentivirus was produced by transfecting HEK293 cells at 80% confluence in 10%DMEM without antibiotics and infecting with packaging plasmids 350ng mDLg/pRRE, 350ng pRSV-Rev, 250ng MD2.G, and 1 μg of each pLKO.1 AREG shRNA plasmid using FuGENE (according to manufactures protocol). Cells were incubated for 12–14 hrs at 37°C at which time media was changed to 10%DMEM with antibiotics. Virus was harvested 48 and 72 hrs post infection, centrifuged at 1500 rpm for 5 min and filtered through a .45micron filter. Recipient cells were seeded at 1×10^6 cells per 10 cm dish, treated with 8 $\mu\text{g/ml}$ polybrene and infected with 48 hr virus harvested from HEK293 cells. Cells were incubated for 12 hrs and new media with 1 $\mu\text{g/ml}$ puromycin was added for 2 weeks before functional assays were performed and 4–10 days before RNA extraction.

Real-time RT-PCR

Total RNA was isolated from subconfluent cells using an RNeasy kit (Qiagen, Valenica, CA, USA) and reverse-transcribed into cDNA using the Superscript III First-Strand Synthesis kit (Invitrogen, Calsbad, CA, USA). Primer sets specific to approximately 100bp sequences of target genes and a control gene (PUM1) used were designed and synthesized by Invitrogen. Primer sets were: AREG F5'/GTGGTGCTGTCGCTCTTGATA3' R5'/ACTCACAGGGGAAATCTCACT3' BTC F5'/TTCCTGTGTGGTGGCAGAT3' R5'/CCTTTCCGCTTTGATTGTGT3' EGF F5'/CGCAGGAAATGGGAATTCTA3' R5'/TCCACCACCAATTGCTCATA3' TGFA F5'/TCGCTCTGGGTATTGTGTTG3' R5'/GGGAATCTGGGCAGTCATTA3' HB-EGF F5'/GGCAGATCTGGACCTTTTGA3' R5'/CCCCTTGCTTTCTTCTTTC3' EPGN F5'/CCCAGCAAGCTGACAACATA3' R5'/CTCATGGTGAATGCACAAG3' EREG F5'/CTGCCTGGGTTTCCATCTTCT3' R5'/GCCATTTCATGTCAGAGCTACACT3' RHOB F5'/ATCCCCGAGAAGTGGGTCC3'

R5'CGAGGTAGTCGTAGGCTTGA3'. Real-time RT-PCR was performed in 25 μ l reactions using 96-well plates, 100 to 200 ng cDNA, and the FastStart SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany). The reactions were done in replicates of three using the Bio-rad iQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA). Calculation of the $\Delta\Delta CT$ values was performed as previously described (Livak and Schmittgen, 2001). Briefly, for each cell line, the average number of cycles for PUM1 control primer reactions to reach threshold fluorescence was calculated and subtracted from the average number of cycles for AREG primer reactions to reach threshold fluorescence, the differences are raised to the power of -2 and then the negative inverse was taken.

Immunoblotting

Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% NP40, 10% glycerol, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 20 μ g/ml leupeptin. Protein concentrations were equalized using the Bradford method. For whole cell lysates, Laemmli sample buffer was added and the samples were boiled. Western blotting was performed as previously described (Kumar-Sinha et al., 2003). Protein from whole cell lysates was loaded onto 15% SDS-polyacrylamide gels for AREG detection, and for EGFR detection lysates were loaded onto 7.5% SDS-polyacrylamide gels. After transferring proteins to (polyvinylidene difluoride membranes), blots were probed with required antibodies: anti-EGFR antibody 31G7 (Zymed Laboratories), phosphor-tyrosine antibody P-Tyr (EMD Chemicals), anti-AREG antibody AF262 (R & D Systems), phospho-tyrosine specific antibodies (Cell Signaling Technologies) or anti-B-Actin (Sigma-Aldrich) and visualized by enzymatic chemiluminescence (Pierce, Thermo Fisher Scientific).

Enzyme-linked Immunosorbent Assay (ELISA)

Conditioned medium was obtained from cells grown in 6-well plates. An AREG DuoSet ELISA (R&D systems, Minneapolis, MN, USA) was used to measure AREG medium concentration. High binding ELISA plates were coated with 3 μ g/ml AREG antibody in sterile phosphate-buffered saline (PBS) overnight at room temperature. Absorbance was measured on a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). Cells were lysed, and nuclei were counted with a Z1 Coulter Counter (Beckman Coulter, Brea, CA, USA) for normalization. Samples were done in triplicate.

Cell Proliferation Assays

Cells were seeded on day 0 in 6-well plates at 1.0×10^4 cells/well. Either 1 μ g/ml AREG Ab, 1 μ g/ml EGFR Ab, 1 μ g/ml EGF Ab, 1 μ g/ml EPGN Ab, 1 μ g/ml IgG Mouse Ab, and 1 μ g/ml IgG Goat Ab, or 0.5 μ mol/L Iressa was added daily. After 7 days of treatment, plates were washed with PBS three times and agitated on a rocker table with 0.5 ml of a HEPES/MgCl₂ buffer (0.01 M HEPES and 0.015 M MgCl₂) for 5 min. Cells were then lysed for 10 min using a BRETOL (ethyl hexadecyldimethylammonium) solution, and the nuclei were counted using a Z1 Coulter Counter (Beckman Coulter, Brea, CA, USA). Day 1 cells were counted for seeding efficiency. All experiments were done in triplicate.

Cell Invasion Assay

Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA) were rehydrated with Dulbecco's modified Eagle's medium for 1 h in a 37°C incubator. 2.5×10^5 of MCF10A cells in SFIHE medium and SUM149, SUM-149sh4, SUM-149shPId, SUM-149shNS cells in SFIH medium were added to the upper chamber of both control and rehydrated Matrigel invasion chambers. 5% fetal bovine serum was added to media in the bottom chamber as a chemoattractant. After 24 h, membranes were fixed and stained using the Hema 3 Staining System (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Membranes were

allowed to dry and then were placed onto slides for visualization. Cells on Matrigel membranes were counted from six microscopic fields after 24 h. Percentage of invasion was calculated by dividing the mean number of cells on the invasion membranes by the number of cells seeded for each cell line. Experiments were repeated three times.

Soft Agar Assay

A bottom layer of 1:1 Ham's F-12 serum-free medium to 1% agarose was poured and allowed to solidify. A 1-mL suspension of 1.0×10^5 cells in a 0.3% agarose solution was plated into six-well plates and fed once a week by adding 3mL medium on top of the soft agar. Cells were then counted at weeks three and four with GelCount automated mammary colony counter (Oxford Optronix, Oxford, UK).

In vitro Mammosphere assay

For the *in vitro* mammosphere assay, cells were grown in a serum-free mammary epithelial growth medium (MEBM Basal Medium, Lonza, Walkersville, MD, USA) supplemented with B27 (Invitrogen, Carlsbad, CA, USA) 1 μ g/ml hydrocortisone, 5 μ g/ml insulin, 5 μ g/ml β -mercaptoethanol, and 10ng/ml epidermal growth factor; 10,000 cells were plated on a six-well ultra low attachment plate (Corning Inc., Acton, MA, USA) and 1ml of medium was added every 3 days. After 7–10 days, the mammospheres were moved to adhering dishes, allowed to attach with 2% FBS, stained with 1X crystal violet for 1 min, washed, and counted after 2–4 days.

Illumina microarray analysis

RNA was isolated from SUM-149, SUM149sh4, SUM-149shPId, and SUM-149shNS cells in triplicate. Quantity measurement of all RNA samples was measured by analysis with the NanoDrop 1000 (Agilent Technologies, Waldbronn, Germany). Expression levels for each cell line were determined by microarray analyses using the Illumina human Ref12v3 chip (Illumina, San Diego, CA, USA). Data were processed for quality control and normalized across compared arrays by quantile normalization. Data were exported from GenomeStudio after performing the needed quality control checks and uploaded into GeneSpring. The data were then quantile normalized, transformed to median baseline, and analyzed using Ingenuity Pathway analysis. Using an un-paired T-test, 952 of 24,927 genes were found to be significantly different in expression at the $p < 0.05$ level between SUM-149sh4 and SUM-149shNS cells. An additional statistical test, Rank-Prod was also performed which identified 655 genes that were significantly different at the $p < 0.05$ level. The genes described in this paper were present in both gene sets. Genes identified using these statistical tests were imported into the Ingenuity Knowledge Base for further analysis. The raw data of the microarray analysis have been deposited in the GEO database (GSE26079).

Results

AREG Knock-down Slows Proliferation of SUM-149 cells

Our lab has previously shown that SUM-149 breast cancer cells are dependent on EGFR activation for proliferation and depend on a self-sustaining AREG/EGFR autocrine loop promoting proliferation (Willmarth and Ethier, 2006). Given that AREG is primarily responsible for EGFR activation, we wanted to investigate the effects of AREG knock-down on these phenotypes in the SUM-149 cells. Using five pLKO.1 shRNA constructs specific to AREG, and a non-silencing (SUM-149shNS) vector from OpenBiosystems, we knocked-down AREG expression in the SUM-149 cells. Among the five constructs tested, three constructs induced the highest level of AREG knock-down, yielding approximately 15 to 60-fold reduction in AREG mRNA levels. The sh4 construct induced the most dramatic

knock-down resulting in a 40–60-fold decrease in AREG mRNA expression, as determined by real-time RT-PCR analysis (Fig. 1A). In attempt to achieve an even further fold-change in AREG knock-down, the three best constructs (viruses sh2, sh3, and sh4) were pooled (shPld) and used to infect the SUM-149 cells. By real-time RT-PCR, we did not observe any additional AREG knock-down by pooling the three constructs as compared to infection with the sh4 virus alone (Fig. 1B). As a result, all additional experiments were performed using SUM-149sh4 and SUM-149shPld cells, as compared to SUM-149shNS cells, which were infected with a non-silencing control shRNA.

To test the effects of shRNA on AREG protein expression, we quantified the amount of AREG secreted into the conditioned media by an enzyme-linked immunosorbent assay (ELISA). Conditioned media was removed from 80% confluent cells, cells were counted, and the media was analyzed using an AREG specific ELISA kit (R&D Systems). Secreted AREG was un-detectable in the conditioned media of the knock-down cells, while the SUM-149 parental and SUM-149shNS controls secreted approximately 100pg/ml AREG/100,000 cells over 24hrs (Fig. 1C). To measure the presence of cellular AREG protein, western analysis was performed with the parental and knock-down cells. Western blot analysis demonstrated a dramatic decrease in each of the AREG isoforms (Fig. 1D). However, a faint band representing the 43 kDa AREG isoform was detected in the SUM-149sh4 and SUM-149shPld cells. Next, we investigated the rate of proliferation in the control cells and each of the knock-down populations. Growth assays following infection with the sh2, sh3, and sh4 and treatment with the selecting antibiotic, puromycin, resulted in the greatest decrease in cell proliferation relative to the SUM-149shNS control (Fig. 2A). Doubling times (DT) were determined by counting cells at five time points over a 94 hr period. SUM-149 and the SUM-149shNS cells had a DT of 27 and 29 hrs, respectively, while SUM-149sh4 and SUM-149shPld cells had DTs of 36 and 42 hrs, respectively (Fig. 2B). These results indicate that a 40–60-fold knockdown of AREG reduced, but did not completely block proliferation of SUM-149 cells.

Continued proliferation of AREG knock-down cells is EGFR dependent

Despite the high levels of AREG knock-down, SUM-149sh4 and SUM-149shPld cells continued to proliferate, although at a slower rate than the parental cells. To address the continued proliferation of the AREG knock-down cells, we first investigated the status of EGFR in these cells. We performed EGFR western blot analysis on the parental and knock-down cells. Results shown in figure 3A demonstrated an increase in steady-state EGFR protein levels in the AREG knock-down cells compared to SUM-149 and SUM-149shNS (Fig 3A). Consistent with the increase in EGFR protein expression we observed a further accumulation of EGFR to the plasma membrane by immunofluorescence in the AREG knock-down cells (Supplementary Fig. 1). Western analysis of specific EGFR tyrosine phosphorylation sites including, Y845 (phosphorylated by src), and autophosphorylation sites Y992, Y1068, Y1086, and Y1148 indicated that EGFR remained active in the knock-down cells (Fig. 3B), which is consistent with continued slower proliferation (Fig. 2B).

Next, we investigated the importance of EGFR signaling in the proliferation of the knock-down cells using inhibitory antibodies, and the small molecule EGFR inhibitor gefitinib. Data shown in figure 3C shows that exposure of the parental cells to gefitinib or EGFR blocking antibody for seven days inhibited cell growth by more than 80% compared to growth in their normal cell culture media. Additionally, the residual cell proliferation observed in the AREG knock-down cell lines was also inhibited by the presence of the EGFR inhibitors. These results indicate that the proliferation observed in the AREG knock-down cells is dependent on EGFR signaling. Furthermore, in one experimental group, an AREG neutralizing antibody was incubated with the AREG knock-down and control cells

for seven days (Fig. 3C). Unlike the EGFR antibody, the AREG antibody did not further inhibit cell proliferation in the knock-down cells, while it did reduce growth in control cells.

There are seven known ligands that bind to and stimulate EGFR; EGF, AREG, BTC, HB-EGF, TGF- α , EREG, or EPGN. It was previously determined that SUM-149 cells synthesize EREG (Willmarth and Ethier, 2006). To investigate the possible expression of other EGFR ligands in SUM-149 AREG knock-down cells, real-time RT-PCR analysis was performed. Data in figure 4A show that the expression of four of the EGFR ligands was concomitantly down-regulated as a result of AREG knock-down. However, two EGFR ligands, EGF and EPGN were not altered in their expression in the AREG knock-down cells (Fig 4A). To determine if the observed down-regulation of ligands in the AREG knock-down cells was due to reduced EGFR signaling, we analyzed EGF-family ligand expression by real-time RT-PCR in SUM-149 cells before and after treatment with gefitinib. After 24 hours of treatment with gefitinib, the same ligands that were down-regulated in AREG knock-down cells were reduced in their expression relative to non-treated SUM-149 cells (Fig. 4B). These data are consistent with the hypothesis that decreased expression of AREG and other EGFR ligands in the AREG knock-down cells is in response to the decrease in EGFR activation, and not to off target effects of the shRNAs. Indeed, BLAST analysis of the AREG shRNA sequences did not reveal any similarity to sequences present in other EGFR ligands..

Since AREG knock down did not decrease expression of EGF or EPGN, we investigated possible proliferative effects of endogenous EGF or EPGN on the growth of knock-down and parental cells by performing growth assays using neutralizing antibodies for each of the ligands. For seven days cells were treated with AREG, EGF, or EPGN neutralizing antibodies or control antibodies, gefitinib, or an EGFR inhibitory antibody. The experiment was performed using cells at different passage levels following AREG knock-down. Figure 4C shows that at early passage after AREG knock-down (<p4) EGF or EPGN neutralizing antibodies had no effect on growth of the knock-down cells. When the growth assays were repeated with cells at a later passage after AREG knock-down (>p20), EGF and EPGN neutralizing antibodies reduced cell growth by about 20% in the SUM-149sh4 and SUM-149shPId cells, while growth of SUM-149 and SUM-149shNS cells was not affected (Fig. 4D). However, exposure to AREG neutralizing antibodies also resulted in a decrease in growth of late passage SUM-149, SUM-149shNS, and AREG knock-down cells. To confirm these results, real-time RT-PCR analysis demonstrated that AREG mRNA expression in SUM-149sh4 and SUM-149shPId cells at passage 18 after knock-down was only two-fold lower than control cells as compared to 40–60-fold lower at early passage (Fig. 4E). These data suggest that AREG knock-down slows proliferation of SUM-149 cells, but as the cells are passaged their growth rate increases through increased expression of AREG and other EGF-like ligands.

Knock-down of AREG Affects Cancer-associated Phenotypes

The ability to grow in soft agar is a hallmark of cancer transformation. Therefore, we measured the soft agar colony forming ability of parental SUM-149 cells, SUM-149sh4, SUM-149shPId, and SUM-149shNS cells. Figure 5A shows that SUM-149sh4 and SUM-149shPId cells formed 50% fewer colonies in soft agar after 3 weeks than SUM-149 cells (Fig. 5A, Supp Fig. 2). However, given longer growth periods (four weeks), AREG knock-down cells were able to produce a similar number of colonies as the SUM-149 and SUM-149shNS controls (Fig. 5B, Supp Fig. 2), suggesting that the smaller number of colonies observed at three weeks reflects the slower growth of the knock-down cells and not their ability to survive under anchorage independent conditions.

To determine whether AREG shRNA expression affects phenotypes of cancer stem cells, we performed mammosphere colony forming assays. Cells were plated at 10,000 cells/well in ultra low attachment plates, cultured for one week, transferred to standard tissue culture plates, and allowed to attach for two days before being stained and counted. When the mammosphere assay was performed using the same growth factor-free medium used to maintain the control and knock-down cells, AREG knock-down cells showed a decrease in mammosphere colony formation (Fig. 5C). However, when parental and knock-down cells were grown in stem cell medium which contains EGF, there was no difference in the number of mammospheres that developed. We interpret these results to indicate that the ability of SUM-149 cells to form mammospheres is not influenced by the presence or absence of AREG. Previously, ALDH1 has been suggested to be a marker of breast cancer stem cells and particularly in SUM-149 cells (Charafe-Jauffret et al., 2010; Charafe-Jauffret et al., 2009). To determine if the percentage of ALDH1 positive cells was influenced by AREG knock-down, we performed Aldefluor assays. Results of these experiments were consistent with the mammosphere assay and indicated that AREG knock-down did not decrease the proportion of ALDH1 positive SUM-149 cells (Data not shown).

Next, we investigated the invasive capacity of the AREG knock-down cells in a Matrigel invasion assay (BDBioscience). In these experiments, 25,000 SUM-149, SUM-149sh4, SUM-149shPld, or SUM-149shNS cells were seeded on top of a Matrigel matrix in Boyden chambers and incubated with their serum containing growth media below the Matrigel for 24 hours. MCF10A cells were used as a negative control and showed little invasion through the matrix, while, SUM-149 and the SUM-149shNS cells were highly invasive. The data in figure 6A shows that both SUM-149 AREG knock-down cell lines exhibited a dramatic decrease in the number of cells that invaded through the matrix relative to the parental and control cells (Fig. 6A). Indeed, AREG knock-down decreased the invasive capacity of the SUM-149 cells by greater than 75%. Taken together, these results indicate that whereas AREG knock-down has a marginal effect on SUM-149 cell soft-agar colony formation and no effect on the expression of stem cell phenotypes, AREG knock-down has a dramatic effect on the motile and invasive capacity of SUM-149 cells.

Effect of AREG knock-down on gene expression

To examine the influence of AREG knock-down on the transcriptome of SUM-149 cells, we performed DNA microarray analysis using Illumina micro-arrays. SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells were grown to 80% confluence and RNA was isolated one day after feeding. The RNA was reverse transcribed and hybridized to the DNA array. All arrays were performed in triplicate. Analysis of the microarray data indicated a statistically significant difference in expression of 952 genes between SUM-149sh4 and SUM-149shNS cells. Of these, 659 genes were greater the 1.3 fold different between the two cell lines, and 381 genes exhibited a greater than 1.5 fold difference. Ingenuity Pathway analysis of the 952 gene set identified cell death (173 genes) and cellular movement (110 genes) as the top two biological functions influenced by AREG knock-down. With respect to cell movement, AREG influenced expression of several genes that are part of canonical pathways associated with cellular motility and invasion including; integrin-linked kinase signaling, focal adhesion kinase signaling, caveolar mediated endocytosis signaling, WNT signaling and Notch signaling (Table 1). Given our observation of the influence of AREG knockdown on motility and invasion as shown in figure 6, the identification of changes in specific genes associated with motility and invasion such as several integrins, vimentin, and MMPs (Table 1) were consistent with those experimental observations. Up-regulation of two specific genes, DKK1 and RHOB, which have been implicated in cancer cell motility and invasion (Jiang et al., 2004; Pukrop et al., 2006) were chosen for validation experiments. Data in figure 7 shows that changes in expression level of each gene by real-time RT-PCR

were consistent with the micro-array results, and western blot analysis demonstrated an increase in expression of RHOB associated with AREG knockdown (Fig. 7A,B,C). In several studies, DKK1 and RHOB have been implicated as negative regulators of cellular motility (Jiang et al., 2004; Pukrop et al., 2006). The expression of RHOB and DKK1 was low in SUM-149 cells and was increased approximately 10-fold in the AREG knock-down cells (Fig 7B,C). Thus, results of microarray analysis of SUM-149sh4 and SUM-149shNS cells are consistent with the biological experiments with respect to motility and invasion, and also point to other important pathways that could be regulated by AREG in these cells.

Discussion

Over expression of EGFR correlates with advanced stage and a poor prognosis in many types of cancer, including breast cancer (Salomon et al., 1995a). Neal et. al. (Neal et al., 1985) found EGFR expression correlates with poor differentiation and invasion in transitional cell carcinoma of the bladder. Additionally, over expression of EGFR is accompanied by autocrine regulation of its stimulating ligands (Voldborg et al., 1997). Our lab has shown that AREG promotes proliferation of SUM-149 breast cancer cells through the expression of an AREG/EGFR autocrine loop (Willmarth and Ethier, 2006). The present work examined transformed phenotypes in SUM-149 cells following knockdown of AREG using lentiviral AREG shRNAs. We found that AREG knock-down failed to completely inhibit cell proliferation, although it did slow the growth-rate of cells. In addition, knock-down did not significantly inhibit the cells' ability to form colonies in soft agar nor did it affect the expression of stem-cell phenotypes as determined by mammosphere colony formation and Aldefluor staining. However cells with AREG knock-down exhibited a dramatic decrease in their invasive capability. Moreover, microarray data were consistent with this observation and identified a number of genes associated with invasive and motile phenotypes that were altered in expression by AREG knock-down.

The observation that dramatic levels of AREG knock-down did not completely block growth of SUM-149 cells, was unexpected. These knock-down cells, while expressing undetectable levels of AREG by ELISA and western blot, continued to express tyrosine phosphorylated EGFR. The increase in steady-state levels of EGFR protein in the knock-down cells, as detected by western blot, is consistent with reduced EGFR activity in these cells. Further, EGFR remained localized to the cell surface in the knock-down cells (Supp Fig. 1). However, EGFR remained active and was responsible for the continued proliferation of the knock-down cells as exposure to either EGFR neutralizing antibodies or small molecule inhibitors completed the inhibition of growth of the knock-down cells.

We observed that AREG levels steadily increased in the knock-down cells with passage after infection with lentiviral AREG shRNA vectors. This finding, coupled with the residual proliferation and EGFR activation in the knock-down cells suggests that small sub-populations of cells were present that continued to express low levels of AREG, and that these cells increased in proportion with each passage. Interestingly, this low-level AREG mediated cell growth was not affected by AREG neutralizing antibodies. If this interpretation is correct, it would explain the continued EGFR activation observed in the population of cells that were responding to paracrine growth factors secreted by this sub-population. This interpretation also has implications for the ability of breast cancer cells to respond to very low levels of EGFR ligands, particularly when EGFR accumulates on the cell surface.

We were surprised to find that knock-down of AREG resulted in concomitant decreases in expression of other EGFR ligands. Previously, we showed that AREG mRNA expression in SUM-149 cells is regulated by EGFR activation (Willmarth and Ethier, 2006). Results

presented here extend that finding and demonstrate that reduced EGFR activation, whether via AREG knock-down or EGFR inhibition, results in decreased transcription of several EGFR ligands, not just AREG.

Despite the inability to completely block proliferation of SUM-149 cells following AREG knock-down, we did find that these cells had dramatically reduced their invasive capacity. Numerous studies have shown that EGF stimulation of EGFR promotes cell invasion and motility in various cancers including glioblastoma, squamous cell carcinoma, bladder, gastric, renal, prostate, and breast cancer (Libermann et al., 1984; Neal et al., 1985; Ozawa et al., 1987; Price et al., 1996; Schlegel et al., 1994; Toi et al., 1990; Tokunaga et al., 1995; Turner et al., 1996). On the contrary, there have been few studies reporting AREG promotion of invasion. Invasion associated with AREG has been described *in vitro* in breast and head and neck cancers. In two different studies, squamous cell carcinomas have been shown to secrete AREG in response to G protein-coupled receptor (GPCR) ligand stimulation resulting in cell proliferation, motility, and invasion (Gschwind et al., 2003; Zhang et al., 2006).

In normal epithelial tissue, AREG is expressed during development of the embryonic lung (Schuger et al., 1996) and in the pubertal mammary gland during ductal morphogenesis and terminal end bud (TEB) formation (Ciarloni et al., 2007; Luetke et al., 1999). EGFR and AREG interactions occur in the stroma thereby stimulating the proliferation of nearby epithelial cells in the ducts and TEBs. Similarly, embryonic lung branching of the epithelial trees depends on the presence of AREG as well as the presence of the extracellular matrix (ECM) (Schuger et al., 1996). The important role of AREG/EGFR interactions in tissue development and morphogenesis is consistent with the pathological role of AREG in mediating motility and invasion when inappropriately expressed in cancer cells.

Mechanisms proposed for AREG/EGFR induced cellular invasion focus on the altered expression of matrix metalloproteases (MMPs). These proteases play a role in embryonic development and growth, while in cancer they function in breaking down the basement membrane barrier. In metastatic breast cancer cells (SKBR-3), an increase in MMP-9 expression modulates AREG promotion of invasion (Kondapaka et al., 1997). Additionally, inhibition of AREG by anti-sense cDNA in NS2T2A1 mouse mammary tumor cells inhibited MMP-9 expression (Ma et al., 2010). Microarray data comparing gene expression in AREG knock-down cells to cells infected with the non-silencing control are consistent with the report of Ma et al., in that we also found MMP-9 expression was decreased in SUM-149sh4 knock-down cells (Table 1). Our gene expression profiling data also indicated a role for AREG in the regulation of expression of other genes that play a role in invasion such as RHOB and DKK1. Rho GTPase's, regulate cytoskeletal rearrangements during cell invasion and motility in inflammatory breast cancer (IBC) cells (Lin and van Golen, 2004), and RhoB expression has been shown to be dramatically reduced as tumors become more aggressive (Adnane et al., 2002; Forget et al., 2002; Mazieres et al., 2004). DKK1, which acts as a negative regulator of WNT signaling, has also been implicated in regulating the invasive phenotype (Pukrop et al., 2006). Thus, our observation of dramatic up-regulation of DKK1 in AREG knock-down cells is consistent with a role of WNT signaling in modulating the invasive phenotype.

In summary, our results with AREG- knock-down versions of SUM-149 cells not only point to roles of the autocrine ligand in maintaining proliferation of the cells in an autocrine manner, but provide further support for the hypothesis that AREG activated EGFR is a major driver of invasion in breast cancer cells. Furthermore, our results suggest that the invasive phenotype is a result of the altered signaling that occurs in cell surface localized EGFR, which occurs as a direct result of AREG-EGFR interaction, and is in contrast to what

occurs with other EGFR ligands, particularly EGF, which drives internalization and degradation of the receptor. It would be constructive to test our findings in additional cell lines where the cells rely on AREG as the SUM-149 cells do and in an experimental *in vivo* model. Further work is required to understand the mechanistic basis for EGFR stability and cell surface localization of AREG activated EGFR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. AREG Knock-down in SUM-149 cells

SUM-149 cells were stably infected with various AREG shRNA lentiviral expression constructs. Levels of AREG mRNA expression in cells infected with each individual construct and the combined sh2, sh3, sh4 constructs (shPld) was measured by real-time RT-PCR (A&B). SUM-149 mRNA expression was set to one. Secreted AREG was measured by ELISA (B). Conditioned media was harvested after 24 hours from the SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells. The cells were counted and the concentration of AREG was normalized to cell number. AREG protein expression in the whole cell was measured by western blot (D).

Figure 2. AREG knock-down decreases the rate of proliferation in SUM-149 cells

(A) Infected cells were treated with 1ug/ml puromycin 12 hours post-infection (Day1) and counted at days 4, 7, and 10. B) Cells at low passage following shRNA infection were seeded at 1×10^4 cells per well and counted at approximately 24 hour intervals for 94 hours, and doubling times were calculated. Error bars represent the standard deviation of three replicates in the experiment.

Figure 3. Continued proliferation of AREG knock-down cells is EGFR dependent (A) Western blot showing EGFR protein levels and (B) phosphorylation at specific EGFR tyrosine residues during normal growth of MCF10A, SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells. (C) SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells were seeded at 1×10^4 cells per well. The first bar in each group represents the number of cells on day one. The next four bars show the number of cells after seven days in their normal growth media or with everyday exposure to 1 μ g/ml EGFR Ab, 0.5 μ mol/L Iressa, or 1 μ g/ml AREG Ab. Error bars represent the standard deviation of three replicates in the experiment.

Figure 4. The role of EGF family ligands in growth of AREG knock-down cells

(A) Relative mRNA expression by real-time RT-PCR of AREG, EREG, EGF, TGF- α , BTC, HB-EGF, and EPGN in the SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells. (B) Relative mRNA expression by real-time RT-PCR of AREG, EREG, TGF- α , BTC, HB-EGF, and EPGN in the SUM-149 and SUM-149 cells treated with 0.5 μ mol/L Iressa for one or 24 hours. (C) Bars show growth of early passage cells (<p4) and (D) late passage cells after AREG knock-down (>p18) Cells were seeded at 1×10^4 cells per well. The first bar in each group represents the number of cells on day one. The next four bars show the number of cells after seven days of growing in their normal growth media or with everyday exposure to 1 μ g/ml IgG mouse Ab, 1 μ g/ml IgG goat Ab, 1 μ g/ml AREG Ab, 1 μ g/ml EGF Ab, 1 μ g/ml EPGN Ab, 1 μ g/ml EGFR Ab or 0.5 μ mol/L Iressa. Error bars represent the standard deviation of three replicates in the experiment. (E) Relative mRNA expression by real-time RT-PCR of AREG in late passage knock-down cells (>p18) with SUM-149 parental cells normalized to one.

Figure 5. Effect of AREG knock-down on soft agar colony formation (A&B) and mammosphere growth (C)

The bars represent the number of colonies that grew in soft agar after three weeks (A) or four weeks (B) of growth divided by the number of cells seeded (1×10^5). Cells were counted with GelCount, automated mammalian cell counter. Error bars represent the standard deviation of three replicates in the experiment. (C) Cells were seeded at 1×10^4 cells in ultra-low attachment plates in mammosphere media with (IHE) or with out (IH) 10 ng/ml of exogenous EGF. The bars represent the number of spheres counted by visualization with a microscope. Error bars represent the standard deviation of three replicates in the experiment.

Figure 6. AREG knock-down inhibits cellular invasion

(A) MCF10A, SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells were seeded in serum free media on a Matrigel matrix and incubated at 37°C for 24 hours with serum containing media as the chemoattractant. The bars represent the average number of cells that invaded the matrix divided by the number of cells seeded (2.5×10^4 cells). Error bars represent the standard deviation of three replicates in the experiment. (B) Photos represent one field of view after staining invading cells.

Figure 7. Validation of increased expression of RHOB and DKK1 in AREG knock-down cells
 (A) Western blot showing RhoB protein expression in parental and knock-down cells. β Actin is used as a loading control. Relative mRNA expression by real-time RT-PCR of (B) RHOB and (C) DKK1 mRNA expression with SUM-149 or SUM-149shNS parental cells normalized to one.

Table 1

Summary of genes regulated by AREG knock-down in SUM-149 cells

Six signaling pathways associated with cell motility and invasion are listed in bold. The top three genes up and down regulated in each pathway are listed beneath each signaling pathway as determined by Ingenuity Pathways Analysis

Symbol	Fold Change	Gene Name
Caveolar mediated endocytosis signaling		
ITGB5	-1.832	integrin, beta 5
FLOT1	-1.475	flotillin 1
ITGB2	-1.296	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
ITGB4	1.479	integrin, beta 4
ITGA5	1.908	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
CAV1	2.112	caveolin 1, caveolae protein, 22kDa
Glioma invasion signaling		
ITGB5	-1.832	integrin, beta 5
PLAUR	-1.614	plasminogen activator, urokinase receptor
MMP9	-1.538	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
RND3	1.632	Rho family GTPase 3
PIK3R1	1.7	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
RHOB	1.924	ras homolog gene family, member B
Integrin Signaling		
ITGB5	-1.832	integrin, beta 5
MYL12A	-1.297	myosin, light chain 12A, regulatory, non-sarcomeric
ITGB2	-1.296	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
ITGA5	1.908	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
RHOB	1.924	ras homolog gene family, member B
CAV1	2.112	caveolin 1, caveolae protein, 22kDa
Integrin linked kinase signaling		
VIM	-2.819	vimentin
ITGB5	-1.832	integrin, beta 5
MMP9	-1.538	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)

Symbol	Fold Change	Gene Name
SNAI2	3.166	snail homolog 2 (Drosophila)
PPAP2B	3.222	phosphatidic acid phosphatase type 2B
MUC1	3.386	mucin 1, cell surface associated
Notch Signaling		
CNTN1	-2.024	contactin 1
HES7	-1.445	hairy and enhancer of split 7 (Drosophila)
DLL3	-1.421	delta-like 3 (Drosophila)
DTX2	1.189	deltex homolog 2 (Drosophila)
PSEN2	1.213	presenilin 2 (Alzheimer disease 4)
NCSTN	1.519	nicastatin
Wnt Beta-Catenin Pathway		
SOX9	-1.861	SRY (sex determining region Y)-box 9
CD44	-1.363	CD44 molecule (Indian blood group)
CSNK1D	-1.185	casein kinase 1, delta
WNT5A	2.076	wingless-type MMTV integration site family, member 5A
MMP7	5.236	matrix metalloproteinase 7 (matrilysin, uterine)
DKK1	7.548	dickkopf homolog 1 (Xenopus laevis)

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