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Amphiregulin (areg) And Epidermal Growth Factor (egf): Disparate In Egfr Signaling And Trafficking

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AMPHIREGULIN (AREG) AND EPIDERMAL GROWTH FACTOR (EGF): DISPARATE IN EGFR SIGNALING AND TRAFFICKING

by

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Advisor Date

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DEDICATION

For Tressa Jo VanAnda, whose memory and friendship was my inspiration to study and change what we know about cancer. For my mother, Carolyn, who believed in and urged me to peruse my dreams, and whose love, support, and encouragement have been essential to my success and a source of inspiration. To my father, David, who never judged and always supported my decisions. For my sisters, Dayna and Renee, who have been my emotional anchors and my biggest cheerleaders, not only throughout graduate school, but throughout my entire life. To my entire family and all my friends, I am grateful for your listening, understanding, and encouragement. To all of these people I thank you for giving me confidence in myself and for helping me realize my full potential, may you also be motivated and encouraged to reach your dreams.

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INTRODUCTION

1.1 Epidermal Growth Factor Receptor (EGFR)

A. Discovery and Structure

In 1962 Stanley Cohen reported the isolation of the factor responsible for early development of the incisors and eyelids of mice. He proposed that secretions from the salivary gland contained the growth promoting factor, which he termed epidermal growth factor (EGF) (Cohen, 1962; Schlessinger, 2000). His work began the investigation of EGF and the search for its receptor. Membranes prepared from A-431, human epidermoid carcinoma cells, incubated with ^{125}I-EGF resulted in the binding of EGF to the 150 kDa epidermal growth factor receptor (EGFR). It was later discovered that EGF binding to EGFR resulted in phosphorylation of the membranes (Carpenter, 1979). Because the EGF-dependent kinase activity and the substrates for phosphorylation co-purified with EGFR Cohen noted that EGF-induced kinase activity could be an entity of the receptor (King et al., 1980). EGFR was the first identified receptor tyrosine kinase (RTK). RTKs are a category of transmembrane proteins that undergo ligand-dependent conformational changes in the presence of extracellular ligands. This results in the activation of an intracellular domain with the ability to catalyze the enzymatic transfer of a γ-phosphate from ATP to tyrosine residues in protein substrates. EGFR is the model by which most RTK-induced cellular processes in eukaryotes are studied (Schlessinger, 2000).

In 1984 Ullrich et. al. cloned and sequenced the complete human EGFR precursor from A-431 cells, which revealed direct homology to the avian erythroblastosis virus (AEV) v-erbB oncogene (Ullrich et al., 1984). The v-erbB oncogene is located on chromosome 7p11-13 and spans 110kbp of DNA. The gene contains 26 exons and transcribes a gene that translates into a 1210 amino acid (AA) precursor protein that undergoes cleavage resulting in a mature 1186 AA protein. There are 12 possible N-linked glycosylation sites accounting for 20% of the 170 kDa protein's molecular weight (Jorissen et al., 2003).

EGFR is one member of the ErbB family (ErbB2, ErbB3, ErbB4) of RTKs. Through structural analysis, EGFR, like each of its family members, was found to have four primary domains: a putative signal sequence (24AA), an extracellular domain (621 AA), a transmembrane domain (23AA), and a cytoplasmic domain (542AA) (Ullrich et al., 1984). The signal sequence directs the post-translational transport of EGFR to the cell surface, but is cleaved off resulting in the mature from of EGFR. The extracellular domain of EGFR, and each of the ErbB family members, contains four sub-domains (I, II, III, IV) (Garrett et al., 2002; Ogiso et al., 2002). Activation of ErbB receptors is initiated by the binding of a ligand to the extracellular domain of the receptor. Ligand binding is associated with sub-domains I, at the amino-terminal, and III. Sub-domains I and III are flanked by the Cysteine-rich sub-domains II and IV (Lax et al., 1989; Lax et al., 1988). Sub-domains II and IV, in the presence of a ligand, are able to exhibit an auto-inhibitory interaction modulating the angle and affinity of the ligand binding cleft (Cho and Leahy, 2002; Garrett et al., 2002; Ogiso et al., 2002; Schlessinger, 2000). The

various binding affinities of EGFR ligands may be explained by the conformational change and change in orientation of the ligand binding domain (Cho and Leahy, 2002). Additionally, sub-domain II, with possible hinge-like action, creates an open conformation necessary for receptor dimerization, and sub-domain IV is necessary for EGFR localization to the caveolae/rafts in the plasma membrane (Garrett et al., 2002; Heldin, 1995; Ogiso et al., 2002; Yamabhai and Anderson, 2002). Each receptor has a transmembrane domain, which plays a passive role in signal transduction, but may have a more important role in receptor dimerization (Heldin, 1995; Ullrich and Schlessinger, 1990). Just inside the plasma membrane the transmembrane domain is linked to the juxtamembrane region. It is the juxtamembane portion of EGFR that anchors the transmembrane domain to the plasma membrane (Earp et al., 1995). The cytoplasmic domain is comprised of the tyrosine kinase domain and the c-terminal tail containing numerous tyrosine phosphorylation sites (Ullrich and Schlessinger, 1990). In addition to tyrosine phosphorylation, EGFR is phosphorylated at serine (Ser) and threonine (Thr) residues.

B. ErbB family members

All ErbB family members in their inactive state exist as monomers on the plasma membrane. Each family member is capable of undergoing homo- and hetero-dimerization, a necessary step for activation and down-stream signaling.

ErbB2 was discovered due to its homology to EGFR; in fact, EGFR and ErB2 exhibit the highest homology among all family members (Coussens et al., 1985; King et

al., 1985). ErbB2, which has no identified ligand, contains a constitutively exposed dimerization loop (Garrett et al., 2002; Ogiso et al., 2002). In this regard, ErB2 is the predominant dimerization partner for each of the ErbB family members and is able to form dimers spontaneously resulting in cell transformation due to over expression alone (Graus-Porta et al., 1997; Klapper et al., 1999; Lemmon, 2009). On the contrary, Erb3, discovered in 1989, has low intrinsic kinase activity (Dubois and Guyot, 1994). This suggests that ErbB2 and ErbB3 require interactions with other family members, as compared to being able to contribute to linear signaling pathways (Yarden and Sliwkowski, 2001). ErbB4, which has kinase activity, was the last family member to be discovered (Plowman et al., 1993) and has identifiable ligands, the nergulins (Stove and Bracke, 2004).

C. EGFR Activation

The majority of research on mechanisms involved with EGFR activation and signaling has utilized the A-431 cell model, from which the human receptor was originally identified. EGFR activation is initiated by ligand binding to the extracellular domain of the receptor. There are seven ligands (to be discussed) that bind to and activate EGFR: epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), amphiregulin (AREG), heparin binding-epidermal growth factor (HB-EGF), betacellulin (BTC), epiregulin (EREG), and epithelial mitogen (EPGN). Upon ligand binding, EGFR homo-dimerizes (with another EGFR monomer) or hetero-dimerizes (with another ErbB family member) resulting in autophosphorylation of the intracellular

cytoplasmic domain thus increasing the enzymatic activity of the tyrosine kinase domain (Hubbard et al., 1998; Schlessinger, 2000). EGFR bound with ligand undergoes a conformational change allowing for dimerization and exposing tyrosine residues, lying on an "activation loop" in the catalytic domain of the protein kinase domain, to ATP and protein substrates. The activated EGFR tyrosine kinase domain catalyzes the transfer of the γ-phosphate of ATP to tyrosine residues in substrate proteins, including the C-terminal and kinase domains of EGFRs dimerization partner (Fig. 1.1) (Hubbard et al., 1998; Schlessinger, 2000; Ushiro and Cohen, 1980; Yarden and Schlessinger, 1987).

Phosphorylation of EGFR occurs at six tyrosine sites (Y701, Y845, Y891, Y920, Y976) in the kinase domain and six tyrosine sites (Y992, Y1045, Y1068, Y1086, Y1101, Y1148, Y1173) in C-terminal domain, of which some are auto-phosphorylation sites (Jorissen et al., 2003). These events may directly initiate signaling events or provide docking sites for adaptor molecules with Src-homology (SH2) or phosphotyrosine binding (PTB) domains (Pawson and Gish, 1992). The docking sites allow for binding of a variety of adaptor molecules: growth-factor receptor bound 2 (Grb2) and phospholipase C (PLC)- gamma, phosphatases: SHP-1 and PTB-1B, and kinases: Src and Abl (Fig. 1.1) (Sebastian et al., 2006). In one case, it was demonstrated that the C-terminal domain is not necessary for full activation of EGFR signaling pathways when EGF is the activating ligand. However when AREG induces EGFR activation the c-terminal domain is required for mitogenic signaling. The differences observed in ligand activation have been attributed to EGF/EGFR promotion of ErbB2 hetero-dimeriztion as compared to AREG/EGFR (Wong et al., 1999). This would suggest that the tyrosine residues in the

kinase domain have more importance in EGFR signaling. Although many of the tyrosine sites are autophosphorylated, non-tyrosine kinases (NTRK) such as c-src are able to phosphorylate EGFR by associating with phosphorylated EGFR, and subsequently phosphorylating EGFR at other sites. However, NTRK phosphorylation does still require ligand binding and receptor dimerization (Lombardo et al., 1995; Stover et al., 1995).

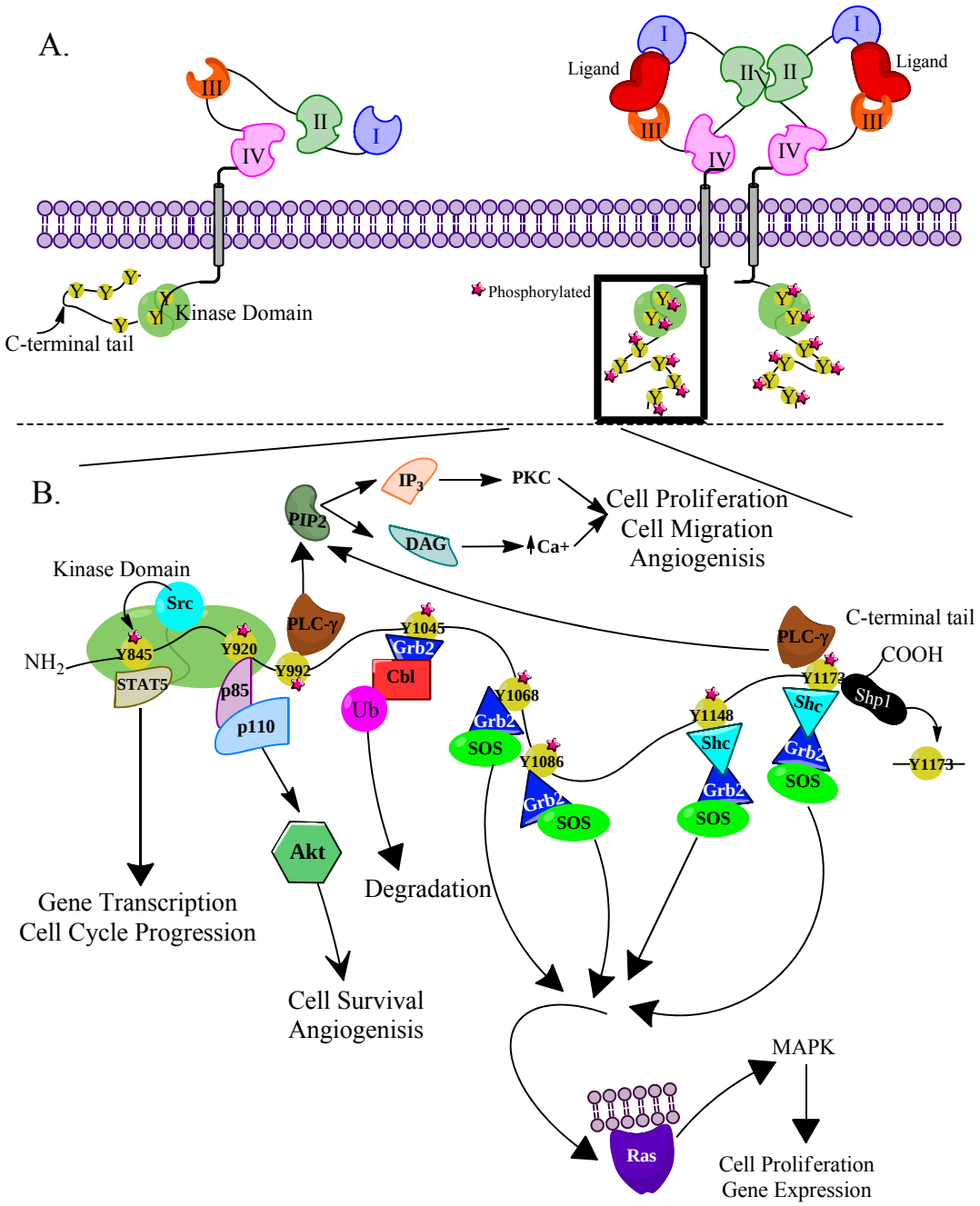


Figure 1.1: EGFR Structure, Activation, Signaling Pathways

A. In the basal state, EGFR exists as an inactive monomer (and possible as an inactive pre-dimer). Ligand binding results in a conformational change exposing tyrosine residues on the C-terminus to be phosphorylated. B. Tyrosine phosphorylation of the kinase and C-terminal domains allows for adaptor molecules to bind and initiate various signaling cascades.

Although it is generally accepted that EGFR kinase activity requires both ligand binding and dimerization, there are conflicting studies supporting the idea that ligand binding is required for receptor dimerization. It has been reported that unligated EGFR exists as predimers on the cell membranes (Clayton et al., 2007; Gadella and Jovin, 1995; Sako et al., 2000; Teramura et al., 2006). This provides evidence for the aggressive nature of cellular transformation and suggests EGFR activation and dimerization are independent events (Van de Vijver et al., 1991; Yu et al., 2002).

D. EGFR signaling

EGFR associates with numerous proteins, hence activates multiple signaling pathways. After ligand binding, tyrosine phosphorylation events occurring on the tyrosine kinase domain and the C-terminal tail provide binding sites for adaptor molecules containing SH2 and PTB domains (Pawson and Gish, 1992). Binding of a specific adaptor molecule promotes a distinct signaling pathway or pathways.

The Ras/Raf/MEK/MAPK pathway is the most extensively studied EGFR signaling pathway. Signaling from this pathway results in a cascade of events predominantly correlated with mitogenisis. Following ligand binding, EGFR recruits adaptor molecule Grb2. Grb2 interacts directly with EGFR at Y1068 and Y1086 or indirectly by binding to Y1173-bound adaptor molecule; Shc. Grb2 then recruits a G-protein activating protein (GAP), son of sevenless (SOS), to EGFR and activates H-, N-, or K- Ras proteins (Buday and Downward, 1993; Lowenstein et al., 1992; Sieh et al., 1994). Activated membrane-associated Ras, in turn, activates, the protein kinase, Raf,

which then activates the MAPK pathway. Mitogen activated extracellular signal regulated kinase (MEK1/2) is activated via phosphorylation that then activates kinases ERK1/2 (MAPK). Activated MAPK ultimately results in the phosphorylation of nuclear transcription factors, such as c-Myc, c-fos, c-jun, Elk1, and ribosomal subunit kinase (RSK), involved in the regulation of cellular proliferation (Fig 1.1) (Marshall, 1994; Marshall, 1996; Pawson, 1995).

In addition to the MAPK pathway, EGFR activates the pro-survival phosphatidylinositol 3-kinase Ia (PI3K Ia)/AKT signaling pathway. PI3K is composed of a regulatory and catalytic subunit that form heterodimers: p85α and p110α, respectively (Carpenter et al., 1990). Upon cellular stimulation, p85α is recruited to Y920 on the C-terminal domain of EGFR. P85α interacts with EGFR via an SH2 domain thus positioning p110α so that it can interact with its substrates and relieving the p85α mediated inhibition of the p110α subunit. p110α is then able to catalyze the production of phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 recruits Protein Kinase B (PKB), also known as AKT, to the cell membrane via its pleckstrin homology (PH) domain (Jones et al., 1991; Stover et al., 1995). AKT becomes phosphorylated on Thr 308, in the kinase domain, by phosphatinositide-dependent kinase 1 (PDK1). In addition, for full AKT activation Ser 473, on the regulatory tail, needs to be phosphorylated. The mechanism, by which Ser 473 is modified is not clearly defined. Indeed it had been hypothesized that S473 may be autophosphorylated, or modified by PDK-2 or integrin-linked kinase (ILK). Recently, it has been accepted that S473 modification is a result of mTORC2 (Delcommenne et al., 1998; Nicholson and Anderson, 2002; Sarbassov et al.,

2006; Toker and Newton, 2000). Activated AKT promotes cell survival through phosphorylation and inhibition of the pro-apoptotic proteins Bad and Caspase-9 (Fig. 1.1) (Cardone et al., 1998; Datta et al., 1997; Franke et al., 1997).

Phospholipase C gamma (PLC-γ) is a member of a family of enzymes catalyzing the hydrolysis of PIP2 into secondary messengers: phosphatidic acid,1,2-diacylglycerol (DAG) and inositol 1,3,5-triphosphate (IP3), thereby contributing to cell migration and pro-proliferative signaling pathways (reviewed in (Rhee et al., 1989)). PLC-γ interacts directly with EGFR at Y992, Y1173, and indirectly through binding with Grb2-associated protein 1 (Gab1). Gab1, in addition to interacting with PLC-γ, is capable of interacting with PI3K subunit p85α (Holgado-Madruga et al., 1996).

Moreover, EGFR is capable of activating signal transducers and activators of transcription (STAT) pathways (Darnell et al., 1994). STAT activation, by cytokine signaling is mediated by Janus kinase (JAK) dependent pathways. However, STAT activation by EGFR signaling is mediated by JAK independent pathways (Andl et al., 2004; Kloth et al., 2003). JAK, like Src, is a NRTK. In the JAK independent pathway, ligand-dependent EGFR activation induces phosphorylation of STAT1 at Y701 and STAT3 at Y705. These events lead to STAT dimerization. The complex translocates to the nucleus activating transcription of genes associated with cell survival (Andl et al., 2004; Leaman et al., 1996). In some instances STATs can be constitutively associated with EGFR, however their activation is dependent on EGFR tyrosine kinase activity (David et al., 1996; Olayioye et al., 1999; Xia et al., 2002). More recently, Yang et. al., in the JAK-independent pathway, found STAT5b to associate with Y845 on EGFR,

possibly down-stream or independent of Src kinase (Kloth et al., 2003; Yang et al., 2008).

Src is non-receptor tyrosine kinase that also plays a role in EGFR signaling. Src phosphorylates EGFR at Y1101 and Y845 (Maa et al., 1995; Tice et al., 1999). Phosphorylation at EGFR-Y845 promotes EGFR-induced mitogenesis, and the response to phosphorylation of EGFR-Y1101, a residue not conserved in the ErbB family in vivo, remains unclear (Biscardi et al., 1999; Stover et al., 1995; Tice et al., 1999). Still, the exact location where src binds is unknown; however, it is thought to bind in the kinase domain.

E. EGFR trafficking, localization, and degradation

EGFR in its basal state is predominantly localized to the plasma membrane in lipid rafts/caveolae (Yamabhai and Anderson, 2002). Inactive receptors are constitutively internalized, transported to early endosomes (EE), and in most cases recycled back to the membrane. The half-life of inactive EGFR is 10-14 hours in normal epithelial cells, while in transformed cells the half life is much longer (20-48 hours) (Wiley, 2003). EGF activated EGFR, via ligand binding and receptor dimerization, enhances EGFR internalization (Sorkin and Goh, 2008; Wiley et al., 1991). This acceleration is due to endocytosis of EGF/EGFR complexes through clathrin-coated pits. Clathrin-mediated endocytosis (CME) is a process whereby EGFR is internalized. Notably, EGFR was shown to undergo internalization via a slower, non-clathrin endocytosis (NCE) pathway as well. The pathway by which EGFR endocytoses is

dependent on the concentration of EGF. In general, EGFR is internalized by the CME pathway when EGF concentrations are low and the NCE pathway when EGF concentrations are high (Sigismund et al., 2005).

Upon EGFR activation, a Grb2/Cbl complex is recruited to phosphorylated Y1045. Cbl proteins are E3 ubiquitin ligases containing a phosphotyrosine binding domain and a C3HC4 RING finger. The Grb2/Cbl/EGFR interaction promotes the recruitment of ubiquitin conjugating enzymes in order to mono-ubiquitinate EGFR. The ubiquitination is recognized by Esp15, Esp15R, and Epsin proteins associated with AP-2. The receptor and associated ligand are then internalized via clathrin coated pits and localized to EE. In the case where EGFR is recycled, the ligand dissociates from the receptor promoting EGFR's return to the plasma membrane via recycling endosomes. When the fate of EGFR is degradation, EGFR remains in the EE. The EEs mature into mutivesicular vesicular bodies (MVB) and late endosomes (LE) where EGFR interacts with a ternary complex composed of EPS15, signal transduction molecule (STAM), and hepatocyte-growth-factor regulated tyrosine-kinase substrate (HRS) (Katzmann et al., 2003). The associated EGFR complex is directed to tumor susceptibility gene-101 (TSG1) and the endosomal sorting complex required for transport-I (ESCRT) complex and EGFR is incorporated into internal vesicles in the MVBs. The MVB fuses with a lysosome, consisting of a highly acidic pH, where EGFR and its associated ligand are degraded (Fig. 1.2) (Sorkin et al., 1991). In one study it has been shown that EGFR can be polyubiquitnated and degraded in the proteosome (Levkowitz et al., 1999).

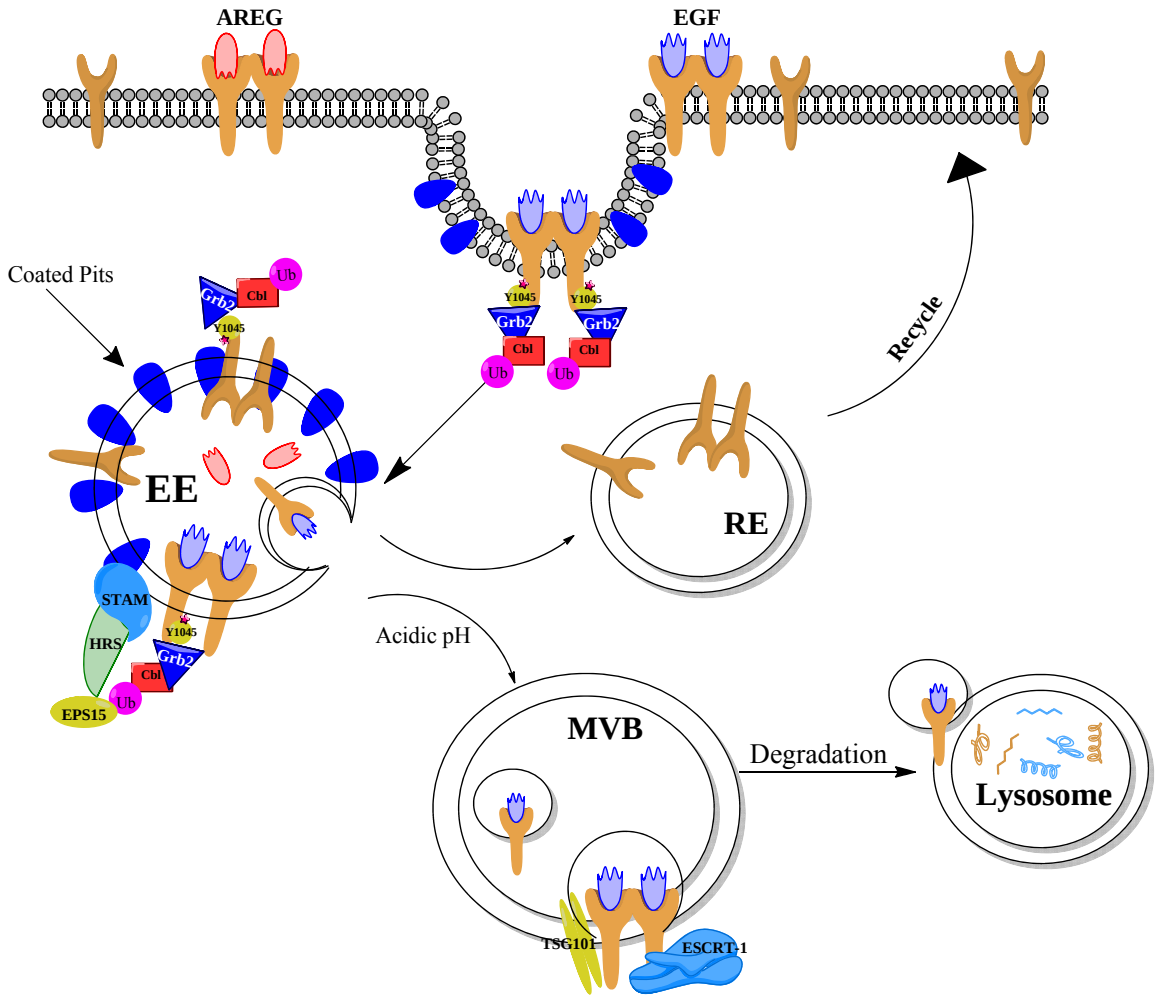


Figure 1.2: EGFR Internalization, Trafficking, Localization
Inactive EGFR is internalized and recycled back to the membrane. When EGFR is stimulated by EGF EGFR is ubiquitinated by cbl and transported to EE. The receptor associated with a complex and moves to the MVB and lastly to the lysosome for degradation. AREG stimulation of EGFR induces EGFR internalization and trafficking to the RE where EGFR is recycled back to the cell surface.

Most of the studies regarding EGFR trafficking have been done with EGF. Not as much is known about trafficking and EGFR localization when EGFR associates with its

other growth factors. TGF-α stimulation of EGFR is the second-most well understood mechanism of EGFR trafficking. TGF-α, unlike EGFR, rapidly dissociates from EGFR in the EE due to the low pH (Ebner and Derynck, 1991). At this time, EGFR is de-ubiquitinated and recycled back to the cell surface (Decker, 1990; Longva et al., 2002). A recent paper by Roepstorff et. al. studied the differential effects of EGFR ligands on endocytic sorting. In this study it was shown that EREG stimulates internalization followed by rapid EGFR recycling to the cell surface. Coinciding with the recycling phenotype, Y1045 phosphorylation and ubiquitination levels were low. In contrast, BTC stimulation resulted in persistent Y1045 phosphorylation, c-Cbl recruitment and EGFR ubiquitnation thus supporting EGFR degradation (Roepstorff et al., 2009).

Studies done in the SUM-149 breast cancer cell line demonstrated AREG stimulation of EGFR resulted in low levels of Y1045 phosphorylation, internalization, and ubiqutination (Willmarth and Ethier, 2008). Moreover, AREG promotes EGFR to be rapidly recycled to the plasma membrane (Baldys et al., 2009). Interestingly, AREG is highly resistant to the acidic pH present in the EE, suggesting that relatively low binding affinity of AREG may cause the dissociation and recycling of EGFR (Roepstorff et al., 2009).

In addition to proteins having the capability of inducing EGFR endocytosis, some proteins such as Sprouty2 have been shown to negatively regulate EGFR internalization by interacting with c-Cbl. Sprouty2 associates with Grb2 and c-Cbl at the RING domain inhibiting E3 ligase function, thereby reducing EGFR ubiquitination and internalization (Hanafusa et al., 2002; Rubin et al., 2003). Cool-1, Tbc1d3, GAPex5, intersectin, and

CIN85 are among other c-Cbl interacting proteins that interrupt EGFR endocytosis (Feng et al., 2006; Martin et al., 2006; Soubeyran et al., 2002; Su et al., 2007; Szymkiewicz et al., 2002; Wainszelbaum et al., 2008). Other proteins that can either inhibit or promote EGFR internalization, aside from c-Cbl, include: Na+/H+ exchanger regulatory factor 1 (NHERF1), Ymer, Spartin, and Cdc42-associated tyrosine kinase 1 (ACK1) (Bakowska et al., 2007; Lazar et al., 2004; Shen et al., 2007; Tashiro et al., 2006).

F. EGFR and Cancer

EGFR is a proto-oncogene. Mice deficient in EGFR exhibit defects ranging from death at mid-gestation, to survival for up to three weeks, depending on the genetic background of the mutant mice. Surviving mice had abnormalities in the liver, skin, lung, brain, and GI tract prior to early death (Miettinen et al., 1995; Sibilia et al., 1998). Characteristic of an oncogene, EGFR is essential to normal development and physiology in the cell, but when altered by mutations or over expression, EGFR induces cellular transformation. The v-erbB viral oncogene, lacking most of the extracellular domain, has direct homology to human EGFR as seen after EGFR was cloned and sequenced (Ullrich et al., 1984). It was this line of evidence that implicated EGFR in tumor progression (Downward et al., 1984).

Classically, cancer transformation has been described by the ability of a transformed cell to acquire six “hallmarks” of cancer. These hallmarks include: cell invasion and metastasis, sustained angiogeneisis, limitless replicative potential, evading apoptosis, self-sufficiency in growth signals, and insensitivity to anti-growth signals

(Hanahan and Weinberg, 2000). Now, ten years later, cellular transformation is continually described by these hallmarks, but with the addition of six more defining characteristics: evading immune surveillance, metabolic stress, proteotoxic stress, mitotic stress, oxidative stress, and DNA damage stress (Luo et al., 2009). EGFR regulates many of these characteristics in a variety of cancers.

Aberrant EGFR activity can contribute to *in vitro* transformation and *in vivo* tumorgenisis. This may be the consequence of one or more of the following: EGFR over expression and/or mutation, alterations in dimerization, increased ligand expression or release, disrupted trafficking and turnover of inactive receptors, or deficiency in inactivating phosphatases (Blume-Jensen and Hunter, 2001; Salomon et al., 1995a; Sebastian et al., 2006). Over expression of EGFR occurs by amplification and/or increased transcription. EGFR over expression without EGFR amplification is one of the most frequently observed mechanisms in EGFR mediated cancer progression (Athale and Deisboeck, 2006). SUM-149 breast cancer cells are one example where this mechanism is observed along with the presence of an AREG/EGFR autocrine loop, thereby leading to EGFR stimulation by AREG and EGFR over expression (Willmarth and Ethier, 2006). Indeed another frequently observed mechanism of EGFR over expression is the presence of a TGF-α autocrine loop (TGF-α induces EGFR recycling) (El-Obeid et al., 2002).

i. EGFR in breast cancer

EGFR expression is observed in head and neck, breast, renal, lung, colon, ovarian, prostate, pancreatic, bladder, and brain cancers. For the purpose of this dissertation the focus will be on breast cancer. In the 1980's experiments carried out by Sainsbury and co-workers first identified an inverse relationship between EGFR and Estrogen Receptor (ER) status (Sainsbury et al., 1987). More over, this group found a significant association between ER and EGFR expression and tumor size and differentiation; EGFR-positive breast cancers showed significantly worse overall and relapse-free survival (Sainsbury et al., 1987). It became clear that the histological sub-type of breast cancer could indicate the prognosis of the disease (Sainsbury et al., 1988). By ^{125}I-labelling EGF, three sub-types of breast cancer were identified; one, EGFR-positive and ER-negative, another EGFR-negative and ER-positive, and lastly a group that was EGFR-positive and ER-positive. As seen in their previous studies, overall and relapse-free survival was nearly 2 years shorter for the EGFR-positive tumors (Harris et al., 1989). In 1992, Harris et. al. found the EGFR and ErbB2 had adverse additive effects and that ErbB2 was a marker of poor prognosis and lack of response to endocrine therapies (Harris et al., 1992).

The work of Harris et al. led to the elucidation of breast cancer sub-types by the use of high-throughput molecular biology techniques. Based on hierarchical clustering of 65 breast cancers compared to normal breast tissue, breast cancer is categorized into four subtypes: normal-like, luminal (A and B), ErbB2 positive, and basal-like (Perou et al., 2000). These subtypes have been validated by Sorlie et al. in 2001 and 2003, further

characterizing the luminal type into two subtypes, types A and B (Sorlie et al., 2001; Sorlie et al., 2003).

Normal-like breast tumors, as expected, have genetic expression similar to those of normal basal epithelial cells and adipose cells (Perou et al., 2000). Luminal breast cancers are defined as two groups, A and B (Sorlie et al., 2003). Luminal A cancers are 96% positive for estrogen receptor (ER) alpha expression and contain high levels of luminal cell keratins 8/18 (Perou et al., 2000). Luminal B cancers along with ER expression include the expression of ErbB2 (Sorlie et al., 2001). While luminal A cancers have a better prognosis than luminal B cancers, both contribute to the significant decline in breast cancer mortalities observed since the 1990s. The expression of ER in the luminal subtype and the introduction of targeted therapies with selective ER modulators (SERMs), such as tamoxifen, are likely the factors responsible for the better prognosis. ErbB2 positive breast cancers are defined by the expression of ErbB2, Grb7 and little, if any, ER (Perou et al., 2000). These cancers tend to have a worse prognosis than luminal breast cancers (Sorlie et al., 2001). However, due to ErbB2 targeted therapy with Trastuzumab, a monoclonal antibody, ErbB2-positive tumors have a better prognosis than the last subtype, the basal-type (Romond et al., 2005; Sorlie et al., 2003).

Basal-type breast cancers represent approximately 15% of all breast cancers and continue to have the worst prognosis (Perou et al., 2000). They are characterized by the expression of cytokeratin 5/6 and 17, fatty acid-binding protein 7, integrin β4 and laminin. Typically, basal-type breast cancers lack the expression of ER, ErbB2, and progesterone (PR). Therefore, as of today, patients are not able to undergo targeted

chemotherapies. Many of the characteristics observed in triple negative, basal-type breast cancers are due to BRCA1 mutations, making a portion of the basal-type a hereditary disease (Sorlie et al., 2003).

ii. Targeting EGFR in breast cancer

EGFR is expressed in 14-91% of breast cancers (Reviewed by (Grandis and Sok, 2004)). The large variance is likely due to the variety of techniques: autoradiography, immunoctyochemistry, immunoenzymatic assay, and gene transcript analysis, utilized in analyzing EGFR expression in breast cancer. By immunocytochemistry, EGFR has been observed to be over-expressed in 60% of basal-type breast cancers (Livasy et al., 2006). Beginning in the 1980s, EGFR was targeted in cancer therapeutics. In 1990, the first clinical trial of an anti-EGFR agent was initiated. In lung cancer patients, monoclonal antibody 225 (Mab225/cetuximab) was found to inhibit EGFR activity. Anti-EGFR antibodies are designed to bind to the extracellular domain of EGFR, therefore preventing ligand binding and cell proliferation (Grunwald and Hidalgo, 2003). Following the development of cetuximab, a number of other monoclonal antibodies entered the clinic: ABX-EGF, EMD7200, and h-R3 (Crombet-Ramos et al., 2002; Rowinsky et al., 2004; Vanhoefer et al., 2004). After the development of monoclonal antibodies, a new class of chemicals, anilinoquinazolines, with similar effects were produced (Ward et al., 1994). These small molecule inhibitors, known as tyrosine kinase inhibitors (TKIs), block the activation of EGFR by interacting with the tyrosine kinase domain resulting in inhibition of EGFR autophosphorylation (Ciardiello et al., 2000). It was not until the 21st century

when the EGFR TKI gefitinib (Iressa) was used in clinical trials and showed promising anti-tumor activity (Fukuoka et al., 2003; Kris et al., 2003). Gefitinib was approved for treatment in non-small cell lung carcinoma (NSCLC) in Japan and one year later the FDA approved gefitinib in the United States. However after clinical trial data demonstrated no significant survival benefit with the use of gefitinib, the drug lost FDA approval and was removed from the market. Similar TKI's such as erlotinib, which can inhibit wild-type EGFR at lower concentrations than gefitnib, are now currently used as they do significantly increase overall survival.

When cancers have EGFR mutations (ie: in lung cancers) there is success in EGFR targeted therapies. In the instance of breast cancer, where EGFR is present in wild-type form, cancers become resistant to the present targeted therapies. (Arteaga, 2003; Osborne et al., 2003). EGFR activation, signaling, trafficking, and degradation mechanisms are complex. A deeper understanding of these mechanisms will advance the field for treatment options in not only breast cancer, but a diverse amount of epithelial cancers.

G. EGFR Ligands

Currently there are six known ligands, aside from AREG, that bind to and activate EGFR. All ligands are type I transmembrane proteins composed of common extracellular, transmembrane, and cytoplasmic domains. The sequence homology between all ligands is approximately 25% (Harris et al., 2003). These differences are

accounted for by the presence of N-terminal extensions, heparin binding domains, differential glycosylation sites, and other biochemical characteristics.

i. Epidermal Growth Factor: EGF (1960)

EGF was the first EGFR ligand discovered in the 1960s. Cohen et. al. isolated EGF, which induced premature eyelid opening and tooth eruption, from the submaxillary gland of mice (Cohen, 1962). EGF is unique compared to the other ligands in that it has nine EGF-like domains (David et al., 1996; Olayioye et al., 1999; Xia et al., 2002). It is located on chromosome 4q25, spanning 99.4 kb of DNA. After post-translational modifications, the mature EGF protein is composed of 53 residues with a molecular weight of about 6 kDa (Kajikawa et al., 1991; Taylor et al., 1970). EGF is produced in a variety of human tissues and in human milk (Connolly and Rose, 1988). Northern Blot and immunohistochemistry studies have demonstrated EGF mRNA and protein expression in adult epithelial cells and the gastrointestinal tract (Connolly and Rose, 1988; Kajikawa et al., 1991). Mice lacking EGF have developmental effects in prostatic epithelial bud formation (Abbott et al., 2003).

ii. Transforming Growth Factor-alpha: TGF-α (1978)

TGF-α was the second EGFR ligand to be found. Its discovery was the result of protein isolation from the conditioned medium of murine sarcoma virus transformed cells (de Larco and Todaro, 1978). TGF-α is located on chromosome 2p11-13, spanning 70-100 kb of DNA. Mature TGF-α yields a 50 residue protein, ranging from 6-20 kDa in

size due to post-translational modifications (Teixido and Massague, 1988). Expression of TGF-α has been demonstrated during embryogenesis and in numerous adult tissues, especially those involved in regeneration and epithelial stem cell populations (Kudlow and Bjorge, 1990). TGF-α -/- mice are viable, but have lens and retinal abnormalities along with a noticeable phenotype change that is demonstrated by waviness of their coat and whiskers (Berkowitz et al., 1996; Mann et al., 1993).

iii. Heparin Binding-Epidermal Growth Factor: HB-EGF (1991)

Initially, HB-EGF was isolated from the conditioned medium of macrophage-like U-937 cells (Higashiyama et al., 1991). It is a unique family member in that membrane anchored HB-EGF acts as a specific receptor for the diphtheria toxin (Iwamoto et al., 1994; Naglich et al., 1992). HB-EGF is located on chromosome 5q23 and spans 15.28 kb of DNA (Freeman et al., 1997; Nishi and Klagsbrun, 2004). The mature form of the protein comprises 68 to 87 residues and has a molecular weight of 4-22 kDa following post-translational modifications. Expression of HB-EGF has been observed in a variety of mammalian tissues, such as bladder, smooth muscle, skin, heart, and lung; however it is not expressed in endothelial cells (Abraham et al., 1993; Freeman et al., 1997). HB-EGF -/- mice form enlarged and malformed semilunar and atrioventricular heart valves and poorly differentiated lungs (Iwamoto et al., 2003; Mine et al., 2005).

iv. Betacellulin: BTC (1993)

BTC was isolated from the conditioned medium of a mouse pancreatic β insulinoma (Shing et al., 1993). A novel characteristic of BTC, along with HB-EGF, is its ability to bind both EGFR and ErbB4 (Dunbar and Goddard, 2000; Elenius et al., 1997; Riese et al., 1996). BTC not only activates two of the ErbB family members, but is capable of initiating all possible combinations of heterodimeric ErbB receptors (reviewed by (Dunbar and Goddard, 2000)). BTC is located on chromosome 4q13-q21 and spans 48.4 kb of DNA (Harris et al., 2003). BTC protein is 32 kDa in size after post-translational modifications and is composed of 80 residues (Shing et al., 1993). BTC is expressed in most adult tissues, but is especially high in the pancreas, liver, kidney, and small intestine (Sasada et al., 1993; Seno et al., 1996). In addition, expression is found in bodily fluids including milk (Dunbar et al., 1999). Mice lacking BTC expression were viable and did not appear to have any growth abnormalities, even in the pancreas (Jackson et al., 2003).

v. Epiregulin: EREG (1995)

EREG was first isolated from the conditioned medium of the mouse tumor cell line NIH3T3/clone T7. EREG does not bind heparin (Toyoda et al., 1995b). EREG is located on chromosome 4q13.3 and spans 4.8 kb of DNA (Toyoda et al., 1997). The molecular weight of the mature protein, as seen by radiolabelling conditioned media, is 5 kDa representing 46 AA residues (Toyoda et al., 1995a; Toyoda et al., 1997). Compared to the other EGFR ligands, EREG is unique in that it has relatively low levels of expression in normal adult tissue. However, EREG expression was found in peripheral

blood, macrophages, and the placenta (Toyoda et al., 1997). EREG -/- mice were viable and proved to have no abnormalities in growth or reproduction, but were more susceptible to transformation when intestinal damage was induced with dextran sulfate sodium (Lee et al., 2004).

vi. Epithelial Mitogen: EPGN (2001)

The epithelial mitogen, EPGN, was identified from a mouse keratinocyte cDNA library due to its homology to EREG (Strachan et al., 2001). It is the most recent EGFR ligand to be discovered. It is a unique growth factor in that it elicits high mitogenicity while having low affinity to EGFR (Kochupurakkal et al., 2005). EPGN's transcript spans 5kb of DNA and is located on chromosome 4q13.3, only 25kbp away from EREG's open reading frame (Kochupurakkal et al., 2005). The mature protein is processed to 86 residues and has a molecular weight of 8 kDa. EPGN is expressed in numerous mammalian tissues, most highly in tissues of the developing embryo such as tongue papillae, dorsal root ganglion, and in hair follicles (Kochupurakkal et al., 2005). The *in vivo* effects of EPGN have been observed through generation of transgenic mice over expressing EPGN (Dahlhoff et al., 2010). These mice expressed enlarged sebaceous glands. Knock-out mice remain to be studied.

1.2 Amphiregulin (AREG)

A. Discovery and Structure

AREG is a heparin-binding molecule 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).

Structurally AREG's amino-terminus was characterized by cleavage of an Edman degradated, N-glycancase treated, reduced, and S-pyridylethylated AREG (NG-SPE-AREG). Two amino-terminal sequences emerged; one being six amino acids shorter (78AA) than the larger (84AA) sequence (Shoyab et al., 1989). The 84AA sequence has a lower binding affinity to EGFR and is the higher yielding (20%) sequence, as compared to the 74AA sequence. The carboxyl-terminal sequence was described by cleavage of NG-SPE-AREG with carboxypeptidase P. Both the 78 and 84 AA sequences have similar carboxyl-terminals, and both are biologically active (Shoyab et al., 1989). Directly following Shoyab's initial characterization of AREG, molecular cloning techniques were utilized to further define AREG. Total cellular RNA was isolated from MCF-7 cells and cDNA of AREG was transcribed and analyzed. The 84 AA corresponded to the mature secreted form of AREG (Plowman et al., 1990).

AREG was compared to other EGF-like growth factors and proteins, and like all family members, AREG's bioactive domain is characterized by six essential cysteine

residues, spaced: $CX_7CX_4CX_{10}CX_1CX_8C$, where C represents cystenines and X can be any AA (Fig. 1.3) (Shoyab et al., 1989). Additionally, AREG has 38% homology with EGF and 32% homology with TGF-α (Shoyab et al., 1989). AREG was found to have six exons and five introns spanning 10.2 kb of genomic DNA (Plowman et al., 1990). Exon one contains a 210bp 5'UTR and signal peptide: exon two contains the amino-terminal precursor: exon three contains a very basic and hydrophilic amino-terminal portion and the first two loops of the EGF-like region: exon four contains the third loop of the EGF-like motif and the transmembrane domain: exon five contains the cytoplasmic region: and lastly, exon six contains the 262bp 3'UTR (Plowman et al., 1990). AREG is located on chromosome 4q13-4q21 in close proximity to genes BTC and EREG (Plowman et al., 1990; Sasada et al., 1993; Toyoda et al., 1997). This supports the hypothesis that EGFR ligands evolved through gene duplication events.

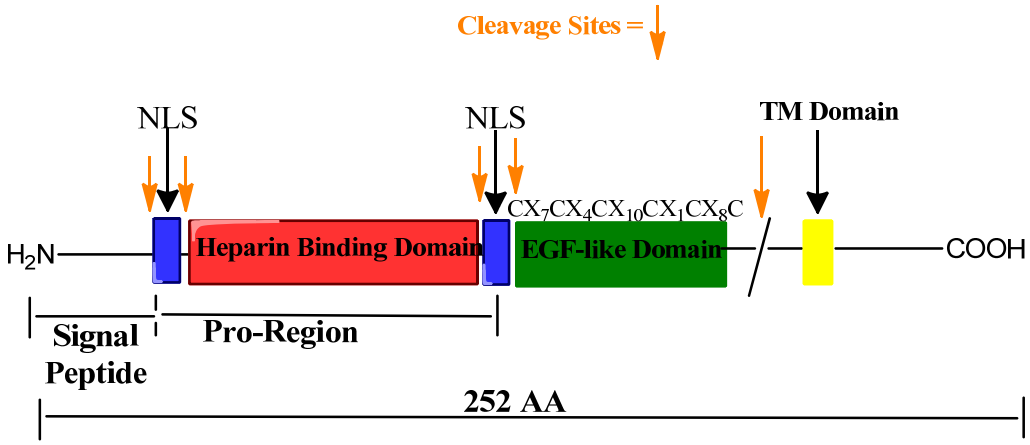


Figure 1.3: AREG Structure and Cell Processing
The structure of the 50 kDa pro-AREG, the location of the nuclear localization signals (NLS), Heparin binding domain, EGF-like domain, and the transmembrane (TM) domain. AREG is processed by ADAM17/TACE at various sites indicated by the orange arrows.

B. AREG isoforms

AREG is synthesized as a 252 amino acid transmembrane precursor that requires proteolytic cleavage for secretion (Fig. 1.3). Proteolytic processing is an important step in EGFR activation by AREG. Tumor necrosis factor alpha (TNF-α) converting enzyme (TACE) has been implicated in the shedding of AREG, TGF-α, HB-EGF, and EPGN from the plasma membrane (Hinkle et al., 2004; Merlos-Suarez et al., 2001; Peschon et al., 1998; Sahin and Blobel, 2007). TACE is a member of the integral membrane proteins and is a Disintegrin and Metalloproteinase (ADAM). It was discovered in the search for the protease responsible for ectodomain shedding of TNF-α (Black et al., 1997). The development of TACE -/- mice demonstrated, *in vivo*, the proteinase's essential role in ectodomain shedding of structurally and functionally diverse membrane-bound proteins (Peschon et al., 1998). The TACE-/- mutation proved to be lethal prior to birth in most cases. Fetuses were examined, and found to have severe epithelial structure developmental defects similar to EGFR-/- and TGF-α-/- mice (Peschon et al., 1998). Similar to defects observed in AREG-/- and HB-EGF-/- mice, TACE-/- mice showed defects in epithelial lung branching, ductal branching in the mammary gland, and heart valve development (Jackson et al., 2003; Luetteke et al., 1999).

Due to proteolytic processing by TACE/ADAM17 and other post-translational modifications, there are several forms of AREG. Cleavage results in two soluble forms of either 78 or 84 amino acids in length, which range 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 protein has four domains: a 43 AA N-terminal pro region, an EGF-like domain, a transmembrane domain, and a cytoplasmic domain. AREG's N-terminal pro region includes the pro-domain, two putative nuclear localization signals (NLSs), and a heparin binding domain. This pro region is rich in prolines, serines, and threonines, making this region highly hydrophilic. Similar to other EGFR growth factors, the N-terminal region has N-linked and O-linked glycosylation sites (Shoyab et al., 1989). The status of AREG protein glycosylation is not important for the bioactivity of the protein, but may be important in the stability, solubility, resistance to proteolysis, or localization of AREG (Rademacher, 1998).

The heparin binding domain (residues 26-44), located within the in pro-region, is necessary for proper folding and secretion of a bioactive AREG. Deletion of the pro-region prevents AREG secretion (Thorne and Plowman, 1994). The heparin binding domain is unique to AREG and HB-EGF. Heparin sulfate proteoglycans (HSPGs) are located on the plasma membrane. The binding of AREG to HSPGs is essential to AREG-triggered mitogenic signaling by EGFR (Johnson and Wong, 1994). HSulf-1, responsible for desulfation of glycosaminoglycans, such as AREG and HB-EGF, is down-regulated in breast and ovarian cancers (Lai et al., 2003); this results in an increased affinity for heparin binding ligands adding to autocrine and paracrine proliferation signals (Narita et al., 2007).

Lastly, within the pro region there is a tetrapeptide sequence, ARG-LYS-LYS-LYS, repeated two times at residues 26-29 and 40-43. This sequence constitutes the NLSs (Shoyab et al., 1989); however the role of AREG in the nucleus continues to remain unclear. NLSs appear to be important in full signaling and co-translocating bound receptors to the nucleus to participate in gene regulation (Jans and Hassan, 1998). This observation corroborates the hypothesis that EGFR functions as a transcription factor in the nucleus (Lin et al., 2001). More recently, truncated pro-AREG has been shown to interact with A-type lamin at the inner nuclear membrane where it participates in chromatin organization and control of transcription (Isokane et al., 2008).

The EGF-like domain is located within 25 residues of the transmembrane domain (Fig. 1.3). Cleavage occurs between the two domains, promoting autocrine and paracrine signaling. The six-cysteine motif previously described is the central and functional feature of AREG and all EGF-like growth factors. It is this motif that associated with EGFR.

The cytoplamsic domain is required for basolateral sorting, guaranteeing proper AREG signaling as seen in Madin-Darby Canine Kidney cells (MDCK). When the cytoplasmic domain was mutated, 65% of the AREG proteins were sorted to the apical surface. However, cleavage of the ligand was not altered by the mutation, suggesting that the C-terminus is not required for processing of the ligand (Brown et al., 2001).

C. AREG expression/regulation

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). During development in the mouse mammary gland, AREG has been shown to play an important role in terminal end bud (TEB) formation and ductal elongation (Ciarloni et al., 2007; Luetteke 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., 1995; Sehgal et al., 1994; Tsai et al., 2006).

In the human AREG promoter, a cyclic adenosine monophosphate (cAMP)-repsonsive element (CRE), a serum responsive element (SRE), a highly conserved specificity protein 1 element (SP1), WT1 responsive element (WRE), and TATA box have been identified (Du et al., 2005; Lee et al., 1999).

Regulation of AREG expression by the CRE site (-267 to -274 nt) has been observed in a cell line, MSK-Leuk1, derived from a dysplastic leukoplakia lesion when exposed to a saline extract containing tobacco smoke (Du et al., 2005). In addition, AREG expression was induced in a CRE-dependent manner in mouse tissue exposed to whole animal hypoxia (O'Reilly et al., 2006). In this pathway, cAMP, a second messenger, phosphorylates Protein Kinase A (PKA) and activates CRE-binding protein (CREB). CREB is a 43 kDa transcription factor which binds to the CRE sequence and, in the instances described here, induces AREG expression (Reviewed by (Mayr and

Montminy, 2001)). Further evidence for cAMP's induction of AREG expression has been demonstrated in response to Prostaglandin E_2 (PGE_2) in colon cancer, T lymphocytes, prostate cancer, breast cancer, and adrenal cells (Johansson et al., 2004; Shao et al., 2003). Parathyroid hormone (PTH), promoter of bone metabolism, is yet another factor that has been shown to induce AREG expression via the cAMP/PKA/CREB pathway in UMR 106-01, osteoblastic cell line (Qin and Partridge, 2005). Lastly, Wilms Tumor Suppressor (WT1) induces the expression of AREG in U2OS, ostesarcoma cells, through an AREG transcriptional activator (Lee et al., 1999).

Recent work in cells derived from cervical cancer, HeLa, demonstrated for the first time AREG expression to be suppressed in response to a transcription factor binding the AREG promoter. Breast cancer-associated 1 (BRCA1), a breast and ovarian tumor suppressor, functions in: transcription, DNA damage repair, and cell cycle regulation (Mullan et al., 2006). BRCA1 binds to the AREG promoter downstream of the TATA Box (-283 to -233nt) at -202 to -182 and +19 to +122, while depletion of BRCA1 resulted in an increase in AREG transcription (Lamber et al., 2010; Lheureux et al., 2010).

Many other molecules have been shown to regulate AREG expression. These molecules include estrogen, androgen, vitamin D3, HOXB9, cytokines: IL-1α and TNF-α, other EGFR growth factors (EGF and TGF-α), and EGFR itself (Akutsu et al., 2001; Hayashida et al., 2010; Kansra et al., 2004; Kitadai et al., 1993; Martinez-Lacaci et al., 1995; Normanno et al., 1994; Sehgal et al., 1994; Streicher et al., 2007; Woodworth et al., 2005). The pathways by which each factor regulates AREG transcription requires

further elucidation; however it is apparent that a variety of signaling molecules can promote AREG expression in a numerous tissues.

D. AREG/EGFR signaling mechanisms and pathways

i. AREG signaling mechanisms

AREG signals with EGFR via three general mechanisms: autocrine, paracrine, and juxtacrine. Autocrine regulation is observed when a soluble ligand is produced by a cell and stimulates the receptor on the cell from which it was made. On the contrary, paracrine signaling occurs when a soluble ligand is produced by a cell and stimulates its receptor on a different cell, independent of the type of cell from which it was shed. Lastly, juxtacrine signaling is defined by stimulation of a receptor on neighboring cells by a ligand that is bound to the cell surface of a neighboring cell.

An AREG/EGFR autocrine loop was first observed in normal human keratinocytes. Heparan sulfate inhibited AREGs ability to compete for binding with EGF to EGFR and reduced EGFR activity and mitogenisis. This led to the initial hypothesis that AREG regulates EGFR activity through an autocrine loop (Cook et al., 1991). Autocrine regulation of AREG has been seen in normal bronchial epithelial cells and normal urothelial cells (Tsao et al., 1996; Varley et al., 2005). In addition to normal cells, an AREG/EGFR autocrine loop has been observed in cancers, including: hepatocellular, colon, gastric, pancreatic, and breast (Akagi et al., 1995; Castillo et al., 2006; Culouscou et al., 1992; Funatomi et al., 1997; Johnson et al., 1992; Willmarth and Ethier, 2006).

Anklesaria et al. defined juxtacrine signaling in mouse bone marrow stromal cells when they found pro-TGF-α expressed on the membranes of stromal cells was able to bind to and activate EGFR on neighboring hematopoeitic progenitor cells (Anklesaria et al., 1990). These data suggest that membrane-anchored growth factors can stimulate adjacent cells, or signal in a juxtacrine manner. TGF-α was confirmed to signal in a juxtacrine fashion in NRK cells when an uncleavable TGF-α was co-cultured with a layer of EGFR expressing cells above them, inducing cell transformation (Brachmann et al., 1989). HB-EGF and AREG have also been shown to signal by this mechanism. HB-EGF, fixed and unable to be shed, enhanced EGFR signaling. In addition to enhanced EGFR signaling in the presence of un-cleavable TGF-α (Takemura et al., 1997; Yang et al., 2000), SUM-149 breast cancer cells, expressing high levels of AREG, were overlain on top of MCF10A cells deprived of EGF, which increased tyrosine phosphorylation of EGFR suggesting that pro-AREG could stimulate EGFR activity (Willmarth and Ethier, 2006).

Only certain EGF-like growth factors are able to signal in a juxtacrine manner. In a study by Dong, et. al. the EGF binding domain of membrane-bound ligands were combined with other various domains of EGF and HB-EGF. Ligands with the membrane-anchoring domain of EGF required proteolytic cleavage for activity. However, the membrane anchoring domain of HB-EGF was sufficient to induce biological activity (Dong et al., 2005). Therefore, it is hypothesized that the juxtacrine mechanism occurs *in vivo* and may induce different signaling cascades upon activation.

ii. AREG signaling pathways

AREG activates the Ras/Raf/MEK/ERK pathway in a variety of cells including: pancreatic duct cells, normal human keratinocytes, bronchial epithelial cells, and bone (Blanchet et al., 2004; Kansra et al., 2004; Qin and Partridge, 2005; Wagner et al., 2002). Upon AREG/EGFR activation and ERK signaling, Elk1, c-jun, and c-fos have been shown to be activated. The C-terminal tail of EGFR is critical for the expression of these down-stream molecules (Wong et al., 1999).

PI3K/Akt signaling can be activated by AREG/EGFR signaling. This cascade promotes cell survival through the up regulation of active NF-κB and IL-1α (Streicher et al., 2007). Aside from AKT and ERK signaling, AREG has been shown to induce STAT 1, 3, and 5 (Berasain et al., 2005; David et al., 1996). AREG promotes STAT signaling in a JAK independent manner in Hela cells (Berasain et al., 2005). However, the activation of STATs is dependent on EGFR kinase activity, as seen in fibroblasts expressing EGFR tyrosine kinase mutants (David et al., 1996).

The by-products of PLC-γ hydrolysis of PIP2 is IP_3 receptor and DAG. DAG can activate protein kinase C (PKC). Activated PKC has been observed in NSCLC. AREG and insulin-like growth factor were found to activate this pathway by increasing phosphorylation of p90rsk and Bad while inhibiting Bax (Hurbin et al., 2005). In addition to EGFR interactions with PLC-γ, EGFR associates with c-src. C-src up regulates genes associated with cytosekeltal rearrangement of the cell including: focal adhesion kinase (FAK), RhoGAP, cortactin, and Eps-8 (Jorissen et al., 2003). Due to the

observation that FAK activation increases in colon cancer cells AREG has been suggested to promote interactions between c-src/EGFR (Picihard et al., 2006).

AREG has been proven to be involved in the expression of several genes involved in cell cycle progression and the inflammatory response. Cyclin D1 expression is regulated by AREG in pancreatic duct cells and vascular smooth muscle cells (Shin et al., 2003; Wagner et al., 2002). IL-8, IL-6, and IL-1 expression has been demonstrated in human airway epithelial cells, multiple myeloma cells, and breast cancers cells, respectively (Blanchet et al., 2004; Chokki et al., 2006; Mahtouk et al., 2005; Streicher et al., 2007). In two instances, in colon cancer cells and oral squamous carcinoma cells, AREG induced the expression of cycloxygenase-2 (COX-2) (Coffey et al., 1997; Tsai et al., 2006). As discussed previously AREG also regulates the expression of MMPs, including: MMP-2, MMP-9, and other factors involved in matrix degradation: uPA, EMMPRIN, and PAI-1 (Giusti et al., 2003; Silvy et al., 2001).

In addition to FAK and MMPs, genes involved with migration and invasion, AREG induces expression and alters localization of E-cadherin. E-cadherin is an epithelial adhesion molecule and has been implicated in cell motility and invasion. In mouse keratinocytes over expressing AREG, E-cadherin was down-regulated via processing into an 80 kDa form in psoriatic lesions. Localization alterations by AREG have been demonstrated in MDCK cells treated with neutrophils and AREG. AREG promoted the movement of MDCK cells and induced a spindle-like morphology as compared to TGF-α (Chung et al., 2005a; Chung et al., 2005b). SUM-149 breast cancer

cells, which over express AREG, on the contrary, express high levels of E-cadherin at the cell surface (Hoffmeyer et al., 2005).

E. AREG expression in normal tissue

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). In addition, AREG is widely expressed in human tissues including the placenta, ovary, testis, heart, pancreas, spleen, kidney, lung, ovary, colon, and breast (Plowman et al., 1990; Stern, 2003).

Differentiation of the mammary gland is crucial for reproduction in mammals. Development of the mammary gland is unique in that most of its differentiation occurs post-partum. In the developing breast there is a fundamental system of small ducts that grow slowly prior to puberty. Ductal outgrowth begins during puberty in the virgin mouse, resulting in the formation of a ductal tree that penetrates the mammary fat pad. Extensive ductal branching and alveolar growth occurs once the mouse is pregnant. At the end of pregnancy, the alveolar tissue terminally differentiates and the biological process of lactation begins (Stern, 2003).

During puberty in virgin mice, AREG is highly expressed in the mammary gland. AREG expression is associated with the developing alveolar structure found in the epithelial cap cells and terminal end buds (TEBs) of 4-6 week old mice (Kenney et al., 1995). AREG -/- mice developed normal but fewer TEBs that fail to penetrate the mammary fat pad throughout the pregnancy and into lactation. Interestingly, when

AREG, EGF, and TGF-α were knocked-out in combination, the effects of AREG knock-down alone were enhanced and lactogenesis was further compromised. However, when EGF or TGF-α were knocked out alone, ductal growth was normal (Luetteke et al., 1999). This suggests that EGF and TGF-α play a significant role in lactation, while AREG is the ligand responsible for pubertal ductal morphogenesis (McBryan et al., 2008). Supporting these findings, mammary glands from EGFR knock-out (KO) mice were transplanted in the renal capsule of virgin athymic mice, which demonstrated a lack of ductal outgrowth (Wiesen et al., 1999). TEB outgrowth was assessed in other ErbB family member KO models. KO of ErbB3 resulted in mice with lower ductal density and fewer TEBs and epithelial branches (Qu et al., 2006). ErbB2 -/- mice had mammary glands characterized by structural defects in TEBs and a slowly advancing epithelial tree (Jackson-Fisher et al., 2004). These data suggest that AREG promotes ductal morphogenesis through EGFR/ErbB2 and EGFR/ErbB3 heterodimers (Jackson-Fisher et al., 2004; Qu et al., 2006). On the contrary, Ferguson et. al. suggested that ErbB receptors do not typically from hetero-dimers, but produce their ligand-induced homo-dimerization (Ferguson et al., 2000). In the case of AREG, this would suggest EGFR homo-dimers. In another study, the mechanism by which EGFR and AREG are required for ductal elongation was investigated. Using tissue recombination methods from EGFR-/-, AREG-/-, and ADAM17-/- mice it was demonstrated that EGFR is required in the stroma and AREG and ADAM17 are required in the epithelium for the development of the mammary gland. ADAM17-/- tissue inhibited ductal outgrowth, indicating ADAM17 is required for the proteolytic processing of AREG. In addition, in order for

normal development of the ductal tree to occur, crosstalk between the stroma and epithelium is required (Sternlicht et al., 2005).

Estrogen induced proliferation in the developing mammary glands has been shown to be mediated by paracrine AREG signaling. In the mammary gland, ER positive cells are not highly proliferative (LaMarca and Rosen, 2007). It is this population of cells that produce AREG, which mediates epithelial cell proliferation in a paracrine manner. Evidence for this was observed when AREG-/- tissue from mouse mammary glands was transplanted into cleared fat pads from mice with wild-type (WT) tissue. The AREG -/- tissue was able to proliferate when grafted into the WT tissue suggesting paracrine signaling by AREG (Ciarloni et al., 2007).

AREG is the primary regulator of EGFR in mammary gland development and promoter of ductal morphogenesis at puberty. In addition to playing a role in the mammary gland, AREG has important signaling functions in other tissues. In normal epithelial tissue, AREG is expressed during development of the embryonic lung (Schuger et al., 1996). 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). In addition, AREG stimulates epithelial branching and differentiation in embryonic mouse kidney cells (Lee et al., 1999). Furthermore, a role for AREG has been implicated in nerve regeneration, bone formation, and blastocyst implantation (Nilsson and Kanje, 2005; Qin et al., 2005). 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.

F. AREG expression in cancer

i. AREG/Breast Cancer

Considering the functional role of AREG in the developing breast, it is of no surprise that AREG plays a role in breast cancer. AREG's role in breast cancer was illuminated by the finding that expression of AREG protein was higher and more frequent in invasive breast carcinomas than in ductal carcinomas *in situ* and normal breast tissue (Salomon et al., 1995). In a study by LeJeune et. al., 36% of 68 inflammatory breast cancers (IBCs) expressed AREG mRNA and protein as seen by immunocytochemistry or northern and dot blot analysis. Also, AREG expression was observed more frequently in lymph-node-positive samples than in those that were lymph-node-negative. These studies indicated that EGFR and AREG are co-expressed in 35% of EGFR positive tumors (LeJeune et al., 1993). Of 68 infiltrating ductal carcinomas (IDCs) and infiltrating lobular carcinomas (ILCs), 77% expressed AREG. In addition, p53 point mutations correlated inversely with AREG expression (Qi et al., 1994). The large difference observed in the studies by LeJeune and Qi can be explained by the use of a monoclonal versus polyclonal AREG antibody, respectively. Another study analyzing 100 human infiltrating breast carcinomas determined AREG expression in 50% of the samples (Panico et al., 1996). AREG protein secretion was measured in 193 primary breast cancers by enzyme-linked immunosorbent assays (ELISAs). Through this method

of directly quantifying AREG protein expression, 92% of the primary breast cancers expressed AREG. AREG expression correlated with uPA and VEGF in postmenopausal tissues and with PR in premenopausal samples, suggesting a role for AREG in vasculogenisis and metastasis (Desruisseau et al., 2004).

Still, AREG expression has not been shown to correlate with survival. However, AREG has been correlated with tumor grade. Lejeune et. al. and Qi et al. both determined that AREG correlated with lymph-node-positive cancers as compared to lymph-node-negative cancers (LeJeune et al., 1993; Qi et al., 1994). In another study, 63.3% of 84 invasive ductal breast carcinomas were found to co-express AREG and EGFR. The co-expression of these two proteins correlated with large tumor size, node involvement, grade III, inflammatory carcinoma, and absence of ER (Ma et al., 2001). AREG positive staining in peritumoral host cells correlated with clinical outcome. 85% of cases expressing AREG correlated with relapse, where as only 29% of AREG negative cases relapsed (Visscher et al., 1997).

AREG plays a role in breast cancer leading to the progression of the disease by promoting cell proliferation, invasion, and metastasis of epithelial cells. The aggressiveness of tumor cells expressing AREG was suppressed by inhibiting AREG via anti-sense cDNA in NS2T2A1 mouse mammary tumor cells, showing a reduction in tumor formation *in vivo* (Ma et al., 1998). 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, expression of the extracellular matrix metalloproteinase inducer (EMMPRIN), activator of MMP1, 2, 3, and 9, is down-regulated in response to AREG anti-sense treatment of NS2T2A1 (Menashi et al., 2003). In a more recent study inhibition of AREG by anti-sense cDNA in NS2T2A1 inhibited MMP-9 expression (Ma et al., 2010). Bissell and colleagues demonstrated the existence of an autocrine loop promoting a maliganant phenotype mediated by ADAM17. Through the use of 3D culture models of breast epithelial cells, they targeted ADAM17/TACE via siRNA techniques. Inhibiting ADAM17 blocked EGFR signaling induced by AREG and TGF-α. (Kenny and Bissell, 2007). DNA micro-array studies comparing differences in gene expression in terminal duct lobular units (TDLUs) and hyperplastic enlarged lobular units (HELUs) showed a 10-fold up regulation of AREG expression in HELUs. HELUs are abnormally enlarged TDLUs and represent premalignant potential during hormone-dependent breast cancer progression (Lee et al., 2007). These studies identify AREG and EGFR as regulators of metalloproteinases leading to cell invasion and metastasis.

ii. AREG in other cancers/diseases

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., 1995; Sehgal et al., 1994; Tsai et al., 2006;

Yamane et al., 2008). Interestingly, AREG expression is also correlated with inflammatory disease: for example, rheumatoid arthritis, ulcerative colitis, and Chron's disease (Nishimura et al., 2008; Yamane et al., 2008). AREG expression in invasive tumors was correlated with locally advanced inflammatory carcinoma (Ma et al., 2001).

iii. AREG and treatment of cancers

EGFR and AREG are co-expressed in approximately 15% of invasive breast carcinomas (Ma et al., 2001). In phase II clinical trials, the two main classes of EGFR inhibitors, small molecule EGFR TKIs and monoclonal antibodies, lead to tumor regression in 10-20% of NSCLC patients (Fukuoka et al., 2003; Kris et al., 2003). In lung cancer cells expressing WT EGFR, gefitinib and cetuximab showed higher efficacy in cells producing high levels of AREG (>20 pmol/L) relative to cells producing low levels of AREG (<20pmol/L). This effect is thought to occur by cell cycle arrest and inhibition of ERK1/2 signaling. This study suggests that in lung cancer patients expressing WT EGFR, AREG may be a predictor for the use of EGFR-targeted therapies to promote stable disease (Khambata-Ford et al., 2007; Yonesaka et al., 2008). Additionally, in metastatic colorectal cancers, high concentrations of AREG in serum has been correlated with longer progression-free survival as compared to patients with low serum levels of AREG in response to treatment with cetuximab (Khambata-Ford et al., 2007). On the contrary, in MCF7 breast cancer cells, AREG has been shown to be a factor in development of resistance to the platinum based chemotherapeutic drug, Cisplatin. The correlation between Cisplatin resistance, examined by MTT assays, in a

panel of breast cancer and lung cancer cells lines demonstrated that the expression of AREG correlates with resistance only in breast cancer and not in lung cancer (Eckstein et al., 2008).

1.3 Invasion

A. EGFR and AREG

Normal cells undergo physiological processes such as embryonic morphogenesis, wound healing, and immune cell trafficking which require mechanisms of cell migration and invasion. Neoplastic cells use these mechanisms to promote cell entry into the lymphatic and blood vessels, thus promoting growth in distant organs (Reviewed by (Friedl and Wolf, 2003)). Invasion is described as "penetration of tissue barriers, such as basement membrane and interstitial stroma, by cells" (Friedl and Wolf, 2003).

There are four main activities that are associated with the invasive phenotype: cell-cell adhesion, cell-matrix interactions, migration, and proteolysis (Mareel and Leroy, 2003). During invasion, cell-cell adhesions can be weakened (Yamazaki et al., 2005). E-cadherin is one such adhesion molecule that acts as a tumor suppressor. In some instances, loss of E-cadherin stimulates migration and cellular invasion (Bracke et al., 1997; Handschuh et al., 1999). The ECM provides a barrier, substrate, and signal for cell invasion by providing motility factors and regulating survival (Mareel and Leroy, 2003). Cell-matrix interactions occur through molecules such as: integrins; transducers of signals regulating anchorage independent growth, and FAK, a NRTK regulating actin cytoskeleton signaling (Parsons et al., 2000). In order for the cells to invade the matrix,

cells must utilize locomotive factors and migrate (Mareel and Leroy, 2003). Src and PI3K pathways have been demonstrated to be necessary for cell invasion (Empereur et al., 1997). Lastly, proteolysis, a process involving the cleavage of proenzymes, promotes the breakdown of the ECM creating routes for invasive cells to migrate. Invasive cells make MMPs, urokinase-type plasminogen activators, and cysteineproteinases to aid in proteolysis (Mareel and Leroy, 2003).

EGFR over expression was suggested to play a role in tumor progression, however EGFR over expression did not correlate with proliferation; it correlated with progression to cellular invasion. This has been observed in glioblastomas, bladder carcinomas, and gastric carcinomas. Up-regulated EGFR is present in invading brain tumors as compared to non-invasive glimoas (Libermann et al., 1984; Schlegel et al., 1994). In bladder carcinomas, Neal et. al. found EGFR expression to correlate with tumor progression, poor differentiation, and invasion (Neal et al., 1985). Additionally, in gastric tumors non-invading tumors did not express EGFR whereas nearly one third of invasive tumors over expressed EGFR (Kitadai et al., 2000).

The mechanisms proposed for over expressed EGFR's promotion of cell invasion are associated with cell-cell adhesion, cell-matrix interactions, migration, and proteolysis. AREG-induced EGFR has been reported to up-regulate MMP-2 and -9 (Kondapaka et al., 1997; Ma et al., 2010; Menashi et al., 2003). Expression of MMPs alters the ECM to allow the passage of cells to invade the stroma, intravasate a lymph or vascular channel where the cell can circulate and extravasate into tissues at distant sites (Stetler-Stevenson and Yu, 2001). GPCR ligands, such as lysophosphatidic acid (LPA), promote the

proteolytic processing of ligands like AREG, via TACE, leading to EGFR phosphorylation in kidney and bladder cancer cells in vitro via ADAM metalloproteinases, more specifically by ADAM-10, -15, and -17 (Gschwind et al., 2003; Schafer et al., 2004). EGFR transactivation by LPA stimulation promotes cell invasion (Schafer et al., 2004). In addition to LPA, the GPCR ligand gastrin releasing peptide (GRP), induces EGFR phosphorylation followed by src activation of TACE and co-translocation to the cell surface where TACE initiates the subsequent release of AREG, thus facilitating cell invasion in head and neck squamous cell carcinomas (Zhang et al., 2004).

EGF activation of EGFR can lead to cell motility and invasion in renal carcinomas, and squamous cell carcinomas (Price et al., 1996; Shibata et al., 1996). In another study examining MDA-MB 468 breast cancer cells, DU-145 prostate cancer cells, KB oral carcinomas, and NIH3T3 cells over expressing EGFR, EGF-induced de-phosphorylation and reduced kinase activity of FAK, ultimately inducing cancer phenotypes: cell motility, invasion, and metastasis (Lu et al., 2001). Aside from EGFR signaling events inducing the expression of genes that promote invasion, it is hypothesized that EGFR can be activated by matrix components. This was most clearly seen by the expression of decorin, a matrix component that binds to fibrillar collagen (Iozzo et al., 1999).

Additional mechanisms relate to EGFR signaling observed during normal physiological activities. Activated EGFR interacts with PLC-γ. PLC-γ promotes cell motility through the hydrolysis of PIP2 allowing for cytoskeletal rearrangement (Chen et

al., 1994). Treatment with PLC-γ inhibitors prevented invasion of prostate tumor in mice, corroborating PLC-γ's role in tumor invasion in the presence of EGFR over expression (Turner et al., 1997).

In our lab's recent work, an AREG/EGFR autocrine loop has been shown to stimulate cell motility and invasion (Willmarth and Ethier, 2006). Whether AREG stimulation of EGFR or EGFR over expression itself are the causal effects of cellular invasion remains to be elucidated.

B. Other factors promoting invasion

i. RHOB

Ras-homologous (Rho) small guanosine triphosphatases (GTPases) regulate a variety of cellular functions, such as: organization of the cytoskeleton, cell-cycle progression, cell-cell contact, cell polarity, migration, adhesion, cell morphology, gene-transcription, and cell transformation (Bishop and Hall, 2000). As a family member of small G proteins, Rho regulates these cellular processes by working as a molecular switch, rotating between GDP- and GTP-bound states (Boguski and McCormick, 1993). Rho-GTP can effectively interact with an effector molecule to initiate a signaling event specific to the target molecule (Bishop and Hall, 2000). In an inactive state, Rho-GDP is associated with guanosine nucleotide dissociation inhibitor (RhoGDI), thereby inhibiting the spontaneous GDP to GTP exchange (Bishop and Hall, 2000).

RhoB exhibits several characteristics that distinguishes it from its other family members. For example, RhoB is localized to the early endosomes and nuclear membrane

(Michaelson et al., 2001), it promotes intracellular trafficking of cytokine receptors (i.e. EGFR) (Gample, A. 1999, Curr Bio), and it has a rapid response to growth factors (i.e. EGF, TGF-B) and genotoxic stress. These qualities suggest RhoB involvement in cancer progression.

While Rho protein expression has been correlated with disease progression, RhoB appears to function as a tumor suppressor. Loss or reduction of RhoB expression has been observed in the progression of lung cancer, head and neck squamous cell carcinoma, and ovarian cancer (Adnane et al., 2002; Couderc et al., 2008; Mazieres et al., 2004). On the contrary, Fritz et. al. demonstrated an over expression of RhoB protein in normal and tumorigenic tissues (Fritz et al., 2002). In NIH3T3 cells, transfected with RhoB and a serum response element, cell transformation, invasion, and metastasis was suppressed. Experiments aimed to identify RhoB's tumor suppressor activity suggest that RhoB negatively regulates the Ras/PI3K/Akt tumor survival pathway (Jiang et al., 2004).

ii. DKK1

Canonical Wnt signaling is occurs when a Wnt family member binds to a cell surface receptor Frizzled (FRZ). FRZ interacts with a cell surface molecule lipoprotein-receptor related protein (LRP) and axin. Subsequently, disheveled (DSH) proteins are activated resulting in a Wnt/FRZ/DSH/LRP/axin complex. The Wnt/FRZ/DSH/LRP/axin complex inhibits the formation of an axin/GSK-3/APC complex responsible for inhibiting B-catenin degradation. This signaling ultimately results in an increase in B-catenin levels.

Dickkopf-1 (dkk-1), one of four family members of Wnt inhibitors, is a secreted protein involved in embryogenesis and Wnt signaling (Glinka et al., 1998). It was first isolated from Xenopus (amphibian) cDNAs that complemented in the formation of a secondary axis during embryogenesis (Glinka et al., 1998). Dkk-1 is composed of 259 AA and contains a signal sequence and two cystenine-rich domains. In the search for Erb3 ligands, a 35 kDa band was identified by silver staining analysis representing the human dkk-1. Northern blot analysis in fetal and adult tissues identified dkk-1 expression in fetal, kidney, liver, and brain tissue, as well as in adult placenta and prostate tissue (Fedi et al., 1999). In the amphibian embryos, dkk-1 was found to be required for head formation by antagonizing Wnt signaling (Glinka et al., 1998). Biochemical approaches determined that human dkk-1 disrupts Wnt signaling and acts up-stream of B-catenin stabilization, which is associated with the morphological effects of transforming Wnts (Fedi et al., 1999). Mechanistically, Dkk-1 inhibits Wnt signaling by binding to the LRP5/6. Wnt/B-catenin signaling is blocked by LRP5/6 (Mao et al., 2001).

Knockout of Dkk-1 in mice resulted in mice lacking anterior heads and forelimb malformations (Mukhopadhyay et al., 2001). In cancer, levels of secreted Dkk-1 are low, resulting in an accumulation of nuclear β-catenin. Expression of recombinant dkk-1 in MCF-7 breast cancer cells down-regulated macrophage induced invasion in a dose-dependent manner. The decrease in the invasive phenotype was independent of cell proliferation and cellular metabolism (Pukrop et al., 2006).

IV. SUM-149 Breast Cancer Cells

The SUM series of cell lines were developed from patients with breast cancer in our laboratory. SUM-149 cells were isolated from a patient with aggressive, pre-menopausal, locally advanced inflammatory ductal carcinoma. The patient failed all treatment with chemotherapy, radiation, and surgery. The cells are ER- and PR- and express low levels of ErbB2 and ErbB3. Additionally, SUM-149 cells have a BRCA1 mutation, have increased levels of cyclin E, and are basal-type (Elstrodt et al., 2006). Cyclin E is a cyclin expressed during G1 phase and is essential for transitioning through the cell cycle to S phase. SUM-149 cells due to a mutation in hCdc4, a F-box protein, have stabilized expression of cyclin E (Willmarth et al., 2004).

SUM-149 cells are used frequently as they are one of three inflammatory breast cancer cell lines in existence. SUM-149 cells have been shown to be able to grow in soft agar and invade a matrigel matrix *in vitro* (Ignatoski and Ethier, 1999). Moreover, SUM-149 cells express high levels of RhoA and RhoC, E-cadherin, NF-κB and caveolins-1 and -2 (Dong et al., 2007; Hoffmeyer et al., 2005; Pan et al., 2003; Pan et al., 2002; Van den Eynden et al., 2006; van Golen et al., 2002; van Golen et al., 2000).

Our lab found that SUM-149 cells over express constitutively active EGFR without gene amplification. They are EGF independent for growth; however, they require EGFR for proliferation. This was based on the observation that after treatment of the cells with EGFR inhibitor CI-1033, cells no longer proliferated (Rao et al., 2000).

AREG was detected in SUM-149 cells by molecular cloning techniques. RNA was isolated, reverse transcribed into cDNA, and cloned into retroviral vectors that were

subsequently infected in MCF-10 cells growing in the absence of exogenous growth factors (Berquin et al., 2005). MCF10A cells are normal human mammary epithelial cells that spontaneously immortalized (Soule et al., 1990). cDNA was isolated from emerging colonies and analyzed by PCR. Subsequent analysis demonstrated that the growth of SUM-149 cells is regulated predominately by AREG (Willmarth and Ethier, 2006).

SUM-149 cells express a self-sustaining AREG/EGFR autocrine loop and can activate EGFR in a juxtacrine fashion. They over express AREG mRNA and protein and are more invasive and motile than MCF10A cells growing in the presence of EGF (Willmarth and Ethier, 2006). In addition, SUM-149 cells show an increase in the steady state levels of EGFR that localizes to the plasma membrane (Willmarth and Ethier, 2008). Previously it was observed that SUM149 cells have constitutive NF-κB activity (Pan et al., 2003). The activation of EGFR by AREG then generates a positive feedback loop involving IL-1 and NF-κB (Streicher et al., 2007).

The over expression of AREG at the mRNA and protein level, and the presence of a functional AREG/EGFR autocrine loop that is maintaining EGFR activity and contributing to SUM-149 cellular invasion and motility makes these cells a useful model for testing the effect of AREG knock-down. We predicted that by knocking-down AREG we would stop or slow cell proliferation, motility, and invasion. By using SUM-149 and MCF10A cells as our cell models we aimed to examine the effect of AREG knock-down on the cancer-associated phenotypes in order to better understand the role of AREG in cancer progression.

CHAPTER 2

MATERIALS AND METHODS

2.1 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) 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 μmol/L Gefitinib/Iressa (AstraZeneca Pharmaceuticals, Wilmington, DE, USA).

2.2 Cell Culture—SUM149 cells were maintained in Ham's F-12 medium with 5% fetal bovine serum, 5 μg/ml insulin, 2 μg/ml hydrocortisone, 5 μg/ml gentamicin, and 2.5 μg/ml fungizone (5%IH). SUM-149sh4, SUM-149shPld, and SUM-149shNS cells were maintained in 5%IH plus 1ug/ml puromycin. The serum-free base medium for MCF10A and MCF10A+AREG cells was SFIH (Ham's F-12 with 1 μg/ml hydrocortisone, 1 mg/ml bovine serum albumin, 10 mm Hepes, 5 mm ethanolamine, 5 μg/ml transferrin, 10 nm triiodothyronine, 50 nm sodium selenate, 5 μg/ml gentamicin, 2.5 μg/ml fungizone, and 5 μg/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% CO_2.

2.3 Lenti-AREG shRNA infection—Knock-down of AREG expression was performed using Mission TRC human shRNA clone sets (OpenBiosystems, Huntsville, AL, USA). sh1:CCGGGAACCACAAATACCTGGCTATCTCGAGATAGCCAGGTATTTGTGGT TCTTTTTG;sh2:CCGGGCCGACTATGACTACTCAGAACTCGAGTTCTGAGTAGT CATAGTCGGCTTTTTG;sh3:CCGGCCTGGCTATATTGTCGATGATCTCGAGATC ATCGACAATATAGCCAGGTTTTTG;sh4:CCGGGAACGAAAGAAACTTCGACAA CTCGAGTTGTCGAAGTTTCTTTCGTTCTTTTTG;sh5:CCGGCACTGCCAAGTCAT AGCCATACTCGAGTATGGCTATGACTTGGCAGTGTTTTTG. Lentivirus was produced by transfecting human embryonic kidney (HEK293) cells at 80% confluence in 10%DMEM without antibiotics and infecting HEK293 cells with packaging plasmids:

350ng mDLg/pRRE, 350ng pRSV-Rev, 250ng MD2.G, and 1ug of each pLKO.1 AREG shRNA plasmid using FuGENE (according to manufacture's 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 $1x10^6$ cells per 10 cm dish, treated with 8ug/ml polybrene and infected with 48 hr virus harvested from HEK293 cells. Cells were incubated for 12 hrs and new media with 1ug/ml puromycin was added for 2 weeks before functional assays, and 4-10 days before RNA extraction was performed.

2.4 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'TTCACTGTGTGGTGGCAGAT3' R5'CCTTTCCGCTTTGATTGTGT3' EGF F5'CGCAGGAAATGGGAATTCTA3' R5'TCCACCACCAATTGCTCATA3' TGFA F5'TCGCTCTGGGTATTGTGTTG3' R5'GGGAATCTGGGCAGTCATTA3' HB-EGF F5'GGCAGATCTGGACCTTTTGA3' R5'CCCCTTGCCTTTCTTCTTTC3' EPGN

F5’CCCAGCAAGCTGACAACATA3’ R5’CTCATGGTGGAATGCACAAG3’ EREG F5’CTGCCTGGGTTTCCATCTTCT3’ R5’GCCATTCATGTCAGAGCTACACT3’ RHOB F5’ATCCCCGAGAAGTGGGTCC3’ R5’CGAGGTAGTCGTAGGCTTGGA3’ CNTN1 F5’GTGGCACTTACTTGGAGCC3’ R5’GGGGGATCTGTCTTTGCATCTT3’ HEY1 F5’TATCGGAGTTTGGGATTTCG3’ R5’AGATGCGAAACCAGTCGAAC3’ HES7 F5’CGGGATCGAGCTGAGAATAGG3’ R5’GCGAACTCCAATATCTCCGCTT3’. Real-time RT–PCR was performed in 25 ul 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 the test primer reactions to reach threshold fluorescence, the differences are raised to the power of -2 and then the negative inverse was taken.

2.5 Immunoblotting— Cells were grown to 80% confluence in their normal growth media and 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 μg/ml leupeptin for 10 minutes. Cells were scraped and spun down to remove cell material. 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). 50-500ng of 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, Thermos Fisher Scientific, Waltham, MA).

2.6 Enzyme-linked Immunosorbent Assay (ELISA)—Twenty-four hour conditioned medium was obtained from cells grown in 6-well plates. An AREG DuoSet ELISA (R&D systems, Minneapolis, MN, USA), with an AREG antibody specific to the extracellular domain of AREG was used to measure AREG medium concentration. High binding ELISA plates were coated with 3 μg/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.

2.7 Cell Proliferation Assays—Cells were seeded on day 0 in 6-well plates at 1.0×10^4 cells/well. Either 1 μg/ml AREG Ab, 1 μg/ml EGFR Ab, 1 μg/ml EGF Ab, 1 μg/ml

EPGN Ab, 1 μg/ml IgG Mouse Ab, and 1 μg/ml IgG Goat Ab, or 0.5 μmol/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/$MgCl_2$ buffer (0.01 m Hepes and 0.015 m $MgCl_2$) 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.

2.8 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-149shPld, 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.

2.9 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).

2.10 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) 1μg/ml hydrocortisone, 5μg/ml insulin, 5μg/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.

2.11 Immunofluorescence assays —Approximately 1×10^5 SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells were seeded on coverslips and given 5%IH, 5%IH+10ng/ml EGF, of 5%IH+20ng/ml AREG for 24hours. The next day, cells were washed 3 times with ice cold PBS, fixed in 4% para-formaldehyde for 20 min at room temperature, washed 3 times in PBS and then permeabilized with ice cold 0.1% Triton-X in PBS for 2 min. Cells were washed 3 times and then blocked for 1 h in 20% Goat Serum. A 1:1000 dilution of 2 μg/μl anti-EGFR antibody MAB108 was incubated with cells for 1 h. Cells were washed 3 times and incubated for 1 h with an Alexa488-conjugated anti-mouse secondary antibody (Molecular Probes, Invitrogen) at 1:1000

dilution. Cells were then washed 3 times with PBS and once more with distilled water to remove salt. ProLong gold with DAPI was used as a mounting reagent (Molecular Probes, Invitrogen) and coverslips were put on slides for microscopy. Pictures were taken with a 65× oil lens on a Zeiss LSM-310 Laser Scanning Confocal Microscope at the Wayne State University Confocal Imaging Facility.

2.12 Illumina microarray analysis— RNA was isolated from SUM-149, SUM149sh4, SUM-149shPld, SUM-149shNS, MCF10A, MCF10A+AREG, MCF10A-EGF, MCF10A-AREG 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 genes were found to be significantly different in expression at the $p<0.05$ level. An additional statistical test, Rank-Prod was also performed which identified genes that were significantly different at the $p< 0.05$ level. The genes described 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 SUM-149 and MCF10A microarray analysis have been deposited in the GEO database GSE26079 and GSE26264, respectively.

CHAPTER 3

KNOCK-DOWN OF AMPHIREGULIN INHIBIITS CELLULAR INVASION IN INFLAMMATORY BREAST CANCER

3.1 Introduction

The epidermal growth factor receptor (EGFR) is a 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-a), 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 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) (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-translastional 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; Luetteke 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.

3.2 Results

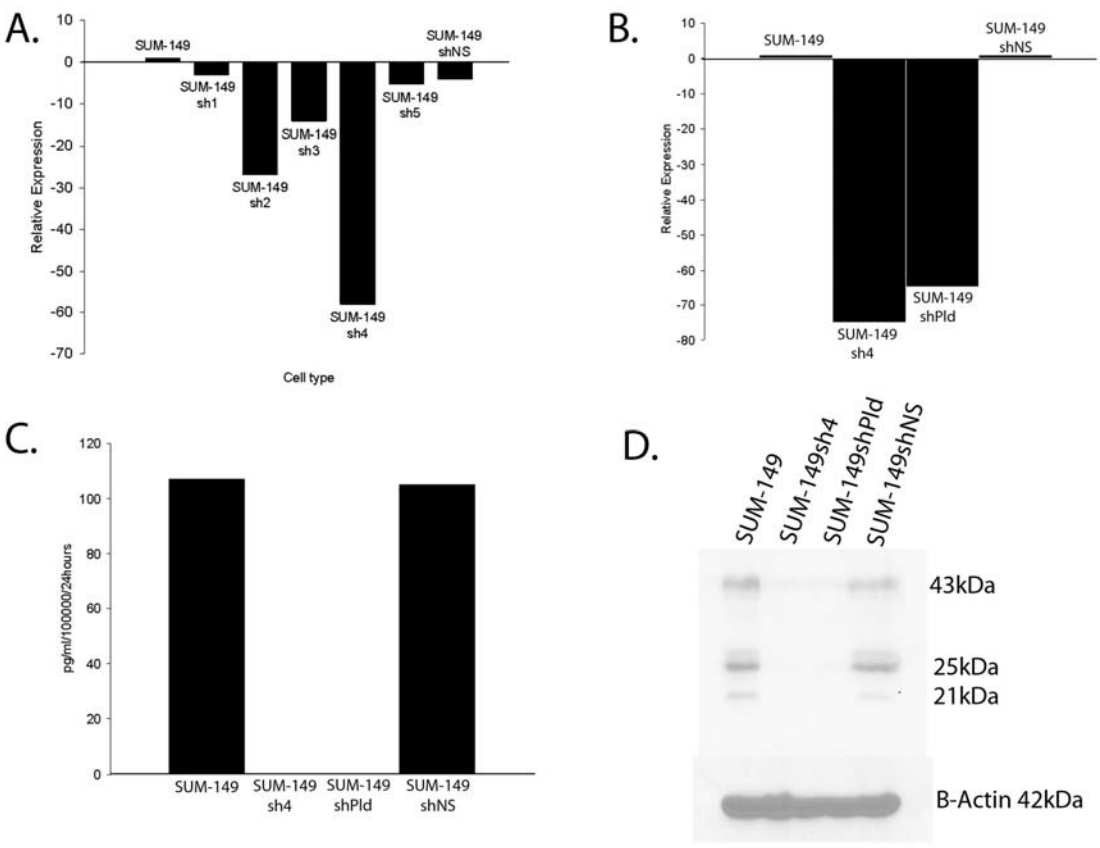
AREG Knock-down Slows Proliferation of SUM-149 cells.

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. 3.1A). 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. 3.1B). 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. 3.1C). 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. 3.1D). 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. 3.2A). 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. 3.2B). These results indicate that a 40-60-fold knock-down of AREG reduced, but did not completely block proliferation of SUM-149 cells.

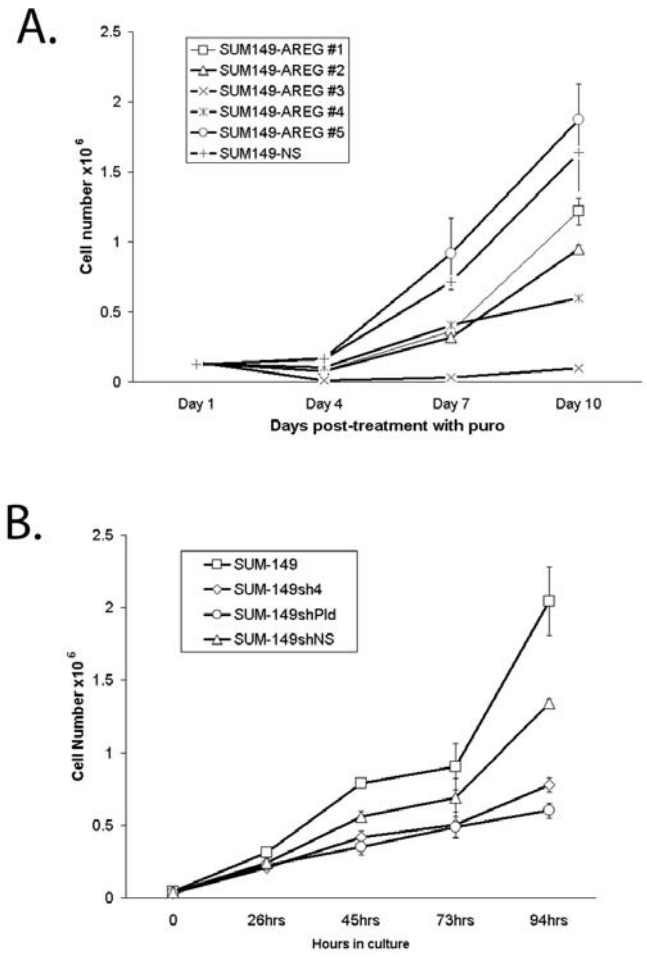
Figure 3.1



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 3.2



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 $1x10^4$ 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.

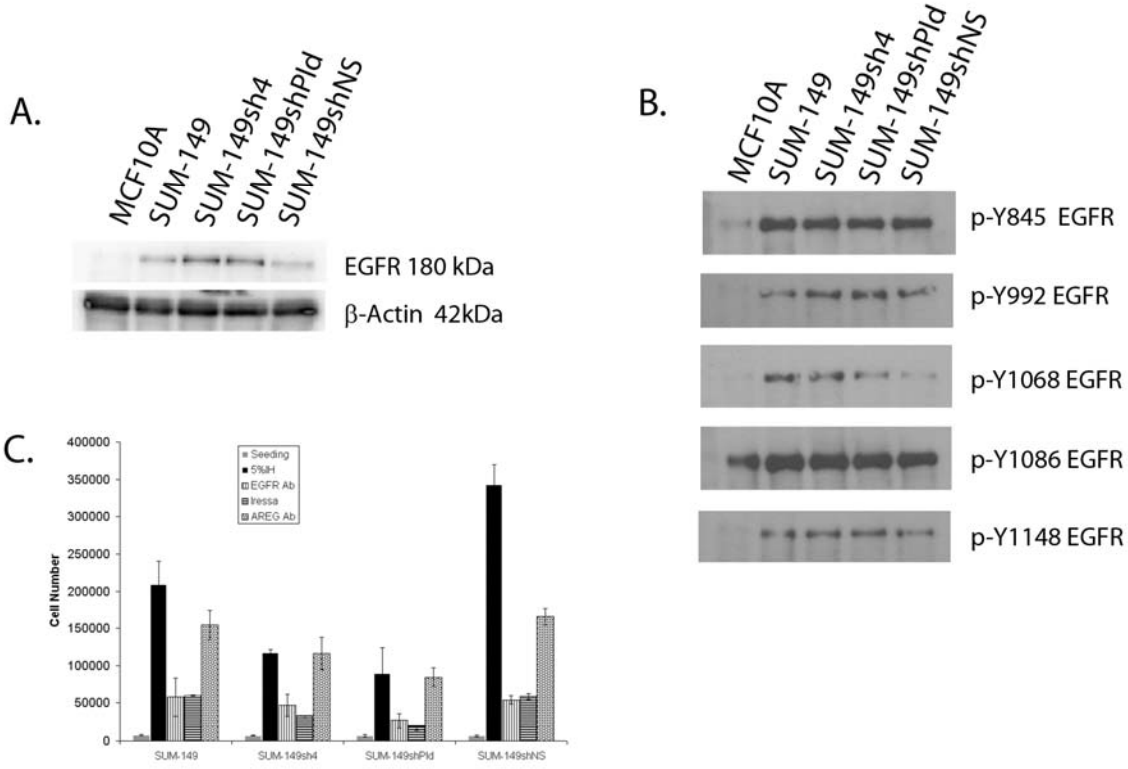
Continued proliferation of AREG knock-down cells is EGFR dependent

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 3.3A demonstrated an increase in steady-state EGFR protein levels in the AREG knock-down cells compared to SUM-149 and SUM-149shNS (Fig. 3.3A). 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 (Fig. 3.4). 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. 3.3B), which is consistent with continued slower proliferation (Fig. 3.2B).

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 3.3C 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. 3.3C). 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.

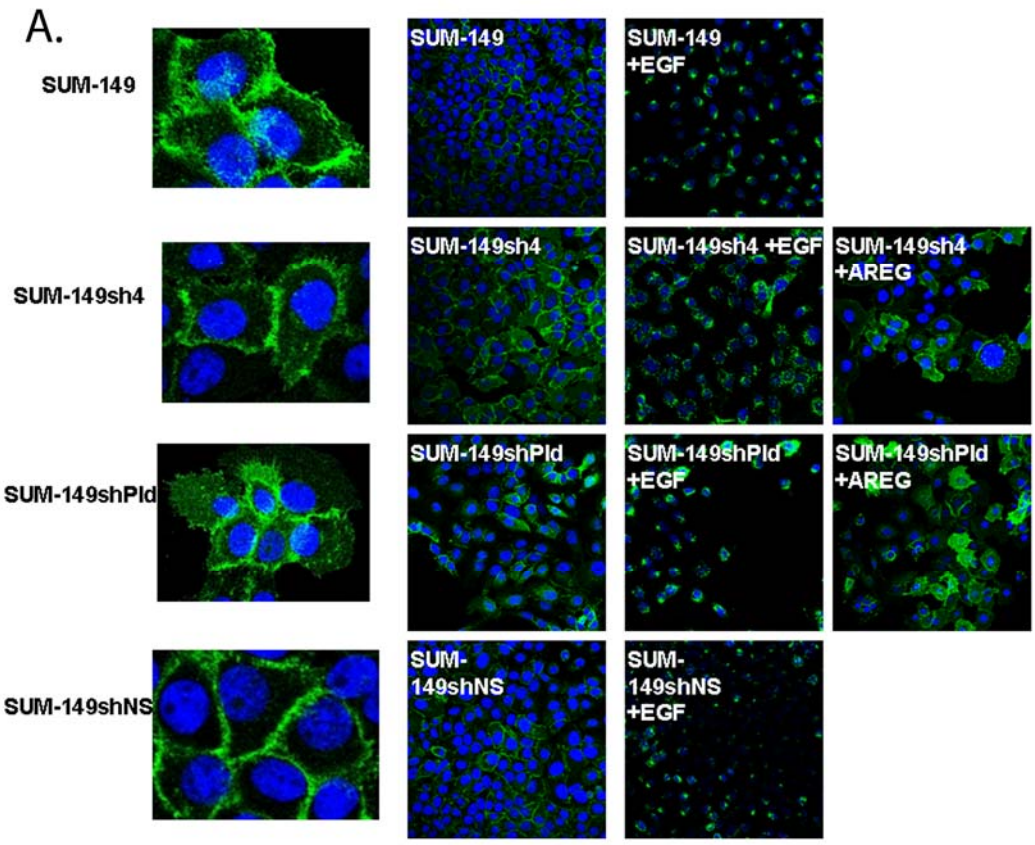
Figure 3.3



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 $1x10^4$ 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 μg/ml EGFR Ab, 0.5 μmol/L Iressa, or 1 μg/ml AREG Ab. Error bars represent the standard deviation of three replicates in the experiment.

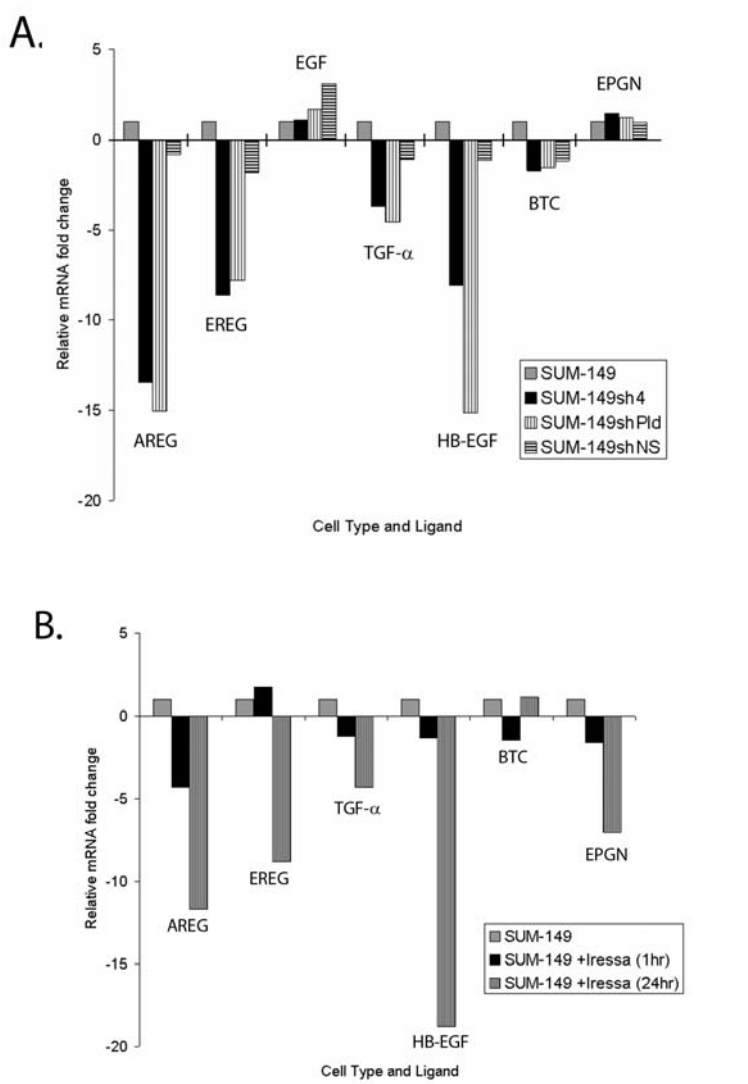
Figure 3.4



EGFR localizes to the plasma membrane and cell-cell junctions after AREG knock-down
(A) EGFR localization in SUM-149, SUM-149sh4 +/- EGF and AREG, SUM-149shPld +/- EGF and AREG, and SUM-149shNS. Cells were permeabilized, fixed, incubated with EGFR Ab, a secondary alexa-fluor Ab, and mounted with DAPI prolong gold. Representative confocal images are shown. Higher magnification of the cells is represented in the panel on the left to show more detailed localization on the membrane and at cell-cell junctions.

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 3.5A 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 3.5A). 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. 3.5B). 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.

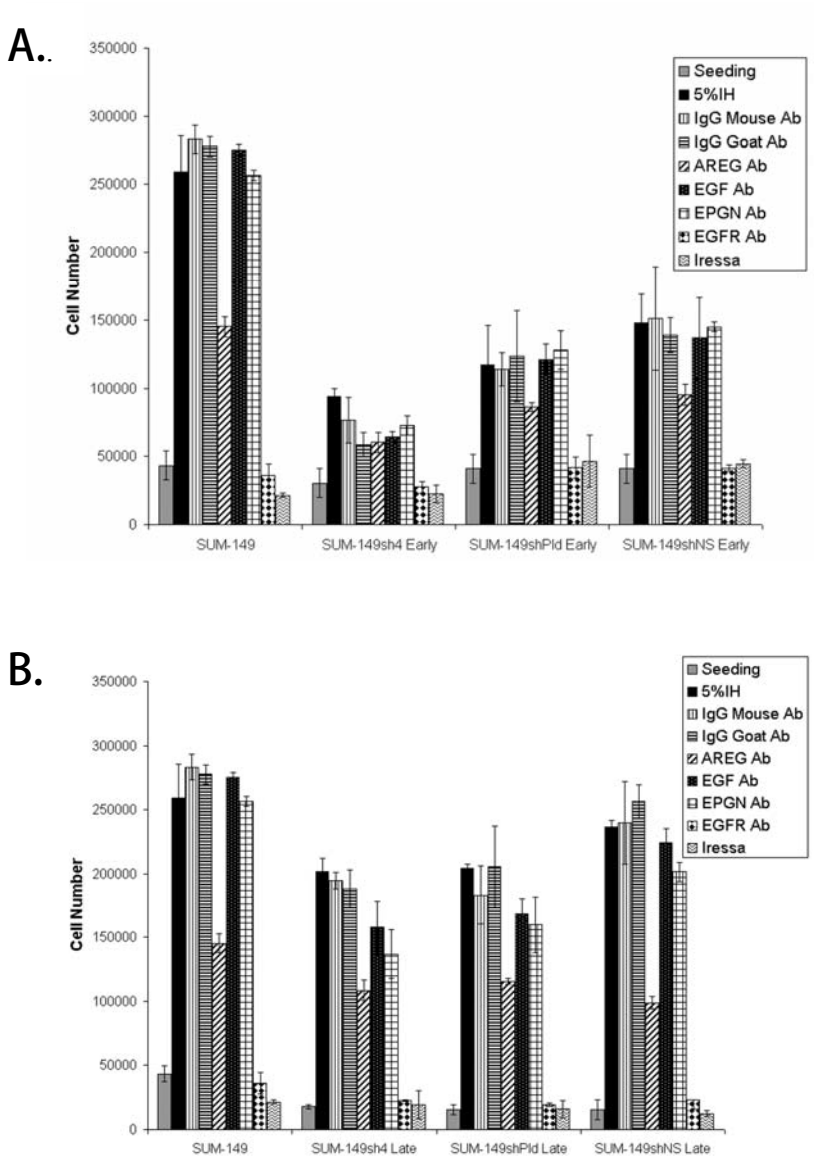
Figure 3.5



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-a, 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-a, BTC, HB-EGF, and EPGN in the SUM-149 and SUM-149 cells treated with 0.5 μmol/L Iressa for one or 24 hours.

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 3.6A 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-149shPld cells, while growth of SUM-149 and SUM-149shNS cells was not affected (Fig. 3.6B). 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-149shPld 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. 3.7). 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.

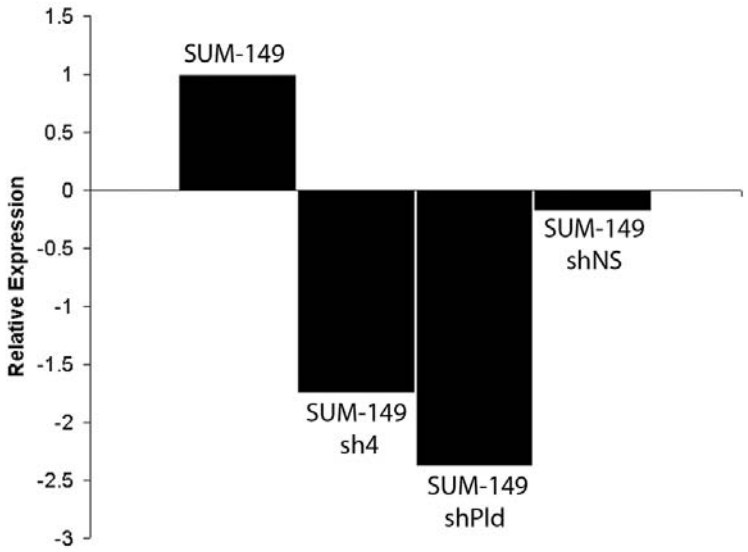
Figure 3.6



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

(C) Bars show growth of early passage cells (<p4) and (D) late passage cells after AREG knock-down (>p18) Cells were seeded at $1x10^4$ 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 1ug/ml IgG mouse Ab, 1ug/ml IgG goat Ab, 1 µg/ml AREG Ab, 1 µg/ml EGF Ab, 1ug/ml EPGN Ab, 1ug/ml EGFR Ab or 0.5 µmol/L Iressa. Error bars represent the standard deviation of three replicates in the experiment.

Figure 3.7

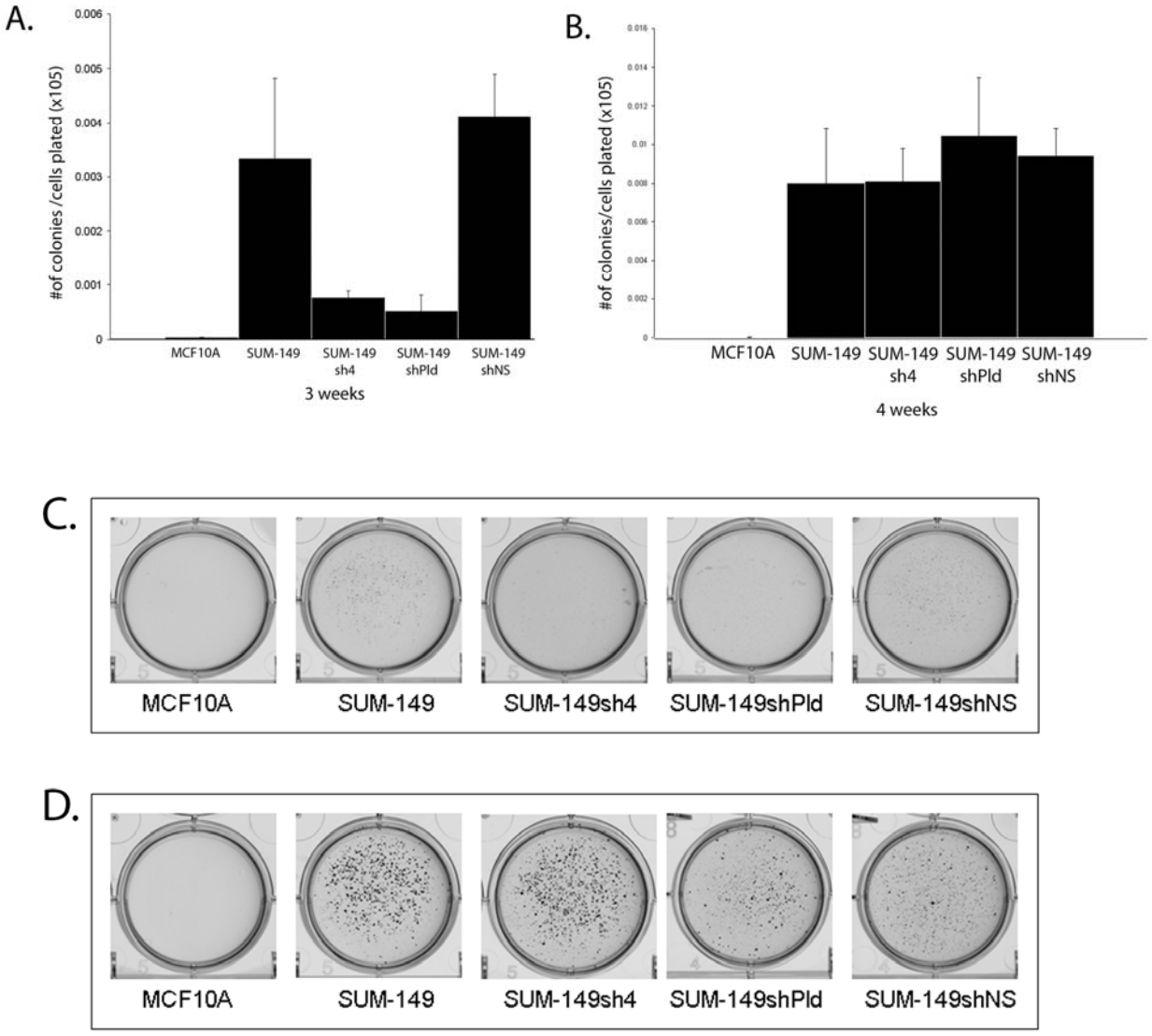


The role of EGF family ligands in growth of AREG knock-down cells
(E) Relative mRNA expression by real-time RT-PCR of AREG in late passage knock-down cells (>p18) with SUM-149 parental cells normalized to one.

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-149shPld, and SUM-149shNS cells. Figure 3.7A shows that SUM-149sh4 and SUM-149shPld cells formed 50% fewer colonies in soft agar after 3 weeks than SUM-149 cells (Fig. 3.8A, 3.8C). 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. 3.8B, 3.8D), 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.

Figure 3.8

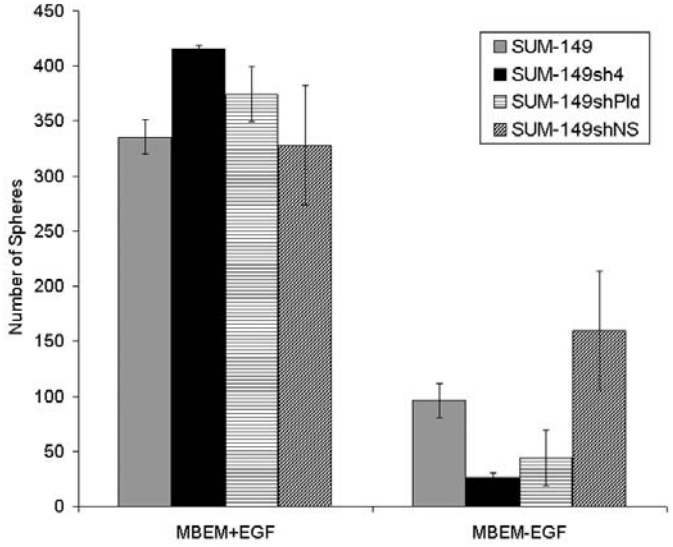


Effect of AREG knock-down on soft agar colony formation

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 ($1x10^5$). Cells were counted with GelCount, automated mammalian cell counter. Error bars represent the standard deviation of three replicates in the experiment. (C) Colonies formed after 3 weeks. (D) Colonies formed after 4 weeks.

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. 3.9). 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).

Figure 3.9

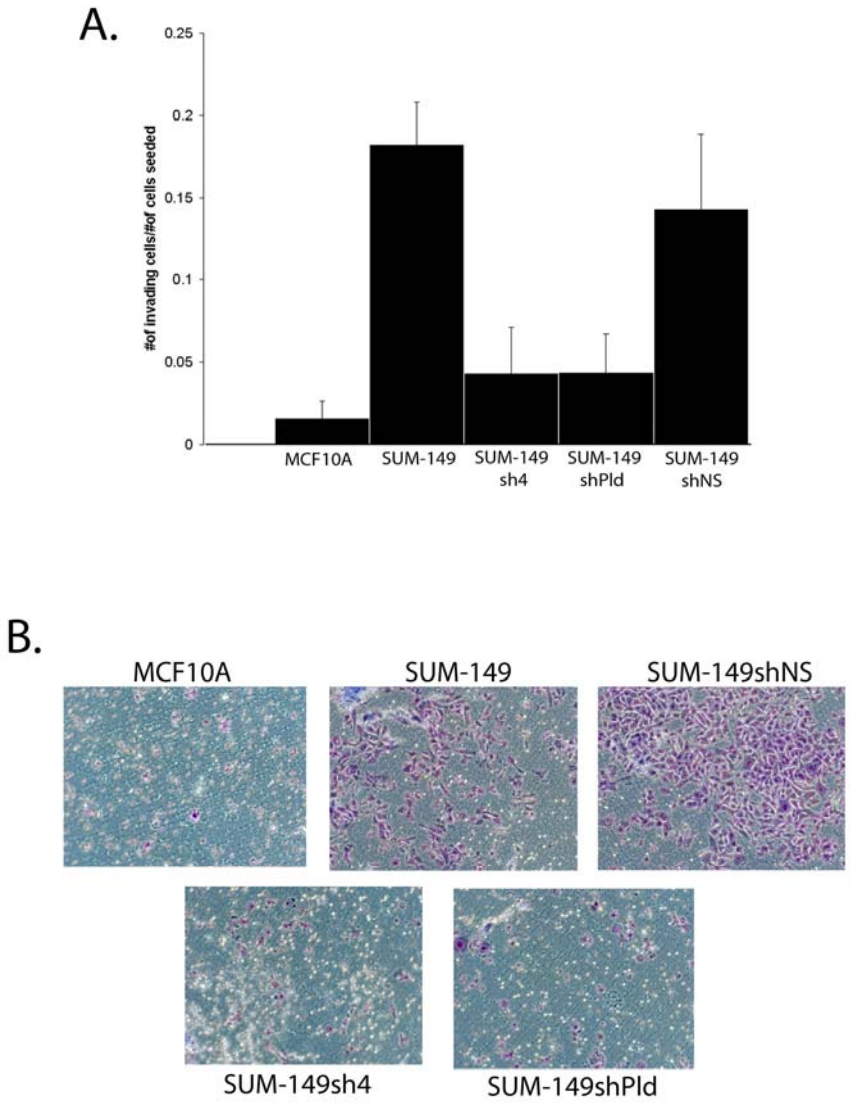


Effect of AREG knock-down on mammosphere growth

(C) Cells were seeded at $1x10^4$ 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.

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 3.10 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. 3.10A,B). 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.

Figure 3.10



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 ($2.5x10^4$ cells). Error bars represent the standard deviation of three replicates in the experiment. (B) Photos represent one field of view after staining invading cells.

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 (IPA) 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 3.1). Given our observation of the influence of AREG knock-down on motility and invasion as shown in figure 3.9, the identification of changes in specific genes associated with motility and invasion such as several integrins, vimentin, and MMPs (Table 3.1) 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 3.11 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 knock-down (Fig. 3.11A,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 3.11B,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.

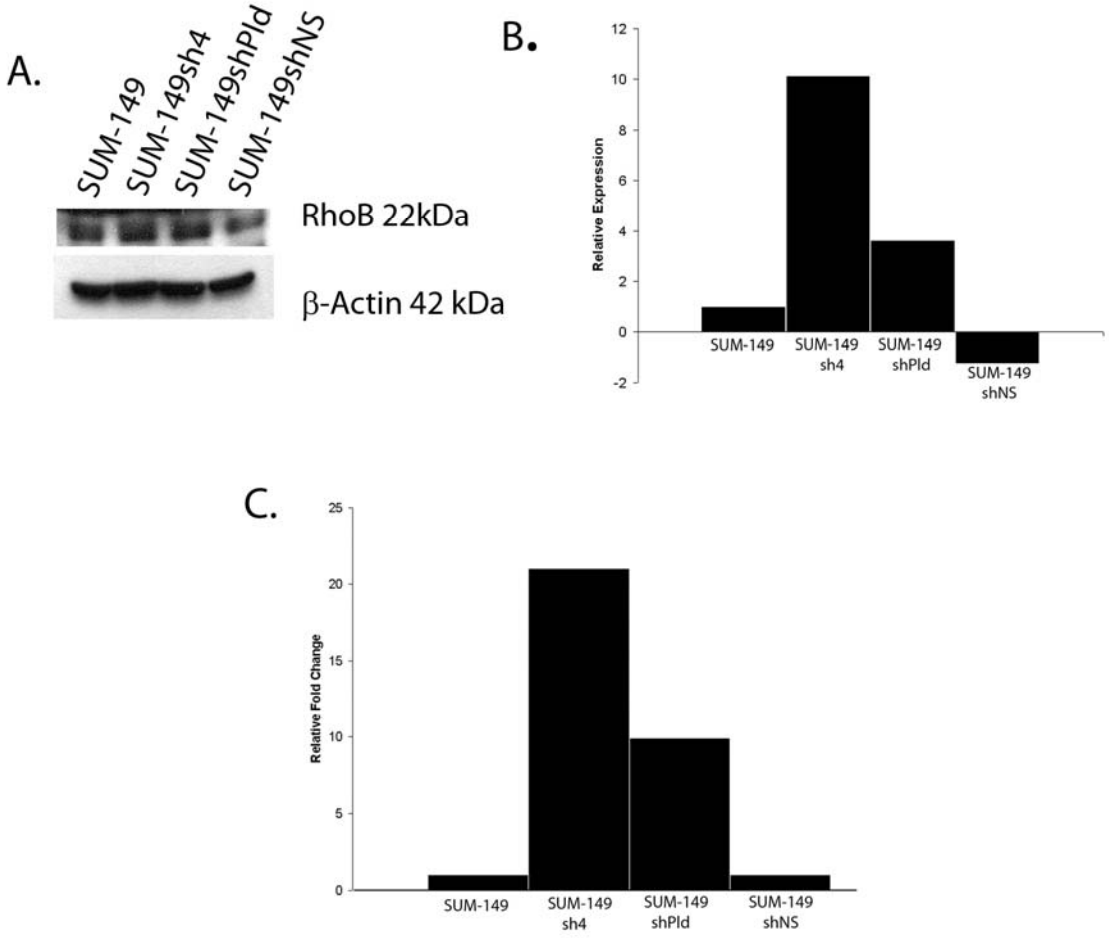
Table 3.1

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 metallopeptidase 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 metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
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	nicastrin
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 metallopeptidase 7 (matrilysin, uterine)
DKK1	**7.548**	**dickkopf homolog 1 (Xenopus laevis)**

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.

Figure 3.11



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.

3.3 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 knock-down 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 (Fig. 3.11). 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; Luetteke 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 (Table1). 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 cytosekeltal 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 or 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.

CHAPTER 4

GENOME-WIDE ANALYSIS OF GENE EXPRESSION REGULATED BY AREG IN NORMAL AND BREAST CANCER CELLS

4.1 Introduction

SUM-149 cells expressing shRNA constructs specific to AREG demonstrated a decrease in cell proliferation, a decrease in cellular invasion, and an increase in the expression of cell surface localized EGFR protein. These results led us to investigate differences occurring in the biology of the cell. By microarray analysis we measured AREG's involvement in various cellular events and processes. We isolated RNA from: SUM-149, SUM-149sh4, SUM-149shPld, and SUM-149shNS cells, reverse transcribed the RNA to cDNA and hybridized the cDNA to an Illumina array (described in Chapter 3).

To address further the biological the role of AREG, we cultured MCF10A cells either with or without EGF or in the presence or absence of exogenous AREG. MCF10A cells are spontaneously immortalized cells derived from mammary epithelial cells (Soule et al., 1990). MCF10A cells were continuously grown in serum free media supplemented with 10ng/ml of EGF (MCF10A) or 20ng/ml of AREG (MCF10A+AREG). Additionally, we withdrew EGF or AREG for 24 hours from MCF10A and MCF10A+AREG cells, respectively. RNA from MCF10A+/-EGF and MCF10A+/-AREG was isolated; cDNA was synthesized and hybridized to an Illumina array. By Ingenuity Pathway Analysis (IPA) we found AREG expression to be associated with

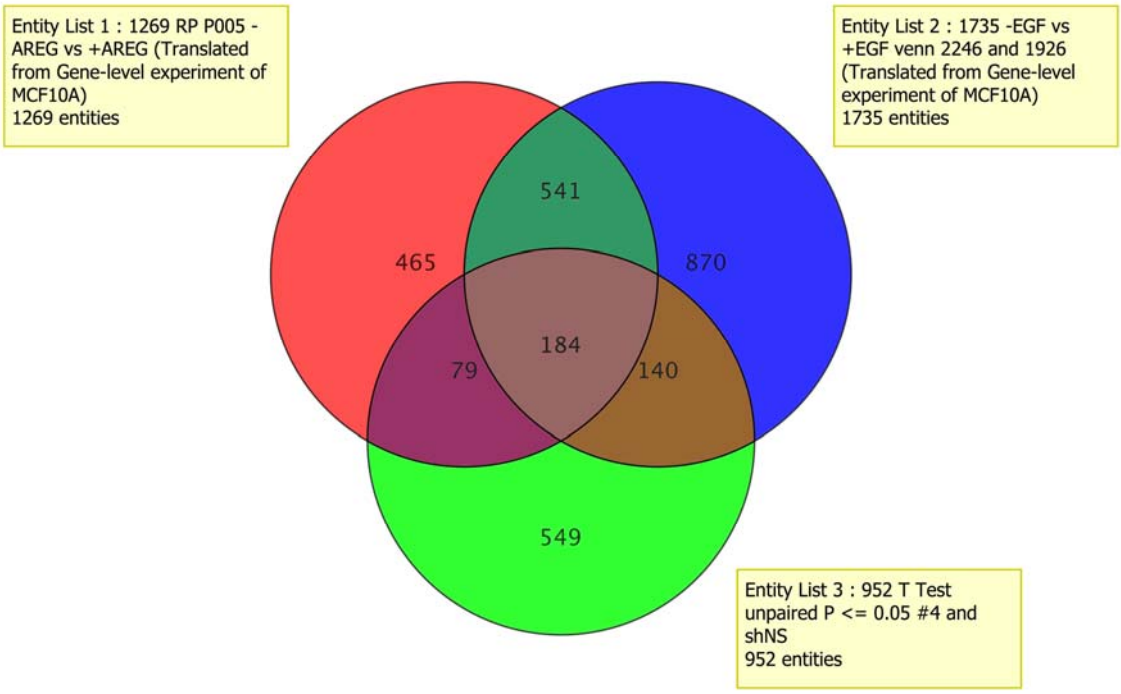
NOTCH signaling in the context of cancer cells only, and with Wnt signaling in both normal and cancer cells. Wnt and NOTCH signaling pathways function in stem cell biology and play major roles in mammary gland development and tumorigenesis (Collu and Brennan, 2007; Molofsky et al., 2004). Wnt signaling alone can cause tumorigenesis; however, recently it has been hypothesized that there is cross talk between two pathways promoting tumorgenesis (Ayyanan et al., 2006).

4.2 Results

Venn diagrams

We performed microarray analysis with three experimental groups, MCF10A+EGF, MCF10A+AREG and SUM-149 cells. MCF10A cells withdrawn of AREG for 24 hours were compared to MCF10A cells growing in the presence of AREG, and, MCF10A cells withdrawn of EGF for 24 hours were compared to MCF10A cells growing in the presence of EGF. SUM-149shNS cells were compared to SUM-149sh4 cells. Figure 4.1 shows a Venn diagram representing the number of genes changed in each gene list and the number of genes that overlap in the three groups as determined by T-Test and RankProd statistical analysis where $p < 0.05$.

Figure 4.1



Venn Diagram

(A) Venn diagram of genes differentially expressed by MCF10A+/- EGF, MCF10A+/- AREG and SUM-149 cells. Expression based on T-test and RankProd statistical analysis. Biological processes and gene lists are represented in Tables (4.2, 4.4, 4.6, and 4.8)

The molecular profile of MCF10A cells regulated by AREG differs from MCF10A cells regulated by EGF. However, there are 725 genes that are commonly regulated by both growth factors (Figure 4.1). The main cellular process regulated by both EGF and AREG in MCF10A cells are: cell cycle, followed by cellular assembly and organization, and DNA replication, recombination, repair (Table 4.1). The top canonical pathways and associated genes regulated by EGF and AREG are listed in Table 4.2. As expected, genes and pathways associated with cell division and thus tissue growth were the most significantly altered by the removal of both EGF and AREG. Cyclins, transcription factors, and cell cycle checkpoint genes were the most significantly affected by AREG and EGF (Table 4.2).

Table 4.1

Molecular and Cellular Functions Regulated by EGF and AREG in MCF10A cells		
Name	p-value	# of molecules
Cell Cycle	**1.59×10^{-31}**	**129**
Cellular Assembly and Organization	2.21×10^{-13}	50
DNA Replication, Recombination, Repair	2.21×10^{-13}	111
Cellular Growth and Proliferation	1.03×10^{-9}	164
Cellular Movement	2.01×10^{-9}	82

Molecular and Cellular Functions Regulated by EGF and AREG in MCF10A cells
Listed are the five most significantly altered cellular functions, the number of genes expressed in each function, and the statistical significance are listed

Table 4.2

Symbol	Fold Change	Gene Name
Mitotic Roles of Polo-Like Kinase		
CDC20	-39.243	cell division cycle 20 homolog (S. cerevisiae)
PRC1	-16.076	protein regulator of cytokinesis 1
CCNB2	-11.985	cyclin B2
CDK1	-9.962	cyclin-dependent kinase 1
CCNB1	-8.233	cyclin B1
PLK4	-7.099	polo-like kinase 4
Role of CHK Proteins in Cell Cycle Checkpoint Control		
CDK1	-9.962	cyclin-dependent kinase 1
E2F2	-6.182	E2F transcription factor 2
RFC4	-5.636	replication factor C (activator 1) 4, 37kDa
CDC25C	-3.901	cell division cycle 25 homolog C (S. cerevisiae)
RFC3	-2.986	replication factor C (activator 1) 4, 38kDa
RFC5	-2.744	replication factor C (activator 1) 5, 37.5kDa
Role of BRCA1 in DNA damage Response		
E2F2	-6.182	E2F transcription factor 2
RFC4	-5.636	replication factor C (activator 1) 4, 37kDa
FANCG	-4.39	Fanconi anemia complementation group G
RFC3	-2.986	replication factor C (activator 1) 4, 38kDa
BARD1	-2.744	BRCA1 associated RING domain
RFC5	-2.744	replication factor C (activator 1) 5, 37.5kDa
Cell Cycle: G2/M DNA Damage Checkpoint Regulation		
TOP2A	-34.175	topoisomerase (DNA) II alpha 140 kDa
CCNB2	-11.985	cyclinB2
CDK1	-9.962	cyclin-dependent kinase 1
CCNB1	-8.233	cyclin B1
CDC25B	-4.891	cell division cycle 25 homolog B (S. pombe)
PKMYT1	-4.413	protein kinase, membrane associated tyrosine/threonine 1
Wnt Beta-Catenin Pathway		
MYC	-2.526	v-myc myelocytomatosis
CCND1	-1.905	cyclin D1
MMP7	2.332	matrix metallopeptidase 7 (matrilysin, uterine)
TGFBR3	3.099	transformaing growth factor, beta receptor III
WNT4	9.755	wingless-type MMTV integration site family, member 4

Summary of genes in specific canonical pathways regulated by EGF and AREG in MCF10A cells

Five signaling pathways associated with cell cycle control and mitosis are listed in bold. The top genes up and down regulated in each pathway are listed beneath each signaling pathway as determined by IPA.

Comparison of MCF10A+AREG cells to MCF10A cells in the absence of AREG for 24 hours resulted in a list of 1269 statistically significant differentially regulated genes (Figure 4.1). These genes are regulated by AREG in the MCF10A model. 465 of these genes are regulated by AREG (Figure 4.1, red). The top cell function regulated by AREG in MCF10A-AREG cells versus MCF10A+AREG gene lists, based on statistics, is cell death followed by cellular movement and cell cycle (Table 4.3). Table 4.3 lists the genes differentially regulated by AREG in some of the statistically significant canonical pathways. Among these pathways are: NRF-2-mediated Oxidative Stress Response, Interferon Signaling, Glutathione Metabolism, Xenobiotic Metabolism Signaling, and Wnt signaling. Genes up and down regulated in the top canonical pathways are listed in Table 4.4. The pathways and genes altered by AREG in MCF10A cells are associated with oxidative stress. Reactive oxidative species (ROS) can cause a significant damage to molecules and cell structures in the cell. NRF-2 signaling induces the transcription of genes influencing oxidative stress, thereby defending the body against oxidative stress (Sykiotis, Science, 2010). Xenobiotics are sources of ROS and glutathione transferases are enzymes that can alleviate/detoxify the ROS stress response. AREG in these cells induces the expression of glutathione signaling moleules to remove the harmful effects of the xenobiotic signaling molecules. In addition to altered ROS signaling Wnt signaling is influenced by the AREG in MCF10A cells.

Table 4.3

Molecular and Cellular Functions Regulated by AREG in MCF10A cells		
Name	**p-value**	**# of molecules**
Cell Death	1.26×10^{-5}	79
Cellular Movement	4.20×10^{-5}	49
Cell Cycle	1.71×10^{-4}	37
Free Radical Transport	2.09×10^{-4}	11
Molecular Transport	2.09×10^{-4}	15

Molecular and Cellular Functions Regulated by AREG in MCF10A cells
Listed are the five most significantly altered cellular functions, the number of genes expressed in each function, and the statistical significance are listed

Table 4.4

Symbol	Fold Change	Gene Name
NRF-2-mediated Oxidative Stress Response		
FOS	-3.664	FBJ murine osteosarcoma viral oncogene homolog
JUNB	-2.192	jun B proto-oncogene
ATF4	-2.161	activating transcriptionfactor 4 (tax-responsive element B67)
SQTM1	2.469	sequestosome 1
MGST3	2.797	microsomal glutathione S-transferase 3
GSTA4	4.192	glutathione S-transferase alpha 4
Interferon Signaling		
IFIT1	1.741	interferon-induced protein with tetratricopeptide repeats 1
PSMB8	1.899	proteosome (prosome macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
JAK1	1.996	janus kinase 1
TAP1	2.021	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
IRF1	3.009	interferon regulatory factor 1
IFIT3	6.298	interferon-induced protein with tetratricopeptide repeats 3
Glutathione Metabolism		
IDH2	2.024	isocitrate dehydogenase 2 (NADP+), mitochondrial
MGST1	2.046	microsomal glutathione S-transferase 1
G6PD	2.282	glucose-6-phosphate dehydrogenase
GSTM1	2.305	glutathione S-transferase mu 1
MGST3	2.797	microsomal glutathione S-transferase 3
GSTA4	4.192	glutathione S-transferase alpha 4
Xenobiotic Metabolism Signaling		
CAMK2B	-8.334	calcium/calmodulin-dependent protein kinase II beta
ALDH1L2	-2.264	aldehyde dehydrogenase 1 family, member L2
PRKCA	-1.862	protein kinase C, alpha
UGT1A10	3.166	UDP glucronosyltransferase 1 family, polypeptide A10
GSTA4	4.192	glutathione S-transferase alpha 4
UGT1A7	7.74	UDP glucronosyltransferase 1 family, polypeptide A7
Ephrin Signaling		
GNG11	-7.916	guanine nucleotide binding protein (G protein), gamma 1
GNA15	-4.82	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)
RAC2	-4.013	ras-related C3 botulinum toxin substrate 2 (rho fmaily, small GTP binding protein Rac2)
HRAS	1.722	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
LIMK2	1.999	LIM domain kinase 2
EPHA4	2.609	EPH receptor A4
Wnt Beta-Catenin Pathway		
RAC2	-4.013	ras-related C3 botulinum toxin substrate 2 (rho fmaily, small GTP binding protein Rac2)
CDH1	1.909	cadherin 1, type 1, E-cadherin (epithelial)
AXIN2	2.884	axin 2
GJA1	3.116	gap junction protein, alpha 1, 43kDa

Summary of genes in specific canonical pathways regulated by AREG in MCF10A cells
Six signaling pathways associated with oxidative stress and ROS signaling are listed in bold. The top genes up and down regulated in each pathway are listed beneath each signaling pathway as determined by IPA.

79 genes were regulated by AREG in both normal (MCF10A) and cancer (SUM-149) cells (Figure 4.1, purple). Cell-to-cell signaling and interaction was the most significantly altered (Table 4.5). In addition, cell movement was a highly significant process that was regulated by AREG in both MCF10A and SUM-149 cells. These analyses corroborate the findings from chapter 3 showing AREG promotes cell motility and invasion in SUM-149 cells. Table 4.6 lists the top canonical pathways and the genes commonly regulated by AREG in SUM-149 and MCF10A cells. These pathways include Clatherin mediated endocytosis and Wnt Signaling; however, the degree of change is greater in the SUM-149 cells than in the MCF10A cells. Genes in these pathways include the integrins, SFRP1, and a frizzled receptor (Table 4.6).

Table 4.5

Molecular and Cellular Functions Regulated by AREG in MCF10A and SUM-149 cells		
Name	p-value	# of molecules
Cell-to-Cell Signaling and Interaction	**2.78×10^{-7}**	**17**
Cell Death	5.83×10^{-6}	20
Cellular Movement	5.92×10^{-6}	14
Cell Cycle	7.54×10^{-5}	12
Cell Growth and proliferation	1.17×10^{-4}	23

Molecular and Cellular Functions Regulated by AREG in MCF10A and SUM-149 cells
Listed are the five most significantly altered cellular functions, the number of genes expressed in each function, and the statistical significance are listed

Table 4.6

Symbol	Fold Change	Gene Name
Clatherin Mediated Endocytosis		
LDLR	-2.887	low density lipoprotein receptor
SH3KBP1	-2.431	SH3-domain kinase binding protein 1
ITGB4	-2.147	integrin, beta 4
ITGB5	-1.954	integrin, alpha 5 (fibronectin receptor alpha polypeptise)
TFRC	1.821	transferrin receptor (p90 CD71)
Wnt Beta-Catenin Pathway		
SOX9	-2.84	SRY (sex determining region Y)-box 9
SFRP1	-2.014	secreted frizzled-related protein 1
FZD8	2.15	frizzled homolog 8 (Drosophila)

Summary of genes in specific canonical pathways regulated by AREG in MCF10A and SUM-149 cells

Five signaling pathways associated with endocytosis and Wnt signaling are listed in bold. The top genes up and down regulated in each pathway are listed beneath each signaling pathway as determined by IPA.

Gene regulation by AREG plays a distinct role in SUM-149 breast cancer cells. 952 genes were altered in their expression after shRNA knock-down of AREG compared to a non-silencing vector (Figure 4.1). The list of genes produced were analyzed by IPA in chapter 3. Table 3.1 (chapter 3) lists the top five signaling pathways altered as a result of AREG knock-down and the top 3 genes up- and down-regulated relative to each signaling pathway.

Of the 952 genes, 549 genes were regulated by AREG only in the SUM-149 breast cancer cells and not the MCF10A cells, 140 genes were regulated by both EGF and AREG in SUM-149 and MCF10A+/-EGF cells, 184 genes were regulated by MCF10A+/-AREG, SUM-149, and MCF10A+/-EGF, and 79 genes were regulated by AREG in SUM-149 and MCF10A+/-AREG cells (Figure 4.1). In the 549 gene list produced the most significant cellular functions affected were cellular movement, cell death, and cell cycle (Table 4.7). The top genes either up- or down-regulated by AREG in SUM-149 cells are listed under each canonical pathway in Table 4.8. The most significantly altered pathway was Agrin Interactions at Neuromuscular Junction. Genes altered in this pathway are predominantly associated with cellular motility, such as ITGB2, CTTN, and ITGA6. The observed decrease in cell invasion and motility following AREG knock-down in SUM-149 cells correlates with the gene lists created by IPA.

Table 4.7

Molecular and Cellular Functions Regulated by AREG in SUM-149 cells		
Name	p-value	# of molecules
Cellular Movement	**1.88×10^{-5}**	**53**
Cell Death	6.79×10^{-5}	84
Cell Cycle	5.65×10^{-4}	24
Cellular Growth and Proliferation	6.05×10^{-4}	89
Cell-to-Cell Signaling and Interaction	7.34×10^{-4}	41

Molecular and Cellular Functions Regulated by AREG in SUM-149 cells
Listed are the five most significantly altered cellular functions, the number of genes expressed in each function, and the statistical significance are listed

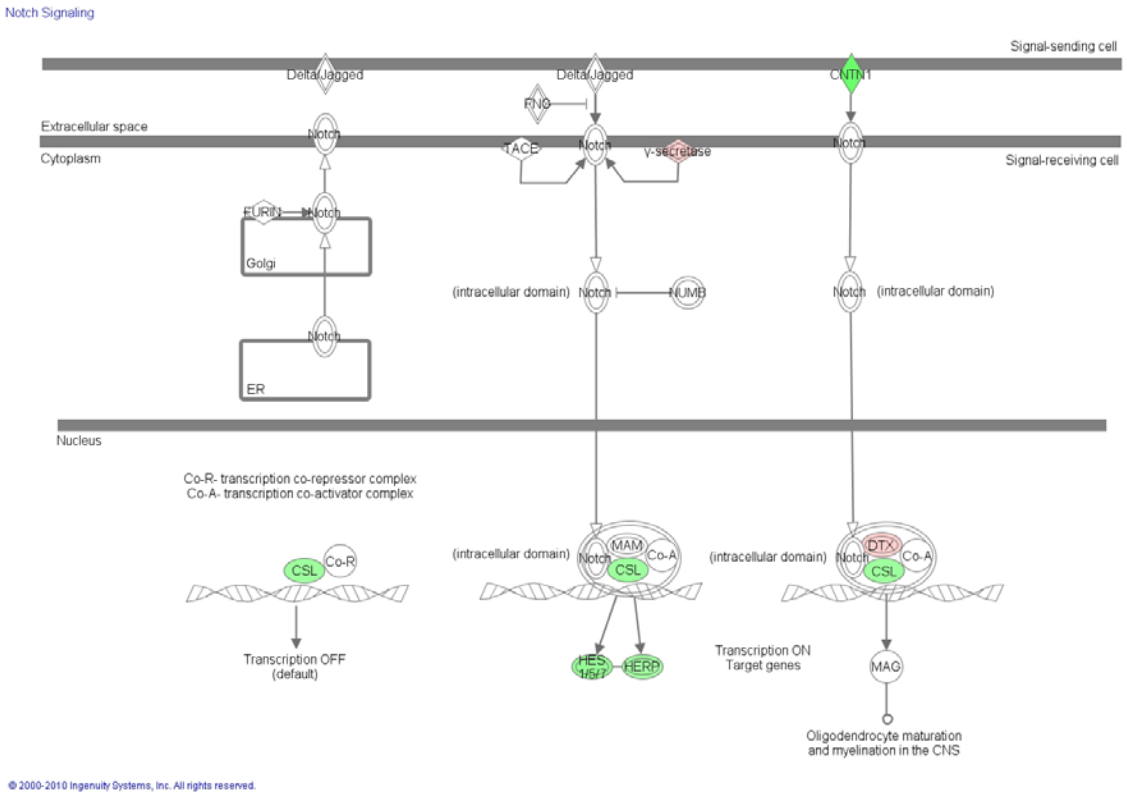
Table 4.8

Symbol	Fold Change	Gene Name
Agrin Interactions at Neuromuscular Junction		
ITGB2	-1.296	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
ITGA6	-1.266	integrin, alpha 6
MAPK1	-1.259	mitogen-activated protein kinase 1
LAMC1	1.178	laminin, gamma 1 (formerly LAMB2)
CTTN	1.309	cortactin
ACTB	1.366	actin, beta
Histidine Metabolism		
SMOX	-1.487	spermine oxidase
ALDH5A1	-1.371	aldehyde dehydrogenase 5 family, member A1
HNMT	-1.314	histamine N-methyltransferase
DBT	1.273	dihydrolipoamide branched chain transacylase E2
ALDH2	1.417	aldehyde dehydrogenase 2 family (mitochondrial)
ALDH3B1	1.521	aldehyde dehydrogenae 3 family, member B1
NOTCH Signaling		
CNTN1	-2.024	contactin 1
HES7	-1.445	hairy and enchancer of split 7 (Drosophila)
RBPJ	-1.336	recombinantion signal binding protein for immunoglobulin kappa J region
HEY1	-1.285	hairy and enchancer of split realted with YRPW motif 1
DTX	1.189	deltex homolog 2 (Drosophila)
PSEN2	1.213	presenilin 2 (Alzheimer disease 4)
ATM signaling		
CDKN1A	-1.718	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
RAD51	-1.354	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)
MRE11A	-1.198	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)
SMC3	-1.156	structural maintenane of chromosomes 3
CREB1	1.212	cAMP responsive element binding protein 1
NFKBIA	1.744	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhbitor, alpha
Wnt Beta-Catenin Pathway		
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

Summary of genes in specific canonical pathways regulated by AREG in SUM-149 cells
Five signaling pathways associated with cell motility, invasion, and NOTCH signaling are listed in bold. The top genes up and down regulated in each pathway are listed beneath each signaling pathway as determined by IPA.

Only in SUM-149 cells did we observe altered NOTCH signaling in response to AREG. NOTCH signaling regulates cell communication in embryonic and adult development. Furthermore we observed the Wnt signaling pathway to be regulated by AREG in SUM-149 cells. Based on the finding that NOTCH signaling was only affected in the SUM-149 cell model and not the MCF10A cell model, we chose to validate molecules in this signaling pathway. Figure 4.2 depicts the NOTCH signaling pathway and the genes altered by AREG in SUM-149sh4 versus SUM-149shNS cells. After AREG knock-down we observed a decrease in gene expression of Contactin1 (CNTN1), a cell adhesion molecule that enhances NOTCH signaling and plays a role in tumor invasion and motility, present on the extracellular surface of a cell. CBF1, Suppressor of Hairless, LAG-1 (CSL), a well characterized transcription repressor, hairy/enhancer of split (E(spI))-related family of transcription factors (HES genes 1,5, and 7, and HEY1), transcriptional targets of CSL, were also down-regulated after AREG knock-down (Purow et al., 2005). The HEY/HES family are NOTCH target genes that counteract or block differentiation. They negatively regulate the expression and/or function of transcription factors influencing the determination of cell lineage (Meier-Stiegen et al., 2010; Schwanbeck et al., 2010). We confirmed the down-regulation of CNTN1, HES7, and HEY1 by real-time RT-PCR (Figure 4.3). HES7 and HEY1 in Hodgkin lymphoma cells have been shown to be over expressed as a result of aberrant NOTCH signaling (Kochert et al., 2010).

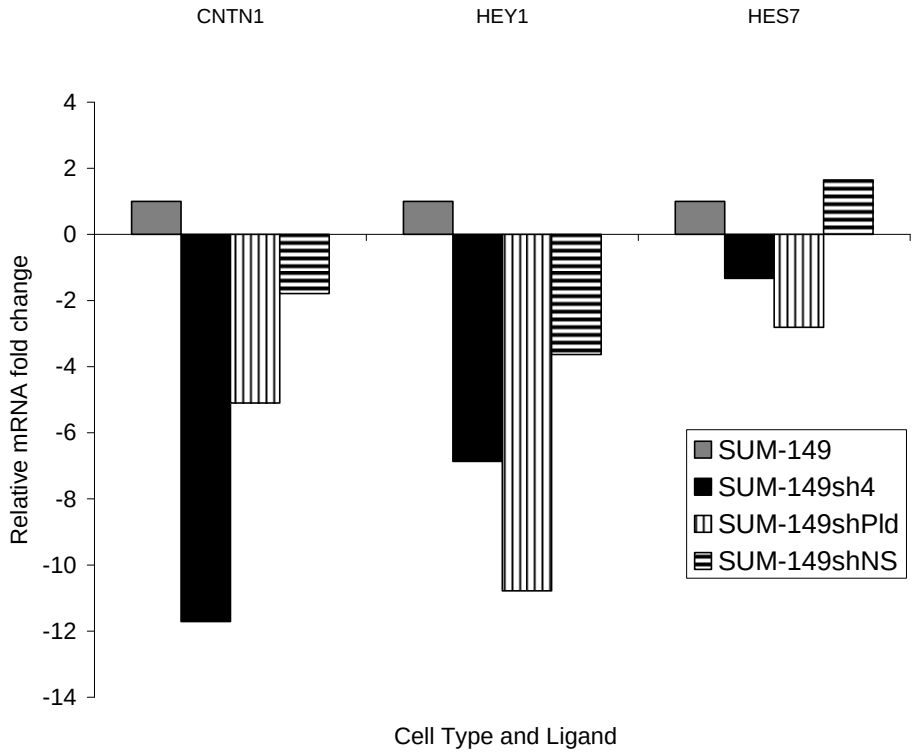
Figure 4.2



NOTCH Signaling Pathway

Genes in the NOTCH signaling pathways that are regulated by AREG only in the SUM-149 cells. Green indicates the gene is down-regulated and red indicates the gene is up-regulated.

Figure 4.3



Validation of decreased expression of CNTN1, HEY1, and HES7 in AREG knock-down cells

(A) Relative mRNA expression by real-time RT-PCR of CNTN1, HEY1, and HES7 mRNA expression in AREG knock-down cells with SUM-149 parental cells normalized to one.

4.3 Discussion

By utilizing IPA we were able to identify signaling pathways that were altered by the expression profiles in figure 4.1. Wnt and NOTCH signaling pathways arose as pathways that were affected in the expression profiles of SUM-149 and MCF10A+/-AREG cells. A recent study, aimed at identifying priming factors contributing to progression from lung dysplasia to cancer identified an up-regulation of AREG and notably, NOTCH and Wnt signaling cascades (Rohrbeck and Borlak, 2009). The identification of these pathways in our system encourages further investigation.

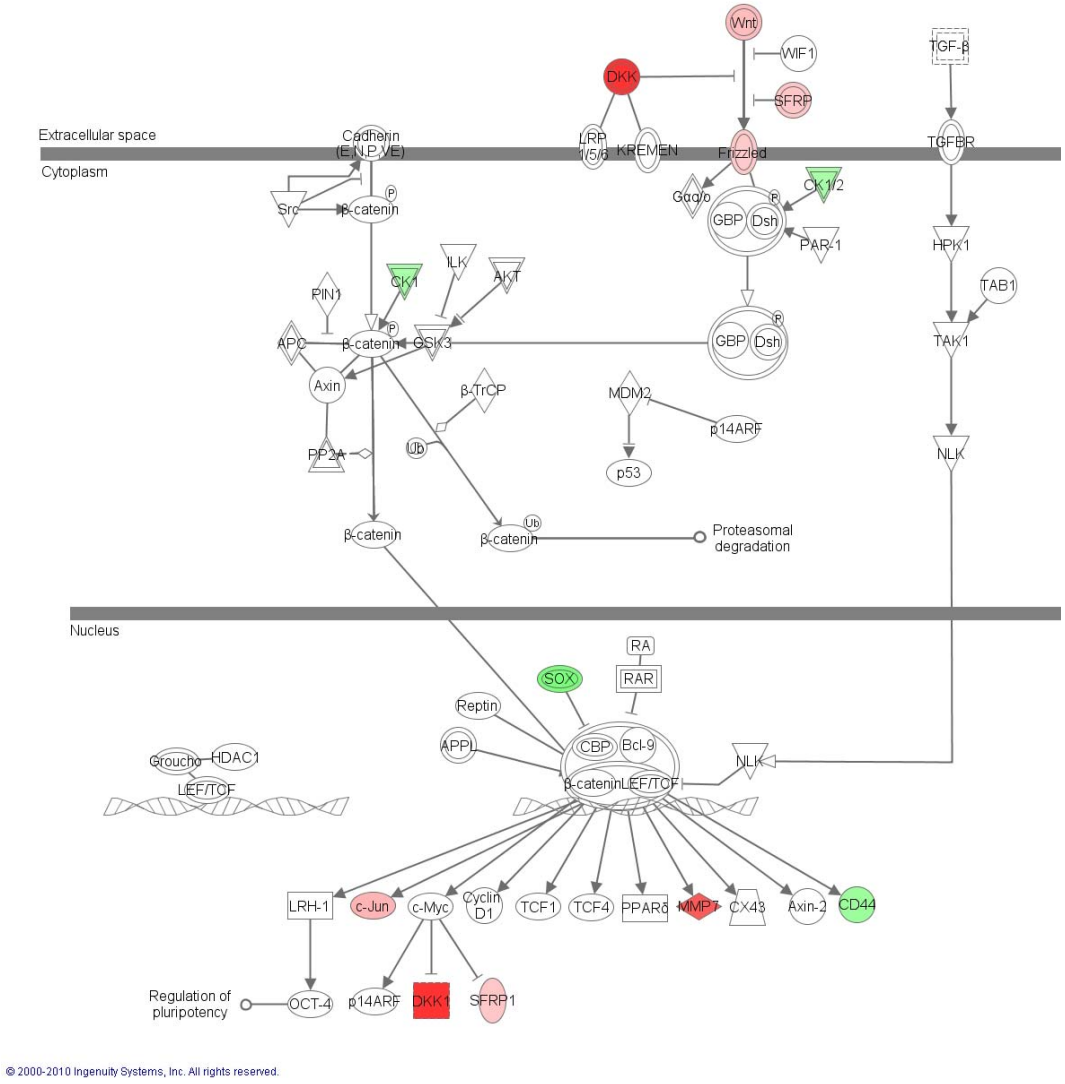
NOTCH signaling occurs when the extracellular domain of the NOTCH receptor on one cell binds to the extracellular domain of a NOTCH ligand on a neighboring cell; therefore, NOTCH signaling requires cell-cell contacts. Interestingly NOTCH signaling was altered by AREG only in the SUM-149 cells (Table 4.7 and 4.8, Fig 4.2 and 4.3). The MCF10A+/-AREG gene profiles did not reflect any changes in NOTCH signaling. This suggests that activation of the NOTCH signaling cascade is contextually dependent, as we only observed its activation in the cancer cell model.

In addition to NOTCH signaling, genes commonly regulated by SUM-149sh4 versus shNS and MCF10A+/-AREG cells influenced the Wnt signaling pathway (Table 4.5 and 4.6, Fig. 4.4). Wnt signaling influences stem cell self-renewal in a variety of epithelial cells (Reya et al., 2001). We observed changes in the Wnt pathway in all of the gene lists, however the change in gene regulation varied. In the Wnt pathway, the level of change in expression of the genes increased in the AREG model compared to the EGF model. Similarly, genes regulated in the SUM-149 cells, especially DKK1, expressed

higher levels of differential expression than the MCF10A+/-AREG gene expression profiles. DKK1, SFRP1, and Wnt were down-regulated in the absence of AREG. The array data was confirmed by analyzing DKK1 mRNA expression by real-time RT-PCR (Fig. 3.10). DKK1 expression was down-regulated in all three cell models, but the expression was down-regulated by approximately one tenth of the expression levels observed when regulated by AREG.

A summary of the flow of genes expression, molecular functions, and signaling pathways are shown in a flow chart in figure 4.5. In cancer cells, the change in expression of negative Wnt regulators is dramatically increased. This suggests that Wnt signaling, in addition to NOTCH signaling, is context dependent.

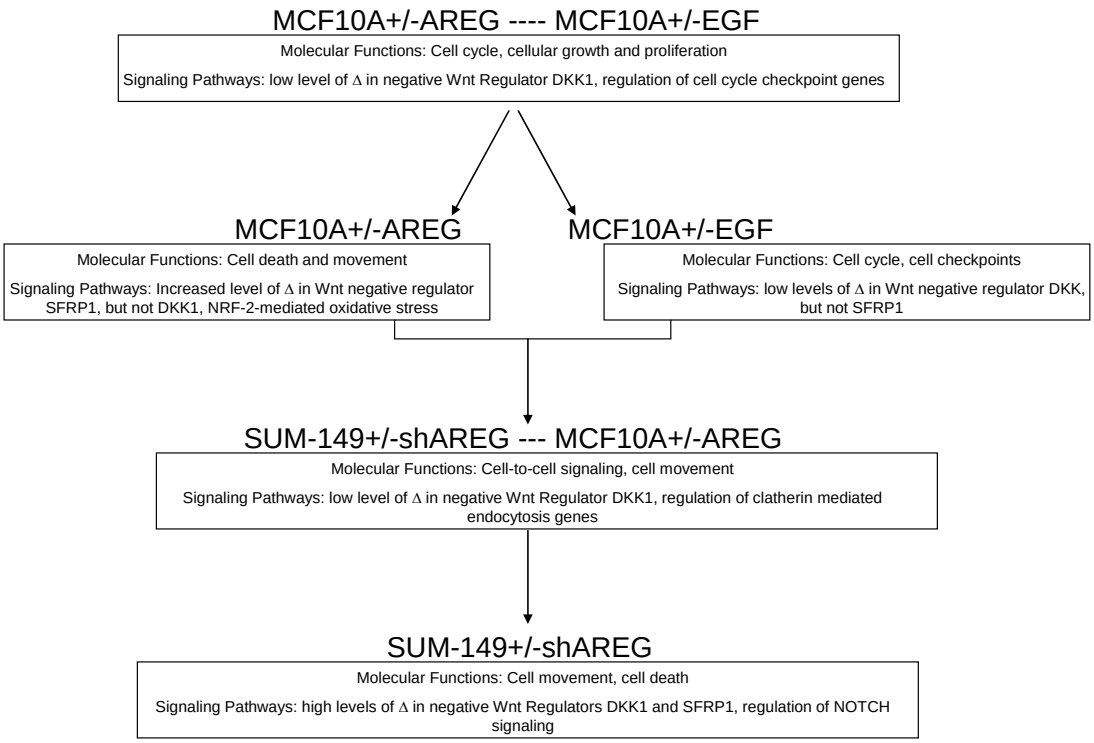
Figure 4.4



Wnt Signaling Pathway

Genes in the Wnt signaling pathways are regulated by AREG in both MCF10A and SUM-149 cells. Green indicates the gene is down-regulated and red indicates the gene is up-regulated. The intensity of the red or green indicates the level of up- or down-regulation

Figure 4.5



Change of Flow in Molecular Function and Signaling Pathway Regulation

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

SUM-149 is an aggressive, highly transformed, primary, triple negative, inflammatory breast cancer cell line. These cells are EGF-independent for growth, motile, and highly invasive (Hoffmeyer et al., 2005; Jia et al., 2004; Willmarth and Ethier, 2006). They express relatively high levels of constitutively active EGFR and are dependent on EGFR for growth (Rao et al., 2000). In order to understand the EGF-independent phenotype and the constitutive activation of EGFR, genes that promoted EGF-independent growth were isolated from MCF10A cells infected with a cDNA library from SUM-149 cells. From these experiments AREG was identified as the gene mediating EGF-independent growth in SUM-149 cells (Berquin et al., 2005).

The discovery of AREG expression in SUM-149 cells and the identification of AREG and EGFR co-over expression in inflammatory breast cancer lead to our focus on AREG (LeJeune et al., 1993; Ma et al., 2001). AREG is over expressed at the protein and mRNA level. Inhibition of the ligand with a neutralizing AREG antibody, preventing ligand binding to EGFR, inhibited cell proliferation suggesting that AREG is the autocrine factor driving the EGF-independent phenotype (Willmarth and Ethier, 2006). In addition, we observed that the synthesis of AREG is also regulated by EGFR signaling. It was the results from these experiments that led us to the identification of an AREG/EGFR autocrine loop in SUM-149 cells. The AREG/EGFR autocrine loop, in addition to regulating cell proliferation, was also found to regulate the localization of

EGFR and the cellular motility and invasiveness of SUM-149 cells (Willmarth and Ethier, 2006). Stemming from the findings of Pan et. al. that SUM-149 cells express constitutively active NF-κB, our lab discovered a NF-κB/IL-α feedback loop interconnected the AREG/EGFR autocrine loop that influences that proliferative, migratory, and invasive capacity of the SUM-149 cells (Pan et al., 2003; Streicher et al., 2007).

These previous experiments led us to knock-down the expression of AREG. Our initial hypothesis, based on the observation that an AREG/EGFR autocrine loop functions in regulating SUM-149 cell proliferation, was that AREG knock-down would inhibit cell proliferation. We found that AREG knock-down, in a polyclonal population, allowed for cells to continue proliferating albeit at a slower rate. However, as cells were passaged the levels of AREG expression increased. In addition, the EGFR levels increased and localized to the cell membrane. This suggested that breast cancer cells are able to respond to very low levels of EGFR ligands, particularly when EGFR accumulates on the cell surface.

Recently, clones were derived from SUM-149sh4 cells. By real-time RT-PCR, clonal derivative SUM-149sh4-c1 showed the greatest knock-down of AREG compared to the SUM-149 parental and SUM-149shNS controls while c5 had the highest levels of AREG mRNA and protein expression (data not shown). Some cells growing in the polyclonal population are producing AREG. This suggests that AREG produced in these cells supports the growth of the entire population by paracrine signaling mechanisms and

that these AREG expressing cells are slowly taking over the culture, hence why we observe increased AREG expression as the cells are passaged.

Given that SUM-149 cells are capable of growing in serum free conditions, SUM-149sh4 clonal derivatives were switched from 5% serum to serum free media. SUM-149sh4-c1 grows slower than the polyclonal SUM-149sh4 cells while SUM-149sh4-c4 and c6 do not proliferate at all and show signs of mitotic catastrophe and apoptosis in serum free conditions (data not shown). It is now our understanding that there may be serum factors that help to promote proliferation of the SUM-149sh4 population. To truly understand AREG knock-down we need to continue our studies using the clonal derivatives grown in serum free media. Future studies should be directed at studying AREG knock-down in other cell lines over expressing AREG, such as the ovarian cancer cell line, KCI-OV1. In addition, it would be valuable to study the invasive capacity and rate of proliferation of the knock-down cells in an *in vivo* model.

Our findings presented in this dissertation highlight the pathological role of AREG in tumor progression. In the past, EGFR's promotion of disease progression has been associated with EGFR when it is either amplified or mutated. In our cell model, AREG drives EGFR accumulation to the cell membrane in the absence of an EGFR mutation or amplification. Moreover, our findings suggest that cell surface EGFR when stimulated by AREG, as opposed to EGF, has a pathological role in promoting tumor invasion.

Previous studies support our finding of AREG's role in enhancing cellular invasion (Gschwind et al., 2003; Zhang et al., 2004). The mechanism(s) by which AREG

can promote invasion is still unclear. The genetic profiles we have obtained in our microarray studies lead us to cell signaling pathways altered in the absence of AREG for further investigation. For example, Wnt signaling is one signaling pathway regulated only by AREG and not by EGF in either normal (MCF10A) and cancer (SUM-149) cells.

Wnt and NOTCH are two signaling pathways, among others like hedgehog, that regulate stem-cell self renewal (Molofsky et al., 2004). Stem cell self-renewal is regulated by the coordination of multiple signaling pathways (Molofsky et al., 2004). Wnt signaling promotes the proliferation and maintenance of stem cells by either canonical (with β-catenin) or non-canonical signaling (Cobas et al., 2004; Reya and Clevers, 2005). Expression of DKK1 and Secreted frizzled-related protein1 (SFRP1), genes involved in the Wnt pathway, inhibit Wnt signaling and, therefore, the proliferation of stem cells (Mao et al., 2002). Removal of either EGF or AREG from normal epithelial cells slightly up-regulates DKK1, but not secreted frizzled receptor protein 1 (SFRP1), and knock-down of AREG in SUM-149 breast cancer cells dramatically up-regulates DKK1 and SRFP1. These data suggest that SUM-149 cells with AREG/EGFR signaling induces Wnt signaling, which promotes stem cell self-renewal, perhaps in concert with NOTCH signaling. Further experiments should analyze the involvement of Wnt signaling and invasion in cells that are responding to an AREG/EGFR autocrine loop

In addition to Wnt signaling, gene lists and signaling pathways derived from microarray data comparing the SUM-149 AREG knock-down to the shNS control shined a light on NOTCH signaling. NOTCH signaling has been suggested to act coordinately

with Wnt signaling to regulate stem cell self-renewal. Interestingly, NOTCH signaling was observed only in the cancer cells and not in the MCF10A cell model.

NOTCH signaling, depending on the context, can inhibit or promote stem-cell renewal (Henrique et al., 1997; Hitoshi et al., 2002). In addition, NOTCH signaling has been observed to promote differentiation (Morrison et al., 2000). Our data suggest that AREG/EGFR signaling is playing a role in the stem cell biology of breast cancer cells by shifting the probability of a symmetric division of two stem cells, therefore maintaining the stem cell population. The down-regulation of the HES/HEY family of stem cell differentiation repressors after AREG knock-down indicates that in the presence of AREG/EGFR signaling stem cells continue to divide and produce more stem cells. This results in expansion of a self-renewing stem cell population resulting in tumorigenesis. Further investigation of context dependent NOTCH signaling in SUM-149 cells as regulated by AREG would be of great interest.

Our studies translate to the clinic, as AREG is a potential bio-marker for aggressive and invasive disease. After EGFR inhibition, the AREG/EGFR axis makes cells more sensitive to radiation (Rao et al., 2000). Additionally, AREG/EGFR sensitizes patients to treatment with cetuximab (Khambata-Ford et al., 2007). Our studies suggest that AREG/EGFR promotes an aggressive and highly transformed form of breast cancer. Recently, high concentrations of AREG in the serum have been suggestive of better disease-specific survival when non-small cell lung patients were treated with EGFR tyrosine kinase inhibitors (Vollebergh et al., 2010). Therefore, patients could benefit

from being screened for the serum levels of AREG and then be given a more appropriate treatment regime.

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ABSTRACT

AMPHIREGULIN (AREG) AND EPIDERMAL GROWTH FACTOR (EGF): DISPARATE IN EGFR SIGNALING AND TRAFFICKING

by

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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 presented dissertation 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 SUM-149 cells. In an additional microarray study, changes in gene expression were analyzed from cDNA transcribed from RNA isolated from MCF10A cells growing in the presence of AREG or EGF and after 24 hours withdrawl of the respective ligand. Genes regulating WNT signaling, but not NOTCH signaling, were altered in the MCF10A cells. Thus, the pathway that AREG/EGFR signaling effects is contextually dependent on the cell type that it is functioning in. We conclude that 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.

AUTOBIOGRAPHICAL STATEMENT

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Andrea received a Bachelors of Arts degree in Biological Sciences from Wayne State University in 2006. She commenced her studies in the Graduate Program in Cancer Biology at Wayne State University School of Medicine in the fall of 2006 under the guidance of Stephen P. Ethier. During her time as a Ph.D. student, Andrea published 2 research articles as first and second author. A major focus of Andrea's research has been on understanding the role of AREG in cancer. After receiving her doctorate, she will peruse a post-doctoral position in molecular biology.