REGULATION OF THE MEK/ERK SIGNALING CASCADE BY ADAM12 IN TRIPLE-NEGATIVE BREAST CANCER CELLS

by

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Abstract

Mitogen-activated protein kinase (MAPK) signaling plays an important role in the proliferation, survival, and therapy resistance of breast cancer cells. Two important protein kinases involved in the MAPK pathway are MEK and ERK. The MEK/ERK signaling cascade can be stimulated by activation of the epidermal growth factor receptor (EGFR) upon binding of EGF-like ligands, which are released from cells by ADAM proteases. EGFR is frequently overexpressed in triple-negative breast cancer (TNBC), a particularly aggressive form of breast cancer. Our analysis of clinical data revealed that high expression of ADAM12, but not other ADAMs, in TNBC is associated with poor patient survival. Thus, we hypothesized that ADAM12 plays a critical role in the progression of TNBC, possibly by stimulating MEK/ERK activity in an EGFR-dependent manner. To test this hypothesis, ADAM12 was knocked-down (KD) in SUM159PT TNBC cells, which express high levels of the endogenous ADAM12 protein. An antibody array assay indicated a significant decrease in the activation of the MAPK pathway in SUM159PT cells after ADAM12 KD. The decrease in MAPK activity was further confirmed by Western blotting using phospho-MEK and phospho-ERK specific antibodies. Additionally, conditioned media from ADAM12-deficient SUM159PT cells failed to support the survival of MCF10A cells, suggesting that ADAM12 KD reduced the release of pro-survival growth factors from SUM159PT cells. Based upon this data, we propose that ADAM12 is a novel regulator of the MAPK pathway and a potential therapeutic target in breast cancer.

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Acknowledgements

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Lastly, I would like to thank my friends and family for being there for me and providing unflinching support, especially when I needed it most.

Dedication

I would like to dedicate this work to my friends, family, and loved ones who have constantly provided me with support and motivation. I could not have done this without them.

Chapter 1 - Introduction

The Biological Role of ADAMs

The ADAM family of metalloproteases

Transmembrane proteins that are a part of the "A Disintegrin And Metalloprotease" (ADAMs) family of zinc proteases are responsible for the cleavage of the extracellular domains of type I and type II membrane proteins. This process of protein cleavage is referred to as "ectodomain shedding" and acts on growth factors, cytokines, adhesion molecules, chemokines, and their respective receptors [Reiss & Saftig 2009; Weber & Saftig 2012; Zolkiewska 2009; Seals & Courtneidge 2003]. So far, 40 mammalian ADAMs have been identified, 20 of which are found in humans (Figure 1.1). ADAMs play fundamental roles in several biological processes, including developmental processes, cell-cell and cell-matrix adhesion, primarily by modulating interactions between membrane proteins [Li *et al.*, 2012; Weber & Saftig 2012].

ADAMs contain a metalloprotease domain that may or may not include an active site, a disintegrin domain, a cysteine-rich domain and/or a EGF-like domain, an α-helical transmembrane domain, and a cytoplasmic domain (Figure 1.2). The cytosolic tails of ADAMs are highly diverse and vary in their length, sequence, and protein-binding capabilities. Additionally, ADAMs contain a secretion signal at their N-terminus, followed by a prodomain that is involved in the protein's proper folding and that is cleaved by furin-like enzymes during the transit through the Golgi complex. Alternative mRNA splicing also results in the formation of soluble isoforms of several ADAMs [Weber & Saftig 2012; Zolkiewska 2009; Seals & Courtneidge 2003]. The multi-domain nature of ADAMs allows them to perform biological functions that include cellular adhesion due to the disintegrin domain, proteolytic cleavage by the

metalloprotease domain, and/or intracellular signaling by means of the cytoplasmic domain [Asayesh *et al.*, 2005; Seals & Courtneidge 2003]. Notably, ADAM12 has been shown to facilitate transforming growth factor β (TGFβ) signaling by stabilizing the TGFβ type II receptor in early endosomes independent of the proteolytic activity of ADAM12 [Atfi *et al.*, 2007].

From the twenty ADAMs found in humans, twelve (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30, and 33) contain a conserved catalytic active site sequence (HEXGHXXGXXHD) (Figure 1.1). The catalytic site forms a globular structure with two subdomains and an active site cleft with a zinc-binding motif. The zinc-binding motif contains three conserved histidine residues surrounding a zinc ion, which is responsible for regulating cleavage specificity. The main regulation of the catalytic activity of the ADAMs occurs through the cysteine-rich region, which contains a hyper-variable region that is in close proximity to the metalloprotease domain [Li *et al.*, 2012; Weber & Saftig 2012]. The proteolytic activity can be inhibited by the prodomain of ADAMs if it is not removed during maturation in the Golgi complex. This is attributable to a conserved cysteine residue that resides in the prodomain that interacts with the zinc ion, preventing the binding of substrates [Wart & Hansen, 1990]. Studies have shown that the EGF-like domain and the disintegrin domain play key roles in the binding of substrates to ADAMs as well [White 2003; Zolkiewska 1999].

ADAMs display cell- and tissue-type specificity. Some ADAM proteins are preferentially expressed in germline cells, while others are present in somatic cells. However, most ADAMs are expressed at rather low levels in adult tissues. Furthermore, ectodomain shedding by ADAMs can regulate the expression of other ADAMs and membrane proteins through activation of signaling downstream of receptors, such as Epidermal Growth Factor Receptor (EGFR) [Chow & Patron 2007; Seals & Courtneidge 2003; Pollheimer *et al.*, 2014].

ADAMs in cancer

Abnormal expression of several ADAMs has been implicated in many diseases, including cancer. Notable ADAMs containing oncogenic potential include ADAM9, 10, 12, 15, and 17, all of which possess proteolytic activity. Studies suggest that the proteolytic activity is one of the main roles of ADAMs in cancer. One of the mechanisms by which these ADAMs promote cancer is suggested to be through the activation of EGFR signaling by the cleavage of membrane-bound EGFR ligands (Figure 1.3) [Weber & Saftig 2012; Zolkiewska 2009; Duffy *et al.*, 2011]. EGFR ligands that are cleaved from their membrane-bound "pro-form" into a "mature" form are released from cells into the extracellular compartment and bind to EGFR in an endocrine, paracrine, or autocrine manner. EGFR activation culminates in increased cellular proliferation, growth, and survival [Higashiyama *et al.*, 2008; Weber & Saftig 2012].

As a result of alternative mRNA splicing, ADAM12 exists as one of two isoforms, the transmembrane ADAM12L, and the secreted ADAM12S. Both isoforms contain similar extracellular domains, but ADAM12S is not membrane-bound and therefore does not contain a transmembrane or cytoplasmic domain [Muggy *et al.*, 2013; Pollheimer *et al.*, 2014; Wewer *et al.*, 2006; Seals & Courtneidge 2003]. ADAM12 is highly expressed during embryogenesis in several tissues, such as skeletal muscle, but the post-natal expression of ADAM12 tends to be low. Importantly, ADAM12 has been found to be upregulated in several cancers including cancer of the breast, lung, bladder, brain, and prostate. The elevation of ADAM12 in these cancers has been suggested to be partly due to epigenetic suppression of a Z-DNA forming negative regulatory element (NRE) in the ADAM12 promoter [Ray *et al.*, 2013; Pollheimer *et al.*, 2014; Ray *et al.*, 2011].

ADAM12 is considered to play a key role in the aggressive and metastatic nature of many cancers. A key example of this is the upregulation of ADAM12 in triple-negative breast cancer (TNBC), which tends to be highly aggressive and metastatic (see Breast cancer subtypes, pg. 10, "*Overexpression of EGFR in Cancer*"). Additionally, ADAM12 has been shown to increase tumor progression by decreasing tumor cell apoptosis and increasing stromal cell apoptosis [Roy *et al.*, 2011; Kveiborg *et al.*, 2005; Muggy *et al.*, 2013; Shao *et al.*, 2014].

Epidermal Growth Factor Receptor Signaling

Overview

Epidermal growth factor receptor (EGFR) is one of four members of the ErbB receptor tyrosine kinase (RTK) family, which consists of ErbB1 (EGFR, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) [Jutten & Rouchop 2014]. EGFR is a 170-kDa transmembrane receptor that contains a glycosylated extracellular-ligand binding domain, a transmembrane α-helix, and an intracellular tyrosine kinase domain [Bazley & Gullick 2005]. EGFR is a key regulator of several biological signaling pathways involved in cellular growth, proliferation, and survival (Figure 1.3).

ErbB receptor signaling

Many ErbB ligands can vary in their affinity and specificity for receptors. Within the extracellular ligand-binding domain of EGFR, there are four subdomains spanning approximately 620 amino acids. These subdomains are referred to as L_1 (I), S_1/CR_1 (II), L_2 (III), and S_2/CR_2 (IV). L_1 and L_2 form the ligand-binding site, whereas S_1 and S_2 are cysteine-rich regions (CR_1/CR_2), which help stabilize the receptor dimers, along with regulating ligand binding affinity (Figure 1.4) [Bazley & Gullick 2005; Walker *et al.*, 2004; Schlessinger 2000].

Ligands are capable of performing endocrine, paracrine or autocrine activation of EGFR on distant cells, neighboring cells, or within the same cell, respectively. EGFR ligand binding induces receptor dimerization and autophosphorylation of several tyrosine residues in its cytoplasmic domain [Schneider & Wolf 2009; Wilson *et al.*, 2009; Vinante *et al.*, 2013].

EGFR ligands include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG), and epigen (EPGN). These are all synthesized as transmembrane proteins that need to be cleaved in order to become biologically active. Contrary to EGFR, ErbB3 and ErbB4 bind distinct isoforms of the neuregulin growth factors (NRG1-4), however, ErbB3 lacks any intrinsic tyrosine kinase activity [Schneider & Wolf 2009; Schlessinger 2000; Wilson *et al.*, 2009]. The extracellular domain of ErbB2 does not bind any ligands, but the receptor is constitutively active and is the most preferred heterodimerization partner for all other ErbB receptors (Figure 1.5) [Schlessinger 2000].

The ability of EGFR to not only homodimerize but heterodimerize with other ErbB receptors allows for diverse biological responses. EGFR dimerization induces phosphorylation of its C-terminal domain at sites such as Y992, Y1045, Y1068, Y1086, S1142, Y1148 and Y1173 [Wilson *et al.*, 2009]. Intracellular signaling proteins that contain Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains interact with phosphorylated tyrosine residues in EGFR. Additional intracellular signaling proteins then interact with SH2 and PTB proteins, linking EGFR activation to internal signaling cascades. An example of such an SH2 domain-containing protein is growth factor receptor-bound protein 2 (GRB2), which is part of the SHC/GRB2/SOS complex that can directly activate downstream targets and promote cell growth

and proliferation (Figure 1.3) [Schneider & Wolf 2009; Wilson *et al.*, 2009; Guo *et al.*, 2003; Wagner *et al.*, 2013].

EGFR signaling can be regulated by a number of positive and negative feedback loops. One such loop involves regulation of ligand availability by the proteolytic cleavage of EGF-like ligands by ADAM proteases, which in turn are induced by EGFR signaling [Avraham & Yarden, 2011]. Src is a non-receptor kinase capable of phosphorylating EGFR tyrosine residues, such as Tyr845, and can regulate the release of EGFR ligands by promoting their cleavage via transmembrane metalloproteases, such as ADAMs [Yu *et al.*, 2014; Mueller *et al.*, 2012]. EGFR can also undergo receptor endocytosis and dephosphorylation to regulate growth factor signaling. Additional regulatory loops include activation of EGFR expression at the transcriptional level, regulation of translation of EGFR mRNA, alternative mRNA splicing, proteolytic processing of the receptor, and intracellular trafficking [Avraham & Yarden, 2011].

ErbB receptors have been considered therapeutic targets in cancers due to overexpression of these receptors in many cancers and their involvement in proliferative signaling pathways [Albanell & Baselga 1999; Masuda et al.., 2012]. EGFR, ErbB2, and ErbB3 all play essential roles in signaling cascades involved in proliferation, survival, and metastasis of breast cancer cells [Foley *et al.*, 2010]. However, even though ErbB4 can bind ligands and induce tyrosine kinase activation, its signaling responses stimulate anti-proliferative responses in mammary epithelial cells [Cook *et al.*, 2008]. Therefore, in triple-negative breast cancer (TNBC) that lacks overexpression of ErbB2, EGFR signaling becomes the main mediator relied upon for transduction of growth signaling important for tumorigenesis.

RAS/RAF/MEK/ERK MAPK downstream signaling pathway

Upon activation of EGFR, the SHC/GRB2/SOS protein complex is recruited to the intracellular domain of EGFR. SOS (son-of-sevenless) is a GDP/GTP exchange factor for RAS, a small GDP/GTP-binding protein that is typically farmesylated and anchored to the plasma membrane. The RAS family is composed of three members, NRAS, HRAS, and KRAS [Wennerberg *et al.*, 2005; Yan *et al.*, 1998; Holderfield *et al.*, 2014; Hancock 2003]. The main differences between the individual RAS proteins are located in the C-terminal hypervariable region (HVR), which assists in the localization of RAS proteins to the plasma membrane. GTP-bound RAS proteins activate several signaling pathways, including the RAF/MEK/ERK, PI3K/Akt, and PLCγ pathways [Yan *et al.*, 1998; Rajalingam *et al.*, 2007; Roberts *et al.*, 2006; Stokoe *et al.*, 1994].

The RAS/RAF/MEK/ERK (MAPK) pathway links signaling from extracellular receptors to the stimulation of transcription factors that act as terminal effectors that regulate gene expression. Activation of the pathway depends predominantly on the stimulation of growth factor receptors, although there are other known stimulators, such as G protein-coupled receptors (GPCRs) [Steelman *et al.*, 2011; Prior *et al.*, 2012; Yan *et al.*, 1998]. Different RAS proteins have different potencies to stimulate distinct pathways; for example, KRAS is a more potent activator of the RAF/MEK/ERK pathway, whereas HRAS is more effective at activating the PI3K/Akt pathway, and NRAS tends to inhibit apoptosis. Aberrant MAPK signaling has been linked to a malignant phenotype. Specifically, *RAS* genes mutations have been frequently found in lung, colon, pancreatic, and thyroid cancers, permitting RAS to remain constitutively active [Prior *et al.*, 2012; Yan *et al.*, 1998; Rajalingam *et al.*, 2007].

Once RAS becomes bound to GTP (GTP-RAS), it can activate RAF, a serine/threonine kinase, which is also known as MAPK kinase kinase (MAP3K). RAF is recruited to the membrane by GTP-RAS and is activated upon binding to GTP-RAS via its RAS binding domain (RBD). The RBD of RAF is considered one of the mechanisms responsible for the membrane localization of RAF. After RAF is activated, homo- or heterodimers are formed involving A-RAF, B-RAF, or C-RAF (RAF-1) [Bondeva *et al.*, 2002; Wojnowski *et al.*, 2000]. These three isoforms of RAF have three functional domains known as CR1, CR2, and CR3 that include the RAS binding domain, the regulatory domain, and the kinase domain, respectively. CR2 has different regulatory phosphorylation sites. Deletion of the CR1 and CR2 domains leads to constitutive activation of RAF [Steelman *et al.*, 2004]. All three RAF kinase proteins can phosphorylate and activate mitogen-activated protein kinase/ERK kinase (MEK1/2), otherwise known as MAPK kinase (MAP2K). B-RAF is considered to be the most potent activator of MEK1/2, whereas A-RAF is considered to be the weakest [Wojnowski *et al.*, 2000; Weber *et al.*, 2001; Yan *et al.*, 1998].

MEK1/2 is a dual-specificity protein kinase, and its preferred downstream target is the extracellular signal-regulated kinase (ERK1/2), otherwise known as MAPK. Activated ERK1/2 (p44/p42) can phosphorylate and activate a copious list of substrates, with several hundred currently known. [Roberts *et al.*, 2006; Yoon *et al.*, 2006; Steelman *et al.*, 2011; Roskoski *et al.*, 2012]. ERK1/2 can affect many cellular activities by translocating to the nucleus upon phosphorylation and regulating several transcription factors, including Ets, Elk-1, and c-Myc. Regulation of these transcription factors allows ERK1/2 to modulate cell proliferation, differentiation, survival, and apoptosis. Additionally, ERK1/2 activation regulates expression of several growth factors and their receptors at the cell surface. Since ERK1/2 can increase the

expression of EGF-like factors, it can potentially regulate the EGFR activity via a feedback loop mechanism. Therefore, EGFR can be both an upstream and a downstream target of the RAS/RAF/MEK/ERK kinase signaling cascade [Roberts *et al.*, 2006; Kim *et al.*, 2010; McCurby *et al.*, 2006; Roskoski *et al.*, 2012]. Overstimulation of ERK1/2 has been shown to be a key event in the growth and progression of cancer. Therefore, cancer therapies that target and inhibit the RAF/MEK/ERK pathway could potentially regulate tumor cell growth and survival and could be considered promising new treatments [Roberts *et al.*, 2006; Downward *et al.*, 2003; Balko *et al.*, 2013].

Activation of ERK1/2 can induce a negative feedback loop where ERK1/2 can phosphorylate certain sites of the regulatory domain of C-RAF and thus either inhibit or enhance the C-RAF activity, depending on the site of phosphorylation. In contrast, phosphorylation of B-RAF or MEK1/2 by ERK1/2 leads to inhibition of these kinases [Holderfield *et al.*, 2014; Steelman *et al.*, 2011]. Additionally, ERK1/2 can phosphorylate SOS and interfere with its coupling to the receptor, thereby inhibiting further RAF/MEK/ERK activation. As well, ERK1/2 can induce transcription of MAP kinase phosphatases (MPKs) that inhibit ERK1/2 by dephosphorylating it [Dhillon *et al.*, 2007].

Interestingly, the RAS/RAF/MEK/ERK pathway can be regulated independently of receptor activation. Alternate regulators include AKT, which can inhibit RAF signaling by phosphorylating residues in its N-terminus [Mendoza *et al.*, 2011]. Phosphatases such as dual-specificity phosphatases (DUSPs) can directly interact with and dephosphorylate ERK1/2 [Giltnane & Balko 2014]. The PI3K and PLCγ pathways can stimulate MEK1/2 activity by acting through protein kinase C (PKC). Other MEK1/2 activators include MAP kinase kinase kinases (MAP3K3, MAP3K8, MAP3K9, and MAP3K10) and multi-lineage kinases (MLK1,

MLK2, MLK3, and MLK4) [Caunt *et al.*, 2015; Carracedo *et al.*, 2008; Mendoza *et al.*, 2011]. PKC can also attenuate EGFR binding affinity for its ligands by phosphorylating Thr654 [Chen *et al.*, 1996].

Overexpression of EGFR in Cancer

In several cancers, such as breast, colon, and lung cancer, the genes coding for ErbB receptors, especially EGFR and HER2, are often mutated and misregulated, Since EGFR is involved in pleiotropic signaling cascades, it controls many cellular functions that are essential for cellular survival [Herold *et al.*, 2013; Wilson *et al.*, 2009; Jutten & Rouschop 2014; Schneider & Wolf 2009; Schlessinger 2000]. Overexpression of EGFR or amplification of the EGFR gene can cause overstimulation of downstream signaling pathways that lead to tumor progression. Therefore, overexpression of EGFR has been correlated with poor patient survival in cancer patients [Schneider & Wolf 2009; Minn *et al.*, 2005; Herold *et al.*, 2013].

Breast cancers can be identified as hormone receptor-positive, which indicates they are positive for the estrogen receptor (ER) and/or progesterone receptor (PR). Another subtype is referred to as HER2-positive, which indicates that there is an overexpression of the HER2 (ErbB2) receptor. Breast cancers that are negative for all three receptors are referred to as triple-negative ($ER^-/PR^-/HER2^-$) [Ueno *et al.*, 2011].

Triple-negative breast cancer (TNBC) represents ~20% of all breast cancers, and currently there are no FDA approved targeted therapies for TNBC. While TNBC typically responds to systemic chemotherapy, most patients experience recurrence and metastases [Dent *et al.*, 2007; Herold *et al.*, 2013]. The invasive and metastatic properties of TNBC are often exacerbated by epithelial-mesenchymal transition (EMT) of tumor cells [Ueno *et al.*, 2011].

EMT is a developmental process, in which epithelial cells acquire a mesenchymal phenotype, allowing them to disseminate from the tumor and migrate to distant locations, therefore achieving metastatic behavior [Sarrio *et al.*, 2008].

Currently there are two strategies for targeting EGFR as a potential therapy. The first one is the direct inhibition of EGFR by blocking the binding site for its ligands. This is accomplished by treatment with anti-EGFR antibodies, such as cetuximab. Cetuximab is an anti-EGFR monoclonal antibody that acts as a competitive antagonist to the EGFR ligands. Cetuximab binds to and blocks the ligand binding domain of EGFR. This inactivates the receptor and potentially leads to the degradation of EGFR. Cetuximab was also shown to activate antibody-dependent cellular cytotoxicity, which may lead to enhanced efficacy of the treatment. Antibody-dependent cellular cytotoxicity is a cellular immune response against cancer cells that possess EGFR bound to the antibodies, like cetuximab [Kocoglu *et al.*, 2016; Doody *et al.*, 2007; Krawczyk *et al.*, 2014]. Currently though, this treatment has not seen success in clinical trials as cancer can acquire resistance to the anti-EGFR therapy. This acquired resistance is thought to be due to alterations in genes that activate the downstream signaling pathway, such as MAPK, which can compensate for the lack of stimulation of EGFR [Emburgh *et al.*, 2014]. Additionally, studies have shown that increased binding affinities of some of the EGF-like ligands could be responsible for the development of resistance, as these ligands may out-compete the antibodies in binding to EGFR [Miller *et al.*, 2015]. The second form of anti-EGFR therapy is the use of small molecule inhibitors that block the tyrosine kinase activity in the intracellular domain of EGFR. These tyrosine kinase inhibitors (TKIs), like Gefitinib or Erlotinib, have not yet shown significant results in clinical trials and they also lead to acquired resistance [Arora *et al.*, 2005; Arteaga *et al.*, 2001; Higashiyama *et al.*, 2008].

In summary, TNBC patients' response to anti-EGFR therapy, like cetuximab or Erlotinib, have lacked efficacy. In TNBC, signaling downstream of EGFR, such as MAPK signaling, is considered vital for cancer progression. Our analysis of clinical data revealed that high expression of ADAM12, but not other ADAMs, in TNBC is associated with poor patient survival (Figure 1.6). Thus, we hypothesized that ADAM12 plays a critical role in the progression of TNBC, either directly through the cleavage and release of EGFR ligands and activation of EGFR and/or potentially indirectly, via protein-protein interactions mediated by the intracellular domain of ADAM12. Therefore, identification of ADAM12 as a potential novel regulator of EGFR may lead to new approaches to target the EGFR pathway in TNBC in the future. It may also help identify patients for whom the current anti-EGFR therapies would be the most beneficial**.** The goal of my work was to test whether ADAM12 is an important regulator of EGFR signaling in TNBC breast cancer cells.

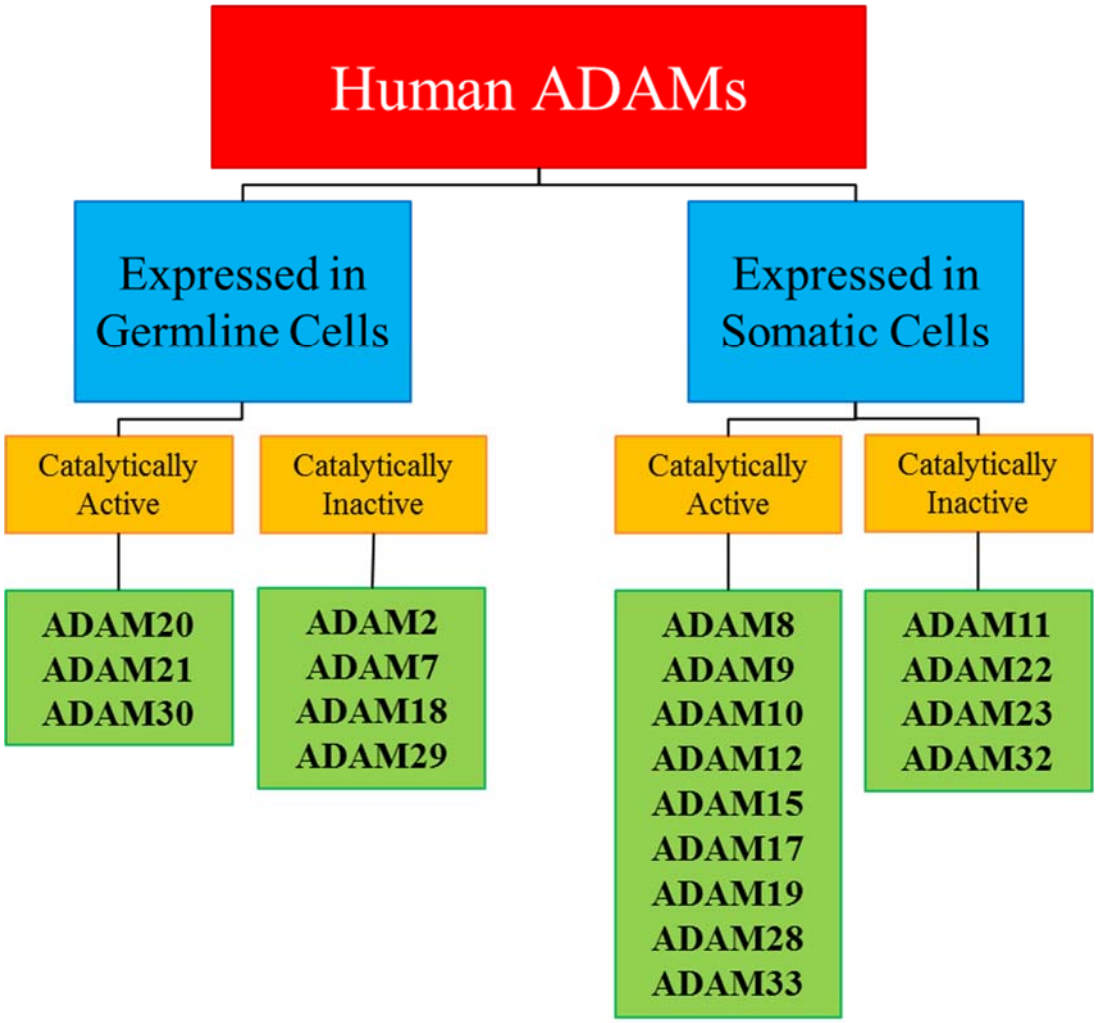


Figure 1.1 Human ADAMs organized by their catalytic activity and site of expression

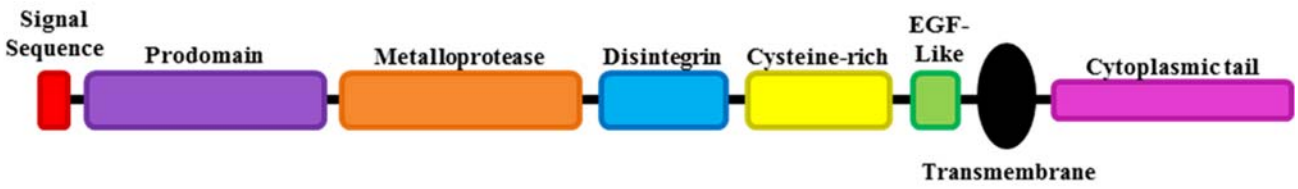


Figure 1.2 Domain organization of human ADAMs

The active form of ADAMs are missing the signal sequence and the prodomain, which are cleaved during transit through the Golgi complex.

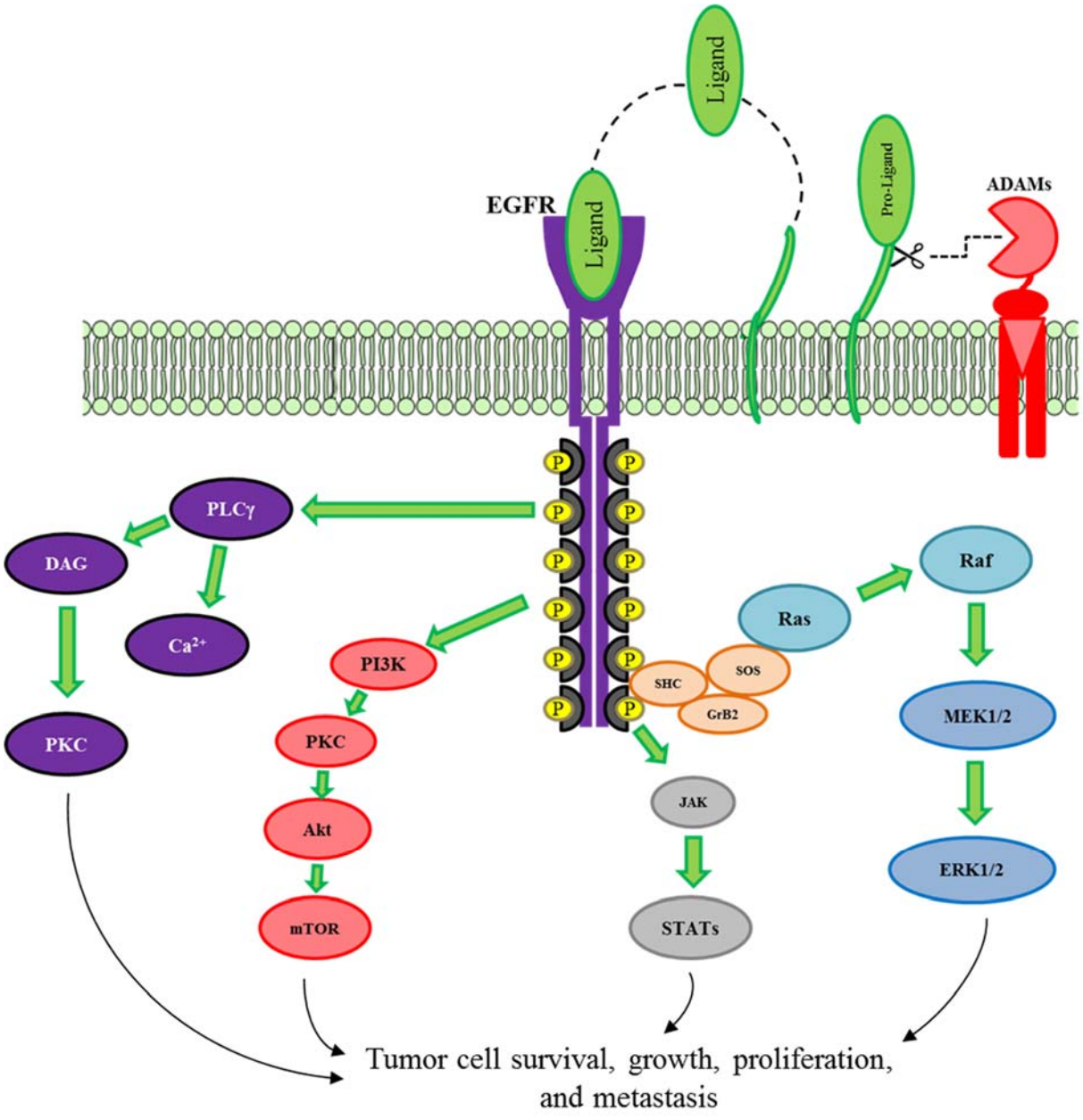


Figure 1.3 Ligand-dependent EGFR signaling

Cleavage of membrane-bound pro-ligands is carried out by ADAM proteases. Solubilized ligands can then bind to EGFR in an endocrine, paracrine, or autocrine manner. Activation of EGFR leads to phosphorylation of the intracellular domain and propagation of signaling cascades.

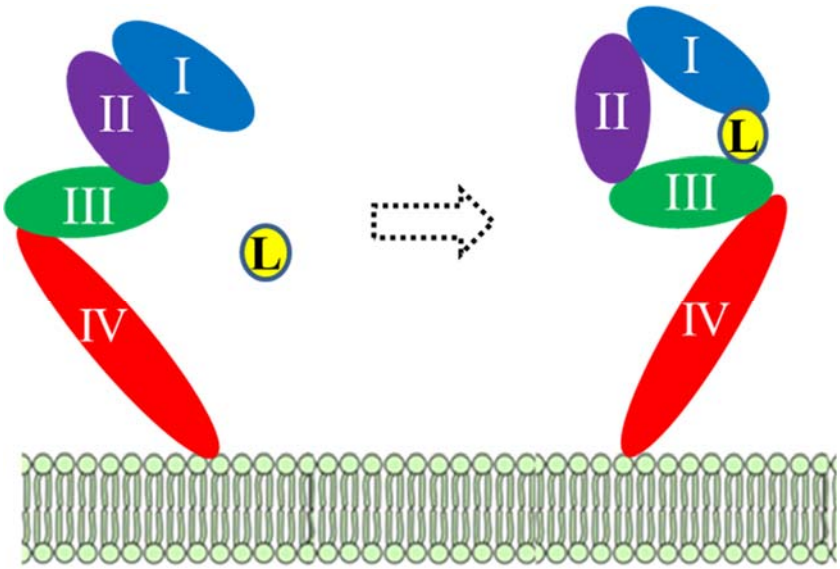


Figure 1.4 EGFR extracellular subdomains (L1 (I), S1 (II), L2 (III), and S2 (IV))

Diagram of the four extracellular subdomains of EGFR. L1 (I) and L2 (III) are involved in ligand (L) binding and S1 (II) and S2 (IV) are cysteine-rich regions involved in receptor stability and they regulate ligand-binding affinity. S1 from one EGFR monomer will interact with S1 from another EGFR receptor to form a dimer.

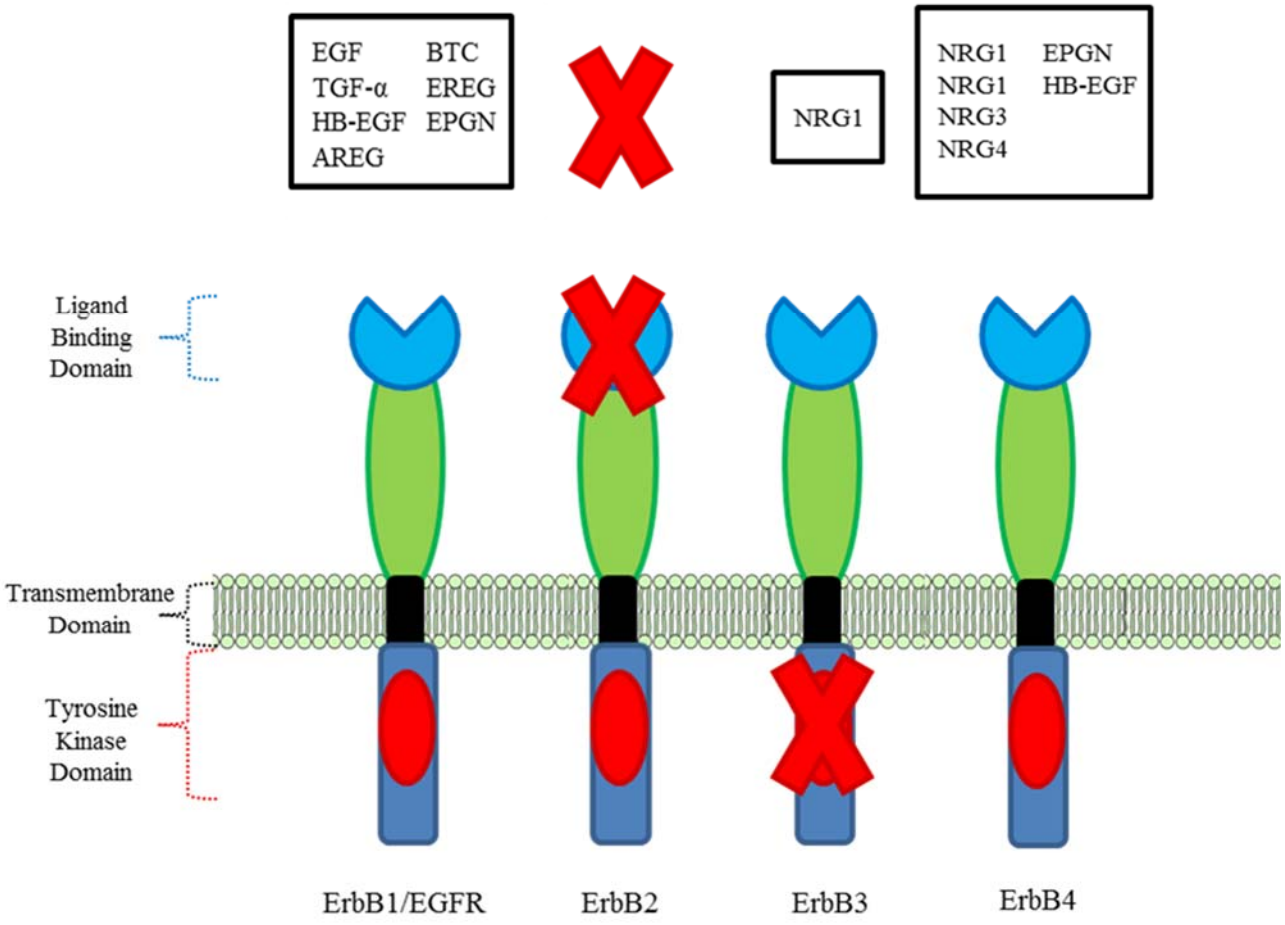


Figure 1.5 ErbB receptors and ligands

Ligand binding domain (light blue), extracellular domain (green), transmembrane domain (black), intracellular domain (dark blue), and tyrosine kinase domain (red) are indicated in the diagram. Ligands are indicated as epidermal growth factor (EGF), transforming growth factor-α (TGF- α), heparin binding EGF (HB-EGF), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG), epigen (EPGN), and neuregulins (NRG1-4). No ligands are known to bind ErbB2, and ErbB3 lacks tyrosine kinase activity, as indicated by the red X's.

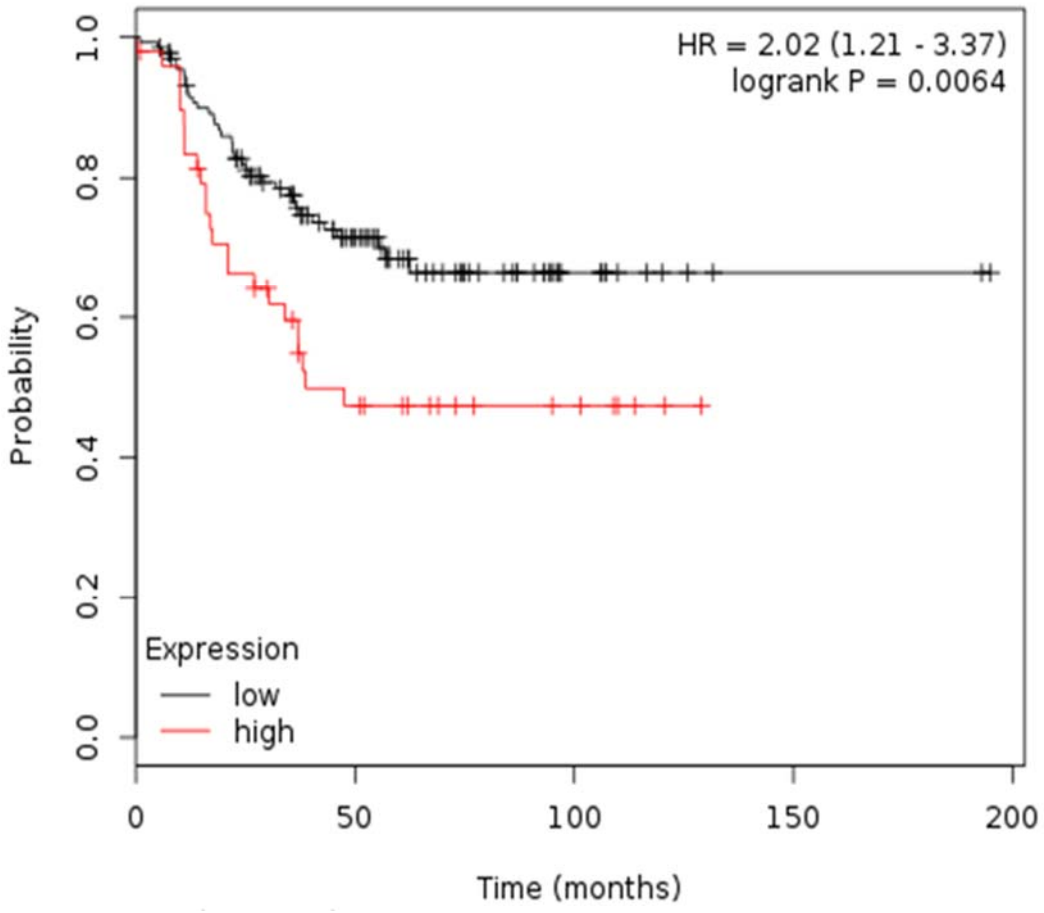


Figure 1.6 High expression of ADAM12 is correlated with poor prognosis in TNBC patients

Kaplan-Meier survival curves based on the expression of ADAM12 in breast tumors from 181 triple-negative breast cancer patients. Patients were ranked according to the expression of ADAM12 and divided into the low expression group (n = 132, cutoff determined by software) or high expression (n = 49, cutoff determined by software), which are shown in black and red, respectively (Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, Szallasi Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients, Breast Cancer Res Treatment, 2010 Oct;123(3):725-31).

Chapter 2 - Materials & Methods

Cell culture

MCF10A cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/F-12 medium supplemented with cholera toxin (100 ng/ml), epidermal growth factor (EGF; 20 ng/ml), insulin (10 μg/ml), hydrocortisone (500 ng/ml), and 5% horse serum (HS). Complete medium contained all supplements, serum-free medium did not contain horse serum or EGF. SUM159PT cells (Asterand) were maintained in Ham's/F-12 medium supplemented with 5% fetal bovine serum (FBS), insulin (5 μg/ml), and hydrocortisone (1 μg/ml). Complete medium contained all supplements. All cell lines were incubated at 37°C under 5% carbon dioxide (CO_2).

Antibodies

Monoclonal rabbit anti-phospho-EGFR antibody (Ab #53A5) was obtained from Cell Signaling Technologies and it was used at a dilution of 1:1000 for Western blotting. Polyclonal rabbit anti-EGFR antibody (Ab #D38B1) was obtained from Cell Signaling Technologies and was used at a dilution of 1:5000 for Western blotting. Monoclonal rabbit anti-phospho-ERK antibody (Ab #D13.14.45) and rabbit anti-ERK antibody (Ab #137F5) were both used at a dilution of 1:2000 for Western blotting and were obtained from Cell Signaling Technologies. Monoclonal rabbit anti-phospho-MEK antibody (Ab #41G9) and rabbit anti-MEK antibody (Ab # D1A5) were both used at a dilution of 1:2000 and were obtained from Cell Signaling Technologies. Other antibodies used were: anti-α-tubulin from Sigma (Ab #DM1A; 1:100,000 dilution) and anti-β1-integrin (Ab# 610467, 1:4000 dilution), both from BD Biosystems. Secondary antibodies were

anti-mouse IgG (Ab #1031.05) and anti-rabbit IgG (Ab #4010.05) from Southern Biotech, both used at a dilution of 1:5000.

Induction of shRNA

SUM159PT_shADAM12 and SUM159PT_shControl cells were incubated in HAM's/F-12 complete medium and treated with 1 μg/ml of doxycycline for four days to induce shRNA. Medium was changed and re-supplemented with 1 μg/ml of doxycycline every two days. Cells were checked for the presence of red fluorescent protein (RFP) using fluorescence microscopy to determine the efficiency of the induction of shRNA expression (as both shRNA and RFP were under the control of a doxycycline-inducible promoter).

Cell survival assay

Cell survival assay was performed by quantitating intracellular ATP levels generated by metabolically active cells using CellTiter-Glo (Promega). MCF10A cells were seeded into 4 sterile 96-well tissue culture plates at ~$1.5x10^5$ cells/ml, 100 μl per well, in complete medium and allowed to attach for 24 hours. After that time, medium was removed, then conditioned media from SUM159PT_shADAM12/shControl cells with or without 1 μg/ml doxycycline treatment were applied to MCF10A cells. After 0, 24, 48, 72 hours, media were removed and 100 μl CellTiter-Glo reagent, diluted 1:1 with serum-free medium, was applied to each well. 96-well plates were then placed on an orbital shaker for 2 minutes and then incubated for 10 minutes in the dark at room temperature. Luminescence was then recorded for each well using Synergy-H1 microplate reader (BioTek). Each time point was normalized to appropriate 0-hour time point to determine fold-change of the luminescence signal.

EGFR signaling antibody array

EGFR signaling was tested by means of semi-quantitative Antibody Array (Cell Signaling Technologies). SUM159PT_shADAM12/shControl cells were incubated in 100-mm culture plates with or without 1 μg/ml of doxycycline for 3 days in order to knock-down ADAM12 expression. Cells were then re-plated into 6-well plates and allowed to attach for 24 hours in the presence or absence of doxycycline. Cells were treated with 1 μM Erlotinib (Cell Signaling Technologies) and/or 50 ng/ml exogenous EGF (Life Technologies) for an additional 24 hours for assay controls. Antibody Arrays were then processed according to the manufacturer's instructions.

Western blotting

Cells were treated with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate (SDC), 0.1% sodium dodecylsulfate (SDS), 5 mM EDTA). Lysis buffer was supplemented with protease and phosphatase inhibitors (1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 5 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 mM 1,10-phenanthroline, 500 mM sodium fluoride, 100 mM sodium orthovanadate, and 100 mM sodium pyrophosphate). Cells were lysed for 15 minutes with rocking at 4°C, and then lysates were collected and centrifuged for 15 minutes at 13,000 rpm at 4°C. After centrifugation, the supernatants were collected and diluted 2:1with 3xSDS sample loading buffer. Proteins were separated in 8% polyacrylamide gels and then transferred to a nitrocellulose membrane. Proteins were visualized by Ponceau S staining. If multiple proteins of different sizes were to be analyzed in the same membrane, the membranes were cut at appropriate positions. The membranes were then blocked for 1 hour in blocking buffer containing 0.3% Tween-20 and 5.0% nonfat dry milk in DPBS. Following blocking, primary

antibodies were diluted in blocking buffer and applied to membranes for overnight incubation. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies were used as secondary antibodies. Bands were visualized using West Pico chemiluminescent substrate (Thermo Scientific) and the Azure™ c500 digital imaging system.

Statistical analyses

Western blots and antibody array were quantified using Azure Biosystems analysis software. All statistical analyses were performed using the GraphPad Prism program.

Chapter 3 - Results

Doxycycline-induced ADAM12 knock-down (KD)

To down regulate the expression of ADAM12 in breast cancer cells, we used a shRNA inducible system. SUM159PT_shADAM12/shControl cells stably transduced with lentiviral shRNA vectors were previously established in our laboratory (Figure 2.1A). We were able to induce the knock-down of ADAM12 upon treatment with 1 μg/ml doxycycline (Figure 2.1B and C). As indicated by qRT-PCR, ADAM12 mRNA was significantly decreased when shADAM12 cells were treated with doxycycline. Conversely, when shControl cells were treated with doxycycline, there was no effect on ADAM12 mRNA expression (Figure 2.1B). The doxycycline-inducible promoter controls red fluorescence protein (RFP) expression, in addition to ADAM12 shRNA. This was shown by flow cytometry, with RFP being expressed in cells only in the presence of doxycycline. Similarly, upon doxycycline treatment, we observed an inhibition of ADAM12 protein expression (Figure 2.1C). Thus, visualization of RFP can be used as an indicator of ADAM12 KD (Figure 2.1D), since both shRNA and RFP are under the control of the same doxycycline-inducible promoter.

Effect of ADAM12 KD on the activation of EGFR

Our previous analysis of clinical data revealed a link between ADAM12 and EGFR in the progression of TNBC. To investigate the effect of ADAM12 on the activation of EGFR, we first focused on the phosphorylation of tyrosine 1173 (Y1173) located in the EGFR cytoplasmic tail. The phosphorylation of Y1173 is involved in the activation of several downstream signaling targets implicated in cell proliferation, including the MAPK pathway (Figure 1.3) [Steelman *et al.*, 2011; Prior *et al.*, 2012; Yan *et al.*, 1998]. In order to study the effect of ADAM12 KD,

SUM159PT_shADAM12 cells were used, as SUM159PT cell line possesses high expression levels of the endogenous ADAM12. Supplementation with 1 μg/ml doxycycline was applied to induce ADAM12 KD, and the efficiency of the KD was determined by examining the expression of RFP using fluorescence microscopy (Figure 2.1D).

Cells were maintained in complete medium, with or without 1 μg/ml doxycycline for 4 days. On day 4, 1 μM Erlotinib was added as a negative control and incubation continued for 24 hours. Erlotinib is a tyrosine kinase inhibitor (TKI) that blocks the activity of the EGFR kinase. On day 5, cells were treated with 20 ng/ml recombinant human EGF as a positive control for a 30-minute stimulation. Treatment with exogenous EGF will lead to receptor activation and phosphorylation of tyrosines in the kinase domain, including Y1173. Cells were then treated with lysis buffer supplemented with protease and phosphatase inhibitors for 15 minutes. Lysates were then collected and Western blot analysis was performed to test for the presence of phospho-Y1173 EGFR. Expression of total EGFR protein was determined and used as a normalization control.

We were not able to detect phosphorylation of EGFR at Y1173 in SUM159PT cells in any of the treatment groups except for cells treated with exogenous EGF (Figure 2.2). Without any detectable basal level of phosphorylation of Y1173, we were not able to address the role of ADAM12 in the regulation of EGFR activity. However, since EGFR contains multiple phosphorylation sites, we cannot exclude a possibility that ADAM12 might regulate EGFR phosphorylation at a different tyrosine residue. Further studies should be performed to determine the potential role of ADAM12 in the regulation of EGFR activity.

EGFR kinase array for pathway activation

To further investigate additional phosphorylation sites in EGFR other than Y1173, we used a commercial antibody array. This array contained four additional antibodies specific for phosphorylated tyrosine/threonine residues in EGFR: T669, Y845, Y998, and Y1068 (Figure 2.3A). Additionally, this array contained antibodies specific for phosphorylated targets downstream of EGFR, allowing us to determine if there was any observable effect in TNBC cells after knock-down of ADAM12. SUM159PT_shADAM12/shControl cells were maintained in complete medium, with or without 1 μg/ml doxycycline for 5 days. On day 4, treatments with 1 μM Erlotinib and/or 50 ng/ml EGF were applied as controls for 24 hours in order to determine the efficacy of the array by its ability to respond to EGF and Erlotinib treatment. We expected the exogenous EGF treatment to show strong activation of EGFR and its downstream targets, whereas Erlotinib should inhibit EGFR activity. Indeed, our results indicated that upon stimulation with 50 ng/ml exogenous EGF, EGFR was phosphorylated at Y845, Y998, and Y1068 (Figure 2.3B). T669 was not activated after EGF treatment. As expected, in addition to the activation of EGFR, targets downstream of EGFR responded to the EGF treatment, including MAPK, PLCγ, and Akt (Figure 2.3B). Both the phospho-EGFR sites and the downstream targets were significantly decreased when cells were additionally treated with 1 μM Erlotinib (Figure 2.3B).

Upon ADAM12 KD, there was no considerable change in the level of phosphorylation of EGFR, with exception for Y845. The Y845 residue was the only site which in fact was phosphorylated at a detectable level, above the background. This residue was also the only site that could be further analyzed and quantified due to the impurity of signal at the other sites from the strong overlapping signal of total MEK1 (Figure 2.3C). To quantify the level of phospho-

Y845, we normalized the level of phospho-Y845 to the total EGFR expression. Our quantification showed a significant decrease in phospho-Y845 level, with ~60% decrease in phospho-Y845 upon ADAM12 KD ($p = 0.002$, n = 2) (Figure 2.3C). Importantly, we observed a decrease in the level of phospho-MEK1/2 (S217/221) as well as phospho-ERK1/2 (T202/Y204) in cells with ADAM12 KD, as compared to control cells. The phospho-MEK1/2 (S217/221) signal was normalized to total MEK1/2. Without a total ERK1/2 antibody in this array, phospho-ERK1/2 (T202/Y204) levels could not be quantified accurately (Figure 2.3A).

We concluded that phospho-MEK1/2 (S217/221) was significantly decreased ($p = 0.03$) when ADAM12 was knocked-down in SUM159PT cells (Figure 2.3C). Additional quantification was performed for PLCγ, Akt, and STAT3, but no difference in phosphorylation levels of these proteins was observed. These results suggest that ADAM12 may play a role in the regulation of MEK/ERK signaling. This regulation may be through modulating the phosphorylation of EGFR potentially at Y845 or other untested residues, or it may be through an EGFR independent mechanism through the cytoplasmic tail of ADAM12.

Change in MEK/ERK signaling after ADAM12 KD

To confirm the results of the antibody array suggesting that MEK1/2 and ERK1/2 phosphorylation is regulated by ADAM12, phospho-MEK1/2 (S217/221) and phospho-ERK1/2 (T202/Y204) protein levels were tested by Western blotting. Therapies targeting MEK1/2 and ERK1/2 activation are of interest due to the link between MEK1/2 and ERK1/2 and the progression of many cancers. MEK1/2 and ERK1/2 are phosphorylated and activated in a kinase cascade that is activated upon stimulation of EGFR by growth factors. We used treatment with 1 μM Erlotinib to block EGFR activation as a negative control since Erlotinib is a TKI and can inhibit EGFR activity and therefore prevent downstream signaling activation, such as MEK1/2

and ERK1/2. Twenty ng/ml EGF was applied as a positive control to induce EGFR activation. SUM159PT_shADAM12/shControl cells were maintained in complete medium for 5 days, with or without 1 μg/ml doxycycline. Erlotinib was applied on day 4 for an additional 24 hours of treatment and 20 ng/ml of exogenous EGF was applied on day 5 for a 30-minute treatment. In the next step, lysis buffer supplemented with protease and phosphatase inhibitors was added to cells. Lysates were collected and Western blot analysis was performed for the detection of phospho-S217/221 MEK1/2 and phospho-T202/Y204 ERK1/2. Expression of total MEK1/2 and total ERK1/2 protein were tested as well in order to allow for normalization of the Western blotting signals. We observed that upon knock-down of ADAM12, there was a comparable decrease in the levels of both phospho-S217/221 MEK1/2 and phospho-T202/Y204 ERK1/2 (Figure 2.4), which is consistent with the results observed in the antibody array assay (Figure 2.3C). Quantification of the relative phosphorylation level of MEK1/2 and ERK1/2 was determined for each sample group. The level of phospho-S217/221 MEK1/2 decreased by ~ 35% upon ADAM12 KD (Figure 2.4). Similarly, the level of phospho-T202/Y204 ERK1/2 decreased by ~ 34% upon ADAM12 KD ($p = 0.0385$, n = 3) (Figure 2.4). These results support our hypothesis that ADAM12 may be involved in the regulation of both MEK1/2 and ERK1/2 activity, and down-regulation of ADAM12 effectively inhibits the phosphorylation of MEK1/2 and ERK1/2.

It is well documented that ADAM12 is capable of cleaving membrane-bound growth factors, such as EGF and EGF-like ligands [Reiss & Saftig 2009; Weber & Saftig 2012; Zolkiewska 2009; Seals & Courtneidge 2003]. However, previous studies have shown that the cytoplasmic tail of ADAM12 can interact with and activate Src [Kang *et al*., 2000; Mueller *et al*., 2012]. Since Src can further phosphorylate Y845 in EGFR, it is possible that ADAM12

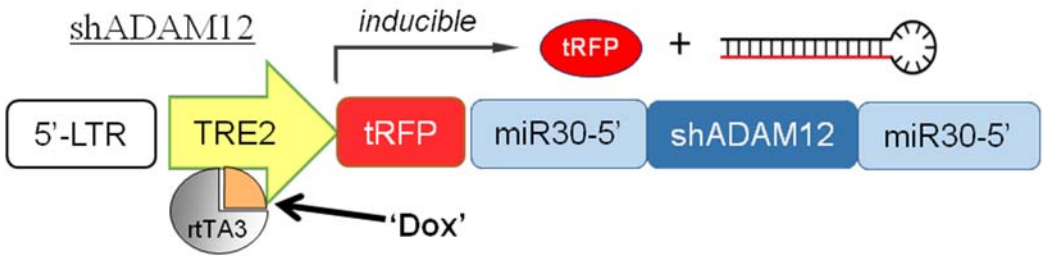
modulates EGFR activation through interactions with Src. Therefore, we wanted to determine if ADAM12 regulation occurs through the cleavage/release of growth factors by ADAM12 or if ADAM12 regulates MEK1/2 and ERK1/2 activity independently of its proteolytic activity, potentially through protein-protein interactions involving its cytoplasmic tail.

Effect of ADAM12 KD on cleaved/released factors and cell survival

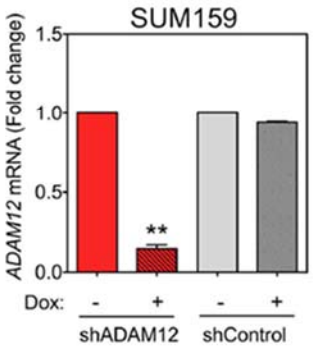
The following CellTiter Glo experiment was designed to indirectly test the importance of ADAM12 in the cleavage/release of these growth factors involved in EGFR signaling. This assay uses intracellular ATP levels as a measure of metabolic activity of viable cells. By determining the survival of cells, with or without the presence of ADAM12, we were able to determine the role ADAM12 may have in maintaining cell survival through cleavage of essential growth factors. Conditioned medium was obtained from SUM159PT cells that were previously incubated in serum-free medium (-serum, -EGF) for 48 hours. Incubation in serum-free medium was vital because in this case any growth factors present in the medium must have been derived from SUM159PT cells. MCF10A cells were then treated with the conditioned medium from SUM159PT cells (Figure 2.5A), and MCF10A cell viability was determined by CellTiter Glo assay. MCF10A cells rely on exogenous growth factors for their survival. MCF10A cells were serum-starved for 24 hours prior to the application of the conditioned medium; therefore, any growth factors required for cellular survival must have come from the conditioned medium of SUM159PT cells. If ADAM12 mediates the cleavage/release of growth factors in SUM159PT cells, then upon ADAM12 KD these cells should release less growth factors into the medium and therefore a decrease in MCF10A survival should be observed. Treatment of MCF10A cells with conditioned medium continued for 0, 24, 48, and 72 hours. The time point for each treatment group was normalized to their appropriate 0-hour time point. As expected, cells treated with

exogenous EGF showed an increasing luminescence signal indicating rising levels of metabolic activity and more viable cells. Conversely, all treatment groups without exogenous EGF showed a decreasing trend in luminescence signal indicating less metabolic activity due to cellular death. MCF10A cells treated with conditioned medium from the ADAM12 KD group showed the poorest survival among all groups, with the largest difference coming at the time point of 24 hours. There was a significant difference in survival between MCF10A cells treated with conditioned medium from SUM159PT cells with intact expression of ADAM12 as compared to the ADAM12 KD group ($p = 0.0494$). Treatment with the ADAM12 KD medium showed a ~35-40% decreased survival after 24 hours, whereas treatment with ADAM12 medium showed only a ~ 10-15% decreased survival after 24 hours, which was comparable to the control groups (Figure 2.5B). These results indicate that ADAM12 mediates the cleavage/release of essential growth factors in SUM159PT cells necessary for the survival of MCF10A cells, most likely through its proteolytic activity.

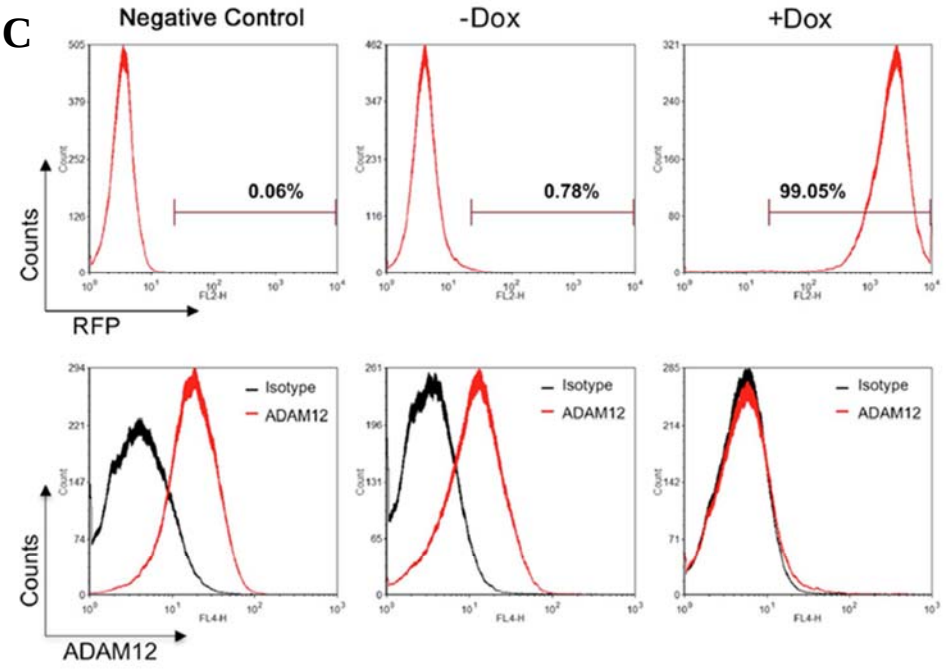
A


shADAM12
inducible
tRFP
+
5'-LTR
TRE2
tRFP
miR30-5'
shADAM12
miR30-5'
rtTA3
'Dox'

B


SUM159
ADAM12 mRNA (Fold change)
1.5
1.0
0.5
0.0
**
Dox: - + - +
shADAM12
shControl

C


Negative Control
-Dox
+Dox
Counts
RFP
0.06%
0.78%
99.05%
Isotype
ADAM12
Counts
ADAM12

D

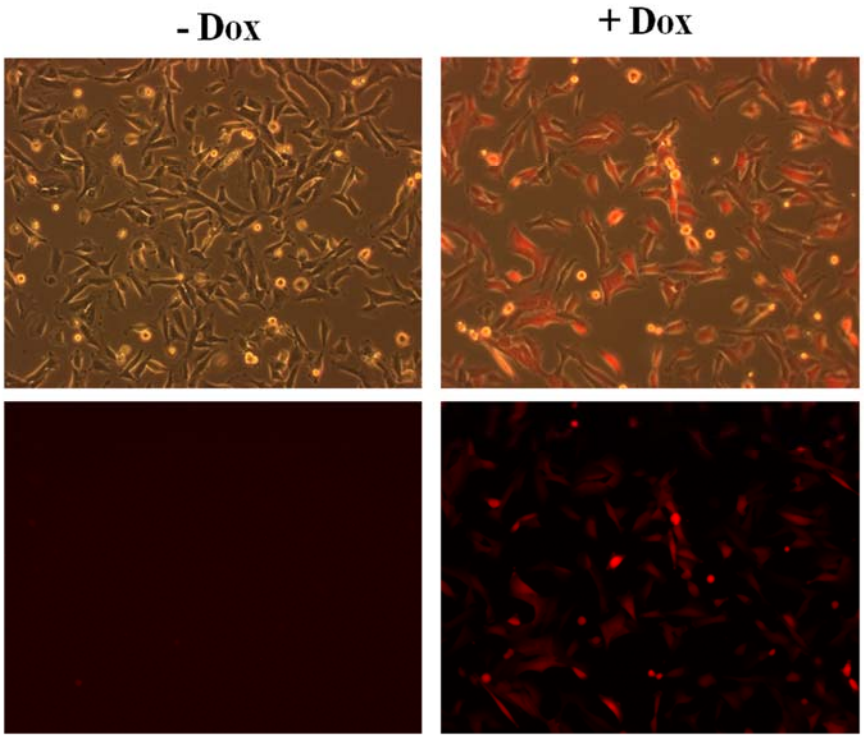

- Dox
+ Dox

Figure 2.1 Doxycycline-induced ADAM12 knock-down (KD)

(A) Diagram of pINDUCER_sADAM12 vector. Treatment with doxycycline (Dox) induces shRNA and RFP expression. (B) qRT-PCR analysis of ADAM12 mRNA expression in SUM159PT_shADAM12/shControl cells treated with or without doxycycline. (C) RFP expression (top; red) and ADAM12 expression (bottom; red) in SUM159PT_shADAM12 cells treated +/- doxycycline and analyzed by flow cytometry. Negative control represents parental SUM159PT cells without shRNA expression. (D) Fluorescence microscopy of SUM159PT_shADAM12 cells with or without doxycycline treatment. RFP expression is an indicator of the induction of shRNA and ADAM12 knock-down.

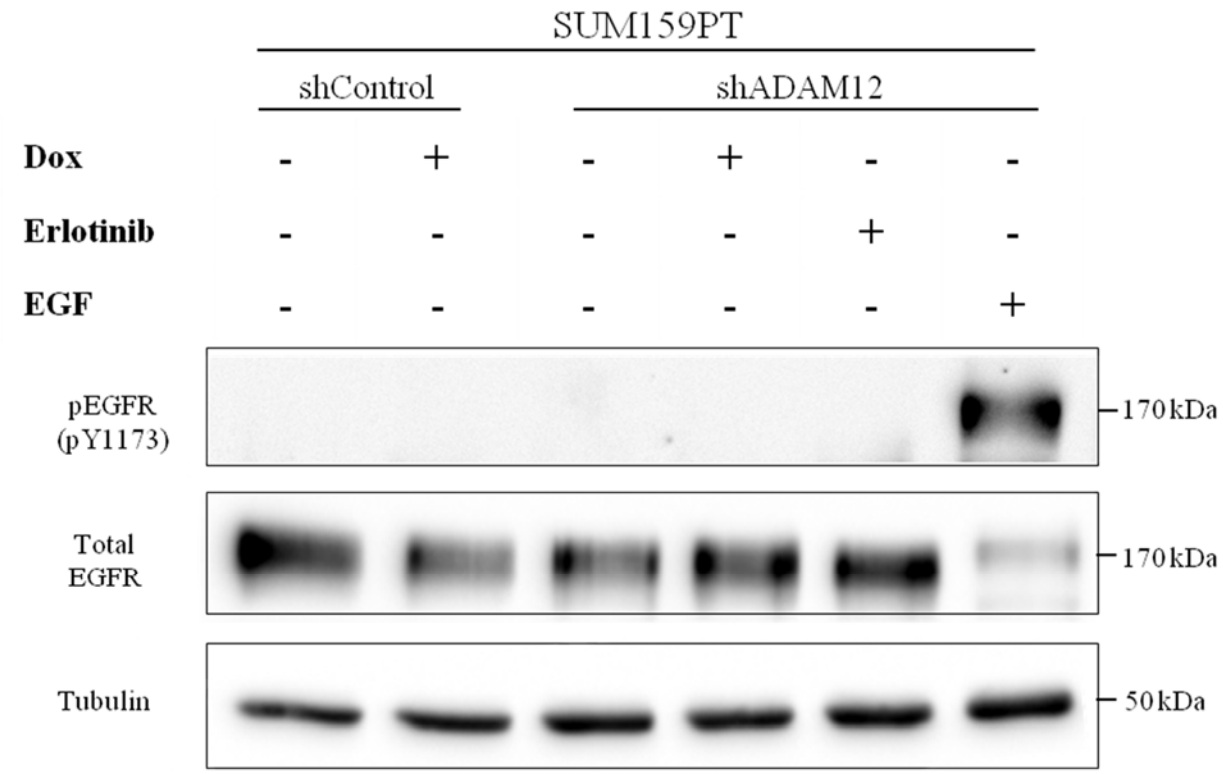


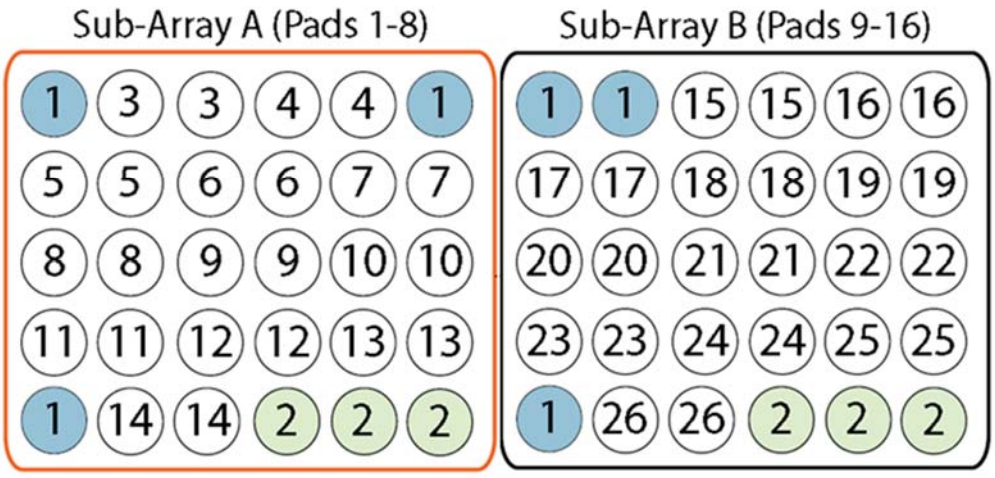
Figure 2.2 Effect of ADAM12 KD on the activation of EGFR

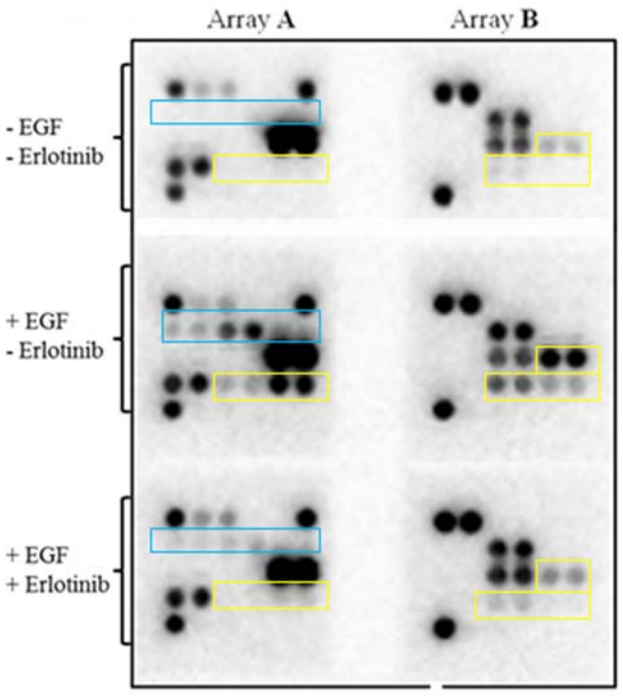
Western blot analysis of pY1173-EGFR and total EGFR in SUM159PT cells. Treatments were with 1 μg/ml doxycycline (4-day), 1 μM Erlotinib (24-hour), and 20 ng/ml EGF (30-minute). Tubulin was used as loading control.

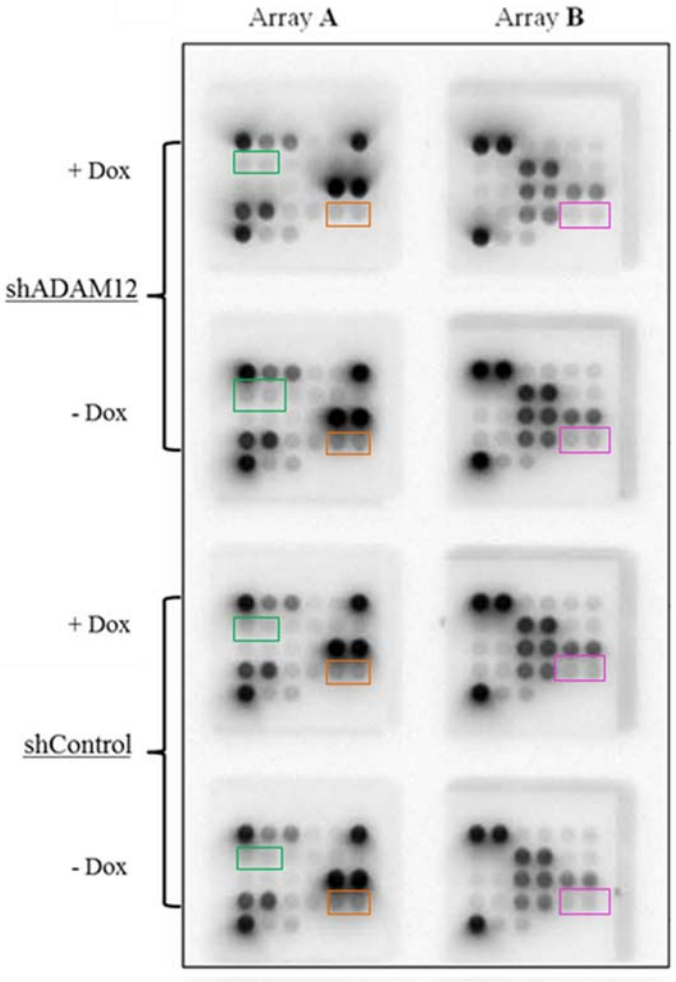
A

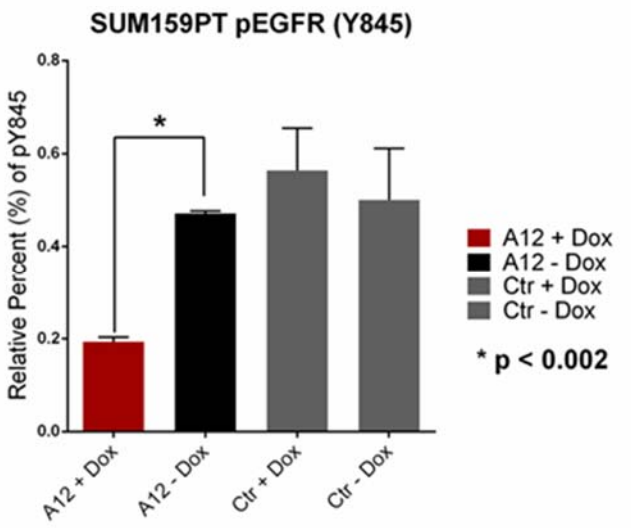
	Target	Phosphorylation Site
1	Positive Control	N/A
2	Negative Control	N/A
3	EGFR	Total
4	EGFR	Thr669
5	EGFR	Tyr845
6	EGFR	Tyr998
7	EGFR	Tyr1068
8	EGFR - L858R Mutant	Total
9	EGFR - E746-A750 Deletion	Total†
10	MEK1	Total
11	MEK2	Total
12	MEK1/2	Ser221
13	MEK1/2	Ser217/221

	Target	Phosphorylation Site
14	Stat3	Tyr705
15	HER2/ErbB2	Total
16	HER2/ErbB2	Tyr1196
17	HER2/ErbB2	Tyr1221/1222
18	Met	Total
19	Met	Tyr1349
20	Met	Tyr1234/1235
21	PLCγ1	Total
22	PLCγ1	Ser1248
23	Akt	Thr308
24	Akt	Ser473
25	Erk1/2	Thr202/Tyr204
26	PARP	Asp214 (Cleavage)




Array A
Array B
- EGF
- Erlotinib
+ EGF
- Erlotinib
+ EGF
+ Erlotinib


Array A
Array B
+ Dox
shADAM12
- Dox
+ Dox
shControl
- Dox


SUM159PT pEGFR (Y845)
Relative Percent (%) of pY845
0.8
0.6
0.4
0.2
0.0
*
A12 + Dox
A12 - Dox
Ctr + Dox
Ctr - Dox
A12 + Dox
A12 - Dox
Ctr + Dox
Ctr - Dox
* p < 0.002

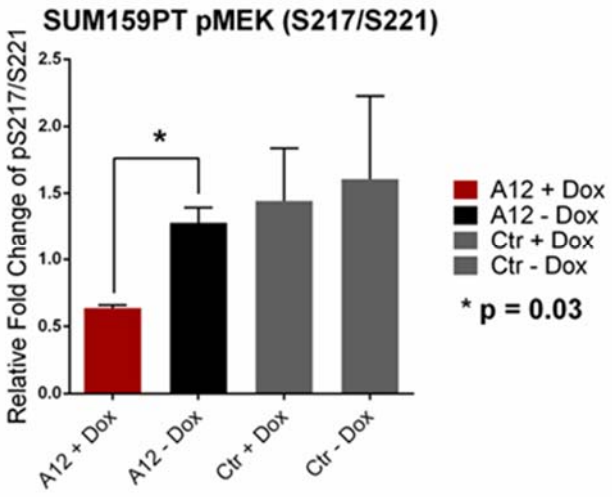

SUM159PT pMEK (S217/S221)
Relative Fold Change of pS217/S221
2.5
2.0
1.5
1.0
0.5
0.0
*
A12 + Dox
A12 - Dox
Ctr + Dox
Ctr - Dox
A12 + Dox
A12 - Dox
Ctr + Dox
Ctr - Dox
* p = 0.03

Figure 2.3 EGFR kinase array for pathway activation

Antibody Array to examine EGFR signaling. (A) The 24 different targets and their respective antibodies, as well as positive and negative controls, are indicated in the top panel. The layout of the array is indicated in the bottom panel. The array is separated into two subarrays (A and B). Subarray A contains antibodies against targets 1-14, Subarray B contains antibodies against targets 1-2 and 15-26. (B) SUM159PT cells were incubated with EGF (positive control) or Erlotinib (negative control). Lysates were collected and processed according to the manufacturer's protocol. Targets indicating EGFR activation are enclosed in yellow boxes, targets downstream of EGFR are indicated in blue boxes. (C) SUM159PT_shADAM12/ shControl cells were treated with doxycycline to down-regulate ADAM12. Lysates were collected and analyzed by Antibody Array (left). Apparent changes in EGFR-Y845 (green), MEK-S217/221 (orange), and ERK-T202/Y204 (purple) after ADAM12 KD were observed. Quantification was performed by normalizing phospho-Y845 and phospho-S217/221 to total EGFR or total MEK1/2, respectively. Quantification of percent of phospho-Y845/EGFR (top-right) ($p < 0.002$; $n = 2$) and relative fold change of pS217/221/MEK1/2 (bottom-right) ($p < 0.03$; $n = 2$) were determined by using Azure Biosystems analysis software.

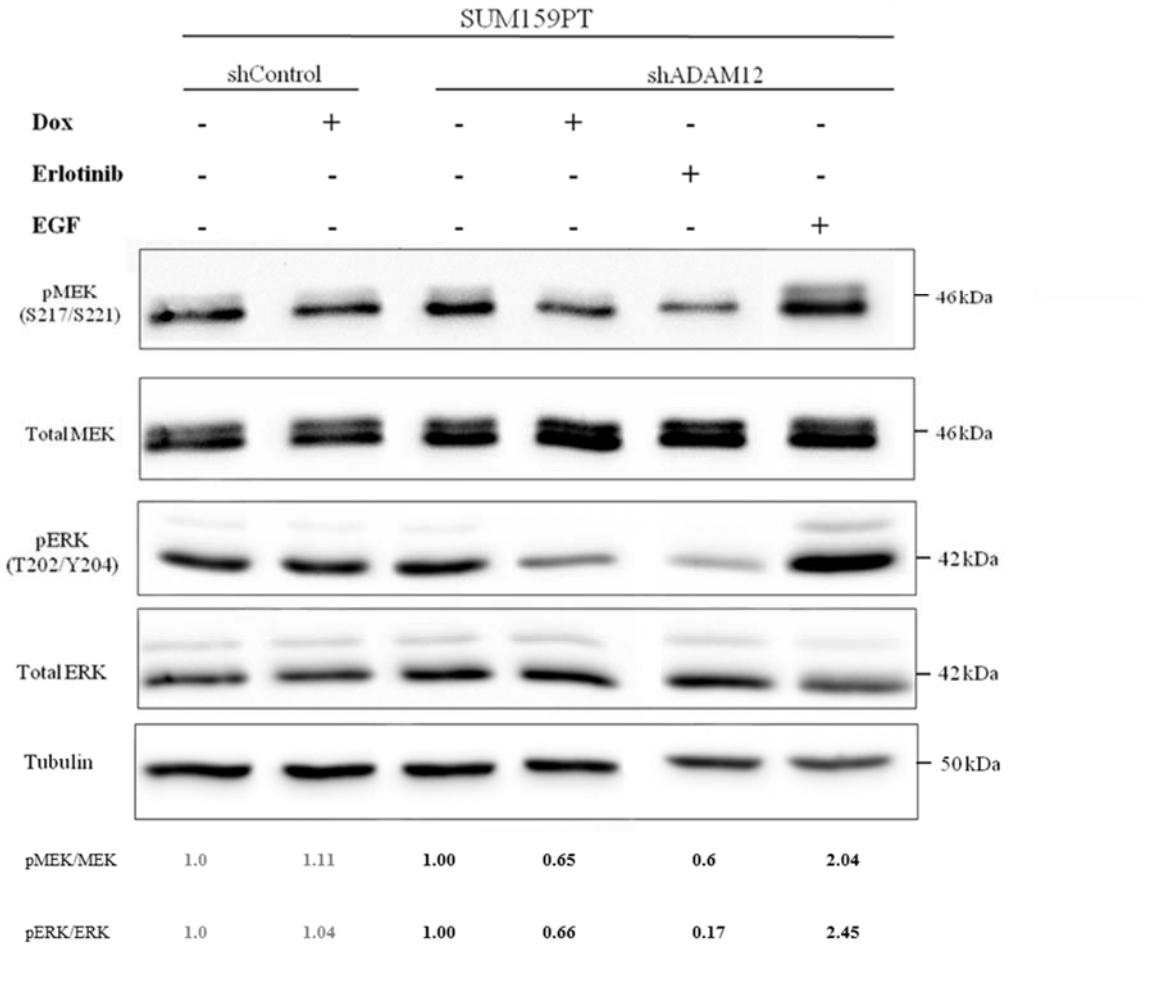
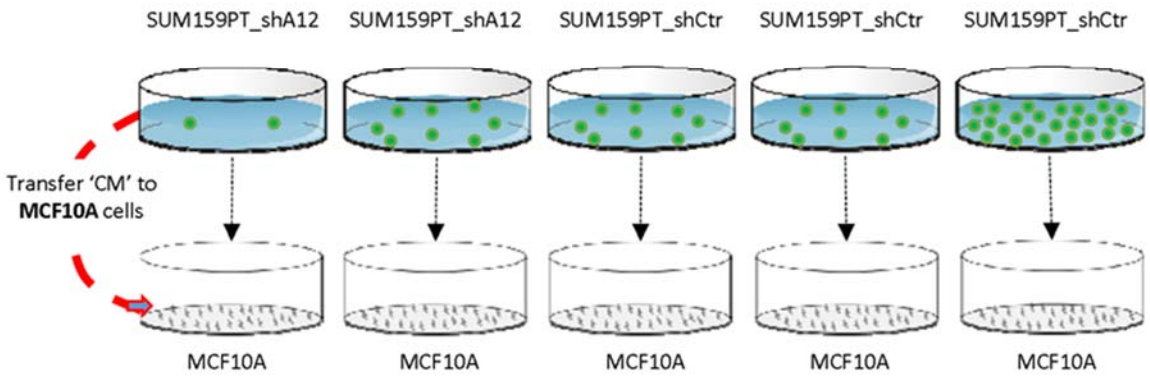


Figure 2.4 Change in MEK/ERK signaling after ADAM12 KD

Western blot analysis shows the phosphorylation status of MEK/ERK in SUM159PT_shADAM12/ shControl cells. Cells were treated with doxycycline in order to induce shRNA targeting ADAM12 or control shRNA. Treatments were with 1 μg/ml of doxycycline, 1 μM Erlotinib (24-hour), and 20 ng/ml EGF (30-minute). Tubulin was used as loading control. Quantification values (bottom) were obtained by normalizing phospho-MEK and phospho-ERK to total MEK and total ERK, respectively, using the Azure Biosystems analysis software.

A

Dox	+	-	+	-	-
EGF	-	-	-	-	+
Serum	-	-	-	-	+



B

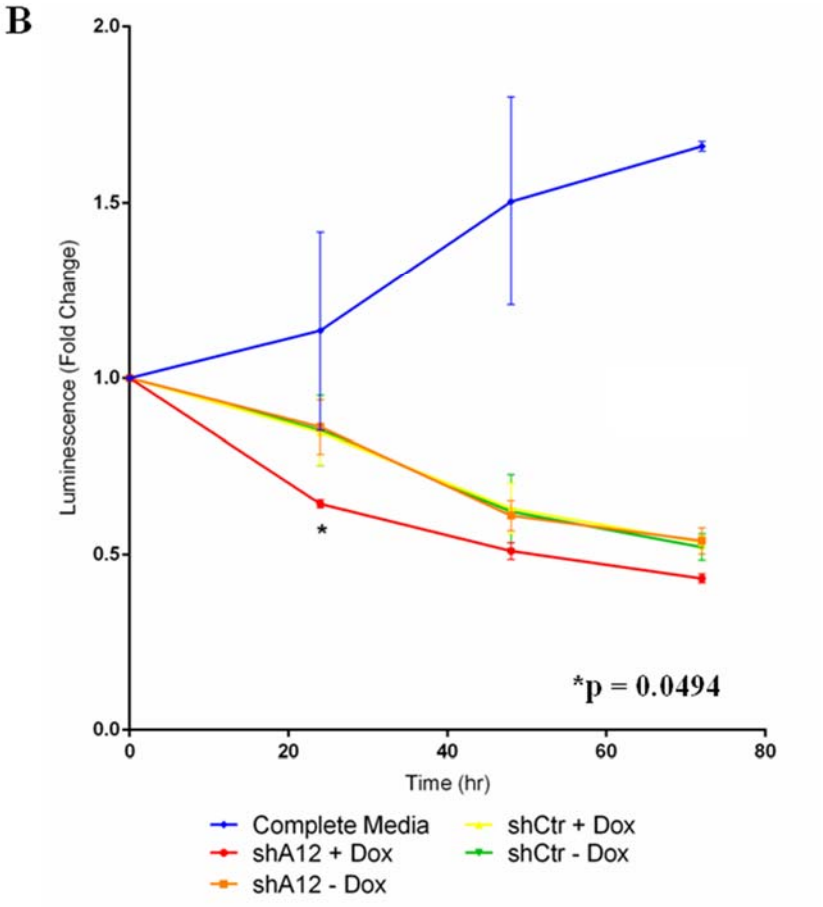
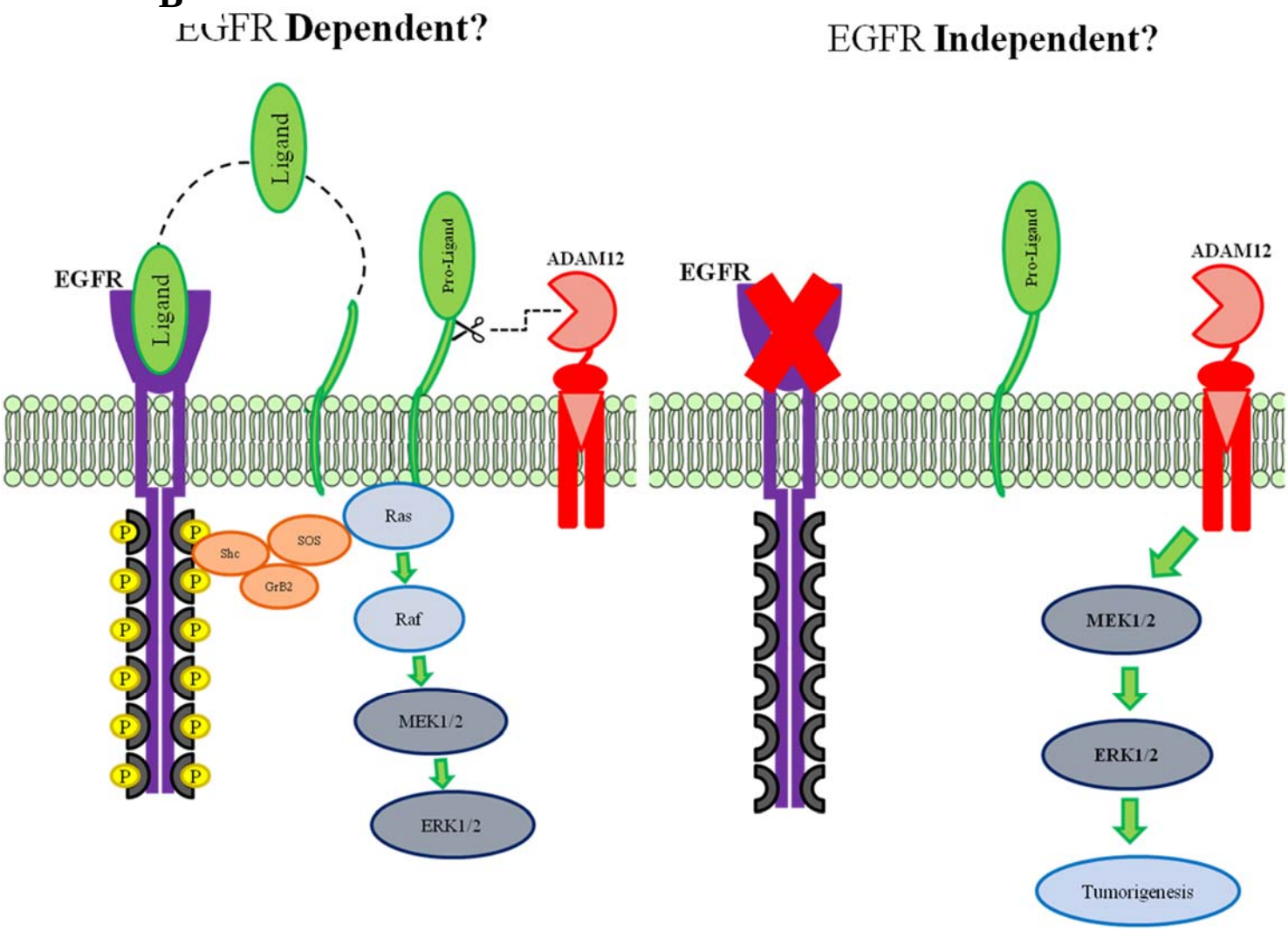


Figure 2.5 Effect of ADAM12 KD on cleaved/released factors and cell survival

(A) SUM159PT_shADAM12/shControl cells maintained in complete medium supplemented with doxycycline were switched to serum-free medium for 48 hours after knock-down of ADAM12. Medium was removed and applied to MCF10A cells that were serum-starved for 24 hours. MCF10A cells were incubated in conditioned media for 0, 24, 48, and 72 hours. (B) CellTiter Glo assay was carried out for each time point. Luminescence fold change was determined by measuring relative changes in luminescent signal from the initial 0-hour time point for each group. A significant decrease in MCF10A survival ($p = 0.0494$; $n = 3$) was observed after 24 hours.

B


EGFR Dependent?
EGFR Independent?
Ligand
EGFR
Ligand
Pro-Ligand
ADAM12
EGFR
Pro-Ligand
ADAM12
P
Shc
SOS
GrB2
Ras
Raf
MEK1/2
ERK1/2
MEK1/2
ERK1/2
Tumorigenesis

C

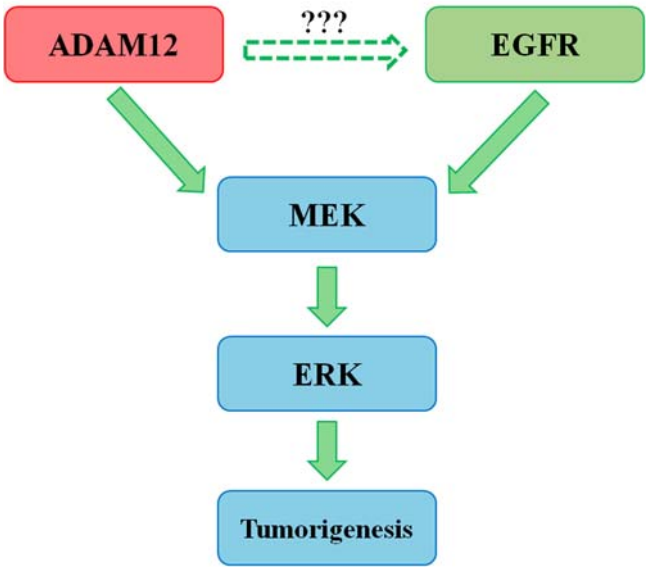

ADAM12
???
EGFR
MEK
ERK
Tumorigenesis

Figure 2.6 Potential ADAM12 mechanisms of MEK/ERK regulation

(A and B) ADAM12 regulation of MEK/ERK signaling in an EGFR dependent manner (A) or EGFR independent manner (B). (C) Current model of ADAM12 regulating MEK/ERK signaling which can control tumor progression. Dashed green arrow between ADAM12 and EGFR indicates that this relationship has not yet been determined; solid green arrows indicate known relationship.

Chapter 4 - Discussion

Our studies show that ADAM12 is a regulator of EGFR-MEK-ERK signaling in triple-negative breast cancer (TNBC) cells and, by knocking-down ADAM12, we hindered MEK-ERK activity. We were not able to detect significant phosphorylation of EGFR at T669, Y998, Y1068, and Y1173 in SUM159PT cells (Figure 2.2 and Figure 2.3C). This is surprising, considering the vital role EGFR plays in the progression of TNBC and the importance of these residues in the activation of downstream effectors associated with cellular proliferation and survival [Wilson *et al.*, 2009; Li *et al.*, 2008; Mueller *et al.*, 2012]. Notably, we detected a basal level of EGFR phosphorylation at Y845 in SUM159T cells (Figure 2.3C) and phosphorylation of Y845 seemed to be positively regulated by ADAM12 expression (Figure 2.3C). This indicates that ADAM12 does play a role in the regulation of EGFR signaling through Y845 in TNBC. However, it is not clear whether ADAM12 modulates Y845 phosphorylation through the cleavage/release of EGFR ligands or through protein-protein interactions mediated by its cytoplasmic tail. ADAM12's ability to activate EGFR through proteolytic cleavage of ligands has been reported before, whereas its potential to modulate EGFR via its cytoplasmic tail is much less studied. Previous reports have indicated an interaction between the proline-rich region of ADAM12 cytoplasmic tail and the SH3 domain of Src kinase and this interaction led to activation of Src [Kang *et al.*, 2000]. It is possible that ADAM12-mediated activation of Src can then lead to the phosphorylation of Y845 in EGFR [Mueller *et al.*, 2012].

We also observed that ADAM12 regulates the activation of the MEK/ERK signaling cascade (Figure 2.3C and 2.4). The mechanism by which ADAM12 modulates MEK/ERK is still not clear. MEK/ERK signaling has been linked to the pathology of several cancers, including

TNBC [Rajalingam *et al.*, 2007; Roberts *et al.*, 2006, Balko *et al.*, 2013; Giltnane & Balko 2014]. EGFR overexpression is thought to play a key role in the oncogenic MEK/ERK signaling in many cancers. Our results suggest that ADAM12 may mediate MCF10A cell survival through the cleavage/release of pro-survival factors from the surface of SUM159PT cells (Figure 2.5). These ligands may participate in EGFR signaling and consequently activating MEK/ERK signaling in MCF10A cells (Figure 2.6A). Another possible explanation might be that ADAM12 knock-down in SUM159PT cells leads to the depletion of other growth factors (not from the EGF family) in the medium or, potentially, to secretion of anti-growth signaling factors. This would suggest that ADAM12 is able to modulate MEK/ERK signaling independently of EGFR, through interactions mediated via its cytoplasmic tail (Figure 2.6B). Further studies of the relationship between ADAM12 and MEK/ERK will need to be performed in order to determine the exact mechanism. Currently, many cancers will acquire resistance to anti-MEK/ERK therapies, therefore, discovering a new approach of targeting MEK/ERK signaling would have strong clinical applications [Roberts *et al.*, 2006; Downward *et al.*, 2003].

If Y845 is the only EGFR residue regulated by ADAM12 in TNBC, this finding is puzzling because no reports have yet shown correlation between Y845 phosphorylation and subsequent activation of MEK/ERK signaling. An alternative hypothesis is that Y845 may not be involved in MEK/ERK activation at all, and instead ADAM12 may be cytoplasmically interacting with a protein other than Src, possibly Grb2. Grb2 is a well-known inducer of MEK/ERK activity through RAS [Prior *et al.*, 2012; Yan *et al.*, 1998; Rajalingam *et al.*, 2007].

Both high activation of the MEK/ERK pathway and high expression of ADAM12 are linked to poor survival among TNBC patients (Figure 1.3) [Minn *et al.*, 2005; Herold *et al.*, 2013]. We have identified a novel role for ADAM12 in the regulation of MEK/ERK (Figure

2.6), possibly through phosphorylation of Y845 in EGFR. Phosphorylation of other tyrosine residues commonly thought of as key targets for EGFR signaling leading to cellular proliferation and survival, did not seem to be affected by ADAM12 KD. Only phospho-Y845, a substrate for Src, was found to be down-regulated upon ADAM12 KD in TNBC. Although an EGFR-independent mechanism is possible, we postulate that ADAM12 might regulate MEK/ERK signaling in an EGFR-dependent manner via Y845, and/or potentially through other residues not yet tested. This mechanism is additionally supported by involvement of ADAM12 in regulating the survival of MCF10A cells in a paracrine manner.

The main limitation of the current study is the use of only one shRNA targeting ADAM12 and a single TNBC cell line. Experiments using multiple shRNAs or siRNAs targeting different regions of ADAM12 are currently being carried out to confirm the results of the current study. Additionally, the use of multiple breast cancer cell lines will be essential to establish the role of ADAM12 in regulation of EGFR/MEK/ERK signaling.

References

1) Albanell, J. & Baselga, J. (1999) The ErbB receptors as targets for breast cancer therapy. *Journal of Mammary Gland Biology and Neoplasia*, 4(4), 337-51.
2) Arora, A. & Scholar E. M. (2005) Role of tyrosine kinase inhibitors in cancer therapy. *Journal of Pharmacology & Experimental Therapeutics*, 315(3), 971-9.
3) Arteaga, C. L. & Johnson, D. H (2001) Tyrosine kinase inhibitors-ZD1839 (Iressa). *Current Opinion in Oncology*, 13(6), 491-8.
4) Asayesh, A., Alanentalo, T., Khoo, N. K. & Ahlgren, U. (2005) Developmental expression of metalloproteases ADAM 9, 10, and 17 becomes restricted to divergent pancreatic compartments. *Developmental Dynamics*, 232(4), 1105-14.
5) Atfi, A., Dumont, E., Colland, F., Bonnier, D., L'helgoualc'h, A., …Theret, N. (2007) The disintegrin and metalloproteinase ADAM12 contributes to TGF-beta signaling through interaction with the type II receptor. *Journal of Biological Chemistry*, 178(2), 201-8.
6) Avraham, R. & Yarden, Y. (2011) Feedback regulation of EGFR signaling: decision making by early and delayed loops. *Nature Reviews. Molecular Cell Biology*, 12(2), 104-17. doi: 10.1038/nrm3048.
7) Balko, J. M., Schwarz, L. J., Bhola, N. E., Kurupi, R., Owens, P., …Arteaga, C. L. (2013) Activation of MAPK pathways due to DUSP4 loss promotes cancer stem cell-like phenotypes in basal-like breast cancer. *Cancer Research*, 73(20), 6346-58. doi: 10.1158/0008-5472.CAN-13-1385.

8) Bazley, L. A. & Gullick, W. J. (2005) The epidermal growth factor receptor family. *Endocrine-Related Cancer*, 12(1), S17-27. doi: 10.1677/erc.1.01032.

9) Bondeva, T., Balla, A., Varnai, P. & Balla, T. (2002) Structural determinants of Ras-Raf interaction analyzed in live cells. *Molecular Biology of the Cell*, 13(7), 2323-33.

10) Carracedo, A. & Pandolfi, P. P. (2008) The PTEN-PI3K pathway: of feedbacks and cross-talks. *Oncogene*, 27(41), 5527-41.

11) Caunt, C. J., Sale, M. J., Smith, P. D. & Cook, S. J. (2015) MEK1 and MEK2 inhibitors and cancer therapy: the long and winding road. *Nature Reviews. Cancer*, 15(10), 577-92. doi: 10.1038/nrc4000.

12) Chen, P., Xie, H. &Wells, A. (1996) Mitogenic signaling from the egf receptor is attenuated by a phospholipase C-gamma/protein kinase C feedback mechanism. *Molecular Biology of the Cell*, 7(6), 871-81.

13) Chow, F. L. & Patron, C. (2007) Many membrane proteins undergo ectodomain shedding by proteolytic cleavage. Does one sheddase do the job on all of these proteins? *IUBMB Life*, 59(1), 44-7.

14) Cook, R. S., Feng, S. M., Strunk, K. E. & Earp, H. S. 3rd (2008) ErbB4/HER4: role in mammary gland development, differentiation and growth inhibition. *Journal of Mammary Gland Biology and Neoplasia,* 13(2), 235-46. doi: 10.1007/s10911-008-9080-x.

15) Corbit, K. C., Trakul, N., Eves, E. M., Diaz, B., Marshall, M. & Rosner, M. R. (2003) Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. *Journal Biological Chemistry*, 278(15), 13061-8.

16) Dent, R., Trudeau, M., Pritchard, K. I., Hanna, W. M., Kahn, H. K., Sawka, C. A., …Narod, S. A. (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. *Clinical Cancer Research*, 13(15 Pt 1), 4429-34.

17) Dhillion, A. S., Hagan, S., Rath, O. & Kolch, W. (2007) MAP kinase signaling pathways in cancer. *Oncogene*, 26(22), 3279-90.

18) Doody, J. F., Wang, Y., Patel, S. N., Joynes, C., Lee, S. P., Gerlak, J., …Hadari, Y. R. (2007) Inhibitory activity of cetuximab on epidermal growth factor receptor mutations in non-small cell lung cancers. *Molecular Cancer Therapeutics*, 6(10), 2642-51.

19) Downward, J. (2003) Targeting RAS signalling pathways in cancer therapy. *Nature Reviews Cancer*, 3(1), 11-22.

20) Duffy, M. J., Mullooly, M., O'Donovan, N., Sukor, S., Crown, J., …McGowan, P. M. (2011) The ADAMs family of proteases: new biomarkers and therapeutic targets for cancer? *Clinical Proteomics*, 8(1), 9. doi: 10.1186/1559-0275-8-9.

21) Emburgh, B. O., Bianchi, A., Nicolantonio, F., Siena, S. & Bardelli, A. (2014) Acquired resistance to EGFR-targeted therapies in colorectal cancer. *Molecular Oncology*, 8(6), 1084-94. doi: 10.1016/j.molonc.2014.05.003.

22) Foley, J., Nickerson, N. K., Nam, S., Allen, K. T., Gilmore, J. L., …Riese, D. J. 2nd (2010) EGFR signaling in breast cancer: bad to the bone. *Seminars in Cell & Developmental Biology*, 21(9), 951-60. doi: 10.1016/j.semcdb.2010.08.009.

23) Giltnane, J. M. & Balko, J. M. (2014) Rationale for targeting the Ras/MAPK pathway in triple-negative breast cancer. *Discovery Medicine,* 17(95), 275-83.

24) Guo, L., Kozlosky, C. J., Ericsson, L. H., Daniel, T. O., Cerretti, D. P., Johnson, R. S. (2003) Studies of ligand-induced site-specific phosphorylation of epidermal growth factor receptor. *Journal of the American Society of Mass Spectrometry,* 14(9), 1022-31.

25) Hancock, J. F. (2003) Ras proteins: different signals from different locations. *Nature Reviews. Molecular Cell Biology*, 4(5), 373-84.

26) Herold, C. I. & Anders, C. K. (2013) New targets for triple-negative breast cancer. *Oncology*, 27(9):846-54.

27) Higashiyama, S., Iwabuki, H., Morimoto, C., Hieda, M., Inoue, H. & Matsushita, N. (2008) Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. *Cancer Science*, 99(2), 214-20. doi: 10.1111/j.1349-7006.2007.00676.x.

28) Holderfield, M., Nagel, T. E. &Stuart, D. D. (2014) Mechanism and consequences of RAF kinase activation by small-molecule inhibitors. *British Journal of Cancer*, 111(4), 640-5.

29) Jutten, B. & Rouschop, K. M. (2014) EGFR signaling and autophagy dependence for growth, survival, and therapy resistance. *Cell Cycle*, 13(1), 42-51. doi: 10.4161/cc.27518.

30) Kim, E. K. & Choi, E. J. (2010) Pathological roles of MAPK signaling pathways in human diseases. *Biochimica et Biophysica Acta,* 1802(4), 396-405.

31) Kocoglu, H., Velibeyoglu, F. M., Karaca, M. & Tural, D. (2016) Clinical efficacy and drug resistance of anti-epidermal growth factor receptor therapy in colorectal cancer. *World Journal of Gastrointestinal Oncology*, 8(1), 1-7. doi: 10.4251/wjgo.v8.i1.1.

32) Krawczyk, P. A. & Kowalski, D. M. (2014) Genetic and immune factors underlying the efficacy of cetuximab and panitumumab in the treatment of patients with metastatic colorectal cancer. *Contemporary Oncology*, 18(1), 7-16. doi: 10.5114/wo.2013.38566.

33) Kveiborg, M., Erohlich, C., Albrechtsen, R., Tinschler, V., Dietrich, N., …Wewer, A. M. (2005) A role for ADAM12 in breast tumor progression and stromal cell apoptosis. *Cancer Research*, 65(11), 4754-61.

34) Li, H., Muggy, S., Qi, Y., Hong, Y., Behbod, F. & Zolkiewska, A. (2012) An essential role of metalloprotease-disintegrin ADAM12 in triple-negative breast cancer. *Breast Cancer Research and Treatment*, 135(3), 759-69. doi: 10.1007/s10549-012-2220-4.

35) Li, x., Huang, Y., Jiang, J. & Frank, S. J. (2008) ERK-dependent threonine phosphorylation of EGF receptor modulates receptor downregulation and signaling. *Cellular Signaling,* 20(11), 2145-55. doi: 10.1016/j.cellsig.2008.08.006.

36) Masuda, H., Zhang, D., Bartolomeusz, C., Doihara, H., Hortobagyi, G. N. & Ueno, N. T. (2012) Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Research and Treatment,* 136(2), 331-45. doi: 10.1007/s10549-012-2289-9.

37) McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., …Franklin, R. A. (2006) Roles of the RAF/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica et Biophysica Acta,* 1773(8), 1263–1284. doi: 10.1016/j.bbamcr.2006.10.001.

38) Mendoza, M. C., Er, E. E. & Blenis, J. (2011) The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends in Biochemical Sciences*, 36(6), 320-8.

39) Miller, M. A., Moss, M. L., Powell, G., Petrovich, R., Edwards, L., …Lauffenburger, D. A. (2015) Targeting autocrine HB-EGF signaling with specific ADAM12 inhibition using recombinant ADAM12 prodomain. *Science Reports*, 5, 15150. doi: 10.1038/srep15150.

40) Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W, …Massague, J. (2005) Genes that mediate breast cancer metastasis to lung. *Nature*, 436(7050), 518–524. doi: 10.1038/nature03799.

41) Mueller, K. J., Powell, K., Madden, J. M., Eblen, S. T. & Boerner, J. L. (2012) EGFR tyrosine 845 phosphorylation-dependent proliferation and transformation of breast cancer cells require activation of p38 MAPK. *Translational Oncology*, 5(5), 327-34.

42) Muggy, S., Li, H., Qi, Y. & Zolkiewska, A. (2013) Alternative mRNA splicing generates two distinct ADAM12 prodomain variants. *PLoS One*, 8(10), e75730.

43) Pollheimer, J., Fock, V. & Knofler, M. (2014) Review: the ADAM metalloproteinases - novel regulators of trophoblast invasion? *Placenta*, 35, S57-63. doi: 10.1016/j.placenta.2013.10.012.

44) Prior, I. A., Lewis, P. D. & Mattos, C. (2012) A comprehensive survey of Ras mutations in cancer. *Cancer Research*, 72(10), 2457-67. doi: 10.1158/0008-5472.CAN-11-2612.

45) Rajalingam, K., Schreck, R., Rapp, U. R. & Albert, S. (2007) Ras oncogenes and their downstream targets. *Biochimica et Biophysica Acta,* 1773(8), 1177-95.

46) Ray, B. K., Dhar, S., Henry, C., Rich, A. & Ray, A. (2013) Epigenetic regulation by Z-DNA silencer function controls cancer-associated ADAM-12 expression in breast cancer: cross-talk between MeCP2 and NF1 transcription factor family. *Cancer Research*, 73(2), 736-44. doi: 10.1158/0008-5472.CAN-12-2601.

47) Ray, B. K., Dhar, S., Shakya, A. & Ray, A. (2011) Z-DNA-forming silencer in the first exon regulates human ADAM-12 gene expression. *Proceedings of the National Academy of Sciences of USA*, 108(1):103-8.

48) Reiss, K. & Saftig, P. (2009) The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. *Seminars in Cell and Developmental Biology*, 20(2), 126-37. doi: 10.1016/j.semcdb.2008.11.002.

49) Roberts, P. J. & Der, C. J. (2007) Targeting the Raf-MEK-ERK mitogen-activated kinase cascade for the treatment of cancer. *Oncogene*, 26(22), 3291-310.

50) Roskoski, R. (2012) ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacological Research*, 66(2), 105-43.

51) Roy, R.., Rodig, S., Bielenberg, D., Zurakowski, D. & Moses, M. A. (2011) ADAM12 transmembrane and secreted isoforms promote breast tumor growth: a distinct role for ADAM12-S protein in tumor metastasis. *Journal of Biological Chemistry,* 286(23), 20758-68.

52) Sarrio, D., Pinilla, S. M., Hardisson, D., Cano, A., Bueno, G. & Palacios, J. (2008) Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Research*, 68(4), 989-97.

53) Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell*, 103(2):211-25.

54) Schneider, M. R. & Wolf, E. (2009) The epidermal growth factor receptor ligands at a glance. *Journal of Cell Physiology*, 218(3), 460-6. doi: 10.1002/jcp.21635.

55) Seals, D. F. & Courtneidge, S. A. (2003) The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes & Development*, 17(1), 7-30.

56) Shao, S., Li, Z., Gao, W., Yu, G., Liu, D. & Pan, F. (2014) ADAM-12 as a diagnostic marker for the proliferation, migration and invasion in patients with small cell lung cancer. *PLoS One*, 9(1), e85936

57) Steelman, L. S., Bertrand, F. E. & McCubrey, J. A. (2004) The complexity of PTEN: mutation, marker and potential target for therapeutic intervention. *Expert Opinion on Therapeutic Targets*, 8(6), 537-50.

58) Steelman, L. S., Chappell, W. H., Abrams, S. L., Kempf, R. C., Long, J., …McCubrey, J. A. (2011) Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging*, 3(3), 192-222.

59) Steelman, L. S., Pohnert, S. C., Shelton, J. G., Franklin, R. A. Bertrand, F. E. & McCubrey, J. A. (2004) JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia*, 18(2), 189-218.

60) Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M. & Hancock, J. K. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science*, 264(5164), 1463-7.

61) Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K. & Ohno, S. (1996) Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. *Journal of Biological Chemistry,* 271(38), 23512-9.

62) Ueno, N. T. & Zhang, D. (2011) Targeting EGFR in Triple Negative Breast Cancer. *Journal of Cancer*, 2, 324–328.

63) Vinante, F. & Rigo, A. (2013) Heparin-binding epidermal growth factor-like growth factor/diphtheria toxin receptor in normal and neoplastic hematopoiesis. *Toxins*, 5(6):1180-1201.

64) Wagner, M. J., Stacey, M. M, Liu, B. A. & Pawson T. (2013) Molecular mechanisms of SH2- and PTB-domain-containing proteins in receptor tyrosine kinase signaling. *Cold Spring Harbor Perspectives in Biology*, 5(12), a008987.

65) Walker, F., Orchard, S. G., Jorissen, R. N., Hall, N. E., Zhang, H. H., Hoyne, P. A,...Burgess, A. W. (2004) CR1/CR2 interactions modulate the functions of the cell surface epidermal growth factor receptor. *Journal of Biological Chemistry*, 279(21), 22387-98.

66) Weber, C. K., Slupsky, J. R., Kalmes, H. A. & Rapp, U. R. (2001) Active Ras induces heterodimerization of cRaf and BRaf. *Cancer Research*, 61(9), 3595-8.

67) Weber, S. & Saftig, P. (2012) Ectodomain shedding and ADAMs in development. *Development*, 139(20), 3693-709.

68) Wennerberg, K. & Rossman, K. L. (2005) The Ras superfamily at a glance. *Journal of Cell Science*, 118(Pt 5), 843-6.

69) Wewer, U. M., Morgelin, M., Holck, P., Jacobsen, J., Lydolph, M. C., …Albrechtsen, R. (2006) ADAM12 is a four-leafed clover: the excised prodomain remains bound to the mature enzyme. *Journal of Biological Chemistry*, 281(14), 9418-22.

70) White, J. M. (2003) ADAMs: modulators of cell-cell and cell-matrix interactions. *Current Opinion in Cell Biology*, 15(5), 598-606.

71) Wilson, K. J., Gilmore, J. L., Foley, J. & Lemmon, M. A. (2009) Functional selectivity of EGF family peptide growth factors: implications for cancer. *Pharmacology & Therapeutics*, 122(1), 1-8. doi: 10.1016/j.pharmthera.2008.11.008.

72) Wojnowski, L., Stancato, L. F., Lamer, A. C., Rapp, U. R. & Zimmer, A. (2000) Overlapping and specific functions of Braf and Craf-1 proto-oncogenes during mouse embryogenesis. *Mechanisms of Development*, 91(1-2), 97-104.

73) Yan, J., Roy, S., Apolloni, A., Lane, A. & Hancock, J. F. (1998) Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *Journal of Biological Chemistry*, 273(37), 24052-6.

74) Yoon, S. & Seger, R. (2006) The extracellular signal-regulated kinase: multiple substrates, regulate diverse cellular functions. *Growth Factors*, 24(1):21-44.

75) Yu, Y., Zhang, M., Zhang, X., Cai, O. Zhiling, Z., …Xu, C. (2014) Transactivation of epidermal growth factor receptor through platelet-activating factor/receptor in ovarian cancer cells. *Journal of Experimental & Clinical Cancer Research*, 33(1), 85.

76) Zolkiewska, A. (1999) Disintegrin-like/cysteine-rich region of ADAM 12 is an active cell adhesion domain. *Experimental Cell Research*, 252(2), 423-31.

77) Zolkiewska, A. (2008) ADAM proteases: ligand processing and modulation of the Notch pathway. *Cellular and Molecular Life Sciences*, 65(13), 2056-68. doi: 10.1007/s00018-008-7586-4.