ROLES OF ADAM12 IN TRIPLE-NEGATIVE BREAST CANCER: REGULATION OF CANCER STEM CELLS

by

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B.S., Tianjin Medical University, China 2004
M.M., Tianjin Medical University, China 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Biochemistry and Molecular Biophysics
College of Arts and Sciences

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Manhattan, Kansas

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Abstract

ADAM12 (A Disintegrin and Metalloprotease 12) is a cell surface protease, which is deregulated in many human diseases. High expression of ADAM12 in triple-negative breast cancers (lacking estrogen receptor, progesterone receptor, and HER2 expression) is associated with poor patient prognosis. My dissertation focused on the understanding of the biological functions of ADAM12 in triple-negative breast cancers. I found that ADAM12 is significantly upregulated in the claudin-low molecular subtype of breast cancer. Claudin-low tumors are typically triple-negative and are enriched in cancer stem cells. Here, I demonstrated that the loss of ADAM12 expression not only decreased the number of cancer stem-like cells *in vitro* but also significantly compromised the tumor-initiating capabilities of breast cancer cells *in vivo*. This is the first evidence showing that ADAM12 might regulate the cancer stem cell-like phenotype in triple-negative breast cancers. I also discovered a novel mechanism of ADAM12-regulated signaling by transforming growth factor β (TGFβ) through modulation of *TGFBR1* mRNA expression in breast cancer cells. Lastly, I characterized the effects of six different somatic mutations in the *ADAM12* gene found in human breast cancers on the intracellular trafficking, post-translational processing, and function of ADAM12 protein. Collectively, the findings of this study support the notion that ADAM12 with catalytically active metalloprotease domain is required for the progression of triple-negative breast cancers.

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Abbreviation

ADAM	a disintegrin and metalloprotease
ALDH	aldehyde dehydrogenase
Bcl-2	apoptosis regulator Bcl-2
BIK	Bcl-2-interacting killer
CaMK	calcium/calmodulin-dependent protein kinase
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
PR	progesterone receptor
ERK	extracellular signal-regulated kinase
JAK	Janus kinase
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor κB
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
PKC	protein kinase C
PyMT	polyoma virus middle T antigen

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Chapter 1 - Introduction

Metalloprotease ADAM12 in Breast Cancer

Overview of the ADAM metalloproteases

A Disintegrin And Metalloprotease is a family of membrane-anchored proteases. ADAMs, together with snake venom metalloproteases (SVMPs) and ADAMs containing thrombospondin motifs (ADAMTs), are members of the M12B adamalysin peptidase subfamily (Jones *et al*., 2016). So far, twenty-two human ADAM metalloprotease have been identified, twelve of which possess full protease activity (Jones *et al*., 2016). A typical ADAM protein possesses a signal sequence, a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich region, an EGF-like domain, a transmembrane domain, and a cytoplasmic domain (Figure 1.1A).

The signal sequence of ADAMs are located at the N-terminus of the protein and lead the proteins into the secretory pathway (Edwards et al., 2008). Right next to the signal sequence is the prodomain of ADAMs, which not only behaves as a self-inhibitor to the metalloprotease site but also acts as an intramolecular chaperone to assure the correct folding of the protease molecule (Roghani et al., 1999). The prodomain of the ADAM metalloprotease is cleaved by furin-like enzymes in the Golgi or is auto-cleaved when passing through the secretory pathway (Howard et al., 2000; Schlomann et al., 2002) or later in the secretory pathway.

Metalloprotease domain connects immediately to the C-terminus of the prodomain. The ADAM metalloproteases are endopeptidases containing His-Glu-Xaa-Xaa-His-Xaa-Xaa-Gly-Xaa-Xaa-His motif, in which Xaa is hydrophobic or threonine (Jongeneel et al., 1989). The three histidine residues bind to a zinc atom and line up with glutamate residue forming the protease catalytic center (http://merops.sanger.ac.uk/cgi-bin/famsum?family=M12). In the catalytic site of

ADAMDEC1, the third histidine residue is replaced by an aspartate residue (His-Glu-Leu-Gly-His-Val-Leu-Gly-Met-Pro-Asp) (Bates et al., 2002).

Eight human ADAMs are catalytically inactive: ADAM2, ADAM7, ADAM11, ADAM18, ADAM22, ADAM23, ADAM29, and ADAM32, suggesting ADAMs could function through direct protein-protein interaction mediated by other domains of the protein (Edwards et al., 2008). The disintegrin domains of ADAMs have been intensively studied due to their potential to interact with adhesion molecule integrins. Studies had proved that ADAMs interacted with specific integrins, such as α2β1, α4β1, α4β7, α5β1, α6β1, α6β4, α9β1, αVβ3, and αVβ5 and that these interactions played roles in cell adhesion and cell-cell interactions (Eto et al., 2002).

The cysteine-rich domain is important for ADAMs substrate interactions. Gaultier's group showed that the Cys-rich domain was required for ADAM13 binding to fibronectin and for ADAM17 mediated IL-1 receptor-II cleavage (Gaultier *et al*., 2002; Reddy *et al*., 2000). Besides, the unique structure formed by the Cys-rich domain and the disintegrin domain via disulfide bonds allowed the cysteine-enriched region facing directly towards the active center of the metalloprotease domain and facilitated the effective cleavage of the substrate (Takeda *et al*., 2006; Janes *et al*., 2005). Since the Cys-rich region has a hyper-variable region (HVR), which is not conserved among different ADAM members, it has been proposed that the structural motif formed by the disintegrin and Cys-rich domain through disulfide bonds could be an essential module or substrate interaction site of ADAMs (Takeda *et al*., 2006; Zhang *et al*., 2015).

The intracellular portions of ADAM proteins contain one or more proline-rich regions, which are potential interacting sites for Src Homology 3 (SH3) domain-containing proteins (such as Src, Yes, Grb, *et al*) (Ebsen *et al*., 2014), suggesting that ADAMs may function as signaling

molecules in a protease-independent manner. The C-terminal tails also regulate the intracellular trafficking of ADAM proteins. The cytoplasmic tail of ADAM10 harbors an arginine-rich region, mutation of which causes the retention of the protein in the endoplasmic reticulum and poor cell surface localization (Marcello *et al*., 2010). Interestingly, there is evidence showing that phosphorylation of the cytoplasmic domain is required for ADAM17 mediated ectodomain cleavage (Killock & Ivetić, 2010; Prakasam *et al*., 2014).

ADAM proteins are glycosylated during the process of synthesis in the endoplastic reticulum and Golgi. Though the exact structure and the function of the sugar moieties still remain unclear, there is evidence showing that the accurate glycosylation is essential for the protein to acquire the full enzymatic activity. Srinivasan and his group showed that the N-glycosylation on Asn91 and Asn612 of ADAM8 were critical for the maturation and surface localization of the protein (Srinivasan *et al*., 2014). ADAM8 with N91Q or N612Q mutations failed to pass beyond Golgi and endoplastic reticulum, respectively.

ADAM12 in breast cancer

ADAM12 is highly upregulated in breast tumor and breast cancer cell lines (Lendeckel *et al*., 2005; Kveiborg *et al*., 2005; Li *et al*., 2012; Kveiborg *et al*., 2008; Li *et al*., 2013) and is also recognized as a candidate cancer gene in breast cancer (Sjöblom *et al*., 2006). Studies have shown that ADAM12 is expressed in the intralobular area of breast tumors and the invading tumor cells (Kveiborg *et al*., 2005; Kveiborg *et al*., 2008). In triple-negative breast cancer, ADAM12 expression level was strongly associated with the activation of EGFR and shorter metastasis-free survival times (Li *et al*., 2012). Besides, ADAM12 was shown to be elevated in claudin-low subtype breast cancers and linked to tumor-initiating phenotype of cancer cells (Li *et al*., 2013) Thus, it is important to understand how ADAM12 is regulated in breast cancer.

Regulation of ADAM12 expression

Regulation of ADAM12 by TGFβ signaling

Transforming growth factor (TGF) β signaling pathway has been shown to regulate the expression of ADAM12 at both the transcriptional and the translational level in breast cancer cell lines (Li *et al*., 2012; Ruff *et al*., 2015). TGFβ-mediated ADAM12 regulation was first reported by Le Pabic and coworkers in human hepatic stellate cells (HSCs), which are activated from the quiescent status by inflammatory or other liver damage stimuli (Le Pabic *et al*., 2003). Le Pabic and coworkers found that ADAM12 expression was significantly elevated by TGFβ treatment in human activated HSCs and in liver cancers (Le Pabic *et al*., 2003; Le Pabic *et al*., 2005). Long term treatment with MAPK inhibitor LY294002, PI3K inhibitor UO126, or mTOR inhibitor Rapamycin decreased the induction of ADAM12 mediated by TGFβ in HSCs, suggesting that TGFβ might modulate ADAM12 gene expression in Smad-independent manner through PI3K/mTOR and MEK/ERK pathways. Beside, TGFβ signaling also mediated the elevated ADAM12 expression triggered by NF-κB signaling (Ray *et al*., 2010).

SnoN, a transcription suppressor of TGFβ signaling, has been shown to be a negative regulator of ADAM12 expression in mouse fibroblast cells (Solomon *et al*., 2010). Upon adding TGFβ, SnoN is targeted by Smad2/3 and Smurf-2 for proteasomal degradation (Deheuninck *et al*., 2009). The process is Smad-dependent and responds shortly after TGFβ treatment. Overexpression of SnoN partially but significantly decreased TGFβ mediated ADAM12 induction, whereas down-regulation of SnoN by shRNA caused a robust increase of ADAM12 at both mRNA and protein levels (Solomon *et al*., 2010).

Regulation of ADAM12 by ErbB2 activation

ErbB2 is a receptor tyrosine kinase and is also known as HER2. Studies have also shown that ErbB2 activation induced ADAM12 expression in human head and neck squamous carcinoma cells (HNSCCs) (Rao *et al*., 2012; Rao *et al*., 2014). Overexpression of ErbB2 resulted in the up-regulation of ADAM12 via ErbB2/PI3K/Akt/mTOR signaling cascade. Conversely, up-regulated ADAM12 promoted the expression of ErbB2 through transcription factor Ets-1 in HNSCC cells (Rao *et al*., 2012). A positive activation loop was proposed between ADAM12 and ErbB2 expression, which might attribute to the tumor malignancy of UV-induced skin cancers and human head and neck cancers (Rao *et al*., 2012; Rao *et al*., 2014). However, no detailed molecular mechanism has been available to explain how mTOR regulates ADAM12 expression and how ADAM12 modulates Ets-1 transcription activity.

Post-transcriptional regulation of ADAM12 by microRNAs

ADAM12-L and ADAM12-S are two ADAM12 isoforms: ADAM12-L represents the long, transmembrane isoform of ADAM12; whereas ADAM12-S has neither transmembrane domain nor cytoplasmic tail and is released from cells after being synthesized (Figure 1.1B). Studies showed that ADAM12-L, but not ADAM12-S, is not only strongly upregulated in the claudin-low molecular subtype of breast cancer but also of prognostic value (Li *et al*., 2013). Since ADAM12-L and -S are encoded by two alternative transcript variants of ADAM12 with differences at 3'-untranslated regions (3'UTRs), it was postulated that the 3'UTRs of ADAM12 might be the target of post-transcriptional regulation. Our recent published data suggested that miR-200b/c and miR-29b/c directly target the 3'UTR of ADAM12-L and negatively regulate the expression of ADAM12-L (Duhachek-Muggy *et al*., 2015).

miR-200b/c are members of the miR-200 family, which has shown to be a negative regulator of breast cancer stem cell properties and an inducer of Epithelial-to-Mesenchymal Transition (EMT) (Schwarzenbacher *et al*., 2013). Down-regulation of the miR-200 family was often observed in breast cancer stem cells, normal mammary stem cells, and embryonal carcinoma cells (Shimono *et al*., 2009). The miR-29 family, which consists of 3 isoforms, has been shown to be downregulated in various types of cancers, including invasive breast cancer (Iorio *et al*., 2005; Garzon *et al*., 2009). Studies showed that miR-29b is involved in the regulation of apoptosis and tumorigenic potential in leukemia, lung, and prostate cancer cells (Mott *et al*., 2007; Garzon *et al*., 2009; Ru *et al*., 2012).

Our results showed that transfection of miR-29b/c mimics resulted in a significant down-regulation of ADAM12-L in SUM159PT and BT549 breast cancer cell lines with no effect on ADAM12-S (Duhachek-Muggy *et al*., 2015). miR-200b/c mimics induced a dramatic decrease of ADAM12 translation in Hs578T cells and reduction of ADAM12-L at both mRNA and protein levels in SUM1315MO2. miR-29b/c and miR-200b/c mimics also inhibited the activity of ADAM12-L 3'UTR reporter. The inhibitory effects disappeared when the targeting sites of miR-29b/c and miR200b/c were mutated. In all, this finding indicates that miR29-b/c and miR-200b/c directly target ADAM12-L 3'UTR and regulate its expression at post-transcriptional level.

Regulation of ADAM12 expression by protein intracellular tranfficking

ADAM12 activity can be regulated at the post-translational level by PKCε, c-Src, and clathrin. PKCε interacted with ADAM12 via regulatory domain and promoted the surface translocation of ADAM12 in a kinase-dependent manner (Sundberg *et al*., 2004). Though it has been shown that PKC activation stimulates the regulated protein secretory pathway (Traub & Kornfeld, 1997), through which ADAM12 translocates to cell surface, no detailed mechanism

model has been proposed which would explain how PKCε increases the surface translocation of ADAM12. Studies have also shown that proteins of the Src family regulate ADAM12 cellular localization (Stautz *et al*., 2010). Grb2 and c-Src bind to ADAM12 cytoplasmic tail at the cysteine-rich region and cause ADAM12 translocation from the near-nuclear area to peripheral punctate structures of the cell (Stautz *et al*., 2012; Stautz *et al*., 2010; Suzuki *et al*., 2000). Clathrin has been found to be involved in the regulation of ADAM12 endocytosis. Down-regulation of clathrin decreased ADAM12 turnover rate and thus enhanced ADAM12 function at the cell surface (Ren *et al*., 2011).

Function of ADAM12 in breast cancer

ADAM12 promotes breast cancer progression via EMT

ADAM12 was shown to be actively involved in epithelial-to-mesenchymal transition (EMT) and thus to promote breast tumor progression and dissemination. EMT is a gene expression reprogramming process that could occur either in physiological or pathological contexts (Lamouille *et al*., 2014). In the context of cancer, EMT features downregulate adhesion molecule expression, enhance migration capability, and increase resistance to apoptosis (Kalluri & Weinberg, 2009). EMT is also closely associated with a subpopulation of cancer cells that possess stem cell-like phenotype and are capable of initiating tumors (Kalluri & Weinberg, 2009; Lamouille *et al*., 2014). Therefore, EMT promotes tumor progression and dissemination. TGFβ is a potent EMT inducer. Long-term treatment with TGFβ not only induces EMT but also leads to a strong upregulation of ADAM12 at both mRNA and protein levels (Atfi *et al*., 2007; Solomon *et al*., 2010; Li *et al*., 2013). On the other hand, ADAM12 was demonstrated to enhance TGFβ signaling by stabilizing TGFBR1 via interacting with TGFBR2, which would

form a tetramer complex with TGFBR1 upon binding with TGFβ (Atfi *et al*., 2007; Gruel *et al*., 2009).

Breast tumors and cell lines with mesenchymal characteristics show potent tumor initiating capability as well as abundant ADAM12 expression. A study of Li and coworkers further demonstrated that ADAM12 was significantly associated with breast tumor-initiating phenotype (Li *et al*., 2013). Recently published data revealed that overexpression of ADAM12 in MCF10A, a breast epithelial cell line, led not only to a dramatic cytoskeleton change but also elevated expression of EMT markers (Ruff *et al*., 2015), suggesting that ADAM12 could facilitate or even induce EMT in breast cells. Interestingly, this evident effect did not require the proteolytic activity of ADAM12 but was associated with the activation of ERK signaling pathway (Ruff *et al*., 2015).

ADAM12 increases apoptotic resistance of breast cancer cells

In vivo studies have shown that ADAM12 might play an active role in breast tumor progression via decreasing the sensitivity of cancer cells to apoptosis (Kveiborg *et al*., 2005; Roy *et al*., 2011). Overexpressed ADAM12-Δcyt increased the apoptotic sensitivity of non-cancerous cells, whereas in breast tumor cells, the exogenous ADAM12 induced the opposite effect: ADAM12 significantly enhanced the apoptosis resistance of breast tumor cells and thus promoted tumor progression (Kveiborg *et al*., 2005). This *in vivo* study further demonstrated that both ADAM12-Δcyt/PyMT and ADAM12S/PyMT transgenic mice had low tumor free rates, increased total tumor mass, and elevated ADAM12 expression in malignant tumor samples. The protease activity of ADAM12 was not required for the acquisition of apoptosis resistance.

In human breast cancer cells, ADAM12-mediated matrix metalloprotease-14 (MMP-14) activation promotes cancer cell survival as well as invasion. ADAM12 overexpression in breast

cancer cells induced MMP14 expression and therefore promoted the degradation of an extracellular matrix component gelatin (Albrechtsen *et al*., 2013). More importantly, ADAM12-mediated MMP14 activation resulted in decreased expression of pro-apoptotic proteins BCL2L11 and BIK, leading to reduced apoptotic sensitivity of cancer cells (Albrechtsen *et al*., 2013). Intriguingly, both effects required neither protease activity nor cytoplasmic tail of ADAM12, suggesting that ADAM12 may function through protein-protein interactions mediated via the extracellular domain. Orthotopic implantation of MCF7 cells with overexpressed ADAM12 further confirmed that ADAM12 facilitated tumor progression and reduced tumor apoptosis *in vivo* (Albrechtsen *et al*., 2013).

ADAM12-mediated growth factor cleavage promotes breast cancer progression and metastasis

ADAM12 sheddase activity is of great importance for breast tumor progression and metastasis. Sonic hedgehog, Delta-like1, HB-EGF, EGF, and β-cellulin are ADAM12 substrates, activating key signaling pathways that regulate cell proliferation, migration, and differentiation (Kveiborg *et al*., 2008; Ohlig *et al*., 2011; Fröhlich *et al*., 2013). HB-EGF, EGF, and β-cellulin are ligands of EGF receptors, activating EGFR signaling pathway, which promotes the growth, migration, survival, and tumor initiation of breast cancer cells (Appert-Collin *et al*., 2015; Lo *et al*., 2006). VE-cadherin (vascular endothelial cadherin), Flk-1 (fetal liver kinase-1), and Tie-2 (receptors of angiopoietin) are expressed in endothelial cells and are required for proper vasculature formation (Carmeliet *et al*., 1999; Maisonpierre *et al*., 1997). ADAM12 was demonstrated to be able to cleave these membrane-associated proteins, suggesting that ADAM12 may promote breast tumor metastasis through affecting blood vessel structure or formation (Olsson *et al*., 2006; Fukuhara *et al*., 2008; Fröhlich *et al*., 2013). Additionally, ADAM12

mediated VE-Cadherin shedding may potentially facilitate cancer cells invasion through blood vessel walls as VE-cadherin regulates vascular permeability (Corada *et al*., 2001; Fröhlich *et al*., 2013).

A study of ADAM12 mutants showed that protease enzymatic activity of ADAM12 is important for cell motility. Cells with overexpressed wild-type ADAM12 migrated faster and showed higher phosphorylation of EGFR than cells with catalytically inactive mutants (Qi *et al*., 2014). More importantly, ADAM12 mutants with impaired protease activity were not included in triple-negative breast cancer and the mutants that appeared in triple-negative breast cancer possessed full enzymatic activity. These results suggest that the protease activity of ADAM12 might be critical for triple-negative cancer progression (Qi *et al*., 2014; Li *et al*., 2012).

ADAM12 induces drug resistance in ERα-positive breast cancer cells

ADAM12 was also shown to induce anti-estrogen resistance in MCF7 cells (Roy *et al*., 2012). Estrogen receptor α (ERα) is a major estrogen receptor isoform, usually overexpressed in ER-positive breast cancer cells, and thereby is routinely used as a protein marker in breast cancer diagnosis (Badve & Nakshatri, 2009). Roy and coworkers observed that overexpression of ADAM12 in ERα-positive MCF7 cells promoted the growth of tumor cells in an estrogen-independent manner (Roy *et al*., 2012). Also, the same study discovered that MCF7 cells with ADAM12 overexpression showed higher phosphorylation of EGFR, insulin-like growth factor-1 receptor (IGF-1R), and MAPK, as well as increased amphiregulin cleavage, suggesting ADAM12-mediated EGFR, IGF-1R, and MAPK activation via amphiregulin cleavage might be the driving force of tumor growth (Roy *et al*., 2012). In all, a large number of previous studies suggest that ADAM12 is actively involved in the breast cancer progression, especially in triple-

negative breast tumors, and that ADAM12 could serve as a potential target for breast cancer treatment.

Role of EGFR Signaling in Breast Cancer

Overview of EGFR signaling pathway

The epidermal growth factor receptor (EGFR) signaling pathway is one of the major cell-cell communication signaling pathways, which is of significant importance in cell fate determination, cell proliferation, cell migration and apoptosis (Yang & Baker, 2003; Yarden & Shilo, 2007). Owing to its critical biological functions, EGFR signaling has been extensively studied in the context of tumorigenesis, progression, and drug resistance.

Receptors and ligands

Receptors

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase whose activation requires the binding of EGF-like ligands. EGFR belongs to the family of four ErbB receptors, which consist of EGFR (also known as ErbB1), ErbB2 (also known as HER2), ErbB3, and ErbB4. Protein structures of the four receptors are similar (Figure 1.2), containing an extracellular ligand-binding domain, a single transmembrane region, and a conserved intracellular tyrosine kinase (TK) domain (Linggi & Carpenter, 2006).

EGFR and ErbB4 require the binding of cognate ligands to form a homo- or heterodimer, which sequentially induces autophosphorylation of the TK domain and activates downstream signaling pathways. ErbB2 behaves as an orphan receptor and does not interact with any ligands (Ward *et al*., 2007). ErbB3 possesses no tyrosine kinase activity. Both of ErbB2 and ErbB3 are

capable of activating signaling pathways through heterodimerization with other ErbB receptors (Citri & Yarden, 2006).

Ligands

The ErbB receptors can be activated by eleven different ligands; epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), β-cellulin (BTC), amphiregulin (AREG), epiregulin (EREG), epigen (EPGN), and neuregulin1-4 (NRG1-4, also known as heregulins) (Schneider & Yarden, 2014) (Figure 1.3). These ligands are small water-soluble protein molecules that share a highly conserved EGF-like motif (Tebbutt *et al*., 2013). Each of the ligands is encoded by an individual gene, synthesized as membrane-anchored precursor, and cleaved by an ADAM protease (Zeng *et al*., 2014; Sahin *et al*., 2004). The involvement of ADAM 9, -10, -12, -15, -17, and -19 in the cleavage of EGFR ligands has been well documented in the literature (Blobel, 2005; Horiuchi *et al*., 2007; Le Gall *et al*., 2009).

ErbB receptors interact selectively with their preferred ligands (Figure 1.2), with the exception of ErbB2, which does not bind any ligands (Linggi *et al*., 2006). Interestingly, though all the ligands are capable of triggering EGFR signaling, the outcomes are often different for different ligands. For example, both AREG and TGF-α are able to induce cell division and proliferation, but only AREG is capable of affecting the distribution of E-cadherin and changing the morphology of kidney cells (Berasain & Avila, 2014).

Expression of EGFR ligands can be regulated at the transcriptional (promoter activity), post-transciptional (mRNA stability), and post-translational levels (sheddase availability and activity) (Berasain & Avila, 2014). Dysregulation of EGFR ligands usually leads to enhanced and extended activation of EGFR signaling and thus is commonly associated with various types of tumors (Taylor *et al*., 2014).

Downstream signaling cascades

Activated EGFR recruits several effectors and sequentially turns on downstream pathways that regulate critical cellular events. Traditional EGFR signaling downstream pathways include: PLC-γ-CaMK/PKC, Ras/Raf/MAPK, PI3K/Akt/mTOR, and STATs (Han & Lo, 2012) (Figure 1.4).

Activation of PLC-γ-CaMK/PKC regulates the expression of pro-apoptotic proteins, such as procaspase-2, -7, -8, and promotes cell proliferation by phosphorylating cdc25c. As a result, PLC-γ-CaMK/PKC plays critical roles in the regulation of cell proliferation, cell death, cell-cell contacts, and secretion (Mochly-Rosen *et al.*, 2012; Litosch, 2015). Mitogen-activated protein kinases (MAPKs) selectively phosphorylate proteins at Ser/Thr/Tyr amino acid residues and regulate cell proliferation, mitosis, cell survival and apoptosis (Pearson *et al.*, 2001). PI3K/Akt/mTOR is one of the critical signaling pathways participating in multiple cellular processes, such as cell proliferation, cell cycle, and apoptosis (Lo *et al*., 2006).

Signal transducer and activator of transcription (STAT) is a family of transcription factors that are actively involved in immune responses, proliferation, and differentiation (Aaronson & Horvath, 2002). Recent studies have shown that STAT3 can be either directly phosphorylated by EGFR/ErbB2 or through EGFR/JAK1/2, and that dysregulation of STAT3 is frequently associated with breast tumor initiation, angiogenesis, and metastasis (Brooks *et al.*, 2014).

EGFR signaling in breast cancer

EGFR signaling promotes breast cancer migration

The capability to migrate is the prerequisite function for cancer cells to invade and metastasize (McLean *et al*., 2005). EGFR signaling alteration is related to advanced stages of

malignancy and metastatic potential of breast cancers (Sainsbury *et al*., 1988; Viale *et al*., 2009; Hohensee *et al*., 2013). Several mechanisms have been proposed to illustrate how EGFR signaling modulates cancer cell migration and invasion, for example, up-regulation of metalloprotease/collegenase expression in breast cancers (Zhou *et al*., 2014; Seoane *et al*., 2015; Kondapaka *et al*., 1997; Navarini *et al*., 2015), activation of focal adhesion kinase (FAK) phosphorylation (Sieg *et al*., 2000), or increase of c-Src activity (Marcotte *et al*., 2012).

EGFR signaling elicits cancer treatment resistance

Increased EGFR signaling is associated with the therapy resistance of ERα+ breast cancer (Massarweh *et al*., 2008; Jin *et al*., 2012; Ciupek *et al*., 2015). Several reports indicate that tamoxifen-resistant ERα+ breast cancer cells frequently show upregulated EGFR/ErbB2 expression and phosphorylation (Shou *et al*., 2004; Arpino *et al*., 2005; Jin *et al*., 2012; Ciupek *et al*., 2015). EGFR/RAS/MAPK and EGFR/PI3K/Akt downstream cascades have been shown to contribute to the chemoresistance in breast cancer cells, which promote breast cancer cell survival via inhibiting apoptosis (Tanabe *et al*., 2003; Shou *et al*., 2004; Polzien *et al*., 2011) and cell-cycle checkpoints (Wang *et al*., 2010; Chakrabarty *et al*., 2013; She *et al*., 2005).

EGFR signaling induces EMT

Accumulating evidence shows that EGFR signaling promotes migration, metastasis, and chemoresistance via inducing EMT. EMT is a transcription reprogramming process that promotes cell transition from the epithelial to mesenchymal-like phenotype, which is characterized by E-cadherin down-regulation and the increase of vimentin (Peinado *et al*., 2007; Barr *et al*., 2008). EMT enables cancer cells to be more motile, to escape the primary tumor site, and generate metastatic lesions at distant sites (Barr *et al*., 2008). Hence, EMT plays an important role in tumor progression and metastasis.

EGFR signaling down-regulates E-cadherin expression via decreasing Caveolin-1 level and increasing Snail promoter activity, which eventually leads to the induction of EMT (Lu *et al*., 2003). EGFR signaling also induces EMT through activation of Signal Transducer and Activator of Transcription 3 (STAT3). EGFR signaling has been denomstrated to stimulate the binding of activated STAT3 to the STAT3 elements region of TWIST promoter, leading to the up-regulation of *TWIST* expression and induction of epithelial-to-mesenchymal transition (Lo *et al*., 2007).

Regulation of breast cancer stem cell by EGFR signaling

Cancer stem cells (CSCs) are a small population of cells characterized as slow cycling, less differentiated, and capable of self-renewal (Scheel *et al*., 2011). Accumulating evidence has shown that cancer stem cell population is accountable for the resistance to chemotherapy, tumor relapse, and metastases (Pinto *et al*., 2013; Park *et al*., 2014; Lee *et al*., 2011). EGFR/ErbB2-mediated STAT3 phosphorylation plays a key role in the regulation of CSCs in breast cancer (Chung *et al*., 2014; Thakur *et al*., 2015; Chung *et al*., 2013; Wei *et al*., 2014).

STAT3 pathway in breast CSC regulation

STAT3 is a member of the STAT transcription factor family, which are downstream effectors responding to extracelluar cytokines and growth factors (Resemann *et al*., 2014). STAT3 regulates the transcription of genes (such as *FOS*, *MYC*, and *MCL1*) involved in inflammation, apoptosis, differentiation, stem cell maintenance, and transformation (Resemann *et al*., 2014; Lo *et al*., 2006). Activated EGFR regulates STAT3 phosphorylation directly or indirectly via JAK1/2, c-Src, or PI3K/Akt/mTOR (Lo *et al*., 2006; Silva, 2004; Zhou *et al*., 2007).

STAT3 not only promotes the conversion of cancer cells from non-stem-like to stem-like cell but also provides the signal for the CSC self-renewal (Thakur *et al*., 2015; Hollmén *et al*., 2015). Expression and activation of STAT3 is elevated in $CD44^{+}/CD24^{-/low}$ and aldehyde dehydrogenase positive (ALDH+) breast cancer stem-like cells (Thakur *et al*., 2015; Chung *et al*., 2013; Lin *et al*., 2013). The similar effect was also observed in tumor-initiating cells in claudin-low breast cancers (Wei *et al*., 2014). Suppression of STAT3 activity or protein expression results in decreased breast cancer stem cell-like cell population *in vitro* and *in vivo* (An *et al*., 2015; Zhu *et al*., 2014; Thakur *et al*., 2015). PI3K/Akt/mTOR is one of the classical EGFR signaling downstream pathways (Lo *et al*., 2006), which has shown to be significantly activated by the STAT3 signaling pathway and therefore regulated in stem-like cell subpopulation in MCF7 breast cancer cells (Zhou *et al*., 2007).

Macrophage regulates breast CSCs

Emerging evidence unveils the involvement of tumor-associated macrophages (TAMs) in the maintenance of CSC subpopulation in breast tumors (Raggi *et al*., 2016). Studies have demonstrated that ER-negative breast cancer cells secrete colony stimulating factor 1 (CSF1) to educate EGF-secreting TAMs, which as a result prepare the microenvironment for tumor invasion (Goswami *et al*., 2005; Nickerson *et al*., 2013). Additionally, TAMs-EGFR-STAT3 axis has been shown to promote breast cancer invasion and initiate cancer stem cell niche (Vlaicu *et al*., 2013). The sphere formation capability of breast cancer cells is enhanced when cells are co-cultured with TAMs. Besides, the increased sphere formation is abolished when the co-cultured cells are incubated with macrophage-interfering reagents (Ward *et al*., 2015). Recently, another signaling cascade of EGFR/STAT3/Sox2 has been proposed by Yang and coworkers, through which murine breast tumors maintain their CSC population (Yang *et al*.,

2013). Altogether, though a large number of studies have been already performed, more experiments are still needed to further determine whether and how the EGF paracrine loop between TAMs and breast tumor cells regulates CSC through STAT3 in human breast cancer.

Role of TGFβ receptors in the regulation of TGFβ signaling

Overview of the TGFβ signaling pathway

Transforming growth factor β (TGFβ) signaling is a ubiquitous and multifunctional pathway, which controls a large variety of cellular and developmental processes. The TGFβ pathway has been demonstrated to be critical for the early embryonic development and to actively participate in the immune response and wound healing processes (Beyer *et al*., 2013; Finnson *et al*., 2013). Thus, it is not surprising that the dysregulation and malfunction of TGFβ signaling is associated with different diseases, such as inflammation, atherosclerosis, neurogeneration, and cancer progression (Gratchev *et al*., 2016; Damato *et al*., 2013; Katsuno *et al*., 2011; Pickup *et al*., 2013).

Ligands and receptors

Ligands

The TGFβ growth factor superfamily contains more than 30 members (Shi & Massagué, 2003; Schmierer & Hill, 2007). According to the similarity of the protein sequences and downstream effector cascades, human TGFβ superfamily growth factors can be grouped into two categories: TGFβs/Activin/Nodal and BMP (bone morphogenetic proteins)/GDF (growth and differentiation factors)/MIS (Muellerian-inhibiting factors) (Figure 1.5) (Schmierer & Hill, 2007). The TGFβ growth factor subfamily has three members: TGFβ1, TGFβ2, TGFβ3 (Shi & Massagué, 2003).

TGFβs are first synthesized as homodimeric pre-proproteins, containing a signal peptide, a pro-domain called latency-associated peptide (LAP), and a domain at C-terminus that becomes mature transforming growth factor-β after proteolytic processing (Khalil, 1999). When the protein is synthesized, the pro-domain is usually cleaved off but it still attaches to the mature TGFβ molecules noncovanlently (Dubois *et al*., 1995). After interacting with latent TGFβ-binding protein (LTBP), the whole complex is secreted from the cell as a large latent complex (LLC) (Rifkin, 2005). Additional steps, such as metalloprotease-mediated cleavage and pH-induced conformational changes, are required to fully activate the latent TGFβ complex and provide important regulatory mechanisms of TGFβ signaling (Ge & Greenspan, 2006; Wipff & Hinz, 2008).

Receptors

TGFβ receptors (TGFβRs) are transmembrane proteins that can be divided into two categories, type I and type II. In the human genome, type I TGFβ receptors have seven members (*ALK1-7*), whereas type II receptors are encoded by five genes (*TGFBR2, ACTR2, ACTR2B, BMPR2, AMHR2*) (Figure 1.5). An extracellular ligand binding domain, a transmembrane domain, and an intracellular Ser/Thr kinase domain are the main structural units of TGFβRs. The extracelluar domains of TGFβRs show diverse structural arrangements, which enable the receptors to interact with different ligands of the TGFβ superfamily (Schmierer & Hill, 2007).

The Ser/Thr kinase domains of type II receptors are believed to be constantly active, and they phosphorylate the GS sequence of type I TGFβ receptors upon binding with their corresponding ligands. Type I receptors, on the other hand, contain a unique and highly conserved GS sequence (TTSGSGSG) at N-terminus of the kinase domain proximate to the transmembrane domain (Massagué, 1998). Phosphorylation of the GS sequence induces a

conformational change of the cytoplasmic tail and therefore increases the selectivity of the kinase domain for the C-terminus of Smad2 (Shi & Massagué, 2003). Activation of TGFβRs follows a certain order. Depending on the ligands, some factors of the TGFβ family (such as TGFβs) bind to the homodimer of type II TGFβR first, then the complex interacts with the type I TGFβR to form a tetramer that activates the signaling pathway. Other TGFβ family ligands, BMPs for example, interact with the type I TGFβR dimer first and then with type II receptors (Schmierer & Hill, 2007).

Smad-dependent and Smad-independent downstream cascades

Smads are the main components of the canonical TGFβ signaling pathway and are classified into three types according to their functions: the receptor-regulated Smads (R-Smads), the common-mediator Smad (Co-Smad), and the inhibitory Smads (I-Smads) (Figure 1.5-1.6). R-Smads have five members, Smad1, -2, -3, -5, and -8. Smad2 and Smad3 specifically transduce signal from TGFβs/activin/nodal, whereas Smad1, Smad5, and Smad8 prefer signaling from BMPs/GDFs (Schmierer & Hill, 2007). R-Smads contain Mad-homology (MH) 1 and MH2 domains, which are responsible for DNA and receptors binding, respectively (Massagué, 2012). Upon the formation of TGFβR tetramer, the activated type I TGFβ receptors phosphorylate R-Smads at the two serine residues at the C-terminus of R-Smads (Figure 1.6-1.7). Subsequently, the phosphorylated R-Smads complexes interact with the co-Smad protein Smad4, translocate into the nucleus, and then modulate the transcription of target genes (Miyazono *et al*., 2000; Shi & Massagué, 2003). The activity of TGFβ signaling can be negatively regulated by I-Smads, Smad6 and Smad7. I-Smads are induced by TGFβ ligands in a R-Smad-dependent manner and are able to terminate TGFβ signaling through promoting the degradation of TGFβRs via

Smurf1/2 (Smad specific E3 ubiquitin protein ligase) or through eliciting TGFβRs dephosphorylation by phosphatase 1 (Miyazono *et al*., 2003; Massagué, 2000).

The TGFβ superfamily also activates downstream cascades through non-Smad-mediated phosphorylation or protein-protein interactions, such as MAP kinase (MAPK) pathways, Rho-like GTPase signaling pathways, PI3K/AKT pathways, and TGFβ activated kinase (TAK) 1/TNFR-associated factor (TRAF) 6 pathways. These Smad-independent pathways and their underlying biochemical mechanisms have been extensively reviewed (Derynck & Zhang, 2003; Zhang, 2009; Choi *et al*., 2012) (Figure 1.7). These non-Smads dependent pathways allow TGFβ signaling participation in the regulation of various cellular and biological processes, such as cell proliferation, differentiation, cell fate determination, apoptosis, and cancer development (Zhang, 2009).

Functions of the TGFβ signaling pathway

The TGFβ superfamily is a pleiotropic cytokine family, which actively participates in various physiological and pathological processes, such as early embryonic and neuron development, cell survival, cell fate determination and differentiation, wound healing, immune responses, and cancer progression. The role of TGFβ in cancer is unique and context-dependent (Massagué, 2012; Akhurst & Padgett, 2015). In normal epithelial cells and early stages of cancers, TGFβ signaling functions as a tumor suppressor, which significantly decreases the proliferation rate of cells. Moreover, loss of TGFβ signaling causes tumor growth at early stages of tumor progression (Massagué, 2012; Harradine & Akhurst, 2006). On the contrary, TGFβ signaling, due to its potent role in the induction of epithelial-to-mesenchymal transition (EMT) (Xu *et al*., 2009), promotes cancer progression, angiogenesis, and malignancy at late stage (Gomes *et al*., 2012; Bierie *et al*., 2009; Janda *et al*., 2002). Many reports have shown that TGFβ

signaling is associated with poor prognosis, high risk of metastasis, and poor therapy response in different types of cancers (Elliott & Blobe, 2005; Biswas *et al*., 2006; Bhola *et al*., 2013; Li *et al*., 2013). Moreover, TGFβ also promotes tumor progression by preparing the microenvironment for tumor growth and dissemination, such as promoting the proliferation of cancer-associated fibroblasts by TGFβ-induced tissue connect growth factor (TCGF) secretion (Pickup *et al*., 2013; Papageorgis & Stylianopoulos, 2015).

Regulation of TGFβ signaling through TGFβ receptors

Regulation of the availability of TGFβ receptors at the cell surface is one of the regulatory mechanisms controlling the activation of TGFβ signaling pathway. Studies have shown that TGFβR levels direct the specificity of the activation and biological functions of TGFβ signaling (Rojas *et al*., 2009).

The regulation of type II TGFβ receptors (TGFβR2) has been well explored due to the fact that these receptors are constitutively active and are required for the activation of type I TGFβ receptors (TGFβR1). Studies have shown that the EGF/PI3K/Akt signaling cascade positively regulates the mRNA and protein levels of TGFBR2 in human dermal fibroblasts, which may contribute to the collagen overexpression in systemic sclerosis (Yamane *et al*., 2002; Yamane *et al*., 2003). At the post-transcriptional level, *TGFBR2* mRNA expression is regulated by miR-337 and miR-153 via targeting the 3'-UTR of the *TGFBR2* gene (Liang *et al*., 2015; Zhong *et al*., 2012). Endocytosis plays an important role in regulating the activity of TGFβ signaling. Depending on the type of the endocytic vehicles, TGFβ receptor internalization can either enhance or attenuate TGFβ signaling (Figure 1.8). Clathrin-mediated receptor trafficking promotes TGFβ signaling, while caveolin 1-induced endocytosis leads to the inhibition of TGFβ signaling (Di Guglielmo *et al*., 2003). Studies further revealed that clathrin-mediated early

endosomes are enriched in a protein called Smad Anchor for Receptor Activation (SARA), which recruits Smad2 and facilitates the binding of TGFβR1 and Smad2 (Hayes *et al*., 2002; Di Guglielmo *et al*., 2003). As a result, the activated TGFβ receptor complexes in these endosomes are still able to transduce the signal to the downstream pathways. Endocytic vehicles formed through lipid raft-caveolar pathway, on the other hand, contain high levels of Smad7 and Smurf2, which lead to the ubiquitination of TGFβ receptors and targeting for the lysosomal degradation, and eventually the termination of TGFβ signaling. In addition, ADAM12 has been shown to increase TGFβR2 in early endosomes and to enhance the stability of TGFβR2 by preventing the receptor from interactions with Smad7 (Atfi *et al*., 2007). However, this study was conducted in ADAM12 overexpression cell models, which are not convincing systems to illustrate the effect of ADAM12 on TGFβ receptors at the endogenous expression level. Thus, ADAM12 knockdown experiments by siRNAs or other methods are required to further confirm the observation that ADAM12 promotes the accumulation of TGFβR2 in endosomes.

TGFβR1 (also known as activin-like kinase 5, ALK5) is a type I TGFβ receptor, phosphorylating R-Smads upon being activated by TGFβR2. Though TGFβR1 is the essential step to activate Smad-dependent signaling pathway, there is very limited knowledge on the regulation of TGFβR1. Similarly to TGFβR2, the TGFβR1 levels at the cell surface can be regulated by endocytosis (He *et al*., 2015; Di Guglielmo *et al*., 2003). Besides, Beclin1, an autophagy and protein trafficking regulatory protein, has been recently reported to facilitate the recycling of TGFβR1 to the cell surface and therefore enhance the TGFβ signaling in neuronal cells (O'Brien *et al*., 2015). Regarding the stability of TGFβR1, salt-inducible kinase (SIK) 1 was shown to promote the degradation of TGFβR1 via interactions with Smad7 in a Smad-dependent manner (Kowanetz *et al*., 2008). In addition, Notch signaling has been shown to up-

regulate the mRNA expression of TGFβR1 through inhibition of neuropilin-1 (Nrp1), a membrane receptor interacting VEGF and other factors (Aspalter *et al*., 2015).

Goals of the Study

In this study, my focus was to better understand the biological role of ADAM12 in breast cancer cells.

- Our previous studies showed that the expression of ADAM12 was significantly up-regulated in breast cancers of the claudin-low molecular subtype and was associated with decreased metastasis-free survival rates in triple-negative breast cancer. Claudin-low tumors are typically triple-negative and are enriched in cancer stem cells (Prat *et al*., 2011). Thus, my goal was to determine whether and how ADAM12 contributes to the cancer stem cell-like features in triple-negative breast cancer cells.
- ADAM12 was shown to enhance TGFβ signaling through interacting with TGFβR2 (Atfi *et al*., 2007) in ADAM12 overexpression cell models. Little effort has been put on illustrating the effect of ADAM12 on TGFβ signaling at the endogenous expression level in breast cancer cells. My goal was to investigate the role of ADAM12 in the regulation of TGFβ signaling in breast cancer cells using inducible shRNA knockdown system.
- In human breast cancer, the *ADAM12* gene is more frequently mutated than other *ADAM* genes that encode catalytically active ADAM proteases. Six different somatic missense mutations had been identified in human breast cancers. My goal was to explore the effects of these mutations on the structure, proteolytic processing, trafficking, and biological function of ADAM12.

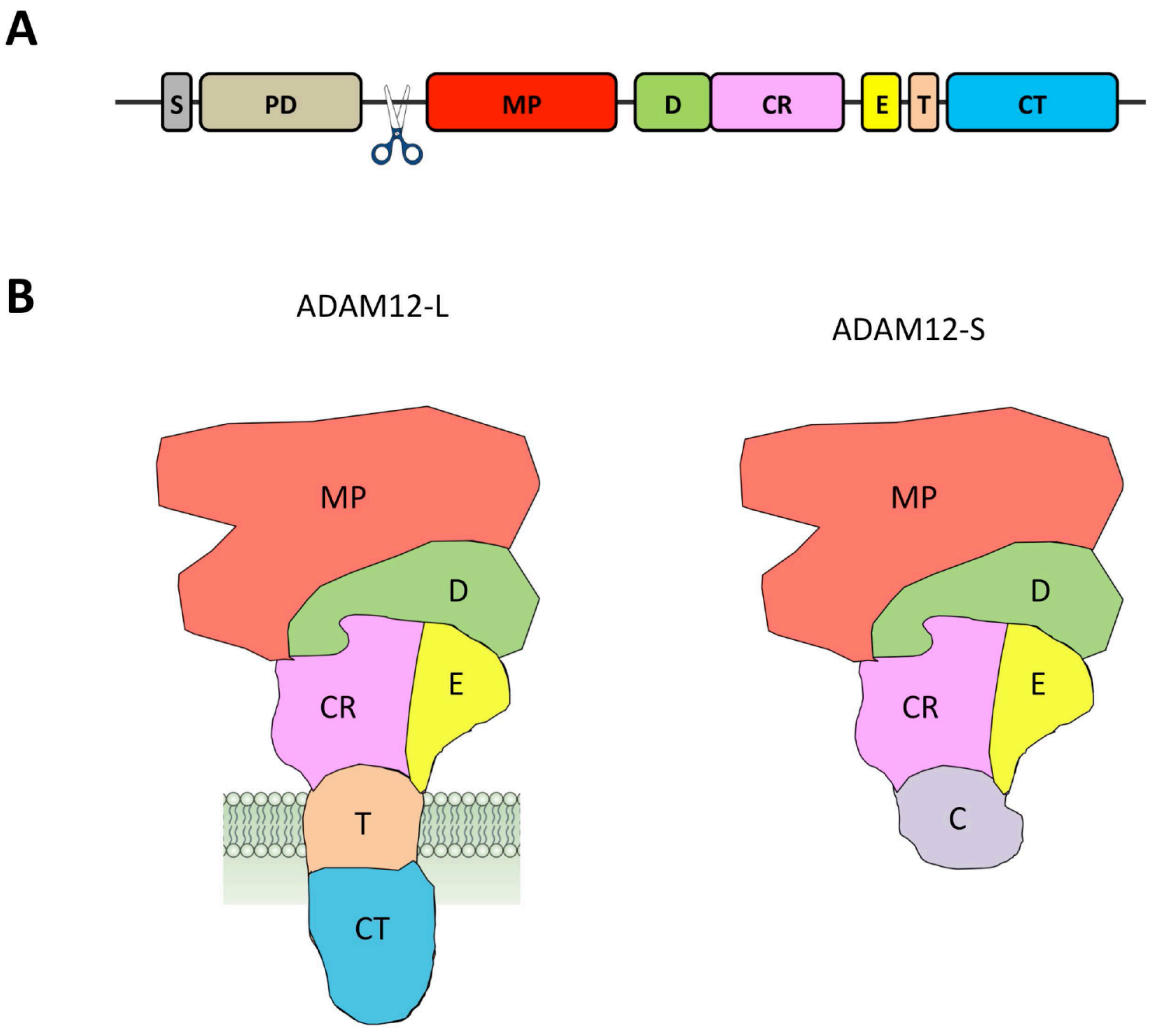

Figure 1.1 Domain organization of ADAM metalloproteases and human ADAM12.

(**A**) A diagram showing the domain organization of the ADAM proteases. The metalloprotease activity requires the removal of the prodomain. The scissors indicate the cleavage of the prodmain of ADAM proteases. (**B**) The schematic diamgrams representing the domain structures of ADAM12-L and ADAM12-S, two major splice variants of human ADAM12. S, signal peptide; PD, prodomain; MP, metalloproteinase domain; D, disintegrin domain; CR, cysteine-rich domain; E, EGF-like domain; T, transmembrane region; CT, cytoplasmic tail; C, C-terminus.

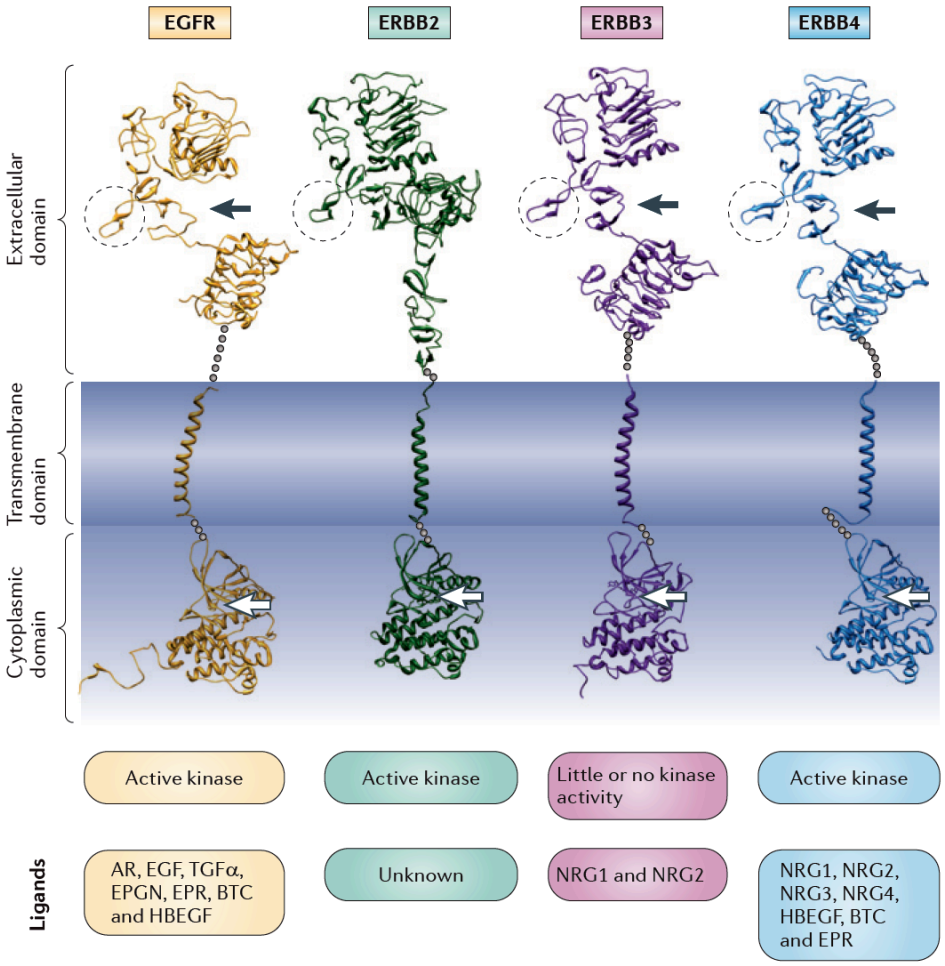

Figure 1.2 Functional and structural features of ErbB receptor family.

Adapted from Yarden *et al*., 2012 with permission from Nature Publishing Group.

ErbB family receptors are transmembrane receptor tyrosine kinases. The black arrows are pointing to the ligand-binding site of each receptor. The binding of the ligands to the receptors induces the conformation change of the extracellular domain and exposes the interaction loop (labeled in dashed circles) for dimerization. ErbB2 can dimerize with no ligand binding because of its unique extracellular protein structure. The ATP-binding clefts are labeled by white arrows. ErbB3 has limited kinase activity. The corresponding ligands of each receptors are provided in the rectangular frames underneath each protein structure.

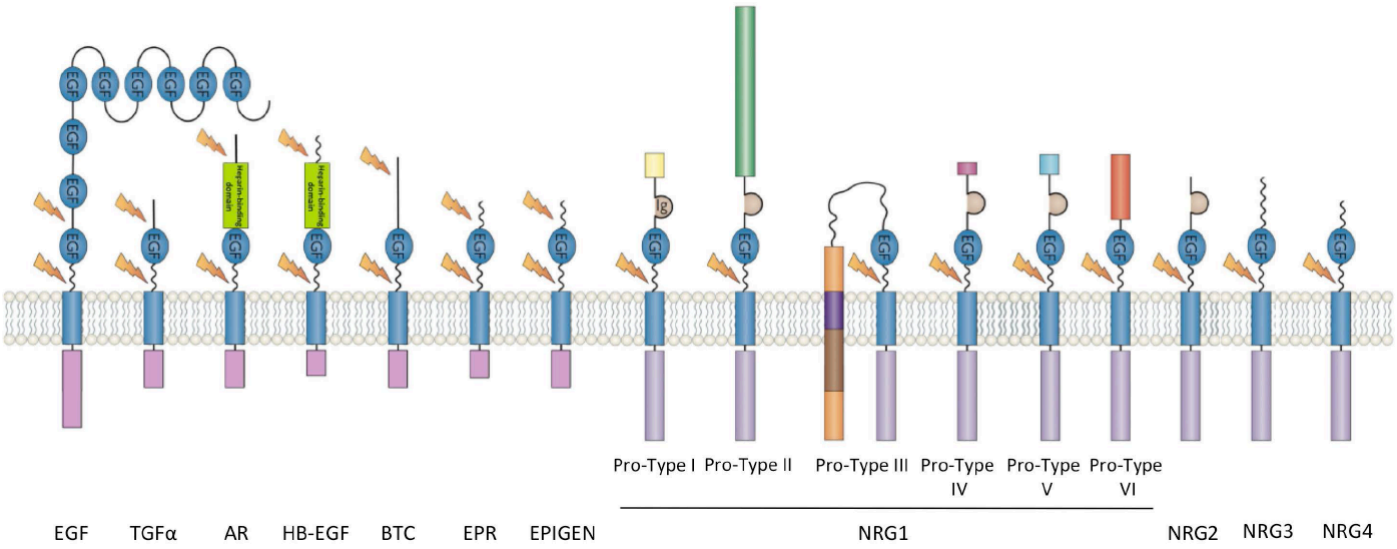

Figure 1.3 Ligands of ErbB family receptors.

Based on Figure1 of Mei *et al*., 2008 & Harris *et al*., 2002 with permission from Nature Publishing Group and Elsevier Ltd.

Ligands of ErbB family receptors are synthesized as transmembrane procursors and cleaved by membrane proteases, such as tumor necrosis factor-α converting enzyme (TACE, also known as ADAM17). The cleavage sites are marked by the yellow lightning symbols in the figure. All the ligands contain EGF-like domain and different N-terminal sequences.

EGF, epidermal growth factor; TGF-α, transforming growth factor-α; AR, amphiregulin; HB-EGF, haprin-binding EGF-like growth factor; BTC, β-cellulin; EPR, epiregulin, EPGN, epigen; NRG, neuregulin.

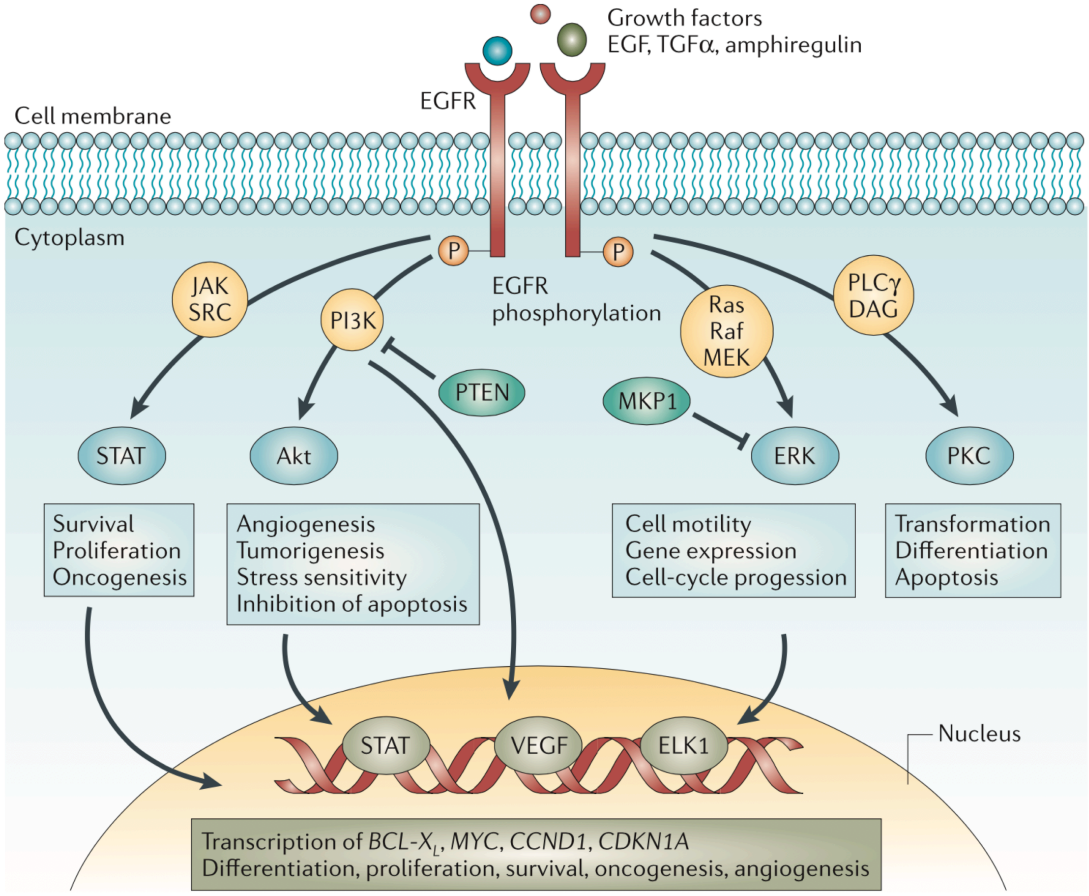

Figure 1.4 A diagram of EGFR downstream signaling pathways.

Adapted from Nyati *et al*., 2006 with permission from Nature Publishing Group.

Main downstream signaling cascades regulated by EGFR are summarized in the figure. Some key negative regulators, such as PTEN and MKP1, are also included.

JAK, Janus kinase; STAT, signal transducer and activator of transcription; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase, PLC, phospholipase c; DAG, diacylglycerol; PKC, protein kinase C; VEGF, vascular endothelial growth factor; ELK1, ETS domain-containing protein Elk-1; CCND1, gene name of protein cyclin D1; CDKN1A, gene encoding p21.

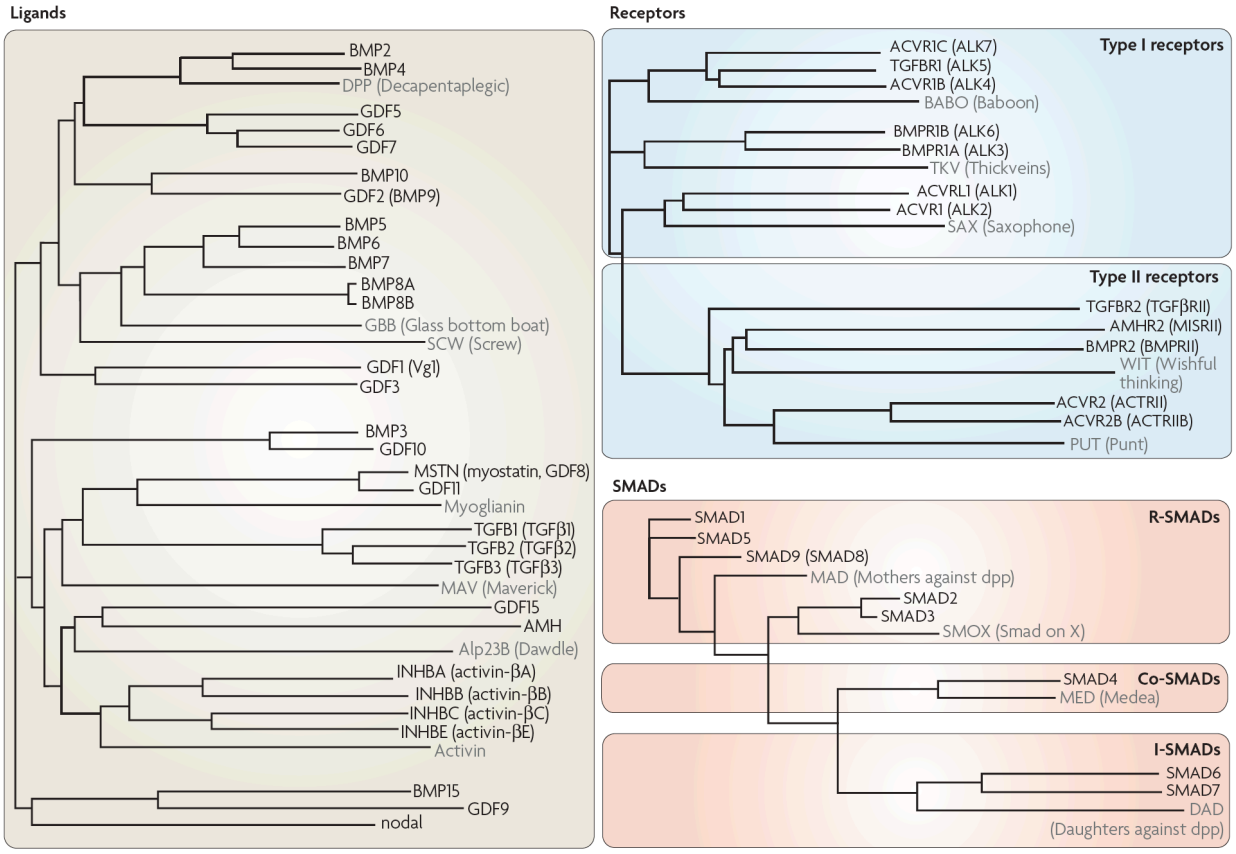

Figure 1.5 Ligands, receptors, and Smads of the TGFβ signaling pathway.
Adapted from Schmierer *et al*., 2007 with permission from Nature Publishing Group.

TGFβ super family growth factors, TGFβ receptors, and Smads proteins are grouped based on their protein sequences. Human proteins are labeled in black, where as D. melanogaster proteins are shown in grey. Alternative names of the proteins are included in parentheses.

ACVR, activin receptor; ALK, activin receptor-like kinase; AMH, Muellerian-inhibiting factor; AMHR2, anti-Muellerian hormone type-2 receptor; BMP, bone morphogenetic protein 4; BMPR, BMP receptor; GDF, growth/differentiation factor; Co-SMAD, common-mediator Smad; I-SMAD, inhibitory Smad; R-SMAD, receptor-regulated Smad; TGF, ransforming growth factor; TGFBR, TGFβ receptor.

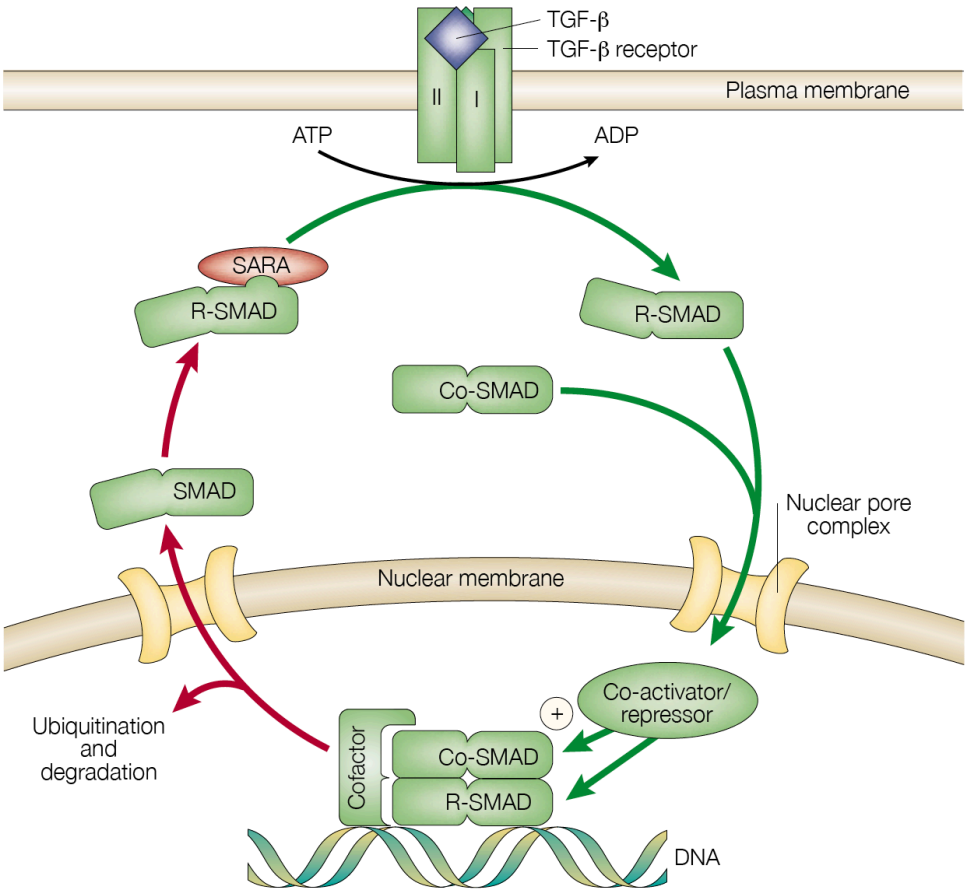

Figure 1.6 A brief review of Smad-dependent TGFβ downstream cascade.
Adapted from Massagué *et al*., 2000 with permission from Nature Publishing Group.

The binding of TGFβ ligands to pre-formed TGFβR homodimers leads to the formation of TGFβR tetramer and the phosphorylation of type I TGFβR homodimer. The activated type I TGFβ receptors phosphorylate R-Smads, which interact with co-Smad protein, translocate into the nucleus, and thus modulate the transcription of target genes (Miyazono *et al*., 2000; Shi *et al*., 2003). The R-Smads/co-Smad complex can be targeted either for degradation or recycling back to the cell surface. The degradation requires ubiquitination mediated by I-Smads and E3 ubiquitin-protein ligase SMURFs. SARA is the scaffold protein that recruits R-Smads to TGFβ receptors (Massagué *et al*., 2000).

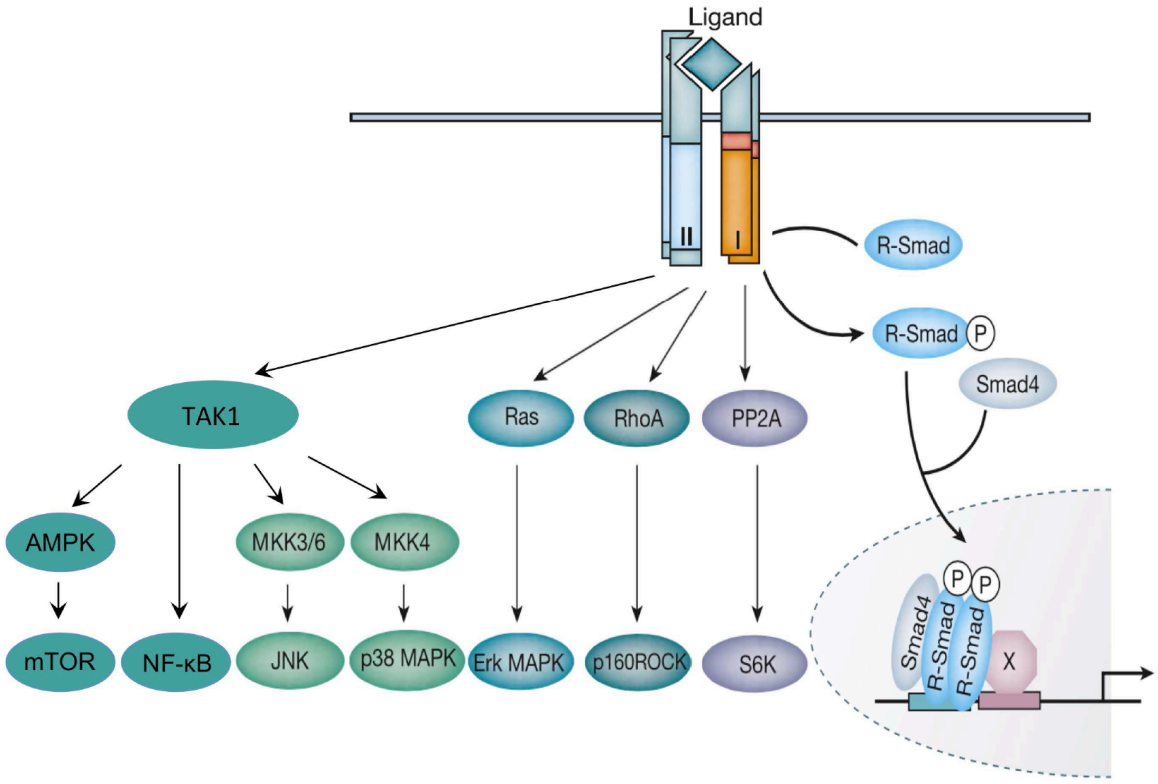

Figure 1.7 A diagram of Smad-independent TGFβ downstream pathways.

Based on Figure 6 from Derynck *et al.*, 2003 with permission from Nature Publishing Group.

TGFβ signaling activates several other signal proteins and their downstream pathways (on the left in green) that do not require involvement of Smads. TGFβ-mediated TAK1 activation induces autophagy and thus plays important roles in the regulation of cell apoptosis and survival (Choi *et al*., 2012; Kang *et al.*, 2011).

TAK, TGFβ-activated kinase; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; MKK, dual specificity mitogen-activated protein kinase kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Erk, extracelluar signal-regulated kinase; ROCK, Rho-associated protein kinase; PP2A, protein phosphatase 2A; S6K, ribosomal protein S6 kinase

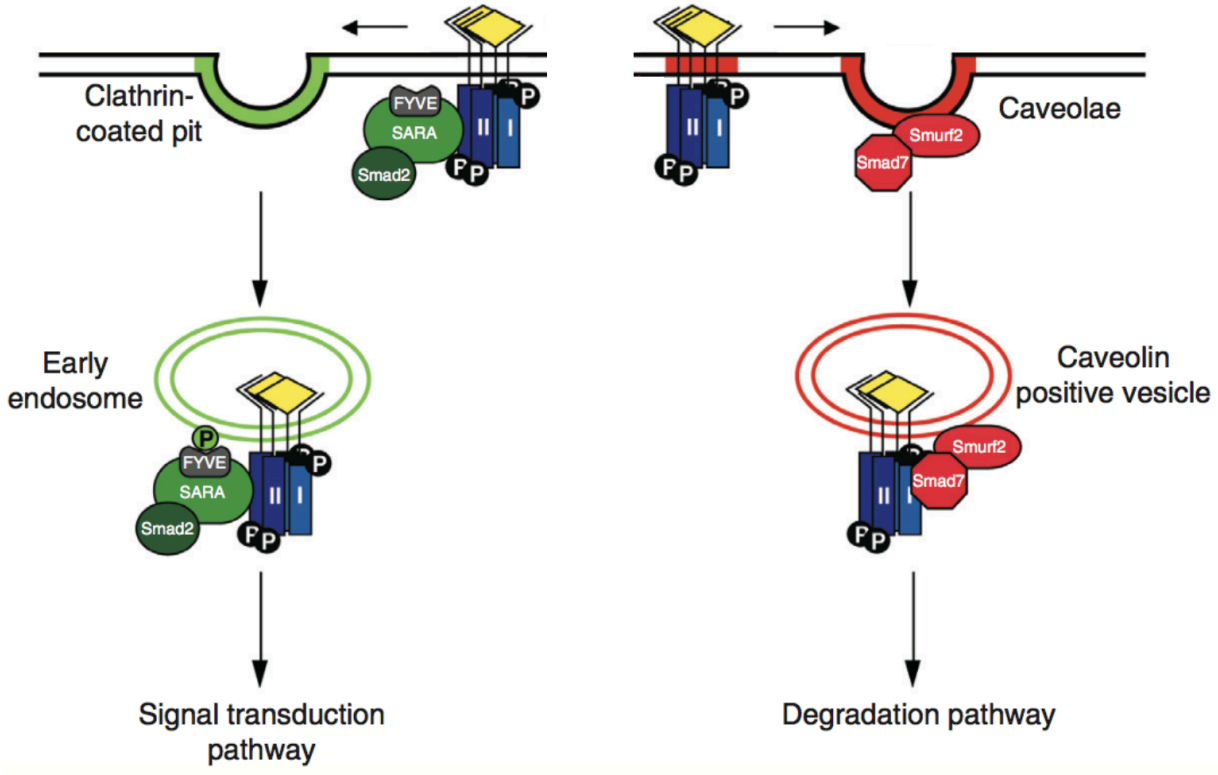

Figure 1.8 Regulation of TGFβ signaling via different TGFβR endocytic pathways. Based on Figure 7 from Di Guglielmo *et al.,* 2003 with permission from Nature Publishing Group.

TGFβ receptors can internalize through two different pathways. Clathrin-mediated endocytosis enhances TGFβ signaling (on the left in green), whereas the lipid raft-caveolar pathway (on the right in red) inhibits the transduction of TGFβ signals.

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Chapter 2 - ADAM12 is a novel regulator of stem-like cells in triple-negative breast cancer

Abstract

Elevated expression of ADAM12 is a prognostic factor of metastasis in triple-negative breast cancers (TNBC) and a predictor of chemoresistance. ADAM12 is up-regulated in claudin-low tumors enriched in cancer stem cell (CSC) markers. Here, we demonstrate that ADAM12 regulates stem-like properties of SUM159PT and Hs578T TNBC cells. We found that ADAM12 knockdown reduced cell migration and invasion, decreased anoikis resistance, and compromised mammosphere formation. ADAM12 knockdown also decreased the $CD44^{+}/CD24^{-/low}$ and ALDEFLUOR+ cell populations and reduced tumorigenesis in mice *in vivo*. RNA sequencing identified a significant overlap between ADAM12- and EGFR-regulated genes. Erlotinib, an EGFR inhibitor, mimicked the effect of ADAM12 knockdown, and exogenous EGF blocked the effect of ADAM12 knockdown on $CD44^{+}/CD24^{-/low}$ populations. These results indicate that ADAM12 supports the CSC phenotype in TNBC cells via modulation of the EGFR pathway.

Introduction

Members of the ADAM family of cell surface metalloproteases catalyze cell context-dependent cleavage within the extracellular domains of transmembrane receptors, growth factor precursors, or adhesion molecules (Jones *et al*., 2015; Edwards *et al*., 2008). ADAM substrates include Notch receptors (Groot *et al*., 2014; van Tetering *et al*., 2009), Epidermal Growth Factor Receptor (EGFR) ligands (Blobel, 2005; Kataoka, 2009), interleukin-6 receptor (IL-6R)

(Schaper & Pose-John, 2015), transforming growth factor-α (TNF-α) (Li *et al*., 2015; Maney *et al*., 2015), E-cadherin (Maretzky *et al*., 2005; David *et al*., 2012), or CD44 (De Falco *et al*., 2012; Hartmann *et al*., 2015). Because ADAMs are often aberrantly expressed or misregulated in human cancers, they may contribute to tumor progression, metastasis, or therapy resistance (Murphy, 2008; Duffy *et al*., 2009).

Among the twelve catalytically active human ADAMs (Jones *et al*., 2015; Edwards *et al*., 2008), ADAM12 possesses unique characteristics that make it an attractive candidate for future targeting in breast cancer. ADAM12 expression is strongly elevated in human breast cancers compared to normal mammary epithelium (Bertucci *et al*., 2006; Kveiborg *et al*., 2005; Lendeckel *et al*., 2005). High levels of ADAM12 mRNA are associated with poor prognosis and decreased metastasis-free survival times in estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and epidermal growth factor receptor 2 (HER2)-negative (triple-negative, TN) breast cancers, but not in HER2-positive or ER-positive tumors (Wang *et al*., 2005; Li *et al*., 2012). ADAM12 expression is induced during epithelial-mesenchymal transition (EMT) in mammary epithelial cells, which is consistent with ADAM12 up-regulation in the claudin-low molecular subtype of breast cancer showing the molecular signatures of EMT (Taube *et al*., 2010; Li *et al*., 2013). Claudin-low tumors, which represent 5-10% of all breast cancers, are typically triple-negative, poorly differentiated, and have an augmented activity of EGFR, SRC, TGFβ and STAT3 pathways (Sabatier *et al*., 2014; Prat *et al*., 2010). Importantly, the gene expression signatures of claudin-low tumors show a significant similarity to the signature of $CD44^{+}/CD24^{-/low}$ mammosphere-forming cells (Sabatier *et al*., 2014; Creighton *et al*., 2009), suggesting an enrichment in cancer stem cell (CSC) or tumor-initiating cell (TIC) features. Breast CSCs are thought to be largely responsible for tumor maintenance, treatment

resistance, and disease recurrence (Baccelli, 2012; Nagaprashantha *et al*., 2011; Medema, 2013). Our previous analysis of two clinical datasets showed that elevated expression of ADAM12 is predictive of resistance to neoadjuvant chemotherapy in ER-negative breast cancer, independent of age, tumor size, grade, and the lymph node status (Li *et al*., 2013). These observations raised a possibility that ADAM12 may serve as a marker or a therapeutic target in breast CSCs in TN breast cancer.

The goal of the current study was to assess a possible contribution of ADAM12 to the CSC properties of TN breast cancer cells. By analyzing the effect of ADAM12 knockdown on cell migration, invasion, anoikis resistance, mammosphere formation, the known CSC markers, tumor formation after xenotransplantation in mice *in vivo*, and global gene expression, we have determined that ADAM12 actively supports the CSC phenotype of TN breast cancer cells. This function of ADAM12 appears to be mediated by modulation of EGFR signaling. Thus, we identify ADAM12 as a novel modifier of the EGFR pathway in TN breast cancer and a potential target in CSC-directed therapies.

Materials and Methods

Reagents and antibodies

ADAM12 siRNA smartpool, ADAM12 siRNA#1 (D-005118-01), ADAM12 siRNA#2 (D-005118-02), control siRNA smartpool #1, control siRNA#3 (D-001206-13), and DharmaFECT1 transfection reagent were obtained from GE Dharmacon. RT2 Profiler arrays were purchased from QIAGEN. Methylcellulose stock solution and growth factor-reduced Matrigel were purchased from R&D Systems and Corning, respectively. ALDEFLUOR kit was obtained from STEMCELL. Apoptosis was measured by Cell Death Detection ELISA kit from

Roche. Erlotinib was obtained from Cell signaling Technology. Vybrant DiO cell-labeling solution, bFGF, B27, and humam recombinant EGF were purchased from Life Technologies. Doxycycline and heparin was obtained from Sigma-Aldrich. Anti-Human ADAM12 mouse monoclonal antibody (#632525) and anti-mouse IgG1 isotype antibody (#11711) were purchase from R&D systems. APC-labeled goat anti-mouse IgG1 antibody was from Jackson ImmunoResearch. PE-conjugated mouse anti-human CD24 antibody (#ML5) and PE-conjugated mouse IgG2aκ isotype control antibody (G155-178) were obtained from BD Pharmingen. APC-conjugated anti-human CD44 antibody (#IM7) and APC-conjugated IgG2bκ isotype control antibody (#eB149/10H5) were purchased form Affymetrix eBioscience.

Cell culture

SUM149PT, SUM159PT, and SUM1315MO2 cell lines were obtained from Asterand (Detroit, MI). BT549 and Hs578T cells were purchase from American Type Culture Collection (Manassas, VA). HEK293T were obtained from Thermo Scientific. SUM102PT and SUM225CWN cells were a gift from Dr. Fariba Behbod (University of Kansas Medical Center). MCF-7 cell line was a gift from Dr. Annelise Nguyen (Kansas State University). SUM149, SUM159PT, and SUM225CWN cells were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum (FBS), 10 mM HEPES, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. SUM1315MO2 cells were cultured in Ham's F-12 medium supplemented with 5% FBS, 10 mM HEPES, 10 ng/ml epidermal growth factor (EGF), and 5 μg/ml insulin. SUM102PT cells were maintained in Ham's F-12 medium containing 5% FBS, 1 μg/ml hydrocortisone, 5 μg/ml insulin, and 1% Penicillin-Streptomycin-Amphotericin B (PSA). BT549 cells were cultured in RMPI1640 medium supplemented with 10% FBS, 1 mM pyruvate, and 0.8 μg/ml insulin. Hs578T and MCF-7cells were cultured in DMEM medium containing 10% FBS and 10 μg/ml

insulin. HEK293T cells were cultured in DMEM medium containing 10%FBS, 6 mM Glutamine, 1% Penicillin/ Streptomycin, and 110 μg/ml sodium pyruvate. Cells were maintained at 37° C under humidified atmosphere containing 5% CO2.

Vectors/virus production and generation of stable cell lines

shRNA targeting human ADAM12 and control shRNA sequences were excised from GIPZ ADAM12 shRNA (V2LHS_11814) or non-silencing lentiviral shRNA control (Cat#RHS4346; Thermo Scientific) and were cloned into pINDUCER10 vector (Addgene) at the XhoI and MluI sites. Lentiviruses were produced by transfecting HEK293T cells with pINDUCER10-shADAM12/shControl, pMD2.G, and psPAX2 plasmids (Addgene) using Mirus TransIT transfection reagent (Mirus). Conditioned medium containing viral partiles was harvested 48h after transfection. SUM159PT cells were plated one day before infection at ~20% confluence. Conditioned medium containing viruses was supplemented with 5 ng/mL polybrene (Sigma) and was added onto SUM159PT cells. Selection of stably transduced cells started 48 hours after infection using 2 μg/ml puromycin and continued for 10 days.

Transwell migration assay

SUM159PT shADAM12 and shControl cells were treated without or with 1 μg/ml Doxycycline for four continuous days. On the 4th day, cells were detached with 0.25% trypsin/1mM EDTA in DPBS, counted by Cellometer ^{TM} AutoT4 (Nexcelom Bioscience), and diluted to $2.5x10^4$ cells/ml. Cells (500μl) were then added to the upper chambers of Transwell inserts. The inserts were placed into lower chambers filled with 700 μl of growth medium and incubated at 37°C for18 hours. After washing the inserts twice with 500 μl DPBS, cells were fixed with 3% glutaraldehyde at room temperature for 20 minutes and stained with 0.5% crystal violet in 20% methanol for 15 minutes. Cells that did not migrate through the pores were

removed using cotton swabs. Migrated cells underneath the inserts were analyzed by light microscopy using 10x magnification and photographed. Numbers of migrated cells in 5 randomly selected areas were counted manually.

Sphere formation assay in methlycellulose

SUM159PT shADAM12 and shControl cells were treated without or with 1 μg/ml Doxycycline for 4 days. Cells were detached with 0.25% trypsin/1mM EDTA in DPBS and counted by Cellometer ^{TM} AutoT4 (Nexcelom Bioscience). Single cell suspension at $1x10^6$ cells/ml were passed through cell strainer and then diluted to $5x10^3$ cells/ml with mammosphere media containing MEMB (Lonza), 20 ng/ml hEGF (Life Technologies), 20 ng/ml bFGF (Life Technologies), 4 μg/ml heparin (Sigma), 1xB27 (Life Technologies), and 1x Penicillin/Streptomycin (Lonza). Cell numbers among different treatments were adjusted within 5% accuracy using CellTiter Glo kit (Promega) according to manufacturer's instruction. Cell suspension at the final dilution ($5x10^3$ cells/ml) was mixed with 3% methylcellulose (R&D Systems) at a ratio of 1:2. The solution was thoroughly mixed by vortexing and incubated at room temperature for 10 min. Cell suspension (500μl 10^3 cells in 1% Methylcellulose) was seeded into one well of 24-well ultra-low attachment plate (Corning). After 7 days, six randomly selected areas from each well were analyzed by light microscopy using a 4x objective and photographed. Numbers of spheres in each image were quantified using ImageJ.

Measurement of cell death

SUM159PT shADAM12 and shControl cells were treated without or with 1 μg/ml doxycycline for 4 days. One thousand suspended cells in 1% methylcellulose were seeded into each well of a 24-well ultra-low attachment plate (Corning), as in the sphere formation assay. Cells were collected at 0, 12, 24, and 48 hours post plating. Each sample was pelleted, washed

twice, lyzed at 4°C for 15 min, and frozen at -20°C. When the lysates of all samples were collected, the Cell Death ELISA kit (Roche) was used to detect the fragmented mono- and oligo-nucleosomes in the lysate. The procedures and analysis were performed according to the manufacturer's instruction.

Flow cytometry

SUM159PT cell and Hs578T cells were transfected with ADAM12 siRNA smart pool or non-targeting siRNA pool (Dharmacon). **ALDEFLUOR assay:** 5 days after transfection, cells were harvested, re-suspended at the density of 10^6/ml in the ALDEFLUOR assay buffer, followed by the addition of 5µl of the ALDEFLUOR Reagent to 1 ml of cell suspension. One half the cells (0.5 ml) were then transferred immediately to a new tube, which contained 10µl of ALDH inhibitor diethylamino-benzaldehyde (DEAB) serving as the negative control for the assay. The other half of cells was incubated without DEAB. Both tubes without and with DEAB were incubated at 37° for 45min. Then, cells were immediately transferred onto ice, centrifuged at 1200 rpm for 5 min at 4°C, and analyzed by flow cytometry using BD Calibur cytometer. **CD24/CD44 assay:** 5 days after transfection, cells were collected in 3% BSA/1xDPBS with calcium and magnesium, and then incubated for 30 minutes on ice with PE-conjugated anti-CD24 antibody and APC-conjugated anti-CD44 antibody. Cells wre analyzed by flow cytometry BD Calibur cytometer. **Erlotinib effect:** SUM159PT cells were treated with 1 µM of Erlotinib in DMSO for 3 days. Cells were harvested and stained with anti-CD24 and anti-CD44 antibodies as described above. **Efect of hEGF on CD24/CD44:** SUM159PT cells were transfected with ADAM12 siRNA #1 (D-005118-01), #2 (D-005118-02), or non-targeting siRNA #3 (D-001210-10-05; Dharmacon) according to the manufacturer's protocol. One set of the transfected cells was treated with 20 ng/ml hEGF. hEGF was added at the moment of transfection. The other set

of cells was incubated with no hEGF. Cells were collected and stained with anti-CD24 and anti-CD44 antibodies, as described above for flow cytometry.

Results

High ADAM12 expression in claudin-low subtype human breast tumor and breast tumor cell lines

To explore the potential function of ADAM12 in breast cancer, we first examined the expression levels of ADAM12 in different molecular subtypes of breast tumors from the human tumor database of Netherlands Cancer Institute (NKI). As shown in Figure 2.1A, ADAM12 mRNA is significantly elevated in the claudin-low subtype breast cancer compared to basal-like, HER2-overexpressing, and Luminal A and B subtypes. A significant increase of ADAM12 expression in claudin-low subtype breast tumors was also observed from the analysis among the data retrieved from The Cancer Genome Atlas (TCGA) (Figure 2.1B). More importantly, the claudin-low subtype breast tumor was shown to have tumor-initiating or stem cell phenotype and features of epithelial-mesenchymal transition (EMT) (Perou CM, 2010).

We further examined ADAM12 protein levels in several breast cancer cell lines. Hs578T, SUM1315MO2, BT549, and SUM159PT cell lines were previously categorized as claudin-low using two different microarray platforms (Prat *et al*., 2013; Neve *et al*., 2006; Chin *et al*., 2006), SUM102PT and SUM149PT were classified as basal-like cell lines, and MCF-7 and SUM225CWN cell lines represent the luminal subtype. Consistent with the results of data mining, our flow cytometry analysis demonstrated that ADAM12 expression at the cell surface was significantly higher in claudin-low cell lines (Figure 2.1C). Basal-like and luminal breast cancer cell lines only showed moderate expression of ADAM12. Together, these data imply that

high ADAM12 expression is associated with the claudin-low molecular subtype breast cancer. Claudin-low cancers are enriched in cancer stem-like features (Prat *et al*., 2010). Given the significant increase of ADAM12 levels in the claudin-low subtype of breast cancer, we decided to examine the biological significance of ADAM12 in this type of breast cancer, using representative triple-negative breast cancer cell lines SUM159PT and Hs578T.

ADAM12 maintains the migratory and invasive properties of TNBC cell

To investigate the role of ADAM12 in migration and invasion of triple-negative breast cancer cells *in vitro*, we established a stable SUM159PT cell line with inducible lentiviral-mediated ADAM12 shRNA expression system (Figure 2.8A). Puromycin-resistant SUM159PT cells were selected for the following experiments. SUM159PT shADAM12 and shControl cells were treated with 1μg/ml Doxycycline (Dox) to induce ADAM12 shRNA. Expression of red fluorescent protein (RFP) was induced approximately 48h post-infection and served as an internal indicator of the expression of shRNAs (Figure 2.8C). Significant decrease of both *ADAM12* mRNA and protein levels was observed 4 days after Dox (Figure 2.8B-C). Proliferation assays showed that doxycycline-inducible ADAM12 knockdown did not affect the growth rate of SUM159PT in 2D culture compared to the cells with non-targeting control shRNA expression after Dox treatment (Data not shown). However, down-regulated ADAM12 expression resulted in a significant reduction of transwell migration of SUM159PT in response to an FBS gradient (Figure 2.2A).

To measure the effect of ADAM12 down-regulation on invasion, we seeded SUM159PT cells with or without ADAM12 KD into Matrigel and observed the formation of invasive branching structures 6 days post-seeding. Matrigel provides a three-dimension environment similar to the extracellular matrix, which allows the cells to attach and grow (Kleinman &

Martin, 2005). The colonies formed by SUM159PT cells with induced ADAM12 shRNA had a more round shape and smooth edges; whereas the colonies formed by cells with ADAM12 expression showed more aggressive branching structures (Figure 2.2B). These results demonstrated that the ability of SUM159PT cell to invade Matrigel was markedly impaired upon ADAM12 knockdown (Figure 2.2B).

Loss of ADAM12 reduces anchorage-independent growth and sphere formation by TNBC cells

Next, we tested the function of ADAM12 in TNBC cells using 3D suspension culture in the presence of 1% methylcellulose. Sphere formation assay showed that decreased ADAM12 expression in SUM159PT cells resulted in reduced mammosphere formation (Figure 2.3C), which indicated that SUM159PT cells with ADAM12 expression possess the ability of anchorage-independent growth. We further examined the effect of ADAM12 on apoptosis induced by detachment. DNA fragmentation, an indicator of the extent of apoptosis, was measured by Cell Death ELISA kit. SUM159PT cells with ADAM12 KD gave a striking increase of DNA fragmentation 24 hours post-seeding in suspension (right panel in Figure 2.3D, * $P < 0.05$).

A time course study of apoptosis showed that SUM159PT cell underwent apoptosis upon detachment. Down-regulation of ADAM12 robustly reduced the ability of SUM159PT cells to resist death under the suspension conditions, as the DNA fragmentation of cells with ADAM12 KD was considerably higher than cells with ADAM12 expression left panel in Figure 2.3D, * $P < 0.05$). These findings are consistent with the results of the sphere formation assay. Altogether, our data showed that ADAM12 plays an indispensible role during maintaining the aggressiveness of triple-negative breast cancer cells in culture.

ADAM12 KD reduces the cancer stem cell subpopulation of TNBC cells

To answer the question of whether ADAM12 would affect the population of tumor-initiating cells in TNBC, we measured the cancer stem cell markers (ALDH and CD24CD44) of two TNBC cell lines, SUM159PT and Hs578T, after ADAM12 knockdown by siRNA. We were first looking at the expression of aldehyde dehydrogenase (ALDH), which is a hallmark of cancer stem cells. Previous studies showed that cancer stem cells have enhanced ALDH enzyme activity. Therefore, we measured the percentage of ALDEFLUOR-positive (ALDEFLUOR+) cells using the ALDEFLUOR kit and flow cytometry. ADAM12 KD diminished the ALDEFLUOR+ subpopulation by ~2 fold (Figure 2.3A-C) in SUM159PT.

To confirm the result of ALDEFLUOR assay, we further examined other cancer stem cell markers, CD24 and CD44, in SUM159PT and Hs578T cells after ADAM12 KD. We found that down-regulated ADAM12 expression resulted in a decrease of $CD44^{+}/CD24^{-/low}$ populations in both cell lines (Figure 2.4A-D). Consistently, the non-cancer stem-like cell population, $CD44^{+}CD24^{+}$ population, increased approximately 4 fold after ADAM12 down-regulation in SUM159PT cells (Figure 2.4C, *** $P < 0.001$) and 1.5 fold in Hs578T cells (Figure 2.4D, * $P < 0.05$). These date are consistent with the results of ALDEFLUOR assay, which suggests that ADAM12 regulates cancer stem-like phenotype in TNBC cells.

ADAM12 KD abrogates tumor-initating potential of TNBC cells *in vivo*

Next, we tested the effect of ADAM12 KD on the tumor-initiating abilities of SUM159PT cells *in vivo* using a limiting dilution assay. Stable SUM159PT cells with Tet-On shADAM12 or shControl systems were treated with Dox (1μg/ml) for 4 days. Single cell

solutions of SUM159PT shADAM12 ± Dox and shControl ± Dox cells were injected into the mammary fat pad of female NOD-SCID mice at different amounts (10^2-10^4 cells, n = 6/dilution, Figure 2.5A). Mice injected with doxycycline-treated cells were fed with a customized diet containing doxycycline. Tumor formation was monitored every three days for three weeks. The tumor-initiating frequency and P values were calculated using the ELDA algorithm (Extreme Limiting Dilution Assay) (Hu & Smyth, 2009). SUM159PT cells with ADAM12 KD exhibited a markedly attenuated tumor occurrence. The frequency of tumor-initiating cells in SUM159PT cells with ADAM12 KD was approximately 4 times lower than that in SUM159PT shControl cells treated with Dox (Figure 2.5A, * P = 0.042).

The effect of ADAM12 KD on the tumor growth rates *in vivo* was investigated by injecting 10^5 cells into the mouse mammary fat pad (n = 6/group). Tumor volumes at the injection sites were checked every three days. Tumors started to develop about 14 days post-injection. The tumor growth rate of SUM159PT with ADAM12 KD (Figure 2.5C, shADAM12 +Dox, *** $P < 0.0001$) was significantly slower than the cells with normal ADAM12 expression (Figure 2.5C, shADAM12 –Dox). In contrast, there was no obvious difference between SUM159PT shControl cells with or without doxycycline treatment (Figure 2.5D). Representative tumor pictures of all four groups are shown in Figure 2.5B and D. In all, these data demonstrate that down-regulation of ADAM12 does not only reduce tumor occurrence, but it also delays tumor growth *in vivo*. These findings also confirmed that the regulation of ADAM12 is biologically significant *in vivo*.

ADAM12 modulates cancer stem cells in TNBC through the EGF signaling pathway

Next, we asked the question of how ADAM12 regulates cancer stem cell phenotype in TNBC. In order to answer this question, we performed RNA-sequencing of SUM159PT

shADAM12 and shControl cells, with and without Dox treatment. RNA extraction and data analysis were performed by Dr. Sara Duhachek-Muggy. To account for possible effects of doxycycline treatment or viral infection on gene expression, all genes that were changed by control treatments were excluded from the analysis (Figure 2.6A). At the end, forty-five genes were found differently expressed after ADAM12 KD in SUM159PT cells (Figure 2.6B). These forty-five genes were analyzed by the Ingenuity Pathway Analysis (IPA) software, which would not only calculate what canonical pathways were enriched in our data set, but would also predict the possible upstream transcriptional regulators that might be the cause of the observed gene expression changes. The statistically significant upstream regulators, for which the Z-score of change was ≥2, are shown in Figure 2.6C. Interestingly, EGFR is one of the regulators discovered by the IPA analysis. Our previous work showed that overexpressed ADAM12 induced higher EGFR phosphorylation in a mouse xenograft breast cancer model and that ADAM12 expression was highly correlated with EGFR phosphorylation in human breast tumors (Li *et al*., 2012). Taken together, these data suggest that ADAM12 might regulate the cancer stem cell phenotype in TNBC through the EGF signaling pathway.

To test this hypothesis, we first examined the $CD24^+CD44^+$ subpopulation in SUM159PT after inhibiting the EGF signaling pathway. Remarkably, $CD24^+CD44^+$ subpopulation was robustly increased by treating SUM159PT cells with 1μM Erlotinib, a potent EGFR inhibitor, for 3 days. (Figure 2.7A). This increase of $CD24^+CD44^+$ by Erlotinib was similar to the up-regulation caused by ADAM12 knockdown using siRNAs (Figure 2.7B), which suggested that ADAM12 down-regulation and Erlotinib might have the same biological consequences. This finding also indicated that reduced ADAM12 expression might have an inhibitory effect on EGF signaling pathway in SUM159PT cells, which was consistent with our IPA upstream regulator

analysis. Thus, to examine this possibility, we tested whether adding exogenous EGF would abolish the increase of $CD24^{+}CD44^{+}$ subpopulation caused by ADAM12 down-regulation. We transfected SUM159PT cells with siADAM12#1, siADAM12#2, and siControl#3 and then cells were treated with and without 20ng/ml EGF for 4 days. Notably, activation of EGFR with exogenous EGF blocked the reduction of $CD44^{+}/CD24^{-/low}$ cells in response to ADAM12 knockdown (Figure 2.7C-D). Thus, ADAM12 seems to act as an upstream activator of EGFR, which in turn promotes the $CD44^{+}/CD24^{-/low}$ stem cell-like phenotype in SUM159PT cells.

Discussion

Genetic studies have proved that TNBCs are heterogeneous (Prat & Perou, 2011). Most of the triple-negative breast tumors fall into either the basal-like (~70%) or the claudin-low subtypes (~25-39%). Claudin-low tumors are mostly TNBCs (61-71%) and marked by the low expression of genes regulating cell-cell tight junctions and epithelial cell adhesion, such as E-cadherin, claudins, and EpCAM (Prat *et al*., 2010). Breast tumors of this subtype commonly feature low luminal/epithelial differentiation, they have the signature of epithelial-to-mesenchymal transition (EMT), and they have potent tumor-initiating capabilities (Prat & Perou, 2011). All these features may contribute, either individually or together, to the enhanced metastatic potential of breast cancer. In this study, we used SUM159PT and Hs578T, triple-negative breast cancer cell lines, which are also of claudin-low subtype signature, to study the role of ADAM12 in tumor-initiating cells of TNBC.

Previously, we found that the level of ADAM12 was strongly associated with decreased metastasis-free survival rate in TNBC and that the expression of ADAM12 was significantly upregulated in TNBC patients with distant metastasis (Li *et al*., 2012). Besides, ADAM12 was

shown to be associated with breast tumor-initiating phenotype, suggesting an active role of ADAM12 in breast cancers with enhanced metastasic phenotype (Li *et al*., 2013). However, our previous studies did not answer the question of whether ADAM12 was the causation of distant metastasis in TNBC nor address a role of ADAM12 in maintaining the BTIC phenotype. Our current report is the first one to demonstrate that ADAM12 may maintain and regulate cancer stem like phenotype in TNBC through the EGFR signaling pathway and can be considered a potential therapeutic target in BTICs.

Patients with TNBC have a higher risk of an early relapse and a tendency of visceral metastasis as well as brain metastasis (Kim *et al*., 2013). In order to metastasize to distant sites, caner cells first need to acquire the ability to invade through the epithelial tissues and penetrate into the blood vessels by undergoing epithelial-to-mesenchymal transition (EMT) (Tsai & Yang, 2013). Then, the transformed cancer cells escaping from the primary site travel along the circulating system to their destination and form metastatic colonies. This process requires the cancer cells not only to survive but also to be able to proliferate upon detachment from the extracellular matrix. Using a Tet-On system, we showed that down-regulation of ADAM12 in SUM159PT cells resulted in a substantial decrease of cell survival under detachment conditions. Besides, the sphere formation assay indicated that ADAM12 also facilitated the proliferation of SUM159PT cells deprived of the extracellular matrix. Based on the fact that the expression levels of ADAM12 have been shown to be strongly associated with the phosphorylation of EGFR in TNBC (Li *et al*., 2012), and the fact that the activation of EGFR signaling triggers the expression of the genes that act against apoptosis and promote cell survival (Han & Lo, 2012), it is reasonable to speculate that ADAM12 confers apoptosis resistance and anchorage-independent growth on TNBC cells by providing pro-survival signals through EGFR signaling pathway and,

therefore, maintain the enhanced metastatic phenotype of TNBC. This hypothesis was supported by Ingenuity Pathway Analysis (IPA) based on our RNA-sequencing data, which showed that the gene expression change pattern of ADAM12 down-regulation is similar as the inhibition of EGFR signaling pathway (Figure 2.6-7).

Scientific literature has shown that tumor-initiating cells are the drive of tumor formation and they are responsible for the tumor relapse. $CD44^{+}CD24^{-/low}$ surface antigenic phenotype and aldehyde dehydrogenase (ALDH) activity are well-established hallmarks of cancer stem-like cells and have been demonstrated to be strongly associated with the tumor/metastasis initiating capabilities of cancer cells (Perou, 2011; van den Hoogen *et al*., 2010; Huang *et al*., 2009; Ponti *et al*., 2005). Using TNBC cell lines SUM159PT and Hs578T, we are the first ones to illustrate that ADAM12 down-regulation alone efficiently decreased the ALDH+ and $CD44^{+}CD24^{-/low}$ subpopulations in TNBC cells. We further confirmed the effect of ADAM12 on tumor-initiating frequency *in vivo* by limiting dilution assay (LDA). Limiting dilution assay is a well-established method designed to quantitatively measure the frequency of tumor-initiating cells in the bulk of original tumor by injecting increasing doses of tumor cells into animals (Ploemacher *et al*., 1989; O'Brien *et al*., 2010). We induced ADAM12 knockdown by a doxycycline inducible shRNA system, injected 100, 1000, 10000 SUM159PT cells into the fourth mammary fat pad of NOD-SCID mice, and monitored tumor formation. Data were uploaded to ELDA software (Hu & Smyth, 2009) to calculate the frequency of SUM159PT cells after eliminating ADAM12. The result showed that the absence of ADAM12 significantly decreased the frequency of tumor initiating cells (Figure 2.5, ~3.8 times lower, $P = 0.042$ compared to the control cells treated with doxycycline), as well as the tumor growth in SUM159PT cells. Altogether, these results suggest

that ADAM12 is not only associated with tumor initiating phenotype, but it is also an active player in the regulation of cancer stem-like cell populations in TNBC.

It has been widely accepted that the EMT program is the main process by which cancer cells acquire stem-like properties. Gupta and his group showed that breast cancer cells acquired $CD44^{+}CD24^{-/low}$ or $CD49f^{high}EpCAM^{-}$ stem-like phenotype while undergoing EMT (Gupta et al. 2009). Besides, breast cancer cells with stem-like traits had high level of EMT-inducing transcription factors, such as ZEB1&2, Snail, and TWIST (Perou, 2011). Given the fact that high expression of mesenchymal markers is one of the main characters of claudin-low breast cancer (Prat *et al.*, 2010) and that the expression of ADAM12 is induced by EMT in breast cancer cell lines (Li *et al*., 2013), we initially hypothesized that ADAM12 regulates stem-like cells in TNBC through TGFβ-mediated EMT. To this end, we first treated SUM149PT, a breast cancer cell line with low mesenchymal features, with TGFβ. No change of $CD44^{+}CD24^{-/low}$ subpopulation in SUM149 cells was observed. Next, we treated SUM159PT cells with SB431542, a potent TGFβ receptor inhibitor, for 3 days, but we were not able to detect any changes in the $CD44^{+}CD24^{-/low}$ subpopulation. Similar results were obtained when SUM159PT cells were transiently transfected with ZEB1 siRNAs for 4 days (data not shown). Interestingly, our IPA analysis based on the RNA-seq result of SUM159PT shADAM12 with and without doxycycline treatment did not identify the TGFβ pathway as one of the upstream regulators. All these results led us to propose that ADAM12 might regulate tumor-initiating cells through a pathway that is different from the TGFβ pathway.

What is the novel pathway that is responsible for the regulation of stem-like phenotype mediated by ADAM12 in TNBC? Interestingly but not surprisingly, our IPA results based on RNA-seq data link the unique role of ADAM12 in stem-like cell regulation in TNBC to the

stimulation of EGFR signaling pathway. These results are consistent with and further support our previous findings that ADAM12 might be the main protease responsible for the activation of EGFR in TNBC. The role of EGFR signaling pathway in solid tumors has been exhaustively studied. PI3K-Akt-mTOR, JAK2-STAT3, PLCγ-PKC, and RAS-MEK-MAPK (Han & Lo, 2012) are the major downstream pathways regulated by EGFR, and these pathways are of importance in the progression, chemoresistance, and metastasis of various types of cancers. Activation of MAPK by EGFR not only promotes the degradation of pro-apoptotic protein BIM-EL, but also increases the expression of pro-survival molecule BCL-X_L (Buchheit *et al*., 2014). Activated mTORC1-p70 (S6K) by the EGFR-PI3K-Akt axis increases the survival rate under detachment conditions by suppressing autophagy (Avivar-Valderas *et al*., 2013). In addition to the fact that ADAM12 levels are correlated with the phosphorylation of EGFR in TNBC (Li *et al*., 2012), it is reasonable to speculate that the activation of EGFR could be the explanation for the increased apoptosis after ADAM12 KD under suspension conditions, which is further supported by the IPA results suggesting that the effect of ADAM12 KD is similar to the inhibition of the mTOR pathway.

In addition to the promotion of anoikis resistance, the EGFR signaling pathway also contributes to the maintenance of the stem-like phenotype of cancer cells. EGFR-activated STAT3 induced TWIST upregulation and therefore promoted EMT by suppressing E-cadherin expression (Lo *et al*., 2007). Studies showed that the EGFR pathway regulated the stem-like traits in nasopharyngeal carcinoma and head and neck squamous cells. Furthermore, Yang's group recently demonstrated that the EGFR-STAT3-SOX2 axis is required for the maintenance of the cancer stem cell phenotype of breast tumor cells (Yang *et al*., 2013). We further explored the effect of EGFR signaling inhibition on $CD44^+/CD24^{-/low}$ subpopulation by incubating

SUM159PT cells with erlotinib, a potent EGFR inhibitor, which impairs the tyrosine kinase activity of EGFR. The effect of erlotinib was similar to the effect of ADAM12 KD. The effect of ADAM12 KD on $CD44^{+}/CD24^{-/low}$ subpopulation was blocked when exogenous EGF was added to the cells.

In the absence of HER2 overexpression in TNBC, it is plausible that the activation of EGFR would be the alternative driving force of cell survival and cancer progression. But the question that still needs to be addressed is how ADAM12 would affect the EGFR signaling pathway in TNBC. Most of the EGFR ligands, like EGF, heparin-binding EGF (HB-EGF), and transforming growth factor (TGF)-α are first synthesized and translocated to the cell surface as transmembrane precursors (Normanno et al., 2006; Han & Lo, 2012). The prototypes of these factors are cleaved and released from the cell surface by ADAMs. Studies showed that ADAM12 was capable of shedding some of the EGFR ligands at the cell surface. A group at MIT has recently demonstrated that ADAM12 could cleave HB-EGF overexpressed in endometriotic epithelial cells (Díaz *et al*., 2013) . Moreover, ADAM12 has been reported to shed HB-EGF in a Notch-dependent manner in head and neck carcinomas, activating EGFR in a paracrine way and promoting cancer cell invasion (Díaz *et al*., 2013). Combined with our previous finding that ADAM12 was the only ADAM member significantly upregulated in TNBC and associated with EGFR activation, we propose that ADAM12 might modulate EGFR signaling pathway in TNBC by cleaving and releasing EGFR ligands from cells. The identification of the ligands cleaved by ADAM12 in TNBC will illuminate the mechanism of the progression and metastasis of TNBC in more depth.

In summary, our data suggest that ADAM12 may be a therapeutic target in TNBC. We showed that ADAM12 expression is abundantly expressed in claudin-low breast tumors at both

mRNA and protein levels. Importantly, we illustrated the important roles of ADAM12 in maintaining the aggressive phenotype of TNBC cells. In addition, our *in vitro* sphere formation assay and *in vivo* limiting dilution assay indicated that ADAM12 down-regulation dramatically decreased the tumor-initiating frequency of SUM159PT cells, suggesting that ADAM12 was required to maintain the tumor-initiating potential of TNBC cells. We further explored the molecular mechanisms and proved that ADAM12 maintained the tumor-initiating population through the EGFR signaling pathway. In all, our studies indicate that ADAM12 is a novel regulator of cancer stem-like phenotype and may serve as a potential diagnostic marker or a therapeutic target in TNBC.

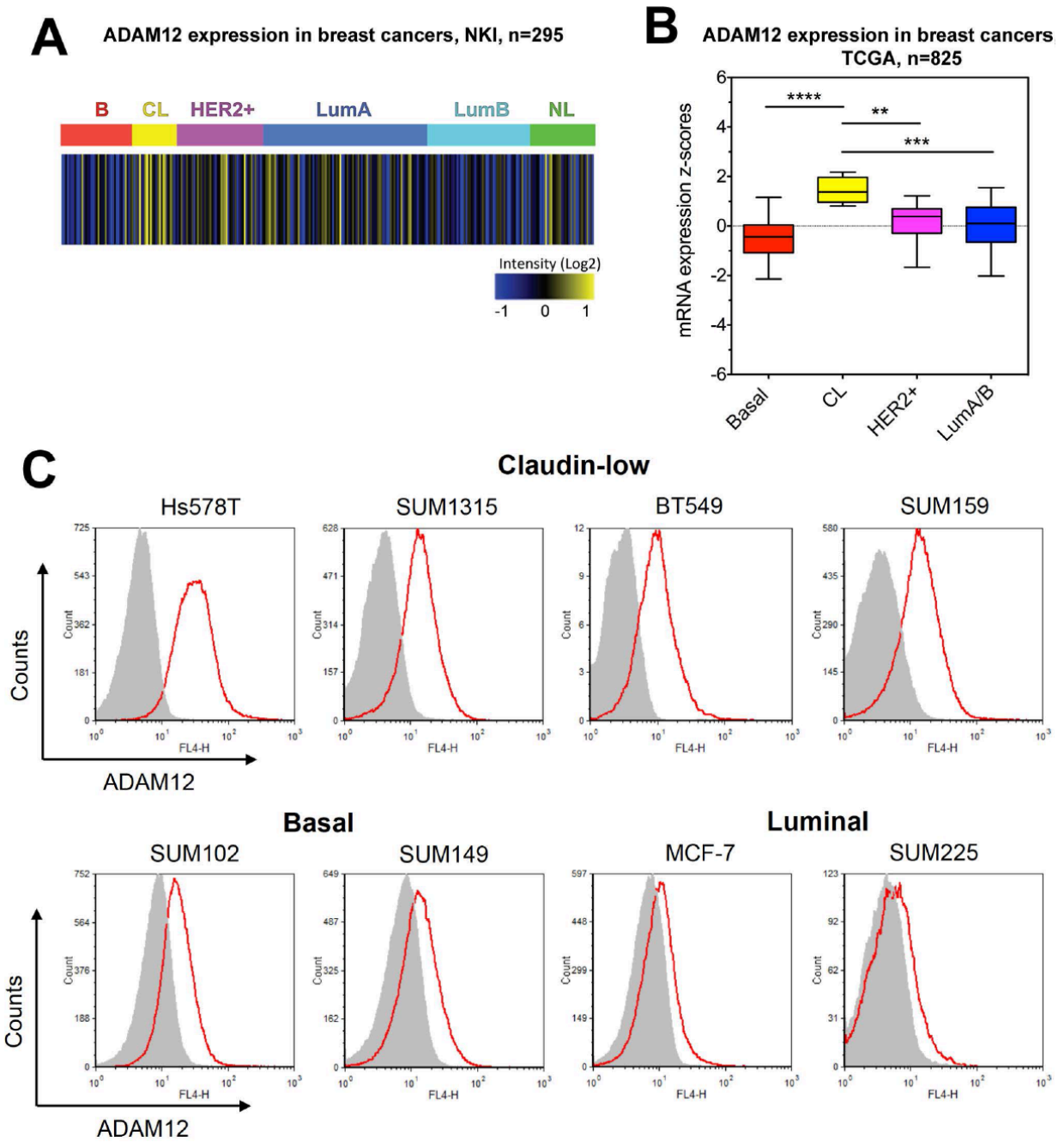


Figure 2.1 ADAM12 expression is upregulated in the claudin-low molecular subtype of breast cancer.

(A) Heatmap showing ADAM12 mRNA expression levels in 295 breast cancer patients from the Netherland Cancer Institute (NKI) database (van de Vijver *et al*., 2002). Molecular subtypes of tumors are: B, basal; CL, claudin-low; HER2+, HER2-enriched, LumA, luminal A;

LumB, luminal B; NL, normal-like. (**B**) Expression levels of ADAM12 in 825 breast cancer patients from the TCGA database. (**C**) cell surface expression of ADAM12 protein in breast cancer cell lines was evaluated by flow cytometry. Hs578T, SUM1315MO2, BT549, and SUM159PT represent the claudin-low subtype, SUM102PT and SUM149PT represent the basal subtype, and MCF-7 and SUM225CWN are classified as luminal (Heiser *et al*., 2012; Prat et al., 2010).

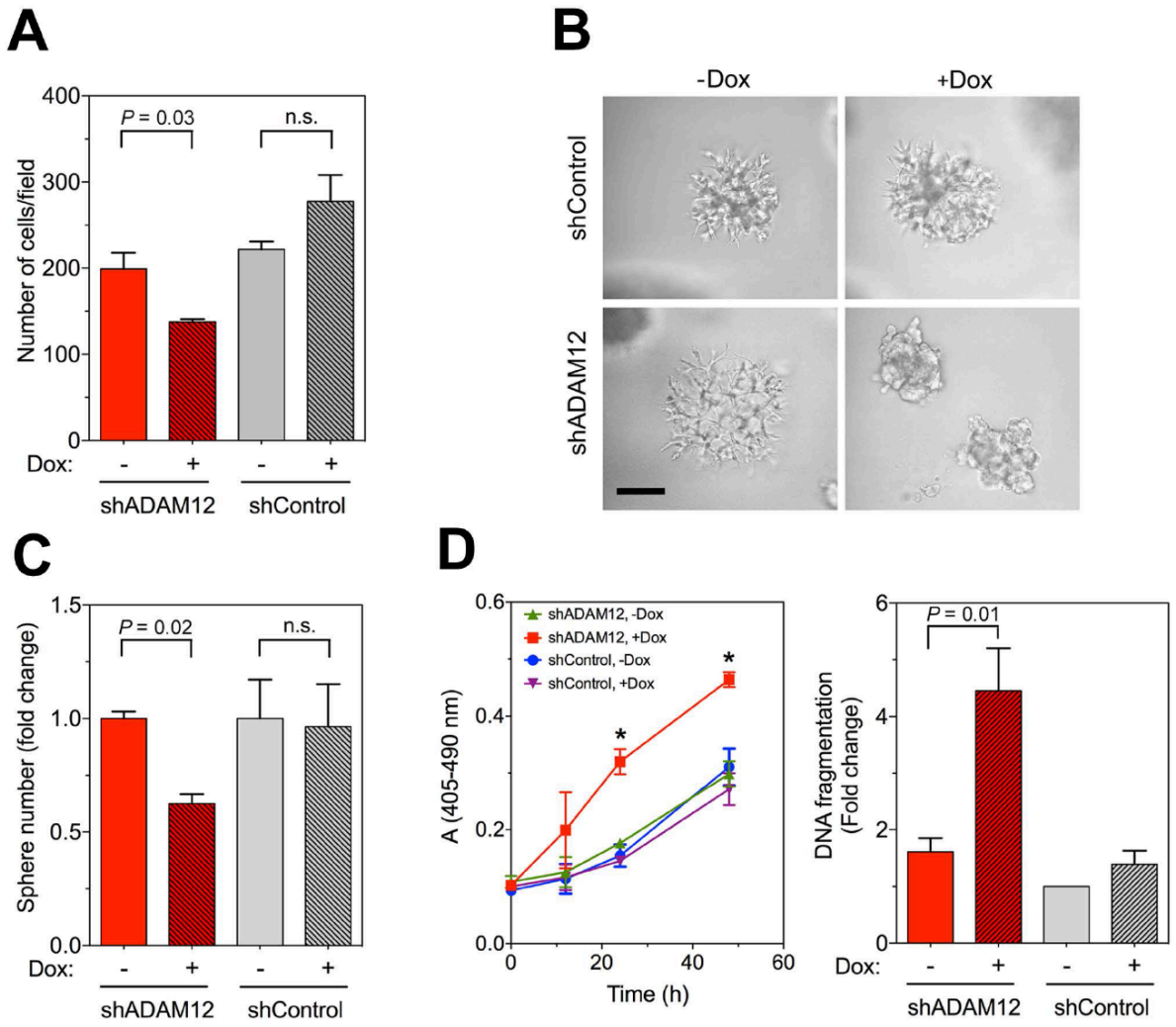


Figure 2.2 ADAM12 knockdown reduces cell migration, invasion, sphere formation, and resistance to anoikis.

SUM159PT cells expressing tet-on ADAM12 shRNA (shADAM12) or control shRNA (shControl) were treated with doxycycline (Dox; 1 μg/ml) for 4 days. (**A**) cell migration was analyzed using Transwell assays. The data are shown as means ± SEM from 3 determinations. (**B**) representative phase-contrast images of SUM159PT cells embedded in 3D Matrigel are shown. Scale bar, 100 μm. (**C**) sphere formation was evaluated after seeding single-cell suspensions in serum-free sphere medium containing 1% methylcellulose into ultra-low attachment plates. After 10 days, spheres were visualized by phase contrast imaging, and the number of spheres with diameters > 50 μm were counted using ImageJ. The results are shown as means ± SEM obtained from 3 independent experiments. (**D**) anokis (apoptotic cell death upon detachment) was assayed using DNA fragmentation ELISA kit, as described in Methods. A time

course of cell death was measured in triplicates (raw data, left), and the mean values of DNA fragmentation, ± SEM, after 24 h of detachment obtained in three independent experiments were calculated (right). *, $P<0.05$, n.s., non-significant.

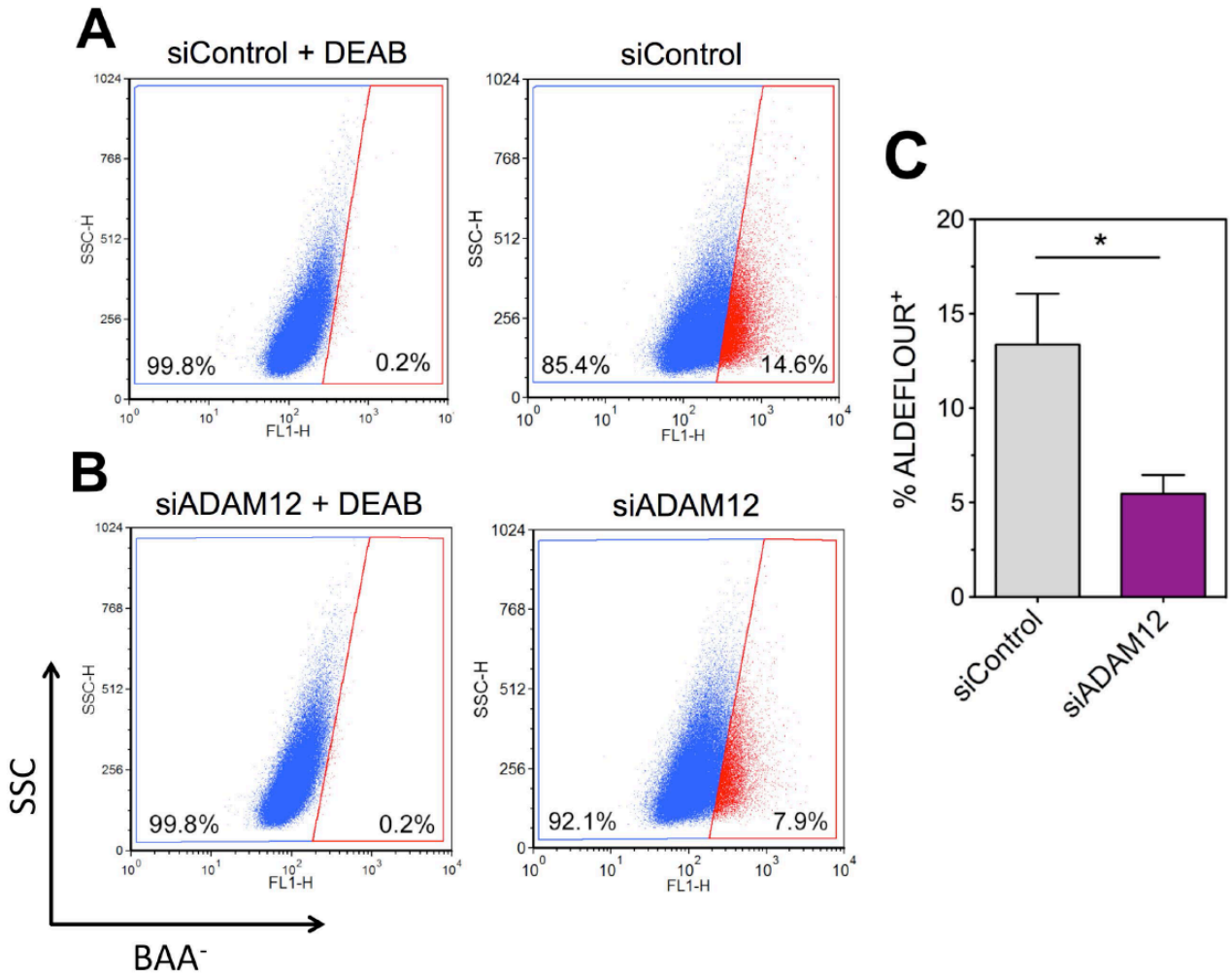


Figure 2.3 ADAM12 knockdown reduces the population of ALDEFLUOR+ cells.

(**A-B**) SUM159PT cells were transfected with a pool of four control non-targeting siRNAs (**A**) or a pool of four ADAM12 siRNAs (**B**). Five days after transfection, cells were stained with ALDEFLUOR reagent and analyzed by flow cytometry, as described in Methods. Plots show side cell scatter (SSC) versus BAA- fluorescence (BODIPY- aminoacetate, an intracellular reaction product of aldehyde dehydrogenase). ALDEFLUOR- (blue) and ALDEFLUOR+ (red) gates were identified after pre-treatment of cells with DEAB, an ALDH inhibitor. (**C**) percentages of ALDEFLUOR+ populations in SUM159PT cells were determined in four independent experiments. The results are shown as means ± SEM; *, $P<0.05$.

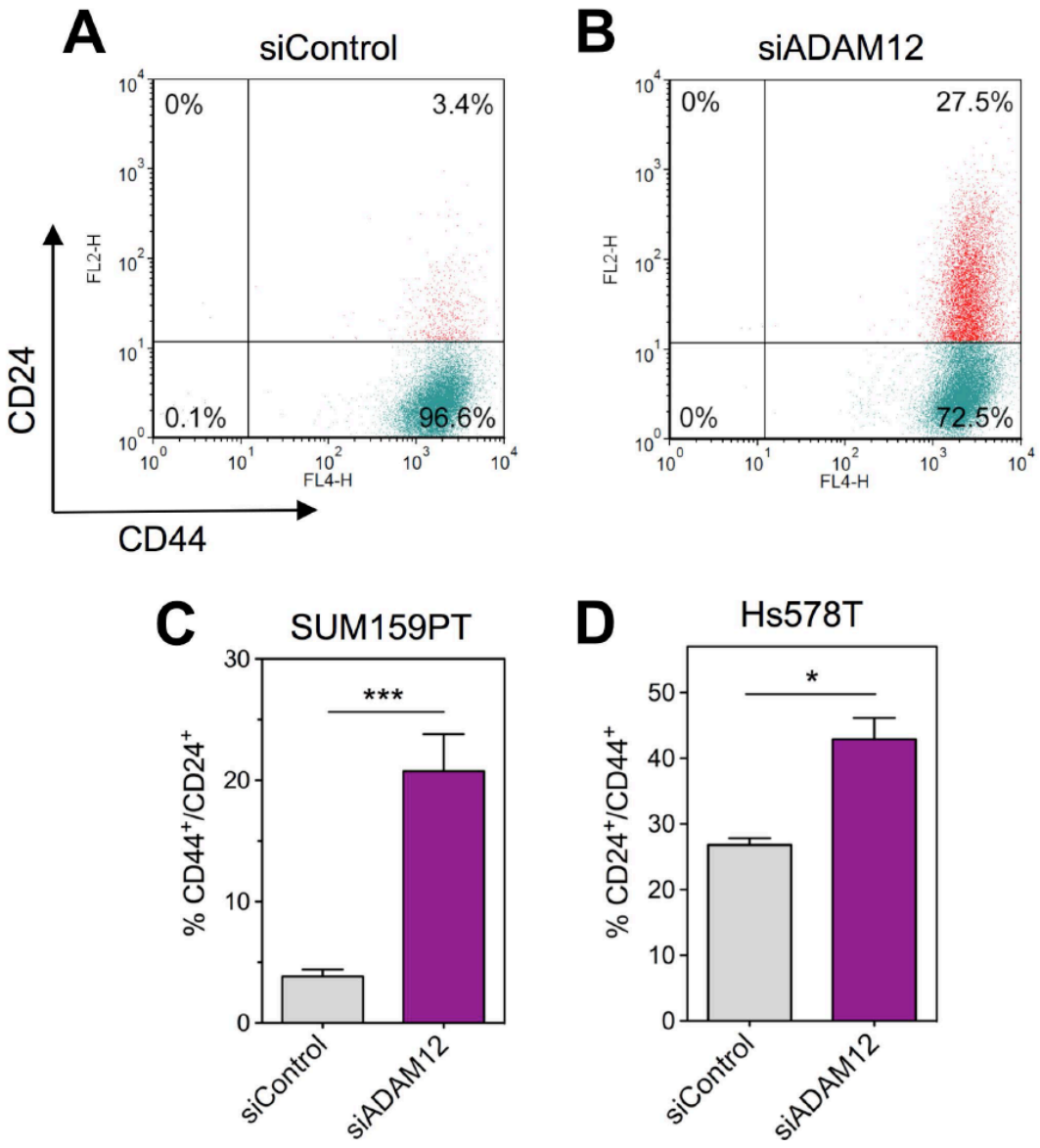


Figure 2.4 ADAM12 knockdown increases the subpopulation of $CD24^+/CD44^+$ cells.

(**A**-**B**) SUM159PT cells were transfected with a pool of four control non-targeting siRNAs (**A**) or ADAM12 siRNAs (**B**). Five days after transfection, cells were stained with PE-conjugated anti-CD24 antibody and APC-conjugated anti-CD44 antibody and analyzed by flow cytometry. $CD24^-/CD44^+$ (green) and $CD24^+/CD44^+$ (red) and populations were identified based on isotype control antibody staining. (**C**-**D**) Percentages of $CD24^+/CD44^+$ populations in SUM159PT cells (**C**) and Hs578T cells (**D**) were determined in three independent experiments. The results are shown as means ± SEM, *, $P<0.05$.

A

Cells	Number of injected SUM159PT cells 1×10^4	1×10^3	1×10^2	BTIC frequency	*P* value	
shADAM12 -Dox	4/6	2/6	0/6	1 per 6808 cells	0.156	0.735
shADAM12 +Dox	2/6	1/6	0/6	1 per 18399 cells		
shControl -Dox	4/6	1/6	0/6	1 per 8447 cells	0.359	0.042
shControl +Dox	4/6	2/6	2/6	1 per 4885 cells		

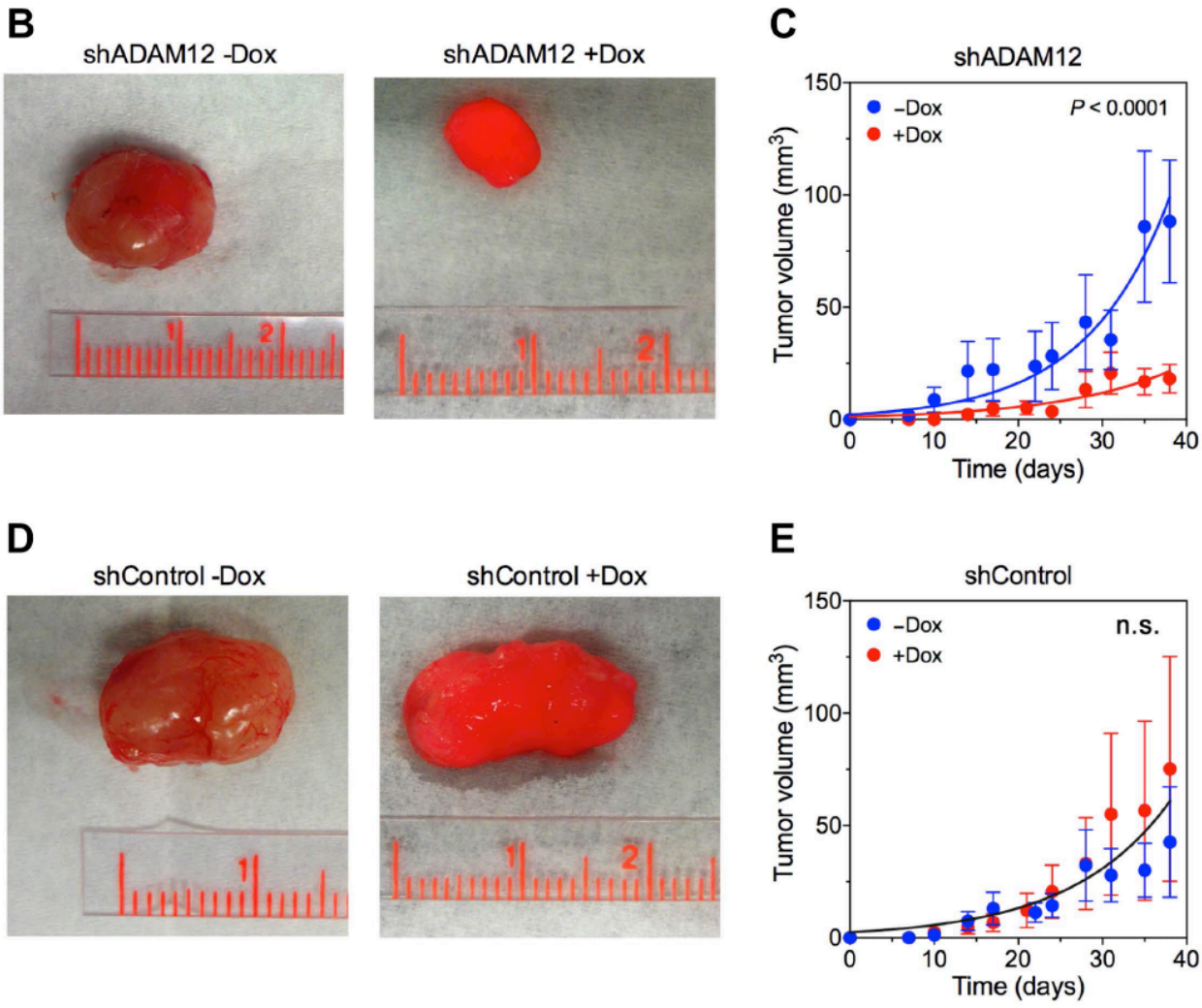
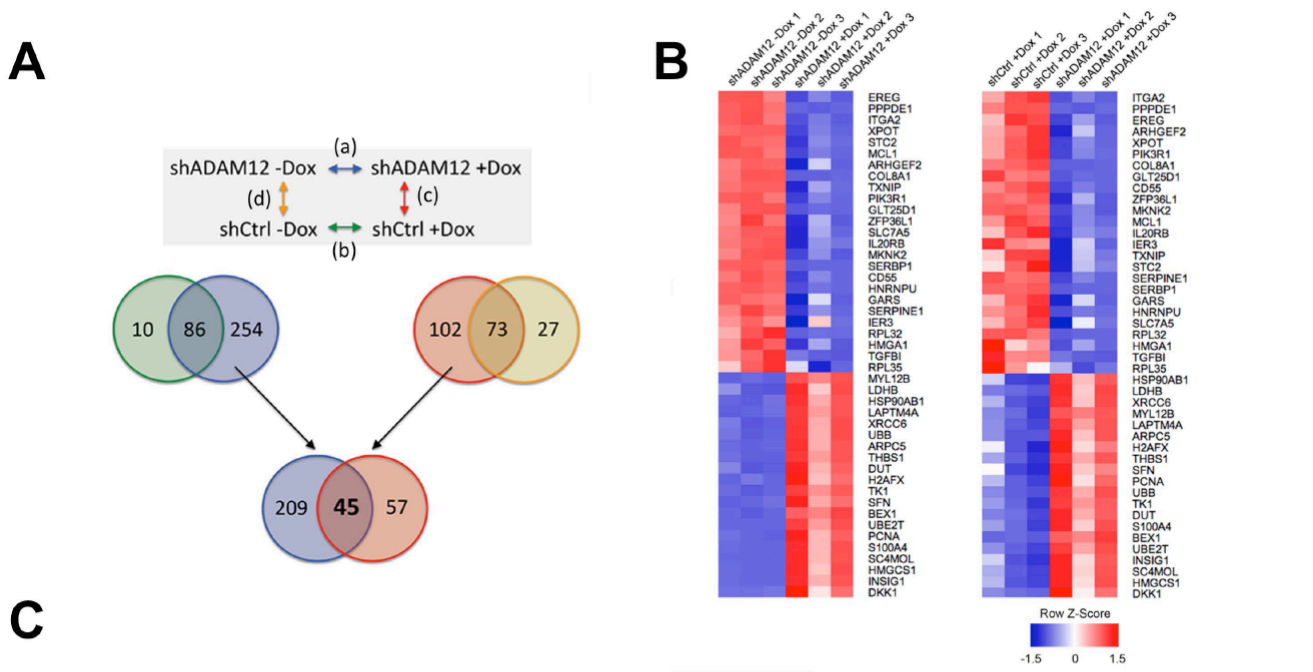


Figure 2.5 ADAM12 knockdown reduces tumor growth in mice *in vivo.*

(**A**) Tumor incidence in the limiting dilution assay. SUM159PT cells expressing tet-on shADAM12 or shControl were transplanted in the fourth mammary fat pad of NOD-SCID mice in limiting dilutions (10,000, 1,000, or 100 cells). To induce shRNA expression, cells were pre-

treated with doxycycline (Dox; 1 μg/ml) for 4 days prior to transplantation, and Dox (2 g/kg) was then continually administered in the diet. The presence of palpable tumors was determined 3 weeks after cell transplantation. The frequency of breast tumor initiating cells (BTICs) and *P*-values for the statistical significance between groups were calculated using extreme limiting dilution analysis (ELDA, http://bioinf.wehi.edu.au/software/elda/) (Hu & Smyth, 2009). (**B-D**) representative tumors formed 38 days after injection of 10^5 cells by SUM159PT_shADAM12 cells (**B**) or SUM159PT_shControl cells (**D**). (**C-E**) tumor growth was monitored for a period of 0-38 days and tumor volumes were evaluated using caliper, as described in Methods. Nonlinear regression curves were fitted using GraphPad software.



Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	P-value of Overlap	Target Molecules in Dataset
LPS	chemical drug	Inhibition	-2.329	9.15E-06	↓CD55, ↓EREG, ↓HMGA1, ↓IER3, ↓IL20RB, ↑INSIG1, ↓MCL1, ↑PCNA, ↓PIK3R1, ↓SERPINE1, ↑SFN, ↑THBS1, ↓TXNIP
SP1	transcription regulator	Inhibition	-2.223	5.73E-05	↓CD55, ↓COL8A1, ↓EREG, ↓HMGA1, ↓ITGA2, ↓MCL1, ↓SERPINE1
EGFR	kinase	Inhibition	-2.180	3.97E-09	↓COL8A1, ↓EREG ,↓IER3, ↓ITGA2, ↓MCL1, ↑PCNA, ↓SERPINE1, ↑THBS1, ↓TXBIP
MTOR	kinase	Inhibition	-2.000	1.54E-03	↑HSP90AB1, ↓MCL1, ↓RPL32, ↑TK1
SMARCA4	transcription regulator	Inhibition	-2.000	2.50E-02	↓EREG, ↓IER3, ↓IL20RB, ↓SERPINE1
NEDD9	other	Inhibition	-2.000	7.08E-07	↓ITGA2, ↓MKNK2, ↓SERPINE1, ↓TXNIP

Figure 2.6 RNA sequencing (RNA-Seq) analysis of the gene expression (GE) changes induced by ADAM12 knockdown in SUM159PT cells.

(This work was done by Dr. Sara Duhachek-Muggy.)

(**A**) A diagram summarizing the design and outcome of the RNA-Seq experiment. Genes differentially expressed in tet-on shADAM12 cells treated *vs* not treated for 6 days with 1 μg/ml Dox are shown in blue (a). Genes differentially expressed in tet-on shControl cells treated *vs* not treated with Dox are shown in green (b); these genes represent a negative control for genes shown in blue. Genes differentially expressed in tet-on shADAM12 *vs* shControl cells treated with Dox are shown in red (c). Genes differentially expressed in tet-on shADAM12 *vs* shControl cells not treated with Dox are shown in yellow (d); these genes represent a negative control for genes shown in red. (**B**) Heatmaps of differentially expressed genes in response to ADAM12 KD. Compared are shADAM12 cells with *vs* without Dox treatment (left) and shADAM12 *vs* shControl cells treated with Dox (right). Only 45 differentially expressed genes common to the

(a) and (c) comparisons, after elimination of genes present in control (b) and (d) comparisons, are included. (**C**) The results of the Ingenuity Pathway Analysis (IPA) Upstream Regulator analysis. Potential upstream regulators with an overlap P-value < 0.05 and an activation $|z|$ score > than 2 are shown.

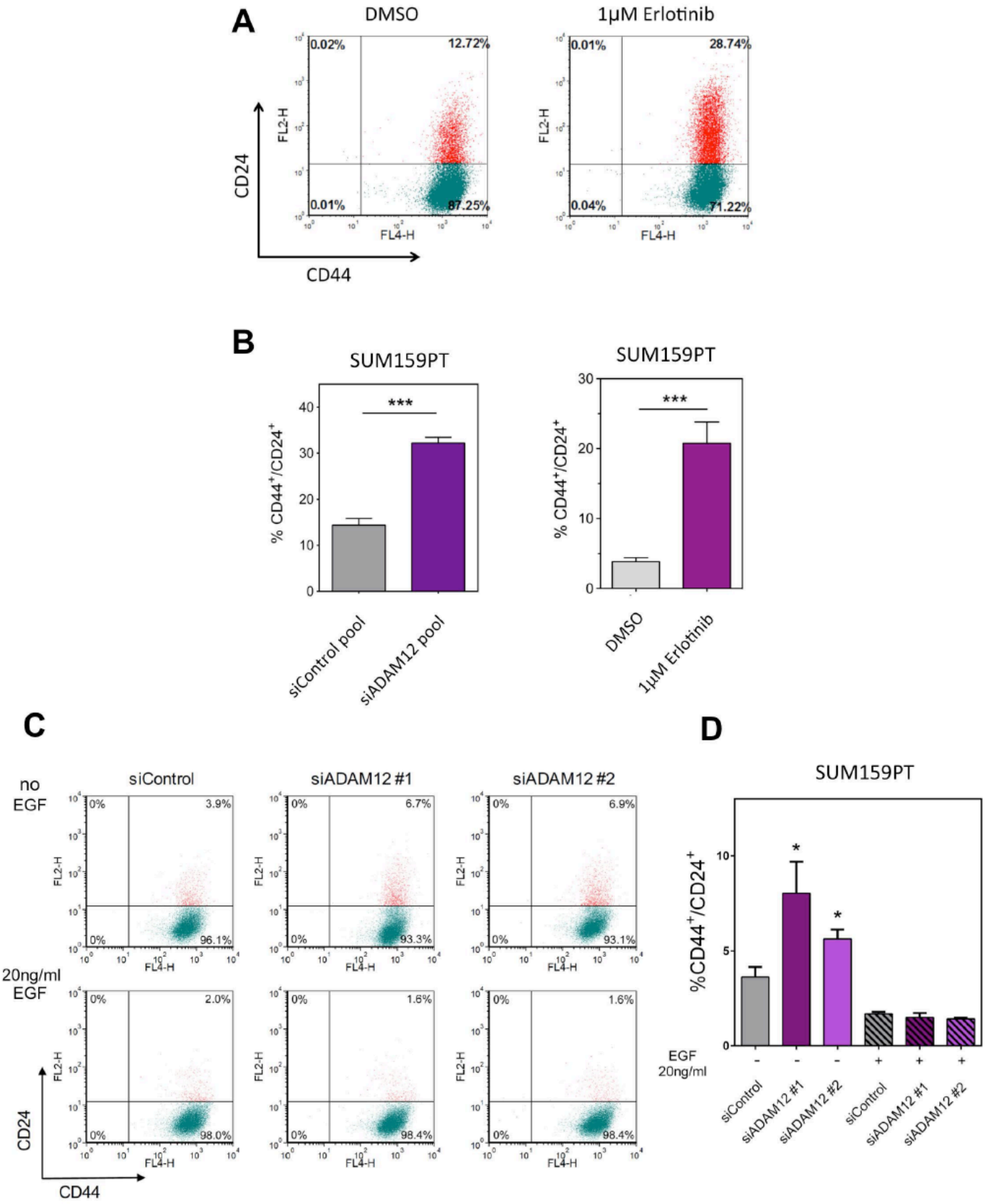

A
DMSO
1μM Erlotinib
0.02%
12.72%
0.01%
87.25%
0.01%
28.74%
0.04%
71.22%
FL2-H
FL4-H
CD24
CD44
B
SUM159PT
SUM159PT
***
***
% CD44+/CD24+
siControl pool
siADAM12 pool
DMSO
1μM Erlotinib
C
D
no EGF
20ng/ml EGF
siControl
siADAM12 #1
siADAM12 #2
3.9%
96.1%
6.7%
93.3%
6.9%
93.1%
2.0%
98.0%
1.6%
98.4%
1.6%
98.4%
SUM159PT
%CD44+/CD24+
EGF 20ng/ml

Figure 2.7 ADAM12 controls the population of $CD24^{+/}$ $CD44^{+}$ cells via regulation of EGFR.

(A-B) Erlotinib mimics the effect of ADAM12 reduction on the population of $CD24^{+}/CD44^{+}$. SUM159PT grown in complete medium were treated for 24 h with 1 μM erlotinib, an EGFR inhibitor. Cell surface expression of CD24 and CD44 was analyzed by flow cytometry. (B) The effect of erlotinib on the $CD24^{+}/CD44^{+}$ populations in SUM159PT cells was quantified based on three independent experiments. (C) Exogenously added EGF blocks the effect of ADAM12 KD on $CD24^{+}/CD44^{+}$ populations. SUM159PT were transfected with siControl or with two different siRNAs targeting ADAM12. Three days later, cells were incubated with or without 20 ng/ml EGF in complete media for additional 24 h and analyzed for CD24 and CD44 expression. (D) The effect of exogenous EGF on $CD24^{+}/CD44^{+}$ populations, determined based on four independent measurements.

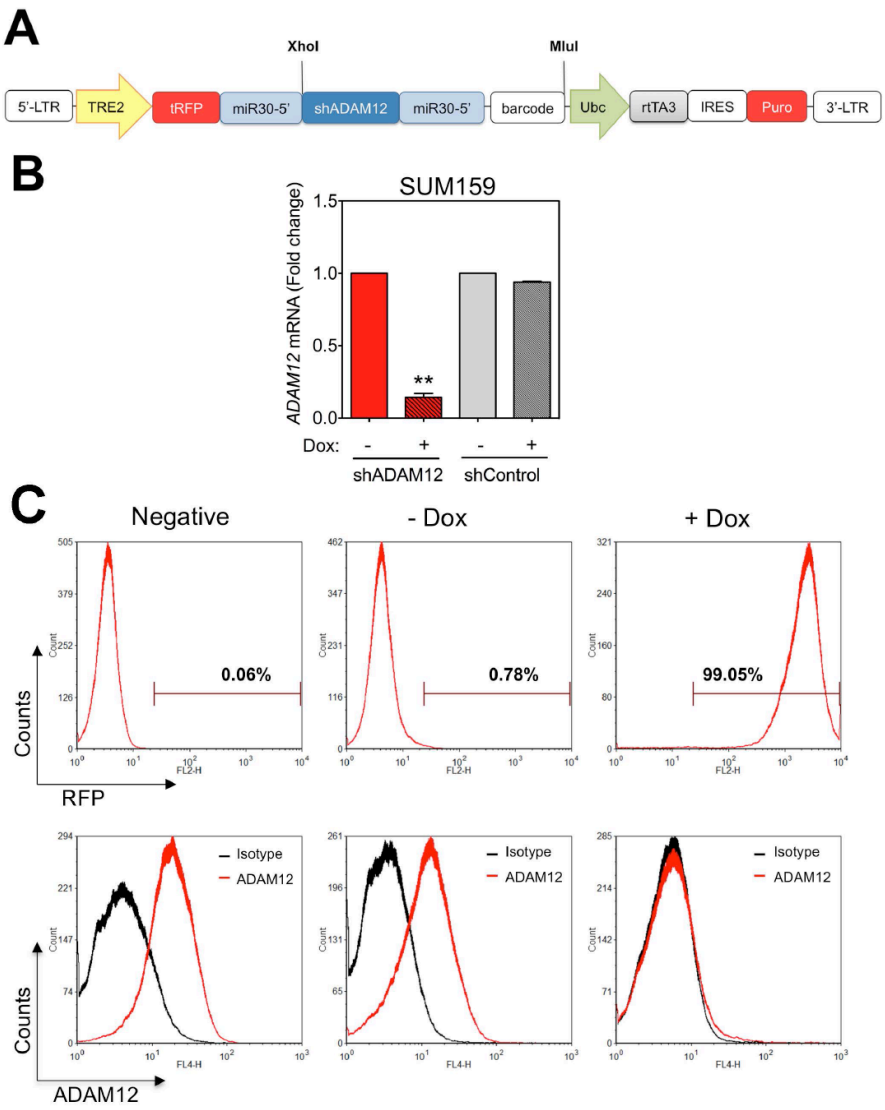


Figure 2.8 Doxycycline-inducible ADAM12 knockdown in SUM159PT cells.

(**A**) Diagram of the doxycycline-inducible lentiviral shRNA construct targeting ADAM12. 5'-LTR, 5'-long terminal repeat; TRE, tetracycline-inducible promoter; tRFP, turbo red fluorescent protein; miR30-5' and shERp57/shPDI/shERp44, micro-RNA-30 adapted shRNA targeting ERp57/PDI/ERp44; Ubc, human ubiquitin C promoter; rtTA3, reverse tetracycline-transactivator 3; IRES, internal ribosomal entry site; Puro, puromycin resistance gene; 3'-LTR, 3'-long terminal repeat. (**B-C**) Validation of ADAM12 knockdown. Stably transduced SUM159PT_shADAM12 and SUM159PT_shControl cells were incubated for 4 days with or without 1 μg/ml of doxycycline. (**B**) ADAM12 mRNA levels were quantified by qRT-PCR. (**C**) Cell surface expression of ADAM12 was examined by flow cytometry.

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Chapter 3 - ADAM12 regulates TGFβ signaling pathway by modulating *TGFBR1* mRNA expression in breast cancer cells

Abstract

TGFβ signaling facilitates breast tumor progression at late stages of the disease through various mechanisms, such as promoting extracellular matrix degradation and angiogenesis. TGFβ induces the expression of ADAM12 at both the transcriptional and translational levels. Interestingly, studies have also shown that ADAM12 overexpression enhances TGFβ signaling through interactions with TGFβR2 in early endosomes. However, little effort has been made to illustrate the effect of ADAM12 on TGFβ signaling at the endogenous ADAM12 expression level in breast cancer cells. In the present study, we investigated the role of ADAM12 in the regulation of TGFβ signaling in breast cancer cells using a doxycycline inducible shRNA knockdown system. The loss of ADAM12 expression significantly decreased the activation of both SMAD-mediated and SMAD-independent TGFβ downstream signaling pathways, and reduced the expression level of TGFβR1. Furthermore, we found that *ADAM12* mRNA levels were strongly associated with the TGFβ gene signature in breast cancer patients. In conclusion, our study describes a novel mechanism by which ADAM12 regulates the activation of TGFβ signaling in breast cancer cells.

Introduction

The TGFβ signaling pathway plays unique and context-dependent roles in cancer progression (Massagué *et al*., 2012; Akhurst & Padgett, 2015). As a tumor suppressor, TGFβ

signaling inhibits proliferation of normal epithelial cells and cancer cells at early stages, whereas in late stage cancers, TGFβ signaling promotes cancer progression, angiogenesis, and malignancy (Gomes *et al*., 2012; Bierie & Moses, 2010; Janda *et al*., 2002). Tumor-promoting functions of TGFβ are related to its capability of inducing epithelial-to-mesenchymal transition (EMT) (Xu *et al*., 2009). Many reports have shown that TGFβ signaling is associated with poor prognosis, high risk of metastasis, and poor therapy response in different types of cancers (Elliott & Blobe, 2005; Biswas *et al*., 2006; Bhola *et al*., 2013; Li *et al*., 2013).

The activation of TGFβ signaling pathway requires the binding of the TGFβ family ligands to the pre-formed TGFβR2 homodimer at the cell surface (Derynck *et al*., 2001). The complex of TGFβ and TGFβR2 further recruits TGFβR1, forming a tetramer complex, which allows the activation of TGFβR1 by TGFβR2 (Massagué & Chen, 2000). Once activated, TGFβR1 phosphorylates the receptor-regulated SMADs and transduces the signaling into the interior of the cells (Massagué & Chen, 2000). Thus, the regulation of TGFβ receptor availability at the cell surface is one of the regulatory mechanisms controlling the activation of the TGFβ signaling pathway (Rojas *et al*., 2009). TGFβR2 has been extensively studied due to the fact that it is constitutively active and is required for the activation of TGFβR1. The expression of TGFβR2 can be regulated at the protein level by endocytosis (Hayes *et al*., 2002; Di Guglielmo *et al*., 2003), as well as at the transcriptional level by EGF/PI3K/Akt pathway and and microRNAs (Yamane *et al*., 2003; Liang *et al*., 2015; Zhong *et al*., 2012). Though TGFβR1 (type I TGFβ receptor) is essential to activate the SMAD-dependent signaling pathway, there is very limited knowledge on the regulation of TGFβR1 (O'Brien *et al*., 2015).

ADAM12 (A Disintegrin And Metalloprotease 12) is a membrane-anchored protease whose expression is positively regulated by TGFβ signaling at both the transcriptional and the

translational level in breast cancer cell lines (Li *et al*., 2013; Ruff *et al*., 2015). A previous study showed that ADAM12 increased TGFβR2 in early endosomes and enhanced the stability of TGFβR2 by preventing the receptor from interactions with SMAD7 and by blocking its degradation (Atfi *et al*., 2007). However, this study was conducted in ADAM12 overexpression cell models, which may not accurately mimic the effect of the endogenous ADAM12 on TGFβ receptors. In the current study, we have studied the role of ADAM12 in the regulation of TGFβ signaling pathway in breast cancer cells using a doxycycline inducible shRNA system to decrease ADAM12 expression. Our study describes a novel mechanism by which ADAM12 regulates the TGFβ signaling pathway through modulating the mRNA level of TGFβR1.

Materials and Methods

Cell cultures and reagents

SUM149 and SUM159PT cells (Asterand, MI) were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum (FBS), 10 mM HEPES, 5 µg/ml insulin, and 1 µg/ml hydrocortisone. SUM1315MO2 cells (Asterand, MI) were cultured in Ham's F-12 medium supplemented with 5% FBS, 10 mM HEPES, 10 ng/ml epidermal growth factor, and 5 µg/ml insulin. BT549 cells (ATCC, VA) were cultured in RMPI1640 medium supplemented with 10% FBS and µg/ml insulin. MCF10DCIS.com (Asterand, MI) were cultured in 1:1 (v/v) Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 containing 15 mM HEPES and supplemented with 5% horse serum and 29 mM sodium bicarbonate. HEK293T cells were cultured in DMEM medium containing 10% FBS, 6 mM Glutamine, 1% Penicillin/ Streptomycin, and 110 µg/ml sodium pyruvate. SUM102PT cell line was a gift from Dr. Fariba Behbod (University of Kansas

Medical Center) and were maintained in Ham's F-12 medium containing 5% FBS, 1 µg/ml hydrocortisone, 5 µg/ml insulin, and 1% Penicillin-Streptomycin-Amphotericin B (PSA).

ADAM12 siRNA smartpool, TGFBR1 siRNA smartpool, control siRNA smartpool #1, and DharmaFECT1 transfection reagent were obtained from GE Dharmacon. Total RNA was extracted using RNeasy kit from QIAGEN. Human recombinant TGFβ1 and methylcellulose stock solution was purchased from R&D System. SYBR Select Master Mix for CFX was obtained from Life Technologies. EZ-link N-hydroxysuccinimide-PEG12-Biotin was purchased from Thermo Scientific. Other chemicals used are polybrene (Sigma-Aldrich), doxycycline (Dox, Sigma-Aldrich), paclitaxel (Sigma-Aldrich), SB-431542 (EMD Bioscience), 4- (2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Thermo Scientific), pepstatin (Roche), leupeptin (Roche), aprotinin (Roche), and 1,10-phenanthroline (Sigma-Aldrich).

Analysis of TGF-β gene expression signature

Genes (n = 147) that are significantly regulated by TGFβ in at least 3 breast cell lines (Padua *et al*., 2008) were used to establish a TGF-β gene expression signature score. Data of up- (n = 98) and downregulated (n = 49) genes in this signature are retrieved from the The Cancer Genome Atlas (TCGA, 593 patients) for the analysis. The TGF-β signature score "*s*" was calculated as:

$$s = \sum_i w_i x_i / \sum_i |w_i|$$

where w is the weight +1 or -1, depending on whether the gene was up- or downregulated in the signature (Bhola *et al*., 2013) and x is the normalized gene expression.

Lentiviral small hairpin RNA (shRNA) and generation of stable cell lines

shRNA targeting ADAM12 and control shRNA sequences were excised from GIPZ Human ADAM12 shRNA (V2LHS_11814) and non-silencing control lentiviral shRNA (Thermo Scientific) and cloned into pINDUCER10 vector (Addgene) at the XhoI and MluI sites. Lentiviruses were produced by transfecting HEK293T cells with pINDUCER10_shADAM12/shControl, pMD2.G, and psPAX2 plasmids (Addgene) using Mirus TransIT transfection reagent (Mirus). Conditioned medium containing viral partiles was harvested 48h after transfection. SUM159PT and BT549 cells were plated one day before infection at ~20% confluence. Conditioned medium containing viral particles was supplemented with 5ng/ml polybrene. Selection of stably transduced cells started 48 hours after infection using 2μg/ml of puromycin and continued for 10 days.

Flow cytometry

SUM159PT/BT549_shADAM12/shControl cells were incubated without or with 1μg/ml Dox for 4 days. Cells were trypsinized into a single cell suspension at 10^6 cells/100μl, washed with 3% BSA/DPBS, and incubated with monoclonal anti-ADAM12 antibody (R&D System; clone# 632525) or isotype control antibody (R&D System; clone #11711) at a dilution of 1:10 for 30 minutes on ice. Cells were then washed 3 times, incubated with allophycocyanin (APC)-conjugated anti-mouse antibodies (Jackson ImmunoRes) for 30 min on ice. Analysis was performed using a BD FACSCalibur flow cytometer.

Luciferase reporter analysis

SUM159PT_shADAM12 cells were treated without or with 1μg/ml Dox for 4 continuous days. Cells were then transfected with SBE4-*Luc* (Addgene), p3TP-*Lux*, or NF-κB-*Luc* (pGL4.32, Promega) reporter plasmids (0.5 μg per well of a 6-well plate) using X-tremeGENE

HP DNA transfection reagent (Roche) at a 2:1 (reagent:DNA) ratio. A *Renilla* luciferase reporter construct, pRL-TK (Promega), was co-transfected with the reporter plasmid as a transfection control. Transfected cells were incubated with TGFβ1for 24 hours (0, 0.5, 1, and 2 ng/ml TGFβ1 was used for SBE4-*luc* and NF-κB-*luc* reporter assays; 2ng/ml of TGFβ1 was applied to cells transfected with p3TP-*lux* reporter). Forty-eight hours after transfection, the cells were washed with DPBS and then lysed using 1X Passive Lysis Buffer (Promega). The lysates were analyzed for firefly and Renilla luciferase using the Dual Luciferase Reporter Assay Kit (Promega), according to the manufacturer's instructions.

Quantitative real time PCR

One microgram of the total RNA was reverse-transcribed using the SuperScript III First Strand Synthesis system (Invitrogen) and oligo(dT) primers. Quantitative real time PCR (qRT-PCR) was performed using 15 µl in a 96-well format on a CFX96 cycler. The final reaction mixture contained 7.5 µl SYBR Select Master Mix for CFX, 6 µl diluted cDNA (1:10 for *ADAM12* and *TGFBR1* analysis and 1:100 for *COL6A3*, *SERPINE1*, *WNT5A*, *β-ACTIN* analysis) and 0.5 µM primers. The primers used were as follows:

Table 3-1 Primer sequences.

Type of primers		Primer sequence
ADAM12-L	F	5'-AGC CAC ACC AGG ATA GAG AC-3'
	R	5'-CGC CTT GAG TGA CAC TAC AG-3'
β- ACTIN	F	5'-TTG CCG ACA GGA TGC AGA A-3'
	R	5'-GCC GAT CCA CAC GGA GTA CT-3'

TGFBR1	F	5’-GCC AGT CCT AAG TCT GCA AT-3’
	R	5’-GGT CTT GCC CAT CTT CAC A-3’
COL6A3	F	5’-GTT CAG AGA GCT TCC CAA CA-3’
	R	5’-TTC AGA CAC AAA ACG AAG CA-3’
SERPINE1	F	5’-ATT GAT GAC AAG GGC ATG GC-3’
	R	5’-TCT GAT TTG TGG AAG AGG CG-3’
WNT5A	F	5’-AGG GCT CCT ACG AGA GTG CT-3’
	R	5’-GAC ACC CCA TGG CAC TTG-3’

The PCR conditions were: 95°C, 15 s; 60°C, 15 s; 72°C, 60 s. At the conclusion of each run, a melt curve analysis was performed to ensure that a single product had been synthesized. The relative expression of ADAM12-L, normalized to ACTIN, was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting

Cells were treated with lysis buffer at 4°C for15 min. Lysis buffer contained 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 5 mM EDTA, 1 mM 4- (2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 5 µg/mL pepstatin, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 10 mM 1,10-phenanthroline. Extracts were centrifuged for 15 min at 13,000 rpm at 4°C. After centrifugation, the supernatants were mixed with 3xSDS sample loading buffer of twice volume as the lysate. For ADAM12 Western blotting, the supernatants were incubated with concanavalin A agarose (Sigma; 50 µl

resin per 1 ml cell lysate) for 2 hours at 4°C post-centrifugation to enrich for glycoproteins. The resin was washed three times and the glycoproteins were eluted with 3xSDS sample loading buffer.

For cell surface biotinylation, cells grown in 6 well-plates were washed with DPBS, then incubated at 4°C for 60 min with 2.5 mM EZ-link N-hydroxysuccinimide-PEG12-biotin (Pierce) in DPBS. Remaining free reagent was quenched using 100 mM glycine. After washing cells several times with DPBS, cell lysates were collected as described above, and a fraction was retained as the input sample. The remaining lysate was allowed to adsorb onto Neutravidin agarose resin (Pierce) for 60 minutes at 4°C. The resin was washed three times with lysis buffer, followed by elution with 3xSDS sample buffer.

Proteins were resolved using SDS-PAGE (8% gel) and were transferred to a nitrocellulose membrane. The membrane was blocked using 5% non-fat milk and 0.3% Tween-20 in DPBS. Primary antibody was diluted in blocking buffer and incubated with the membrane. The dilutions were: anti-ADAM12 antibody (Ab#3394, 1:10,000, Li *et al*., 2012); anti-α-tubulin (DM 1A, 1:40,000 dilution, Sigma-Aldrich); anti-TGFBR1 (V-22, 1:300, Santa Cruz); anti-EGFR (D38B1, 1:5,000), anti-GAPDH (D16H11, 1:100,000), anti-phospho-SMAD2 (Ser465/467, 138D4, 1:1,000) and anti-SMAD2/3 (D7G7, 1:1,000, Cell Signaling). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were used as secondary antibodies. Detection was performed using the SuperSignal West Pico or Femto Chemiluminescent Substrate (Pierce).

Mammosphere formation analysis

SUM159PT_shADAM12 cells were first treated without or with 1 μg/ml Dox for 4 days and then with 10 nM Paclitaxel for 6 continuous days. Cells were detached with 0.25% trypsin/1

mM EDTA in DPBS and counted by Cellometer TM AutoT4 (Nexcelom Bioscience). Single cell suspensions of $1x10^6$ cells/ml were first passed through cell strainer and then diluted to $3x10^3$ cells/ml with mammosphere media containing MEMB (Lonza), 20 ng/ml hEGF (Life Technologies), 20 ng/ml bFGF (Life Technologies), 4 μg/ml heparin (Sigma), 1xB27 (Life Technologies), and 1x Penicillin/Streptomycin (Lonza). Cell numbers among different treatments were adjusted within 95~105% using CellTiter Glo kit (Promega) according to the manufacturer's instructiosn. Cell suspensions at final dilutions of $3x10^3$ cells/ml were mixed with 3% methylcellulose (R&D Systems) at the ratio of 2:1. The solution was thoroughly mixed by vortexing and incubated at room temperature for 10 min. Five hundred μl of the mixture (10^3 cells in 1% Methylcellulose) was seeded into one well of 24-well ultra-low attachment plate (Corning). After 7 days, cells were analyzed using a phase contrast microscopy and a 4x objective, and six randomly selected areas from each well were photographed. The number of the colonies in each image was analyzed by Image J.

Statistical analysis

Pearson and Spearman correlation coefficient were calculated using the GraphPad Prism 6.0 software. All reported *P* values are two-sided. Statistical significance between two data sets was evaluated by Student's *t*-test. *P* values < 0.05 was considered statistically significant.

Results

ADAM12 mRNA is correlated with the TGFβ signature and is induced by TGFβ in breast cancer cells

We first examined the correlation of *ADAM12* mRNA level with TGFβ-responsive gene signature score (Bhola *et al*., 2013) in 593 breast cancer patients (the UNC AgilentG4502A_07,

data available at The Cancer Genome Atlas, http://cancergenome.nih.gov). The TGFβ-responsive gene signature was first evaluated by Padua and his colleagues in four breast cancer cell lines (Padua *et al*., 2008). The signature includes one hundred fifty three genes that were significantly changed by TGFβ in at least two cell lines. Our analysis demonstrated that *ADAM12* mRNA expression was strongly correlated with the TGFβ gene score in breast cancers (Spearman r = 0.5765, $P < 0.0001$) (Figure 3.1A).

Next, we asked whether ADAM12 is a TGFβ responsive gene or a regulator of TGFβ signaling in breast cancer cells. To this end, we measured *ADAM12* mRNA in SUM159PT, SUM149PT, and SUM102PT cells after 3 days of treatment with TGFβ using qRT-PCR. The results revealed that *ADAM12* mRNA levels were significantly increased after TGFβ treatment in all three cell lines ($P < 0.05$) (Figure 3.1B). We also examined ADAM12 protein levels by Western blotting in breast cancer cells (SUM1315MO2, MCF10DCIS.com, SUM159PT, and SUM102PT) incubated with or without TGFβ. Consistent with our qRT-PCR results, ADAM12 protein expression was strongly elevated by TGFβ treatment in all four cell lines (Figure 3.1C) compared to untreated cells. These results suggest that ADAM12 is induced by TGFβ signaling.

TGFβR1 expression is regulated by ADAM12

A previous study has shown that ADAM12 enhances TGFβ signaling via modulation of TGFβR2 in the early endosomes (Atfi *et al.*, 2007). Since the activation of TGFβ signaling requires both TGFβR1 and TGFβR2, we wondered whether ADAM12 regulates TGFβR1 in breast cancers. To address this question, we established SUM159PT and BT549 breast cancer cell lines stably expressing doxycycline inducible ADAM12 shRNA system (Figure 3.2A). Upon treating cells with doxycycline (Dox, 1μg/ml) for 4 days, ADAM12 expression was strongly downregulated in both cell lines (Figure 3.2B).

Interestingly, the amount of TGFβR1 protein (MW~58kDa) was decreased in both SUM159PT (Figure 3.2D) and BT549 cells (Figure 3.2F) after ADAM12 knockdown. The decrease of TGFβR1 protein was more pronounced after TGFβ treatment (Figure 3.2D-G). To confirm the specificity of anti-TGFβR1 antibody, we transfected SUM159PT and BT549 cells with siRNAs targeting TGFβR1. The blot showed that the band at ~58kDa was significantly decreased after treating cells with TGFβR1 siRNAs (Figure 3.2H). Furthermore, we performed cell surface biotinylation assay in BT549_shADAM12 cells after TGFβ treatment to measure the expression of TGFβR1 at cell surface. In agreement with our findings described above, ADAM12 knockdown decreased the amount of biotinylated TGFβR1 almost 2-fold compared to the control cells (Figure 3.2I).

ADAM12 knockdown attenuates TGFβ signaling

To examine the effect of ADAM12 downregulation on TGFβ signaling, we first examined the phosphorylation of SMAD2 induced by TGFβ. ADAM12 knockdown was induced in SUM159PT by doxycycline treatment, which was then followed by treatment with different doses of TGFβ. Western blotting indicated that ADAM12 knockdown attenuated the phosphorylation of SMAD2 in response to TGFβ (Figure 3.3A).

Next, we used dual-luciferase assay as an alternate approach to evaluate the inhibitory effect of ADAM12 knockdown on TGFβ signaling. We co-transfected SUM159PT_shADAM12 cells with the SBE4-*luc* reporter and pRL-TK, a control *Renilla* luciferase reporter. Our result suggested that downregulation of ADAM12 efficiently diminished the increase of the SBE4-*luc* reporter activity in response to TGFβ (Figure 3.3B, $P < 0.05$). Interestingly, a strong decrease of SBE4-*luc* reporter activity was observed in cells with ADAM12 downregulation even in the absence of added TGFβ (Figure 3.3B). The results obtained for another TGFβ signaling reporter,

p3TP-*lux,* was consistent with the SBE4-*luc* reporter showing the compromised reporter activity after reducing ADAM12 expression (Figure 3. 3C).

Studies have shown that TGFβ-TGFβR1 regulates the NF-κB signaling pathway through the activation of TGFβ-associated kinase1 (TAK1) in a SMAD-independent manner (Ataie-Kachoie *et al*., 2013; Gingery *et al*., 2008). Here, we used a NF-κB-*luc* reporter to test whether ADAM12 knockdown would affect the activation of the TAK1-mediated non-canonical TGFβ signaling pathway. Our results demonstrated that SUM159PT cells with ADAM12 downregulation had a significant decrease of the NF-κB-*luc* reporter activation in response to different doses of TGFβ (Figure 3.3D), suggesting that ADAM12 regulates non-canonical TGFβ signaling pathways.

Next, we asked whether ADAM12 knockdown had an effect on TGFβ responsive genes. To answer this question, we checked the expression of several typical TGFβ target genes in SUM159PT cells. *COL6A3*, *SERPINE1*, and *WNT5A* are well-studied TGFβ targeted genes and their protein products play important roles in cell-cell adhesion, fibrinolysis, proliferation, migration, and other cellular events (Verrecchia *et al*., 2001; Otsuka *et al*., 2006; Kikuchi *et al*., 2012). First, we downregulated ADAM12 in SUM159PT cells and then treated cells with TGFβ. At the end of the experiment, total RNA was extracted and TGFβ target gene expression was analyzed by qRT-PCR. The results demonstrated that ADAM12 knockdown significantly reduced the increase of *COL6A3* (Figure 3.3E, $P < 0.01$), *SERPINE1* (Figure 3.3F, $P < 0.05$), and *WNT5A* mRNA (Figure 3.3G, $P < 0.01$) in response to TGFβ. Together, these results suggest that ADAM12 is not only associated with the TGFβ gene signature, but it is also actively involved in the regulation of TGFβ signaling.

ADAM12 regulates TGFβR1 expression through a TGFβ independent pathway

Next, we asked the question of how ADAM12 regulates TGFβR1 expression. First, we tested the effect of ADAM12 knockdown on the stability of TGFβR1 protein. Our cycloheximide chasing experiment showed no significant change of TGFβR1 expression when ADAM12 was downregulated (data not shown). Therefore, we investigated whether ADAM12 knockdown affects *TGFβR1* mRNA levels. Our qRT-PCR results indicated that TGFβ induced a significant increaseof *TGFβR1* mRNA (Figure 3.4A, ~ 2.6-fold, $P < 0.01$). ADAM12 knockdown not only completely diminished the elevation of *TGFβR1* mRNA triggered by TGFβ (Figure 3.4A, $P < 0.0001$) but also was able to decrease the basal level of expression of TGFβR1 mRNA (with the absence of TGFβ) (Figure 3.4A, $P < 0.01$).

Given the observation that *TGFβR1* mRNA was responsive to TGFβ and that the loss of ADAM12 expression impairs the TGFβ signaling pathway, we speculated that ADAM12 might regulate *TGFβR1* mRNA expression through the TGFβ signaling pathway, in a positive feedback loop. To address this hypothesis, we used SB-432542, a potent TGFβR1 inhibitor, to block the TGFβ pathway. Our rationale was that if ADAM12 regulated *TGFβR1* mRNA through TGFβ signaling, the decrease of *TGFβR1* mRNA caused byADAM12 downregulation should not occur in the presence of the inhibitor. Unexpectedly, we observed that ADAM12 knockdown was led to inhibition of *TGFβR1* mRNA expression even in the presence of SB-431542 (Figure 3.4A, with no TGFβ $P < 0.0001$; with TGFβ, $P < 0.05$). Altogether, our results indicated that ADAM12 regulated *TGFβR1* mRNA expression through a novel pathway that did not require an active TGFβR1.

ADAM12 downregulation attenuates the sphere formation induced by TGFβ

We asked about the biological consequence of the regulation of TGFβ signaling by ADAM12. It has been previously shown that TGFβ is a potent EMT inducer and increases the ability of cancer cells to form spheres under suspension conditions (Bhola *et al*., 2013). In addition, Bhola and coworkers showed that paclitaxel (PTX) treatment expanded cancer stem cell population in triple-negative breast cancers through TGFβ signaling. As a result, breast cancer cells after PTX treatment formed higher number spheres (Bhola *et al*., 2013).

In this study, we downregulated ADAM12 expression in SUM159PT cells, treated the cells with TGFβ, and seeded single cell suspensions in 1% methylcellulose in ultra low-attachment plate for sphere formation. We observed that ADAM12 knockdown efficiently diminished the effect of TGFβ on sphere size, further confirming that ADAM12 knockdown compromised cellular responses to TGFβ (Figure 3.5B, $P < 0.05$). When SUM159PT cells were incubated with PTX for 6 days, the surviving cells in which ADAM12 expression was reduced formed fewer spheres (Figure 3.5D) and the resulting spheres were smaller in size (Figure 3.5C). These results suggest that ADAM12 knockdown also impaired cellular responses to the endogenous TGFβ produced in response to PTX.

Discussion

TGFβ signaling is an important promoter of tumor progression in late stage cancers (Gomes *et al*., 2012; Bierie & Moses, 2010; Massagué *et al*., 2012). As a potent EMT inducer, TGFβ signaling causes the loss of cell polarity, equips cells with mesenchymal characteristics, and prepares the microenvironment for tumor dissemination (Xu *et al*., 2009; Pickup *et al*., 2013; Papageorgis *et al*., 2015). Our analysis of the TCGA transcriptomic datasets for a large number

of breast tumors revealed that ADAM12 expression is strongly associated with the TGFβ gene signature. We further demonstrate that the loss of ADAM12 significantly impairs both SMAD-dependent and SMAD-independent TGFβ signaling in breast cancer cells. Our study is the first report showing the role of ADAM12 in the regulation of TGFβ signaling in breast cancer cells, though overexpressed ADAM12 had been previously shown to contribute to TGFβ signaling in mouse myoblasts and liver carcinoma cells (Atfi *et al*., 2007).

TGFβ receptors provide an important regulatory point for the activation of TGFβ signaling pathway (Rojas *et al*., 2009). Much attention has been focused on the regulation of TGFβR2 due to the fact that TGFβR2 is constitutively active and is required for the activation of TGFβR1 (Yamane *et al*., 2003; Liang *et al*., 2015; Zhong *et al*., 2012; Di Guglielmo *et al*., 2003). ADAM12 has been previously demonstrated to enhance TGFβ signaling by increasing the amount of TGFβR2 in early endosomes as well as preventing TGFβR2 from interacting with SMAD7 for degradation (Atfi *et al*., 2007). The results of our current study uncover that ADAM12 positively regulates TGFβ signaling via modulating *TGFBR1* mRNA levels. In the present study, the reduced ADAM12 expression caused a dramatic decrease of TGFβR1 at the protein level (Figure 3.2D-G, I), whereas TGFβR2 did not change (data not shown). At the mRNA level, the downregulation of ADAM12 attenuated *TGFBR1* mRNA levels, and this effect was even more pronounced in the presence of TGFβ. Intriguingly, our results suggest that ADAM12 modulates *TGFBR1* mRNA through a TGFβ signaling-independent manner, because a TGFβR inhibitor could not block the decrease of *TGFBR1* mRNA mediated by ADAM12 downregulation. A possible explanation for the discrepancy between our results and those by Atfi *et al*. is that the latter study was performed using ADAM12 overexpressing cells, which did not accurately replicate the effect of endogenous ADAM12. Besides, we did not observe any

change in TGFβR2 expression after ADAM12 knockdown, indicating ADAM12 might not participate in the stabilization of TGFβR2 in breast cancer cells. The question of whether ADAM12 regulates the expression of TGFβR2 at mRNA level needs to be further studied.

Though TGFβR1 is a critical component of SMAD-dependent TGFβ signaling pathway, the current knowledge regarding the regulation of TGFβR1 is very limited. Studies have shown that endocytosis is a main regulator of TGFβR1 at the cell surface (He *et al*., 2015; Di Guglielmo *et al*., 2003; O'Brien *et al*., 2015). In addition, Notch signaling has been shown to upregulate the mRNA levels of TGFβR1 through inhibition of neuropilin-1 (Nrp1), a membrane receptor interacting with VEGF and other factors (Aspalter *et al*., 2015). Coincidentally, our previous work demonstrated that ADAM12 was able to cleave Notch receptor ligand Delta-like 1 and to activate Notch reporters (Dyczynska *et al*., 2007). More work is needed to unveil the TGFβ independent pathway through which ADAM12 modulates *TGFBR1* mRNA levels.

Given the fact that ADAM12 expression is also regulated by TGFβ (Li *et al*., 2013; Ruff *et al*., 2015), there is an interesting reciprocal relationship between ADAM12 and TGFβ signaling: ADAM12 is not only regulated by TGFβ signaling but it also potentiates TGFβ signaling. This effect is, to an extent, consistent with the observation that ADAM12 expression is particularly elevated in claudin-low subtype breast cancer (Li *et al*., 2013), which is characterized by high mesenchymal signatures and activation of the TGFβ pathway.

In conclusion, our present study describes a new role of ADAM12 in the regulation of TGFβ signaling in human breast cancers. Importantly, the expression of ADAM12 significantly and positively regulates the activation of TGFβ signaling via modulation of *TGFBR1* mRNA in breast cancer cells.

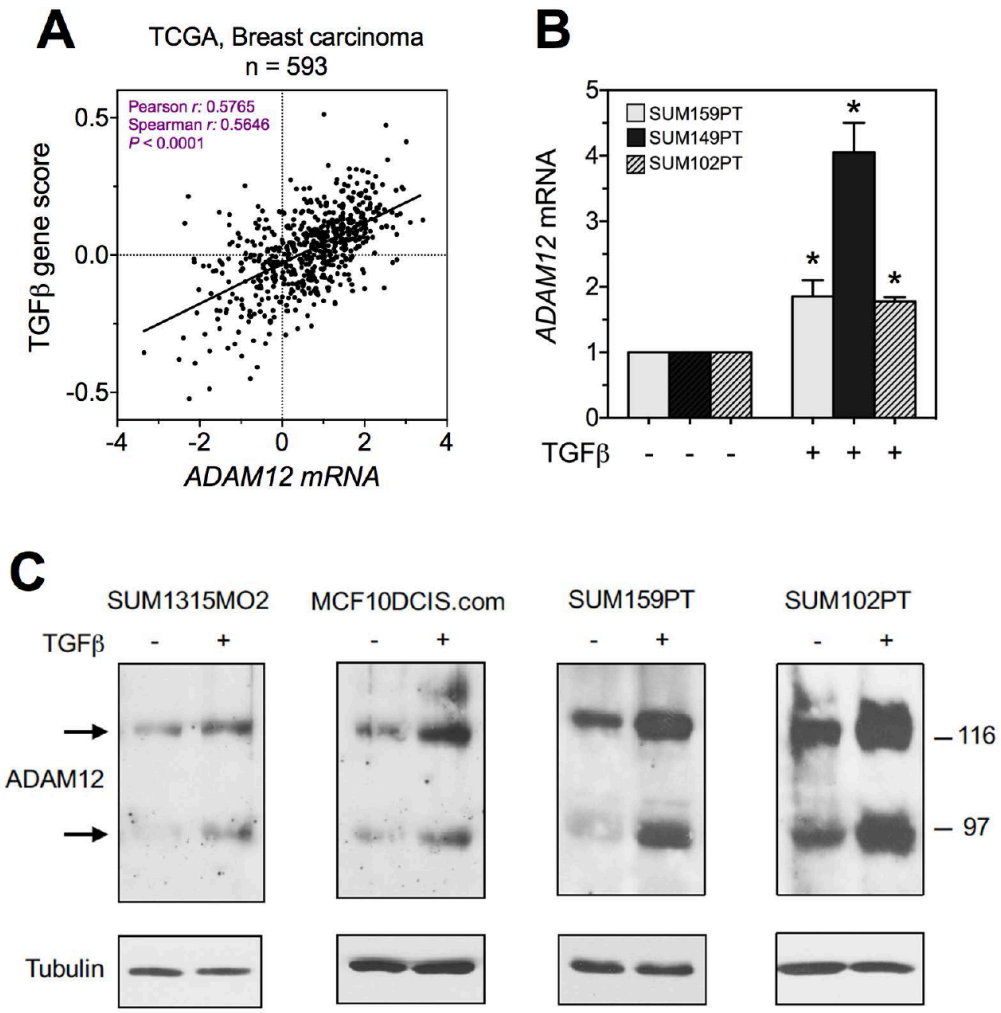
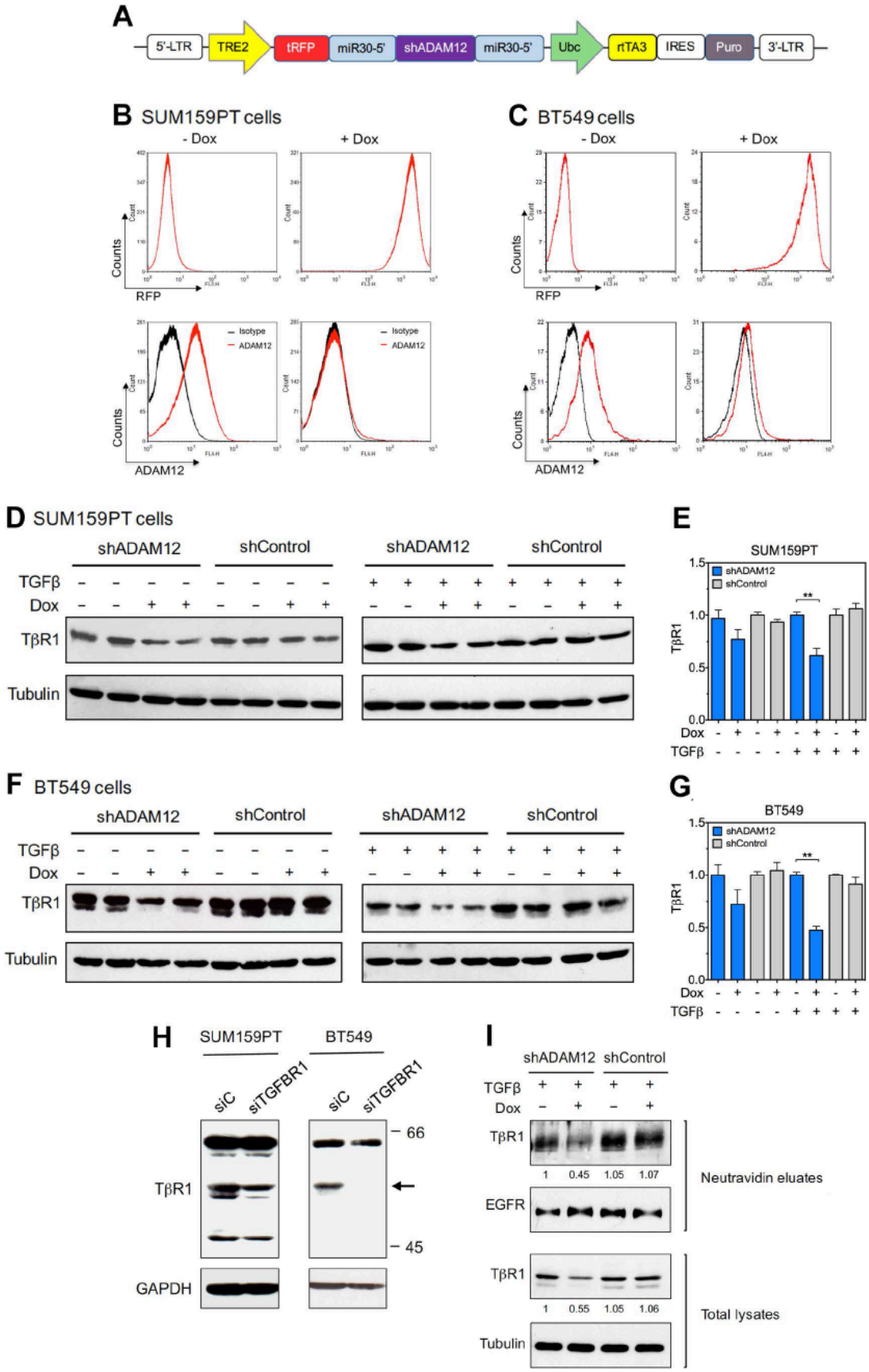


Figure 3.1 ADAM12 expression is positively associated with TGFβ signaling in human breast cancer.

(Panel B was performed by Linda Alyahya.)

(**A**) Correlation analysis between *ADAM12* mRNA expression and the TGFβ gene signature scores of 593 breast cancer patients from the TCGA database. The TGFβ gene expression scores were calculated based on ref. Bhola *et al*., 2013 and Padua *et al*., 2008. (**B**) SUM159PT, SUM149PT, and SUM102PT cells were treated with 2 ng/ml TGFβ for 3 days. *ADAM12* mRNA levels were measured by qRT-PCR and normalized to *β-ACTIN*. Fold change in TGFβ-treated cells *versus* no TGFβ treatment were shown as the average values ± SEM. (n = 3; one sample *t* test, * $P < 0.05$). (**C**) ADAM12 protein levels before and after 3-day treatment with 2 ng/ml TGFβ in different cell lines were detected by Western blotting; Tubulin is a gel-loading control. Arrows indicate the nascent and the mature forms of ADAM12, respectively.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5'-LTR
TRE2
tRFP
miR30-5'
shADAM12
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Ubc
rtTA3
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Puro
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B SUM159PT cells
- Dox
+ Dox
Counts
RFP
Isotype
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ADAM12
C BT549 cells
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EGFR
Neutravidin eluates
TβR1
1 0.55 1.05 1.06
Tubulin
Total lysates

Figure 3.2 ADAM12 downregulation decreases the expression of TGFβR1 in SUM159PT and BT549 cells.

(**A**) A diagram of the doxycycline-inducible lentiviral shRNA construct targeting ADAM12. 5'-LTR, 5'-long terminal repeat; TRE, tetracycline-inducible promoter; tRFP, turbo red fluorescent protein; miR30-5' and shADAM12, micro-RNA-30 adapted shRNA targeting ADAM12; Ubc, human ubiquitin C promoter; rtTA3, reverse tetracycline-transactivator 3; IRES, internal ribosomal entry site; Puro, puromycin resistance gene; 3'-LTR, 3'-long terminal repeat. (**B**, **C**) Validaton of ADAM12 knockdown. SUM159PT (**B**) and BT549 (**C**) cells expressing inducible ADAM12 shRNA (SUM159PT or BT549_shADAM12) were treated with 1 μg/ml Dox for 4 days. ADAM12 expression at the cell surface was measured by flow cytometry. (**D**-**G**) Stably transduced SUM159PT_shADAM12/shControl (**D**, **E**) and BT549_shADAM12/Control (**F**, **G**) cells were incubated for 4 days with 1 μg/ml of Dox, followed by 3-day TGFβ treatment. Cell lysates were analyzed by Western blotting using anti-TGFβR1 (TβR1) antibody; Tubulin is a gel loading control. Band intensities were measured using Image J. The average fold changes in TGFβR1 of SUM159PT (**E**) and BT549 (**G**) cells under each condition are shown as the average value ± SEM. (n = 3; unpaired Student *t* test, ** $P < 0.01$). (**H**) SUM159PT and BT549 cells were transfected with a pool of four siRNAs targeting TGFβR1 or a pooled control siRNAs. Expression levels of TGFβR1were analyzed by Western blotting; GAPDH is a gel loading control. Arrow indicates the position of TGFβR1. (**I**) BT549_shADAM12/shControl cells were sequentially incubated with 1 μg/ml of Dox for 4 days and 2 ng/ml of TGFβ for 3 days. Cells were then incubated with NHS-PEG_{12}-bition, followed by purification of biotinylated proteins using NeutaAvidin beads and Western blotting; EGFR is the positive control for biotinylation and Tubulin is a gel loading control. Band intensities were quantified by Image J.

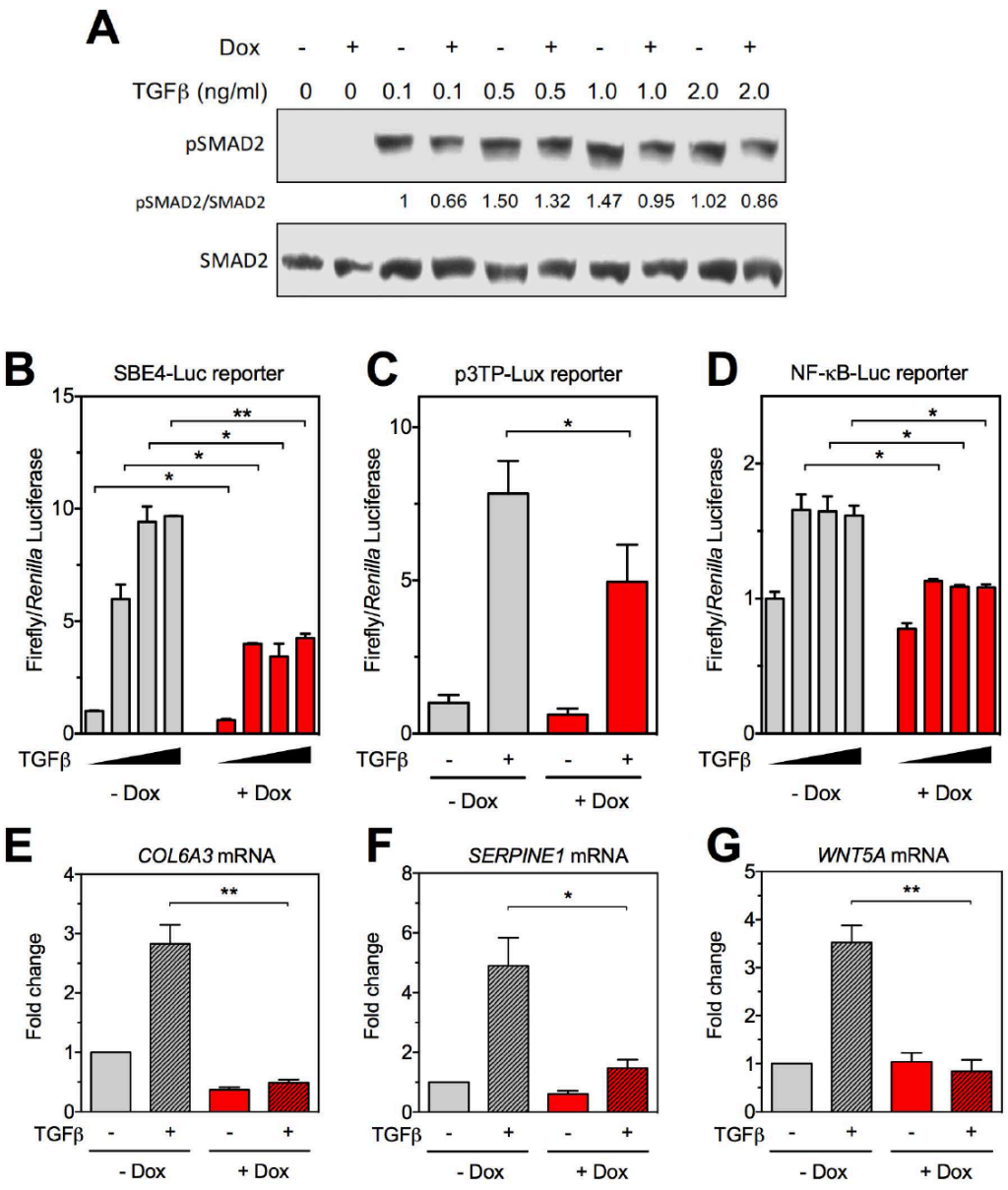

Figure 3.3 ADAM12 knockdown impairs SMAD-dependent and SMAD-independent TGFβ downstream signaling in SUM159PT cells.

(Panel E-G were performed by Dr. Sara Duhachek-Muggy.)

(**A**) Dox pre-treated SUM159PT_shADAM12 cells were incubated with 0, 0.1, 0.5, 1, and 2 ng/ml TGFβ for 3 days. Protein levels of phosphorylated SMAD2 and SMAD2 were analyzed by Western blotting using specific antibodies. (**B-D**) SUM159PT_shADAM12 cells were treated with 1 μg/ml Dox for 4 days and then were transfected with SBE4-*Luc* (**B**), p3TP-*Lux* (**C**), or NF-κB-*Luc* (**D**) reporters, and a *Renilla* luciferase control vector. Transfected cells

were treated with different amounts of TGFβ for 24 hours and were collected for dual-luciferase gene reporter assay 48 hours after transfection. The activities of *Firefly* luciferase reporters were normalized to *Renilla* luciferase activity and were shown as the average value ± SEM (n = 3; unpaired Student *t* test, * $P < 0.05$, ** $P < 0.01$). (**E**-**F**) Dox pre-treated SUM159PT_shADAM12 cells were incubated with 2 ng/ml TGFβ for 3 days. *COL6A3* (**E**), *SERPINE1* (**F**), and *WNT5A* (**G**) mRNA expression levels were analyzed by qRT-PCR and normalized to β-ACTIN and to the control cells (with neither Dox nor TGFβ treatment). The fold changes are presented as the average values of three independent experiments ± SEM. (n = 3; one sample *t* test, * $P < 0.05$, ** $P < 0.01$).

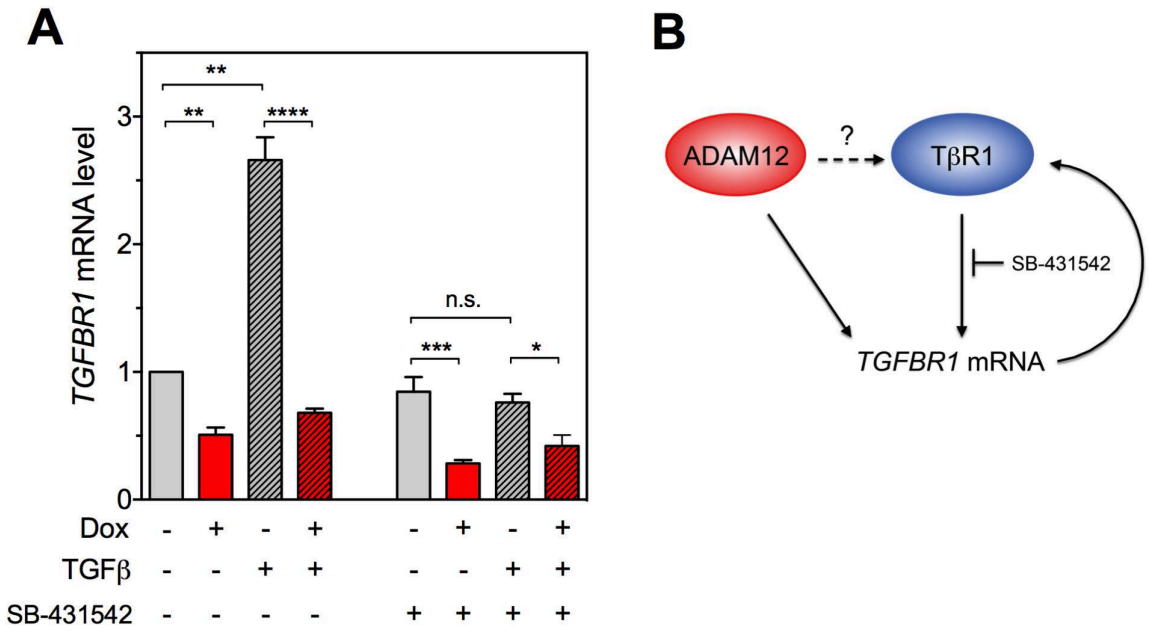

Figure 3.4 ADAM12 negatively regulates the expression of *TGFBR1* mRNA in SUM159PT cells.

(**A**) SUM159PT_shADAM12 cells were treated with 1 μg/ml Dox for 4 days and then incubated with TGFβ or SB431542, an inhibitor of TGFβR1, for 3 days. *TGFBR1* mRNA expression levels were analyzed by qRT-PCR and normalized to *β-ACTIN* and to the control cells (with no treatments). The fold changes in *TGFBR1* mRNA expression are presented as the average values of three independent experiments ± SEM (n = 3; unpaired Student *t* test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). (**B**) A diagram for the regulation of *TGFBR1* mRNA by ADAM12 in breast cancer cells. TGFβR1-mediated TGFβ signaling activation induces the expression of *TGFBR1* mRNA, which can be blocked by TGFβR1 inhibitor SB431542. ADAM12 positively modulates *TGFBR1* mRNA expression in a manner that is independent of TGFβ signaling. More data are needed to exclude the possibility that ADAM12 regulates TGFβ signaling via direct interaction with TGFβR1 at the protein level.

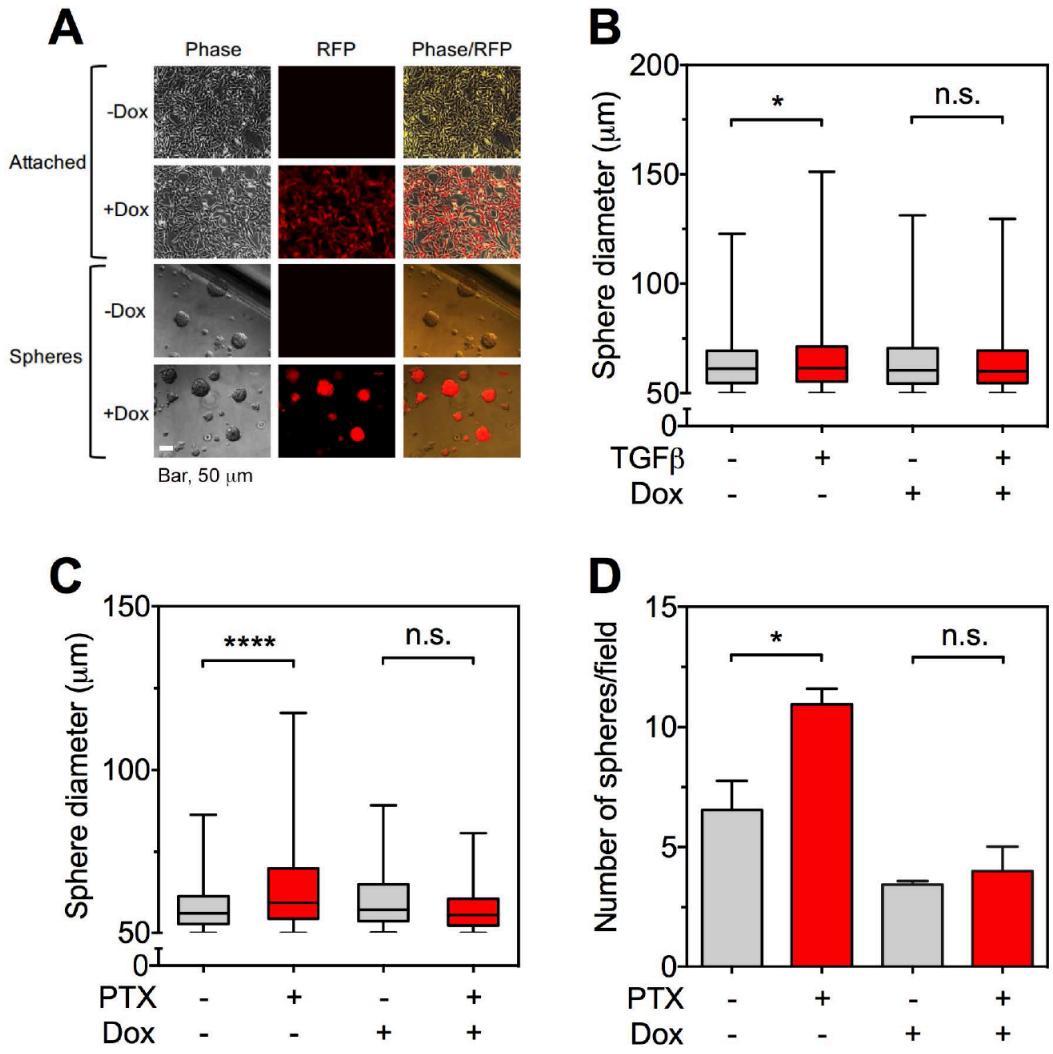

Figure 3.5 The effect of ADAM12 knockdown on mammosphere growth induced by TGFβ.

(**A**) SUM159PT_shControl cells were pre-incubated for 4 days without or with Dox and were further grown for 10 days in mammosphere media in the absence or presence of doxycycline. Representative phase contrast and fluorescent images are shown. (**B-D**) SUM159PT_shADAM12 cells were pre-incubated for 4 days without or with Dox and then were further treated with vehicle, 2 ng/ml TGFβ, or 10 nM paclitaxel (PTX), respectively, for another 6 days. Cells were seeded at the density of 1000 cells/well in a 24-well ultra-low attachment plates. After 7 days, spheres were visualized by phase contrast imaging and counted using Image J. (**B**, **C**) Size distribution of spheres formed after TGFβ (**B**) and paclitaxel (**C**) treatments are shown as box-and-whisker plots. Sphere diameters were determined using Image J. The results

were obtained in 3 independent experiments (n = 3; unpaired Student *t* test, * $P < 0.05$, **** $P < 0.0001$). (**D**) The numbers of spheres formed after paxlitaxel treatment with diameters larger than 50 μm were counted by Image J and are shown as the mean values ± SEM. (n = 3; unpaired Student t test, * $P < 0.05$).

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Chapter 4 - Phenotypic diversity of breast cancer-related mutations in metalloproteinase-disintegrin ADAM12

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Abstract

Six different somatic missense mutations in the human *ADAM12* gene have been identified so far in breast cancer. Five of these mutations involve highly conserved residues in the extracellular domain of the transmembrane ADAM12-L protein. Two of these extracellular mutations, D301H and G479E, have been previously characterized in the context of mouse ADAM12. Three other mutations, T596A, R612Q, and G668A, have been reported more recently, and their effects on ADAM12-L protein structure/function are not known. Here, we show that ADAM12-L bearing the G668A mutation is largely retained in the endoplasmic reticulum in its nascent, full-length form, with an intact N-terminal pro-domain. The T596A and R612Q mutants are efficiently trafficked to the cell surface and proteolytically processed to remove their pro-domains. However, the T596A mutant shows decreased catalytic activity at the cell surface, while the R612Q mutant is fully active and comparable to the wild-type ADAM12-L. The D301H and G479E mutants, consistent with the corresponding D299H and G477E mutants of mouse ADAM12 described earlier, are not proteolytically processed and do not exhibit catalytic activity at the cell surface. Among all six breast cancer-associated mutations in ADAM12-L, mutations that preserve the activity - R612Q and L792F - occur in triple-negative

breast cancers, while loss-of-function mutations - D301H, G479E, T596A, and G668A - are found in non-triple-negative cancers. This apparent association between the catalytic activity of the mutants and the type of breast cancer supports a previously postulated role of an active ADAM12-L in the triple-negative breast cancer disease.

Introduction

Disintegrin and metalloproteinase domain-containing protein ADAM12 is a member of the ADAM family of proteins that mediate cleavage of substrates at the cell surface and/or modulate intracellular signaling pathways (Edwards *et al*., 2008; Weber & Saftig, 2012). ADAM12 is highly up-regulated in human breast tumors (Iba *et al*., 1999; Kveiborg *et al*., 2005; Lendeckel *et al*., 2005; Mitsui *et al*., 2006; Bertucci *et al*., 2006; Turashvili *et al*., 2007; Roy & Moses, 2011). In triple-negative breast cancers (TNBCs, lacking estrogen receptor and progesterone receptor expression and *ERBB2* gene amplification), high expression of *ADAM12-L,* but not *ADAM12-S,* mRNA is associated with poor prognosis (Li *et al*., 2012). *ADAM12-L* and *ADAM12-S* are two different splice variants that encode the long, transmembrane protein isoform ADAM12-L and the short, secreted ADAM12-S, respectively (Kveiborg *et al*., 2008).

Among thirteen different *ADAM* genes that encode catalytically active proteases (Edwards *et al*., 2008), *ADAM12* is the most frequently somatically mutated gene in human breast cancers. As of September 2013, the COSMIC database (Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) listed 6 confirmed somatic missense mutations in the *ADAM12* gene per a total of 1104 unique breast carcinoma samples analyzed. The frequencies of breast cancer-associated missense mutations in other genes encoding catalytically active *ADAMs* were: 1/973 in *ADAM9*, 1/973 in *ADAM10*, 1/1147 in

ADAM17, 1/1010 in *ADAM19*, 1/973 (plus one nonsense mutation) in *ADAM20*, and 1/973 in *ADAM30*. No other missense/nonsense somatic mutations were reported for the remaining *ADAM* genes encoding catalytically active proteases (i.e., *ADAM8, -15, -21, -28, -33*, and *ADAMDEC1*). A relatively high frequency of mutations in the *ADAM12* gene can be attributed to the fact that *ADAM12* is located on human chromosome 10q26.2, in a region capable of forming highly stable secondary structures (Dillon *et al*., 2013).

The six breast cancer-associated mutations in the ADAM12-L protein include the D301H mutation in the metalloproteinase domain, G479E in the disintegrin domain, T596A and R612Q in the cysteine-rich domain, G668A in the epidermal growth factor (EGF)-like domain, and L792F in the cytoplasmic tail (Sjoblom *et al*., 2006; Shah *et al*., 2012; Jiao *et al*., 2012) (Figure 4.1A). We have previously shown that the D299H and G477E mutations in mouse ADAM12 (which correspond to the D301H and G479E mutations in human ADAM12) are loss-of-function mutations that inhibit the intracellular trafficking and proteolytic activation of the nascent ADAM12 protein (Dyczynska *et al*., 2008). The L792F mutation in human ADAM12-L was reported not to affect protein processing, localization, or function (Dyczynska *et al*., 2008; Stautz *et al*., 2012). The other three mutations - T596A, R612Q, and G668A - have been identified more recently (Shah *et al*., 2012; Jiao *et al*., 2012), and their effects on the structure/function of ADAM12 are currently unknown.

Here, we show that there is functional diversity between the three recently identified mutations. While ADAM12-L containing the G668A mutation is largely retained in the endoplasmic reticulum (ER) and is not proteolytically activated in the Golgi, the T596A mutant is properly trafficked and proteolytically processed but is still catalytically inactive. The R612Q mutant is trafficked, processed, active, and is indistinguishable from the wild-type (WT)

ADAM12-L. Taking into consideration all six known breast cancer-associated somatic mutations in ADAM12-L, we note an apparent association between the catalytic activity of ADAM12-L mutants and the molecular characteristic of the tumor. The two mutations that do not have any effect on ADAM12-L activity, R612Q and L792F, occurred in TNBCs. The four mutations that render ADAM12-L inactive, i.e., D301H, G479E, G668A, and T596A, were described in non-TNBCs. This observation further suggests an important role of the catalytically active ADAM12-L in the triple-negative breast cancer disease.

Materials and Methods

Expression Constructs

Retroviral expression vector *ADAM12-L*-pBABEpuro was used for the expression of the wild-type (WT) ADAM12-L protein. The D301H, G479E, T596A, R612Q and G668A point mutations were introduced by site-directed mutagenesis using the QuickChange kit (Stratagene). The entire lengths of all DNA inserts were sequenced to confirm that no other mutations were introduced during mutagenesis. The expression construct of Delta-like 1 (DLL1) in pIRESpuro vector was described earlier (Dyczynska *et al*., 2007).

Cell Culture and Treatment

Human MCF10A mammary epithelial cells (ATCC) were cultured in DMEM/F12 (1：1) supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 20 ng/ml human EGF, 10 μg/ml insulin, 100 ng/ml cholera toxin, and 1% penicillin/streptomycin. Retrovirus production and stable transduction of MCF10A cells with viruses encoding WT or mutant ADAM12-L, or with control viruses bearing empty pBABEpuro vector, were performed as described previously (Li *et al*., 2013). Transient transfections were performed using X-tremeGENE HP transfection reagent

(Roche). For protein stability assay, cells were treated with 10 μg/ml cycloheximide (EMD Millipore) in culture medium for indicated times.

Antibodies

Anti-ADAM12 rabbit polyclonal antibody (Ab 3394) specific for the cytoplasmic domain of human ADAM12-L was developed in our laboratory, as described (Li *et al*., 2012). The remaining antibodies were: anti-ADAM12 mouse mAb (R&D Systems; clone 632525, specific for the extracellular domain of ADAM12-L), anti-KDEL mouse mAb (clone 10C3, Enzo Life Sciences), anti-EGFR (D38B1) XP rabbit mAb (Cell Signaling), anti-phospho-EGFR (pY1173) rabbit polyclonal Ab (R&D Systems), anti-DLL1 rabbit polyclonal Ab (H-265, Santa Cruz Biotechnology), anti-β-actin mouse mAb (clone AC-15, Sigma), and anti-α-tubulin mouse mAb (clone DM 1A, Sigma).

Immunofluorescence

Stably transduced MCF10A cells were plated on glass coverslips placed in 6-well plates. Two days later, cells were fixed with 3.7% paraformaldehyde/DPBS for 20 min, followed by permeabilization with 0.1% Triton X-100/DPBS for 5 min. Coverslips were incubated with anti-ADAM12-L polyclonal antibody (1：500 dilution) and anti-KDEL antibody (1：200 dilution), followed by incubation with rhodamine Red-X-conjugated anti-rabbit IgG antibody, Alexa 488-conjugated anti-mouse IgG antibody, and DAPI. Immunofluorescence was examined using an Axiovert 200 inverted fluorescent microscope.

Flow Cytometry

Cells were trypsinized into a single cell suspension, washed with DPBS containing 3% BSA, and incubated with anti-ADAM12 monoclonal antibody or isotype control antibody (R&D

Systems, both at 1：10 dilution) for 30 minutes on ice. Cells were then washed 3 times, incubated with allophycocyanin (APC)-conjugated anti-mouse antibodies (Jackson ImmunoResearch; 1：100) for 30 min on ice, washed again, and then incubated with 1 μg/ml propidium iodide (PI; BD Biosciences) for viability. Analysis was performed using a BD FACSCalibur flow cytometer. Only the cells negative for PI staining (viable cells) were selected for the ADAM12 analysis.

Cell Surface Biotinylation and Western Blotting

Cells were washed with DPBS, incubated for 60 min at 4°C with 2.5 mM EZ-link NHS-PEG_{12}-biotin (Thermo Scientific), and then washed with ice-cold 100 mM glycine/DPBS. Cellular proteins were extracted with extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl)-benzene-sulfonylfluoride hydrochloride (AEBSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM 1,10-phenanthroline; 0.5 ml buffer/well in a 6-well plate). Cell extracts were centrifuged at 21,000×g for 15 min at 4°C, and supernatants were incubated for 1 h at 4°C with NeutrAvidin sepharose (GE Healthcare; 0.5 ml cell extract/25 μl of resin). The resin was washed three times with extraction buffer, eluted with SDS sample buffer; samples were then resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting was performed using anti-ADAM12 polyclonal (1：20,000 dilution), anti-DLL1 (1：1,000), anti-EGFR (1：5,000 dilution), anti-pY1173 EGFR (1：5,000) primary antibodies and HRP-conjugated secondary antibodies, as described (Li *et al*., 2012). Signal detection was performed using WestPico chemiluminescence detection kit (Pierce).

Endo H Treatment

Stably transduced cells were treated with extraction buffer and centrifuged at 21,000×g for 15 min at 4°C. Supernatants were treated with EndoH$_f$ denaturing buffer (New England BioLabs), boiled, and then treated with Endo H$_f$ (3,000 U), according to the manufacturer's instructions.

Determination of Cell Doubling Times

Stably transduced cells were seeded in 6-well plates at the density of 60,000 cells/well. After 24, 48, and 72 h, cells were detached and counted with Cellometer AutoT4 (Nexcelom Bioscience), in duplicates. Exponential growth curves were fitted to each dataset and the doubling times were calculated using a nonlinear regression function in the GraphPad Prism 5.0 software.

Evaluation of ADAM12-L-mediated Shedding of EGFR Ligands

MCF10A cells stably overexpressing WT or mutant ADAM12-L proteins, or cells stably transduced with empty pBABEpuro vector, were incubated for 16 h in serum-free media. Conditioned media were then collected, pre-cleared by centrifugation, and added to the duplicate wells containing "reporter" empty vector-transduced MCF10A cells that were pre-incubated for 16 h in serum-free medium. After 30 min, cells were washed with DPBS, treated with extraction buffer containing phosphatase inhibitors 50 mM NaF, 2 mM Na_3VO_4, and 10 mM $Na_4P_2O_7$, and analyzed by SDS-PAGE and Western blotting using anti-phospho-EGFR (pY1173) and anti-EGFR antibodies.

Cell Migration Assay

MCF10A cells with stable overexpression of WT or mutant ADAM12-L proteins, or control empty vector-transduced cells, were suspended in MCF10A medium containing 0.1% BSA instead of horse serum, and seeded in the upper chambers of Transwell inserts with a 8-μm pore size polyethylene terephthalate membrane (BD Biosciences), at 2.5×10^4 cells/chamber. The lower chambers contained the full culture medium supplemented with 5% horse serum and 20 ng/ml EGF. After incubation at 37°C for 18 h, cells were fixed with 3% glutaraldehyde in DPBS for 20 min, washed twice with DPBS, and stained with 0.5% crystal violet in 20% methanol for 10 min. Cells at the upper face of the membrane were removed with cotton swabs, and cells at the lower face were examined with an inverted microscope using a 10× magnification, and photographed. Numbers of cells in five random fields were counted, and the mean number of migrated cells for each insert was determined.

ADAM12-L Structure Prediction

The I-TASSER software (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy *et al*., 2010; Zhang, 2008) was used to predict the structure of the extracellular domain of the mature form of human ADAM12-L (amino acids 208-708). The ADAM22 template (PDB: 3G5C) was excluded from the I-TASSER template library due to a compact packing of the metalloproteinase domain against the cysteine-rich domain and steric hindrance of the pseudo-catalytic site (Liu *et al*., 2009), a feature that may be characteristic for catalytically inactive ADAMs.

Statistical Analysis

Fisher's exact test, unpaired *t* test, and linear regression were performed using GraphPad Prism 5.0.

Results

The five breast cancer-associated mutations mapping to the extracellular portion of ADAM12-L are located in different domains and spread over a region spanning more than 300 amino acids (Figure 4.1A). We asked whether these mutations might be clustered in a particular region in the three-dimensional structure of the protein. Since the X-ray structure of human ADAM12-L is not available, we used the I-TASSER protein structure prediction tool (Roy *et al*., 2010; Zhang, 2008) to build a 3D model of the extracellular domain of the active form of human ADAM12-L comprising the metalloproteinase, disintegrin, cysteine-rich, and EGF-like domains. From the model, it is apparent that the cancer-associated mutations are scattered over the entire structure and they do not cluster in a particular region of the protein (Figure 4.1 B).

The three recently identified mutations - T596A, G668A, and R612Q - involve amino acid residues in ADAM12 that are highly conserved between species (Figure 4.1C). The G668 residue is also conserved between human ADAM12 and the most closely related human ADAMs, namely ADAM19, ADAM33, and ADAM15 (Edward *et al*., 2008). In contrast, the T596 and R612 residues are not conserved between ADAM12-L and the other three ADAMs (Figure 4.1D). To study the effects of the T596A, G668A, and R612Q mutations on the functionality of ADAM12-L, we stably expressed WT and mutated ADAM12-L proteins in human MCF10A mammary epithelial cells. Western blotting of total cell lysates with an antibody specific for the cytoplasmic tail of ADAM12-L demonstrated that the proteolytic processing of WT ADAM12-L and the T596A and R612Q mutants was virtually indistinguishable (Figure 4.2A). In all three cases, the mature, processed form of ~90-kDa was easily detected, and its abundance was similar to that of the nascent, full-length form of ~120-kDa. In contrast, processing of the G668A mutant was significantly inhibited. Staining of live

cells with an antibody recognizing the extracellular domain of ADAM12-L and analysis by flow cytometry demonstrated that WT ADAM12-L and the T596A and R612Q mutants were readily detected at the cell surface, whereas the G668A mutant showed much weaker cell surface staining (Figure 4.2B). To further explore the intracellular localization of WT ADAM12-L and the mutants, we performed immunofluorescence staining of permeabilized cells with anti-ADAM12 and anti-KDEL antibody, a marker of the ER. We observed that substantial amounts of WT ADAM12-L and the T596A and R612Q mutants were present in post-ER compartments located at the cell periphery (Figure 4.2C), consistent with cell surface localization of these proteins detected by flow cytometry. In contrast, anti-ADAM12 staining in G668A-expressing cells largely coincided with anti-KDEL staining (Figure 4.2C), suggesting that this mutant was retained, at least partially, in the ER and explaining why it was poorly detected at the cell surface (Figure 4.2B).

The pronounced effect of the G668A mutation on the processing and localization of ADAM12-L is unexpected, given the very conservative nature of the Gly-to-Ala substitution. Thus, the maturation of the G668A mutant in the secretory pathway was further probed with endoglycosidase H (Endo H) and compared to the WT ADAM12-L. When the lysate of WT-expressing cells was treated with Endo H, the mobility of the nascent ADAM12-L increased, while the mature form was more resistant to the Endo H treatment (Figure 4.3A). This result indicated that the mature form, but not the nascent form, progressed through the Golgi compartment, where the resistance of N-linked oligosaccharides to Endo H is acquired. The G668A mutant, represented predominantly by the full-length form, was sensitive to Endo H, further suggesting that this mutant did not efficiently progress beyond the ER. Finally, we performed cell surface biotinylation of intact cells expressing the WT or G668A mutant protein.

In WT ADAM12-L-expressing cells, the mature form was efficiently biotinylated, whereas the nascent form was resistant to the modification (Figure 4.3B). This result indicated that at the cell surface of WT ADAM12-L-expressing cells, the mature ADAM12-L form was much more abundant than the nascent form. In G668A mutant-expressing cells, the extent of cell surface biotinylation of ADAM12-L was considerably lower than in WT ADAM12-expressing cells, further indicating that the G668A mutation impaired trafficking of the ADAM12-L protein to the cell surface.

The next question might be: Why is the G668A mutant inefficiently trafficked to the cell surface and predominantly retained in the ER? We reasoned that this mutant might be misfolded and retained by the protein quality control system operating in the ER. However, using co-immunoprecipitation, we did not detect interaction between the G668A mutant (or WT ADAM12-L) with ER chaperones BiP, Grp94, and calnexin, or with ER stress proteins that assist in proper disulfide formation ERp44, ERp57, ERp72, Ero1, PDI (results not shown). Also, cellular levels of these chaperones/stress proteins were not elevated in G668A-expressing cells. Cycloheximide chase experiments further demonstrated that the G668A mutant was in fact more stable than the WT ADAM12-L protein. Estimated half-life of the G668A mutant was 5.9 h, which was significantly larger than the half-life of WT ADAM12-L (3.3 h, Figure 4.3C). Collectively, these results suggested that although the G668A mutant was retained in the ER, most likely it was not misfolded and it was not subject to rapid degradation.

Next, we asked about the catalytic activity of ADAM12-L mutants. While a mutation causing impaired intracellular trafficking of ADAM12-L is naturally expected to cause a decrease in the enzyme activity at the cell surface (unless it exerts an indirect effect, such as enhancing cell surface expression of a different proteolytic enzyme), a mutation that does not

affect intracellular trafficking and processing can still have an impact on the catalysis or substrate recognition. For comparison, we also included the D301H and G479E mutants in the current analysis. The corresponding D299H and G477E mutations in mouse ADAM12 were shown previously to block the intracellular trafficking and processing of the protein, as well as ADAM12-mediated cleavage of the substrate protein DLL1 (Dyczynska *et al*., 2012). Trafficking, processing, and catalytic activities of human D301H or G479E mutants have not been examined before. The L792F mutation in the cytoplasmic tail of human ADAM12-L was reported to have no effect on ADAM12-L trafficking, processing, or catalytic activity (Dyczynska *et al*., 2012; Stautz *et al*., 2012).

We used three different approaches to evaluate the catalytic activity of the D301H, G479E, T596A, R612Q, and G668A mutants at the surface of MCF10A cells (Figure 4.4A). In the first approach, cells with stable overexpression of WT or mutant ADAM12-L were transfected with a plasmid encoding DLL1. The full-length (FL) DLL1 and the C-terminal fragment (CTF) generated by the proteolytic cleavage were detected by Western blotting, as described previously (Dyczynska *et al*., 2012; Dyczynska *et al*., 2007). We observed that the CTF/FL ratio of DLL1 was higher in cells expressing WT ADAM12-L or the R612Q mutant than in control cells, indicative of the catalytic activity of these ADAM12 proteins toward the DLL1 substrate (Figure 4.4B). In contrast, the CTF/FL ratio was not increased in D301H, G479E, G668A, or T596A mutant-expressing cells compared to control cells, suggesting that these four mutations significantly reduced the ability of ADAM12-L to cleave DLL1.

In the second approach, we focused on ADAM12-L-mediated shedding of EGFR ligands, as this function of ADAM12-L has been recently shown to be important in the biology of TNBC (Li *et al*., 2012). Among different EGFR ligands, ADAM12-L was previously shown to cleave

EGF (Horiuchi *et al*., 2007; Fröhlich *et al*., 2013) and heparin-binding(HB)-EGF (Asakura *et al*., 2002; Diaz *et al*., 2013). Here, the amount of endogenous EGFR ligands shed to the media by MCF10A cells stably overexpressing WT or mutant ADAM12-L proteins was evaluated. Cells were incubated for 16 h in serum-free media, the conditioned media were then transferred to starved "reporter" MCF10A cells, incubated for 30 min, and then the extent of EGFR phosphorylation at Tyr1173, one of the major autophosphorylation sites in response to ligand binding (Olayioye *et al*., 2000), was examined. Conditioned media from WT ADAM12-L or R612Q mutant-expressing cells increased EGFR phosphorylation in reporter cells (Figure 4.4C). In contrast, conditioned media from D301H, G479E, G668A, or T596A mutant-expressing cells did not cause elevation in EGFR phosphorylation (Figure 4.4C), suggesting that these mutants most likely did not efficiently shed EGFR ligands.

In the third approach, we investigated the effect of WT and mutant ADAM12-L expression on cell migration. We showed previously that overexpression of mouse ADAM12 in NIH3T3 cells increased cell migration using scratch wound assay, and that the D299H and G477E mouse ADAM12 mutants were inactive (Dyczynska *et al*., 2008). ADAM12-L has been also found to potentiate the migration of head and neck squamous cell carcinoma cells (Rao *et al*., 2012). However, overexpression of ADAM12-L in breast cancer MCF-7 cells did not affect cell migration (Roy *et al*., 2011), and mouse ADAM12 was reported to inhibit keratinocyte migration or integrin α4β1-mediated CHO cell migration cells (Harsha *et al*., 2008; Huang *et al*., 2005). Thus, the effect of ADAM12 on cell migration appears to be highly context-dependent and may involve distinct mechanisms. In the current study, we used a Transwell assay to assess the effect of mutations in human ADAM12-L on the migration of MCF10A cells. While overexpression of WT ADAM12-L or the mutants did not affect cell growth (Figure 4.5A), the

WT and the R612Q mutant ADAM12-L significantly increased cell migration (Figure 4.5B and C). This up-regulation of cell migration required the catalytic activity of ADAM12-L, because the E351Q mutant-expressing cells migrated at a rate similar to control cells. Importantly, overexpression of the D301H, G479E, G668A, or T596A mutants did not increase cell migration, further indicating that these mutants were either not efficiently targeted to the cell surface or had activities significantly lower than the WT ADAM12-L and the R612Q mutant.

A summary of the functional characterization of breast-cancer associated ADAM12-L mutations, as well as the properties of breast tumors in which each mutation was identified, is provided in Table 4-1. Included in Table 4-1 are also SIFT scores and PolyPhen scores obtained from the Ensembl Genome Browser (www.ensembl.org). SIFT is a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect (http://sift.jcvi.org) (Kumar *et al*., 2009). PolyPhen-2 is a tool which predicts the variation effect on protein function based on physical and comparative considerations (http://genetics.bwh.harvard.edu/pph2) (Adzhubei et al., 2010). SIFT scores ≤ 0.05 and PolyPhen scores ≥ 0.95 designate amino acid substitutions that are predicted to be damaging to the structure/function of a protein. As summarized in Table 4-1, with the exception of the T596A mutation, there is an agreement between the predicted effect of each mutation and the experimentally determined activity of ADAM12-L. Importantly, mutations that do not have impact on the ADAM12-L activity - R612Q and L792F - were found in TNBCs. Mutations that inhibit the ADAM12-L activity - D301H, G479E, G668A, and T596A - occurred in non-TNBCs. Thus, there is an apparent association between the intact activity of ADAM12-L and the triple-negative status of tumors, although this association is only borderline significant due to the small sample size ($P=0.0667$, Fisher's exact test).

Discussion

The current study, together with two other previous reports (Dyczynska *et al*., 2008; Stautz *et al*., 2012), provides an insight into the structural/functional aspects of the currently known breast cancer-associated mutations in ADAM12. These mutations are scattered along the entire length of the protein, and they do not appear to cluster within a specific region in the three-dimensional model of ADAM12-L. Among three novel mutations characterized for the first time in this study, only one - G668A - has a severe impact on the intracellular trafficking and proteolytic processing of the protein. Considering the very conservative nature of the Gly-to-Ala substitution, this finding is somewhat unexpected. However, as Gly is highly flexible and can adopt conformations that are forbidden for other amino acids, it is possible that the replacing Gly with Ala at position 668 is not compatible with the native structure of ADAM12-L. High conservation of Gly668 between ADAM12-L and other related ADAMs (Figure 4.1D) further suggests the importance of a Gly residue at this position.

The G668A mutation adds to the other two mutations that were previously found to cause ER retention and a lack of proteolytic processing of mouse ADAM12, and are now confirmed to inhibit the processing of human ADAM12-L, i.e., D301H and G479E. While we do not find any evidence that these mutants are unfolded and rapidly degraded, they must assume a significantly different conformation from the WT ADAM12-L to be retained in the ER. Clearly, since these three mutants exist mostly as the ~120-kDa precursors, with the inhibitory pro-domain intact, they are expected to be catalytically inactive. The lack of catalytic activity of the D301H, G479E, and G668A mutants has been confirmed here by the DLL1 cleavage assay, by the EGFR activation assay, and by the Transwell migration assay.

The effects of D301H, G479E, and G668A mutations on the structure/function of ADAM12-L agree well with the effects predicted by the SIFT and PolyPhen tools (see Table 4-1). Interestingly, the T596A mutation is predicted to be tolerated by SIFT, but harmful by the PolyPhen-2 algorithm. We find that the T596A substitution does not affect intracellular processing of ADAM12, and this result is consistent with the fact that with Thr596 is poorly conserved between ADAM12-L and other closely related human ADAMs (Figure 4.1D). However, we find that the T596A mutation renders ADAM12-L inactive at the cell surface. Thr596 is not located at the active site and most likely it is positioned distantly from the metalloproteinase domain in the three-dimensional structure of ADAM12 (Figure 4.1B).

It is currently unclear whether the T596A substitution exerts a long-range inhibitory effect on the catalytic site or whether it blocks the interaction of ADAM12-L with its substrates. Overall, we conclude that the D301H, G479E, T596A, and G668A mutations should be classified as loss-of-function mutations.

The most striking observation emerging from this study is the apparent association between the catalytic activity of ADAM12-L mutants and the type of breast cancer. Two mutations that did not have any impact on the catalytic activity of ADAM12-L, R612Q and L792F, were found in triple-negative tumors. In contrast, all four loss-of-function mutations, D301H, G479E, T596A, and G668A, occurred in non-triple-negative tumors. Thus, it appears that loss-of-function mutations in ADAM12-L tend to be excluded from TNBCs. However, a larger patient population and possibly a broader mutation spectrum are needed to test whether there is indeed a significant association between the type of ADAM12-L mutations and the triple-negative status of breast tumors. Interestingly, the *ADAM12* gene is located in the genomic region that has been recently found to be significantly deleted in Luminal B tumors (cytoband

10q26.11, wide peak boundaries chr10:104674916-135534747, q value 0.0056373) (The Cancer Genome Atlas Network, 2012). Low q-values associated with gene amplifications/deletions (typically below 0.25) suggest that amplifications/deletions at a particular locus are enriched by selective pressures (Beroukhim *et al*., 2010). The same study found that the *ADAM12* gene was hypermethylated and showed lower expression in Luminal B tumors than in other types of breast cancer (Beroukhim *et al*., 2010). As Luminal B tumors are estrogen receptor-positive (Creighton, 2012; Beroukhim *et al*., 2010), these results, together with our functional analysis of ADAM12 breast cancer-related mutants presented here, collectively suggest that ADAM12 may play fundamentally different roles in TNBCs and in non-TNBC.

We have recently postulated that ADAM12-L may be the primary protease responsible for the activation of EGFR in early stage, lymph node-negative TNBCs (Li *et al*., 2012). This conclusion was supported by decreased distant metastasis-free survival times of patients with high expression levels of *ADAM12-L*, increased EGFR phosphorylation in a mouse xenograft model of breast cancer, and a strong correlation between the level of anti-ADAM12-L and anti-phospho-EGFR immunostaining in human breast tumor samples. We have also noticed a positive correlation between *ADAM12-L* and *HB-EGF* and *EGFR* in TNBCs, but not in receptor-negative non-TNBCs (Li *et al*., 2012). In estrogen receptor-positive MCF-7 breast cancer cells, overexpression of ADAM12-L promoted estrogen-independent proliferation, and this effect of ADAM12-L was linked to elevated EGFR activation (Roy & Moses, 2011). Furthermore, a recent study demonstrated cancer cells under hypoxia up-regulate ADAM12-L expression, leading to increased HB-EGF shedding, EGFR activation, formation of invadopodia, and cancer invasion (Diaz *et al*., 2013). We believe that these results, together with the analysis of breast

cancer-associated ADAM12-L mutants described here, point to an important role of ADAM12-L in the pathology of triple-negative breast cancer.

Acknowledgments

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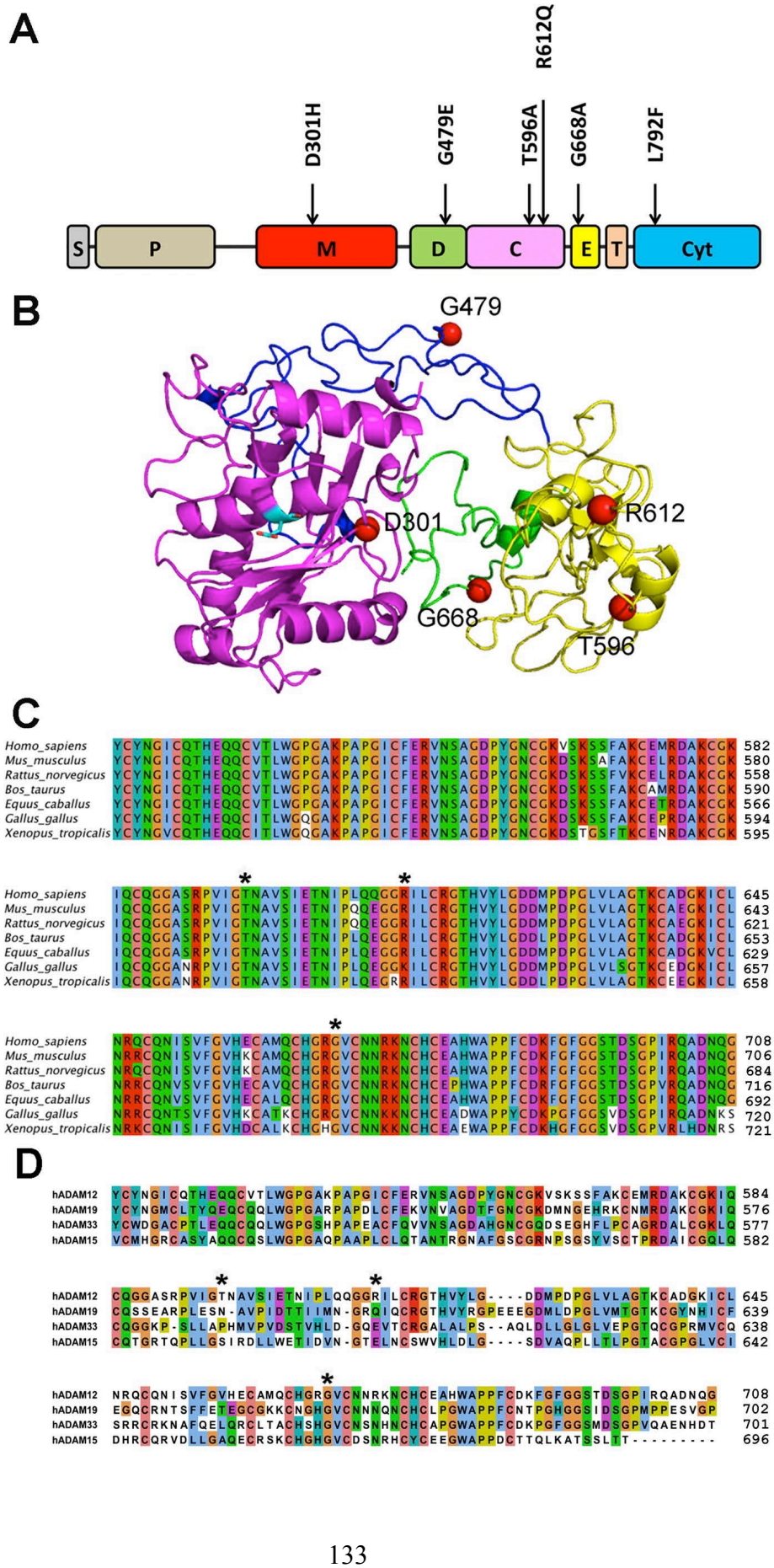
Author Contributions

Conceived and designed the experiments: YQ SDM HL AZ. Performed the experiments: YQ SDM HL. Analyzed the data: YQ SDM HL AZ. Wrote the paper: YQ AZ. Proofread the manuscript: SDM HL.


A
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Homo_sapiens YCYNGICQTHEQQCVTLWGPGAKPAPGICFERVNSAGDPYGNCGKVSKSSFAKCEMRDAKCGK 582
Mus_musculus YCYNGICQTHEQQCVTLWGPGAKPAPGICFERVNSAGDPYGNCGKDSKSAFAKCELRDAKCGK 580
Rattus_norvegicus YCYNGICQTHEQQCVTLWGPGAKPAPGICFERVNSAGDPYGNCGKDSKSSFVKCELRDAKCGK 558
Bos_taurus YCYNGICQTHEQQCVTLWGPGAKPAPGICFERVNSAGDPYGNCGKDSKSSFAKCAMRDAKCGK 590
Equus_caballus YCYNGICQTHEQQCVTLWGPGAKPAPGICFERVNSAGDPYGNCGKDSKSSFAKCETRDAKCGK 566
Gallus_gallus YCYNGICQTHEQQCITLWGQGAKPAPGICFERVNSAGDPYGNCGKDSKSSFAKCEPRDAKCGK 594
Xenopus_tropicalis YCYNGVCQTHEQQCITLWGQGAKPAPGICFERVNSAGDPYGNCGKDSTGSFTKCENRDAKCGK 595
Homo_sapiens IQCQGGASRPVIGTNAVSIETNIPLQQGGRILCRGTHVYLGDDMPDPGLVLAGTKCADGKICL 645
Mus_musculus IQCQGGASRPVIGTNAVSIETNIPQQEGGRILCRGTHVYLGDDMPDPGLVLAGTKCAEGKICL 643
Rattus_norvegicus IQCQGGASRPVIGTNAVSIETNIPQQEGGRILCRGTHVYLGDDMPDPGLVLAGTKCAEGKICL 621
Bos_taurus IQCQGGASRPVIGTNAVSIETNIPLQEGGRILCRGTHVYLGDDLPDPGLVLAGTKCADGKICL 653
Equus_caballus IQCQGGASRPVIGTNAVSIETNIPLQEGGRILCRGTHVYLGDDMPDPGLVLAGTKCADGKVCL 629
Gallus_gallus IQCQGGANRPVIGTNAVSIETNIPLQEGGKILCRGTHVYLGDDMPDPGLVLSGTKCEDGKICL 657
Xenopus_tropicalis IQCQGGANRPVIGTNAVSIETNIPLQEGRRILCRGTHVYLGDDLPDPGLVLAGTKCEEGKICL 658
Homo_sapiens NRQCQNISVFGVHECAMQCHGRGVCNNRKNCHCEAHWAPPFCDKFGFGGSTDSGPIRQADNQG 708
Mus_musculus NRRCQNISVFGVHKCAMQCHGRGVCNNRKNCHCEAHWAPPFCDKFGFGGSTDSGPIRQADNQG 706
Rattus_norvegicus NRQCQNISVFGVHKCAMQCHGRGVCNNRKNCHCEAHWAPPFCDKFGFGGSTDSGPIRQADNQG 684
Bos_taurus NRRCQNVSVFGVHECAVQCHGRGVCNNRKNCHCEPHWAPPFCDRFGFGGSTDSGPVRQADNQG 716
Equus_caballus NRRCQNVSVFGVHECALQCHGRGVCNNRKNCHCEAHWAPPFCDKFGFGGSTDSGPIRQADNQG 692
Gallus_gallus NRRCQNTSVFGVHKCATKCHGRGVCNNKKNCHCEADWAPPYCDKPGFGGSVDSGPIRQADNKS 720
Xenopus_tropicalis NRKCQNISIFGVHDCALKCHGHGVCNNKKNCHCEAEWAPPFCDKHGFGGSVDSGPVRLHDNRS 721
D
hADAM12 YCYNGICQTHEQQCVTLWGPGAKPAPGICFERVNSAGDPYGNCGKVSKSSFAKCEMRDAKCGKIQ 584
hADAM19 YCYNGMCLTYQEQCQQLWGPGARPAPDLCFEKVNVAGDTFGNCGKDMNGEHRKCNMRDAKCGKIQ 576
hADAM33 YCWDGACPTLEQQCQQLWGPGSHPAPEACFQVVNSAGDAHGNCGQDSEGHFLPCAGRDALCGKLQ 577
hADAM15 VCMHGRCASYAQQCQSLWGPGAQPAAPLCLQTANTRGNAFGSCGRNPSGSYVSCTPRDAICGQLQ 582
hADAM12 CQGGASRPVIGTNAVSIETNIPLQQGGRILCRGTHVYLG----DDMPDPGLVLAGTKCADGKICL 645
hADAM19 CQSSEARPLESN-AVPIDTTIIMN-GRQIQCRGTHVYRGPEEEGDMLDPGLVMTGTKCGYNHICF 639
hADAM33 CQGGKP-SLLAPHMVPVDSTVHLD-GQEVTCRGALALPS--AQLDLLGLGLVEPGTQCGPRMVCQ 638
hADAM15 CQTGRTQPLLGSIRDLLWETIDVN-GTELNCSWVHLDLG----SDVAQPLLTLPGTACGPGLVCI 642
hADAM12 NRQCQNISVFGVHECAMQCHGRGVCNNRKNCHCEAHWAPPFCDKFGFGGSTDSGPIRQADNQG 708
hADAM19 EGQCRNTSFFETEGCGKKCNGHGVCNNNQNCHCLPGWAPPFCNTPGHGGSIDSGPMPPESVGP 702
hADAM33 SRRCRKNAFQELQRCLTACHSHGVCNSNHNCHCAPGWAPPFCDKPGFGGSMDSGPVQAENHDT 701
hADAM15 DHRCQRVDLLGAQECRSKCHGHGVCDSNRHCYCEEGWAPPDCTTQLKATSSLTT--------- 696

Figure 4.1 Breast cancer-associated mutations in human ADAM12-L.

(A) A diagram of human ADAM12-L. Six non-synonymous mutations identified in human breast cancers are indicated. S, signal peptide; P, prodomain; M, metalloproteinase domain; D, disintegrin domain; C, cysteine-rich domain; E, EGF-like domain; T, transmembrane region; Cyt, cytoplasmic tail. (B) Model of the extracellular domain of human ADAM12-L generated by the I-TASSER protein structure prediction tool (C-score −0.26, estimated TM accuracy of the model 0.68±0.12) (Roy *et al*., 2010; Zhang, 2008). The metalloproteinase, disintegrin, cysteine-rich, and EGF-like domains are shown in purple, blue, yellow, and green, respectively. Positions of the five amino acids mutated in breast cancers (red spheres) and the side chain of the catalytic residue E351 (cyan sticks) are indicated. (C) Sequence alignment of the cysteine-rich and EGF-like domains of ADAM12 from different species. NCBI RefSeq numbers are: Homo_sapiens, NP_003465; Mus_musculus, NP_031426; Rattus_norvegicus, XP_001054670; Bos_taurus, NP_001001156, Equus_caballus, XP_001490097; Gallus_gallus, NP_001136322, and Xenopus_tropicalis, NP_001035103. (D) Sequence alignment of the cysteine-rich and EGF-like domains of human ADAM12 and the most closely related human ADAMs. NCBI RefSeq numbers are: ADAM19, XP_005266060, ADAM33, NP_079496.1, and ADAM15, NP_997080. In C and D, asterisks indicate three novel mutations in human ADAM12 found in breast tumors (Shah *et al*., 2012; Jiao *et al*., 2012). Clustal X color scheme was applied.

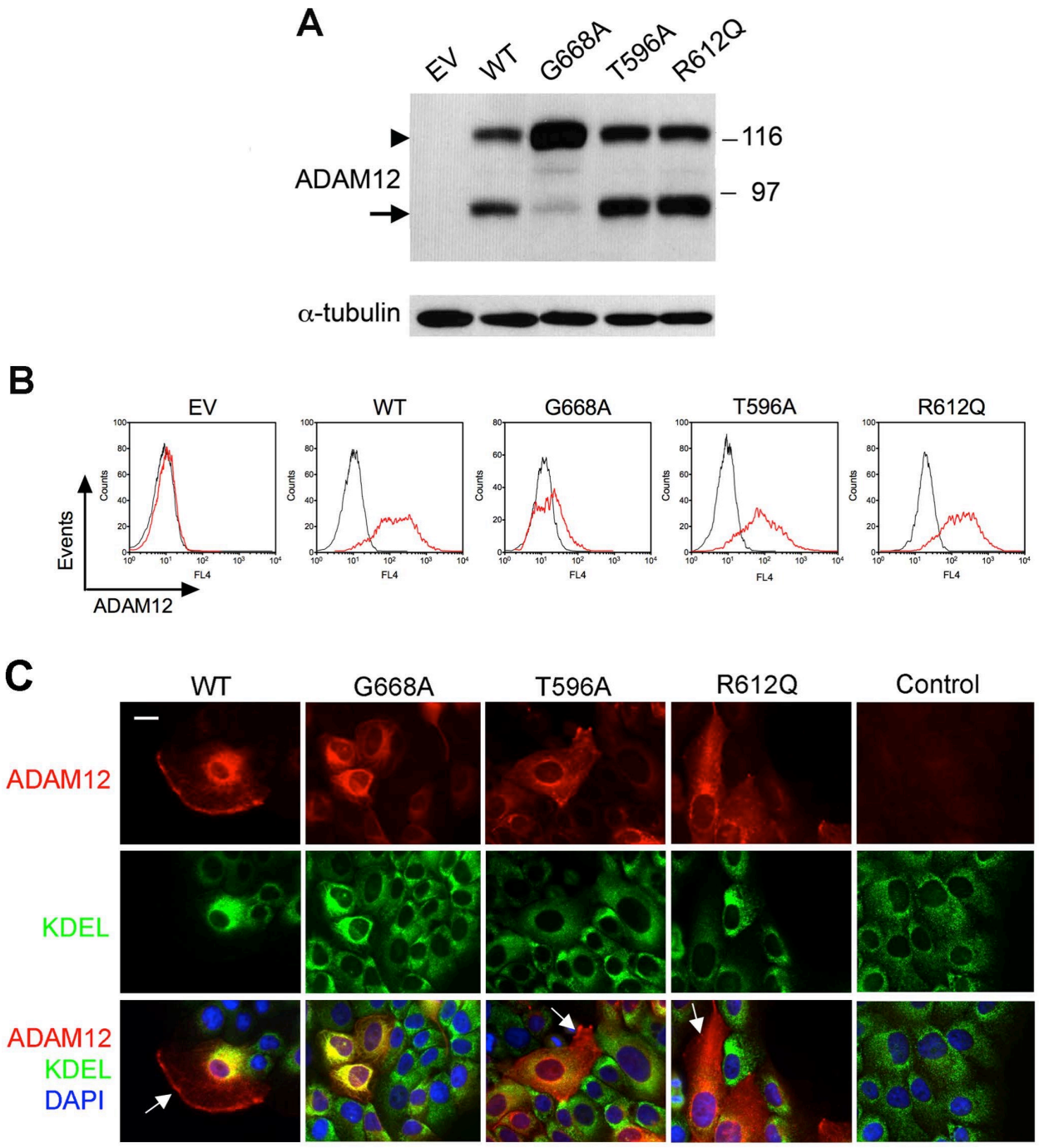

Figure 4.2 The effect of G668A, T596A, and R612Q mutations on the proteolytic processing and intracellular localization of ADAM12-L.

(A) Proteolytic processing of the WT and mutant forms of human ADAM12-L in MCF10A cells. Cells with stable expression of ADAM12-L proteins or control empty vector (EV)-transduced cells were selected with puromycin after retroviral infection. Total cell lysates

were analyzed by Western blotting using antibody specific for the cytoplasmic tail of ADAM12-L. Arrowhead indicates the nascent, full-length, catalytically inactive form, and arrow denotes the mature, processed, catalytically active form of ADAM12-L. (B) Cell surface localization of ADAM12-L was examined by flow cytometry. Live cells were trypsinized and stained with an antibody specific for the extracellular domain of ADAM12-L (red) or with isotype control antibody (black). (C) Intracellular localization of the WT and mutant ADAM12-L proteins. Cells were co-stained with anti-ADAM12 antibody (red), anti-KDEL antibody (endoplasmic reticulum marker; green), and DAPI (blue). Control represents cells expressing WT ADAM12-L, incubated with pre-immune serum instead of anti-ADAM12 antibody. Arrows indicate ADAM12 staining in post-ER compartments. Bar, 20 μm.

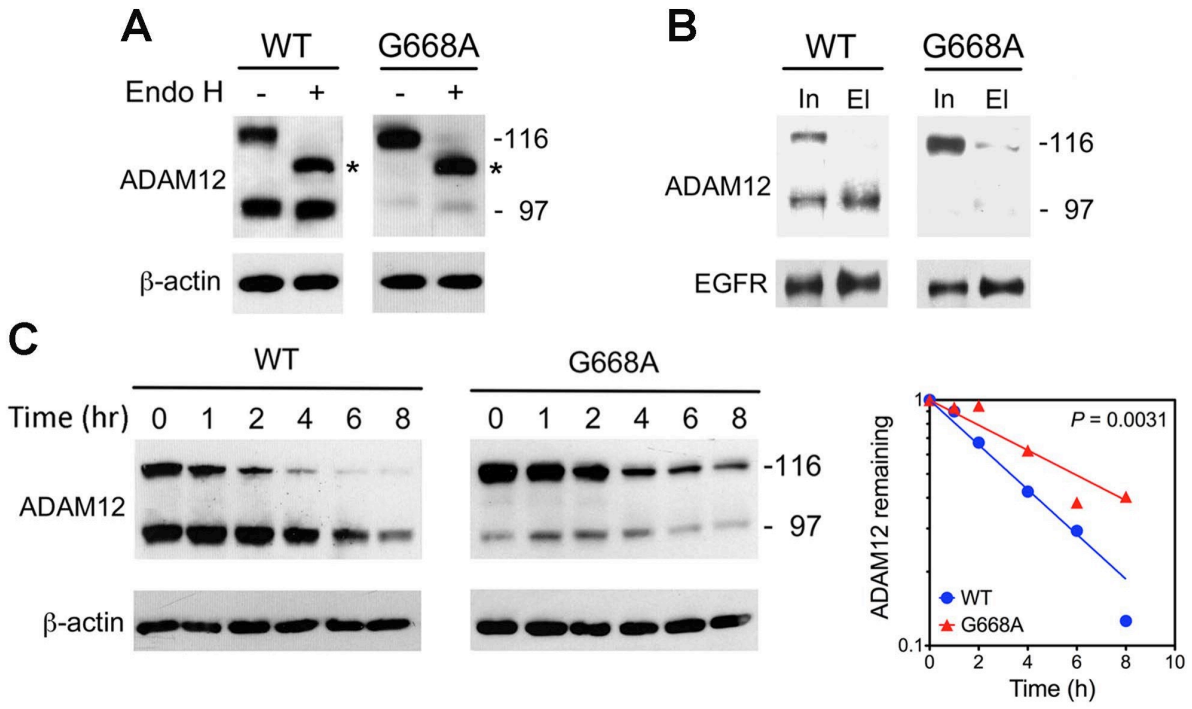

Figure 4.3 The G668A mutation causes retention of ADAM12-L in the endoplasmic reticulum.

(A) Probing the maturation of ADAM12-L proteins in post-ER compartments by treatment with endoglycosidase H (Endo H). Total cell lysates were incubated for 1 h with Endo H, followed by Western blotting with anti-ADAM12 antibody. Asterisk indicates the full-length, de-glycosylated form of ADAM12-L. (B) Cell surface biotinylation of ADAM12-L proteins. Intact cells were incubated for 1 h with membrane-impermeable NHS-PEG12-biotin, followed by isolation of biotinylated proteins using NeutrAvidin beads and Western blotting with anti-ADAM12 antibody. Input (In) refers to total cell lysates prior to Neutravidin binding, and eluate (El) refers to biotinylated proteins that bound to the resin. Biotinylation of epidermal growth factor receptor (EGFR) served as positive control. (C) Protein stability assay. Cells were incubated with 10 μg/ml of cycloheximide for the indicated times, followed by immunoblotting. Band intensities of ADAM12-L (nascent and mature forms combined) were quantified by densitometry, normalized to β-actin, and analyzed using a single exponential decay model. Half-lives of the WT and the G668A mutant ADAM12-L were significantly different (3.3 h vs 5.9 h, respectively, P=0.0031).

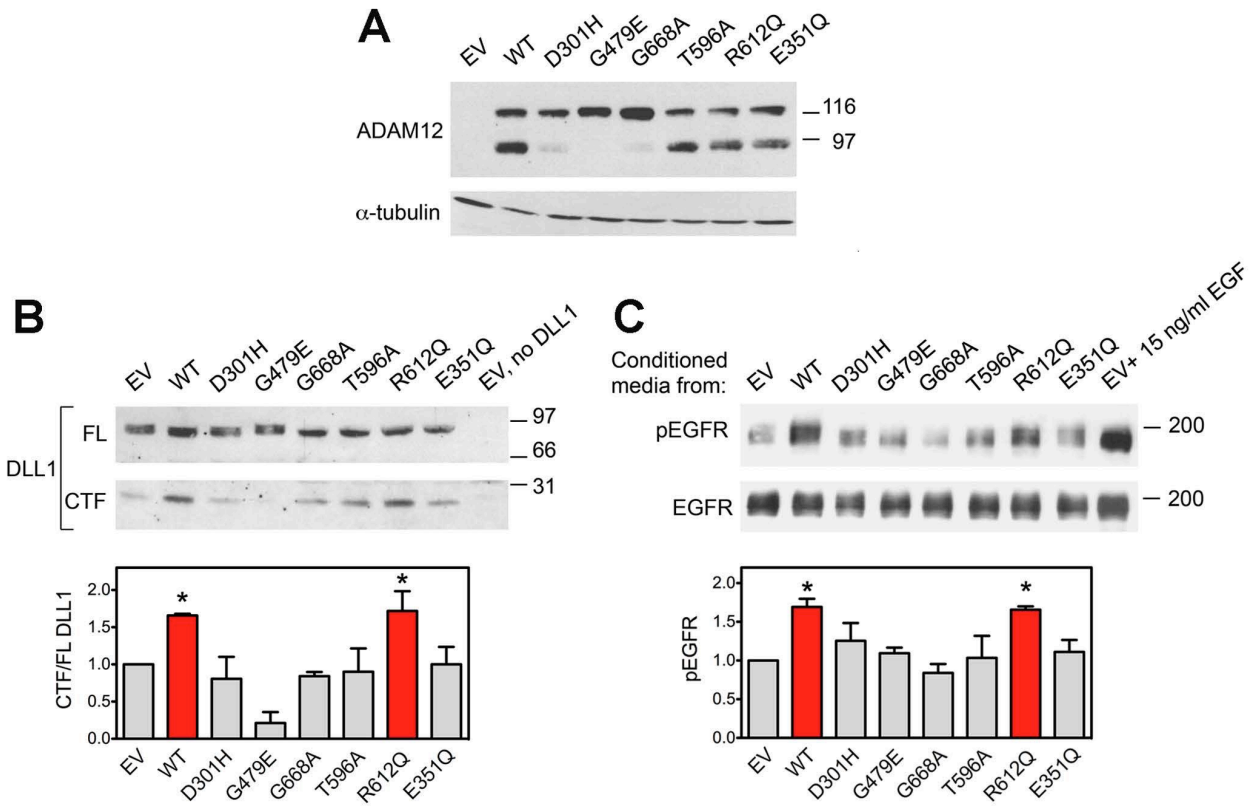

Figure 4.4 Cell-based assays of the catalytic activity of ADAM12-L mutants.

(A) MCF10A cells with stable overexpression of WT or mutant ADAM12-L proteins, or control empty vector (EV)-transduced cells used in assays shown in panels B and C. The E351Q mutant is catalytically inactive due to the mutation at the active site and is used as negative control. (B) Cells shown in panel A were transiently transfected to express a substrate protein Delta-like 1 (DLL1). Cell extracts were subjected to Western blotting with anti-DLL1 antibody. The full-length (FL) DLL1 and the C-terminal fragment (CTF) generated by the proteolytic cleavage are indicated. Band intensities were quantified by densitometry. The experiment was repeated two times, mean values ± SEM are shown. *, $P<0.05$. Notice that the amount of the C-terminal fragment (CTF) of DLL1 is increased in WT- and R612Q mutant ADAM12-L-expressing cells. (C) Cells shown in panel A were serum-starved for 16 h. Conditioned media were then transferred to reporter MCF10A cells that were also pre-starved for 16 h, and incubation continued for 30 min. The level of phosphorylation of EGFR in reporter cells was evaluated by Western blotting using anti-phosho-Y1173 antibody, band intensities were quantified by densitometry, and the extent of phosphorylation of EGFR (pEGFR) normalized to

the total EGFR protein was evaluated. Conditioned media from EV-transduced cells supplemented with 15 ng/ml EGF served as positive control. The experiment was repeated three times, mean values ± SEM are shown. *, $P<0.05$. Notice that EGFR phosphorylation in reporter cells was increased upon adding conditioned media from WT- or R612Q mutant ADAM12-L-expressing cells.

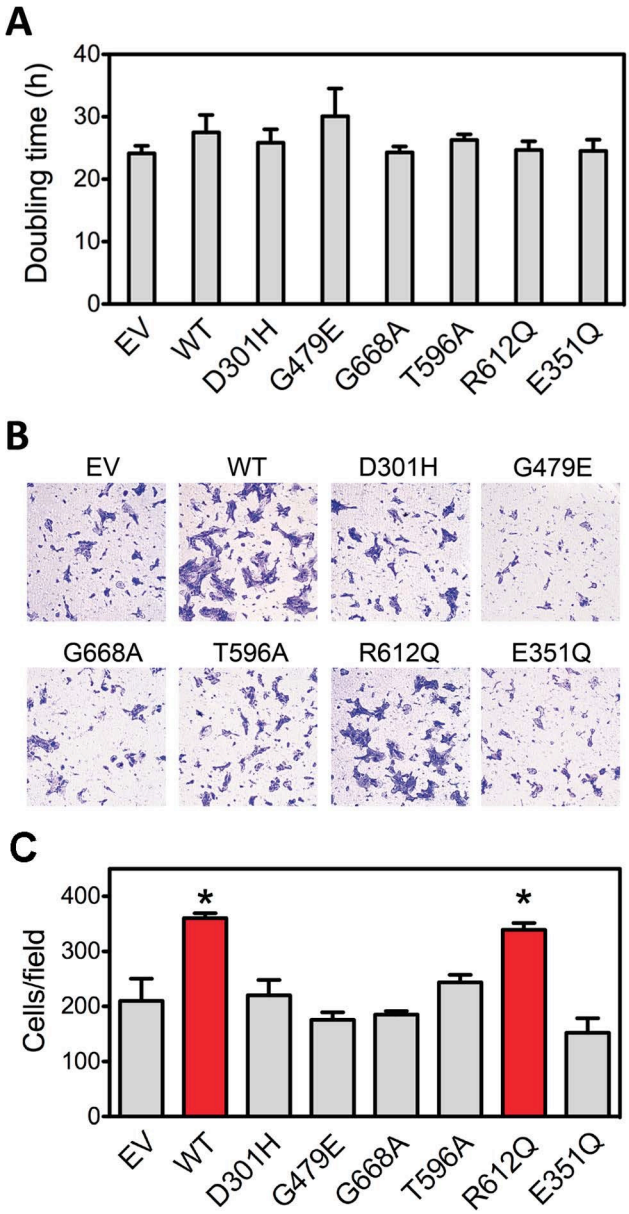

Figure 4.5 The effect of WT and mutant ADAM12-L on cell growth and migration.

(A) Doubling times of MCF10A cells with stable overexpression of WT or mutant ADAM12-L proteins, or control empty vector (EV)-transduced cells. The data are shown as means with 95% confidence intervals based on 3 independent determinations. (B) Cells were analyzed for migration using Transwell assays. Representative images of crystal violet-stained cells present at the lower face of the Transwell inserts are shown. (C) Quantification of the migration assay. The numbers of migrated cells were counted in five random fields for each insert. The data are shown as mean ± SEM from 3 determinations. *, $P<0.05$.

Table 4-1 Summary of breast cancer-associated somatic mutations in ADAM12-L.

Mutation	Molecular characteristics	Sample source	Zygosity	SIFT[a]	PolyPhen[b]	ER retention	Activity	Reference for ER retention/Activity
D301H	non-TNBC	Cultured	Heterozygous	**0**	**1**	++	−	Dyczynska *et al*., 2008 and this study
G479E	non-TNBC	Tumor sample	Heterozygous	**0**	**1**	+++	−	Dyczynska *et al*., 2008 and this study
G668A	non-TNBC	Tumor sample	Unknown	**0.001**	**0.998**	++	−	This study
T596A	non-TNBC	Tumor sample	Unknown	0.09	**0.999**	−	−	This study
R612Q	TNBC	Tumor sample	Unknown	0.28	0.89	−	+	This study
L792F	TNBC	Cultured	Heterozygous	0.35	0.223	−	+	Stautz *et al*., 2012

[a] Predicted effect of each mutation on ADAM12-L function according to the SIFT algorithm (Kumar *et al*., 2009). SIFT scores ≤ 0.05 indicate amino acid substitutions that are predicted to be damaging (shown in bold).

[b] Predicted effect of each mutation on ADAM12-L function according to the PolyPhen-2 tool (Adzhubei *et al*., 2010). Scores represent prediction confidence that a given mutation changes the protein function; scores ≥ 0.95 are indicated in bold.

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Chapter 5 - Global discussion

ADAM12 is a transmembrane protease with multiple biological functions. Studies have indicated that ADAM12 expression is dysregulated (Murphy, 2008; Duffy *et al*., 2009) in different types of cancers, including breast cancer, and is often correlated with poor prognosis (Cheon *et al*., 2015; Li *et al*., 2012; Rao *et al*., 2012). My dissertation focused on understanding the biological functions of ADAM12 in the context of triple-negative breast cancers. I discovered that ADAM12 regulates the cancer stem cell-like subpopulations in triple-negative breast cancers. I revealed a new mechanism of ADAM12-regulated TGFβ signaling through modulation of *TGFBR1* mRNA expression in breast cancer cells. I also studied the biochemical characteristics of six different somatic mutants of ADAM12 and their impact on ADAM12 function.

In Chapter 2, I demonstrated that the loss of ADAM12 not only decreases the percentage of cancer stem-like cells (CSCs) *in vitro* but also significantly compromises the tumor-initiating capability of breast cancer cells *in vivo*, which is the first evidence showing that ADAM12 regulates the cancer stem-like cell population in triple-negative breast cancers. Cancer stem-like cells represent of a small population of cancer cells, having features similar to normal stem cells, such as slow cycling and self-renewal (Reya *et al*., 2001; Al-Hajj *et al*., 2003). Because of these characteristics, cancer stem-like cells are more resistant to chemotherapies and possess the capability of initiating tumor formation, which is responsible for tumor relapse and metastasis in most types of cancers (Nuti *et al*., 2014; Pinto *et al*., 2013). Previously, we found that the level of ADAM12 was strongly associated with the decreased metastasis-free survival rate in TNBC and that the expression of ADAM12 was significantly up-regulated in TNBC patients with distant

metastasis (Li *et al*., 2012). My findings in Chapter 2 not only support this notion but also provide a molecular explanation to the previous observations.

In addition, I also showed that the exogenous EGF diminishes the reduction of CSC subpopulations mediated by ADAM12 knockdown, suggesting that ADAM12 might regulate CSC in TNBC through EGFR signaling pathway. Yet we still need direct *in vivo* or *in vitro* evidences to prove that the activation of EGFR is induced by ADAM12. However, our discovery creates an opportunity to fully outline the roles of ADAM12 in breast cancers. Interesting questions raised in Chapter 2 are how ADAM12 activates EGFR and which of the downstream pathways are modulated by ADAM12 to regulate CSCs. The EGFR pathway is a very complex signaling network. Several EGFR downstream cascades have been already reported to regulate the stem-like cell phenotype of cancer cells, such as JAK1/STAT3 and Ras/Raf/MEK/ERK (also known as MAPK/ERK).

Extracellular signal-regulated kinase (ERK) is a key player in the downstream EGFR signaling pathway and a well-studied CSC regulator in different types of cancers, including breast cancer. Studies have shown that the phosphorylation of ERK promotes the transcription of genes related with CSC features (Fang *et al*., 2005; Balko *et al*., 2013; Ciccarelli *et al*., 2016). Inflammatory cytokine Interleukin-8 (IL8) is one of these genes and it was shown to participate in the regulation of breast CSCs (Singh *et al*., 2013; Singh *et al*., 2013). Our unpublished data shows that the reduced expression of ADAM12 significantly decreased *IL8* mRNA levels in breast cancer cells. Therefore, it would be interesting to check whether ADAM12 would regulate cancer stem like cells by modifying the phosphorylation of ERK proteins.

PI3K/Akt/mTOR signaling pathway has recently emerged as a potential target for inhibiting cancer stem cell activities. The 70-kDa ribosomal protein S6 kinase (p70S6K) is a

serine/threonine kinase downstream of PI3K/mTOR and it regulates cell cycle progression and cell survival (Heinonen *et al*., 2008). PI3K/mTOR/p70S6K was reported to induce cancer stem-like cellular traits in luminal-like breast cancer cells, acute myeloid leukemia, and glioblastoma (Corominas-Faja *et al*., 2013; Altman *et al*, 2011; Lamour *et al*., 2015) In addition, p70S6K is also involved in the MEK/ERK pathway and is able to be phosphorylated by ERK (Chai *et al*., 2015). Studies showed that the attenuation of p70S6K by inhibition of ERK phosphorylation impaired the tumorigenic capabilities of glioblastoma, pancreatic, and non-small lung carcinoma cells, suggesting that the crosstalk between PI3K/mTOR and MEK/ERK pathways may cooperate to maintain the CSC state of cancer cells. (Sunayama *et al*., 2010; Chai *et al*., 2015; Khan *et al*., 2012) Coincidentally, the analysis of our RNA-sequencing data suggests that the effect of ADAM12 down-regulation on the global gene expression is similar to the gene expression pattern induced by the suppression of mTOR signaling. Thus, it is reasonable to postulate that ADAM12 may regulate the CSC phenotype in TNBC through modulating the phosphorylation of p70S6K by both mTOR and MEK/ERK signaling cascades, or by either of the two. In this regard, assessing whether ADAM12 affects the mTOR signaling pathway and the phosphorylation of p70S6K is important to elucidate the subsequent processes through which ADAM12 regulates the CSC traits in triple-negative breast cancer cells.

Another important question that needs to be solved is to understand the function of ADAM12 at the cell surface during the CSC regulation. As a transmembrane protease, ADAM12 cleaves several growth factor precursors, including epidermal growth factor receptor ligands EGF and HB-EGF (Asakura *et al*., 2002; Horiuchi *et al*., 2007). It is likely that the autocrine and paracrine EGFR ligands shed by ADAM12 activate EGFR and its downstream signaling pathways and thereby maintain the mesenchymal and stem cell state of breast cancer. However,

based on the data in Chapter 2, we could not exclude the possibility that ADAM12 may activate EGFR and its downstream pathways though protein-protein interactions. The cytoplasmic tail of ADAM12 contains cysteine-rich region, which is capable of interacting with Src Homolog 3 (SH3) domain containing proteins (Stautz *et al*., 2010). Moreover, another study showed that ADAM12 enhanced PI3K/Akt signaling via the interactions with integrin-linked kinase (ILK), which did not require the metalloprotease activity of ADAM12 (Leyme *et al*., 2012). To address this question, an ADAM12 specific inhibitor or antibody could be applied to block the protease activity and then to test whether ADAM12 is still capable of regulating the cancer stem-like cell populations.

In Chapter 3, I described a new mechanism by which ADAM12 modulates the strenght of TGFβ signaling via the regulation of *TGFBR1* mRNA levels, which is the first study reporting a role of ADAM12 in the regulation of TGFβ signaling at the endogenous ADAM12 expression level. An important question that emerged from this study is: how would ADAM12, a membrane-located protease, regulate the transcription of *TGFBR1* inside the nucleus? What would be the communicating pathway that connects ADAM12 with the transcription machinery of *TGFBR1*? An interesting observation of my study is that ADAM12 regulates *TGFBR1* mRNA independently of TGFβ signaling. Though little is known about the regulation of *TGFBR1*, a recent paper has shown that Notch signaling is involved in the regulation of TGFβR1 at the transcriptional level (Abrahams *et al*., 2015). Coincidentally, our previous work showed that ADAM12 cleaves Delta-like l, a ligand activating Notch receptor (Dyczynska *et al*., 2007). Theses evidences together suggest that ADAM12 may mediate the regulation of *TGFBR1* transcription via the Notch pathway. To test this hypothesis, we need to investigate whether constitutively active Notch would block the down-regulation of *TGFBR1* mRNA induced by the

loss of ADAM12. In another approach, we could use bioinformatics analyses. With the availability of ChIP datasets, the transcription factors that could potentially bind to the promoter region of the *TGFBR1* gene can be predicted by computing softwares (such as the Genome Browser at UCSC and PROMO at IMIM-UPF). The validation of these predicted candidates would further help to pinpoint the upstream signal-transducing routes through which ADAM12 regulates *TGFBR1* mRNA.

There are also some interesting issues not discussed in Chapter 3 but still worthy of investigation; for instance, the protein-protein interaction between ADAM12 and TGFβR1 at cell surface or in other cellular compartments. It is possible that ADAM12 may increase TGFβR1 protein stability and thereby enhance the intensity of TGFβ signaling. Another question is whether the role of ADAM12 in TGFβ signaling is universal in all types of breast cancers or whether this observation is only limited to breast cancers of certain molecular characteristics. TGFβ signaling is important for breast cancer cells to maintain the mesenchymal state and to acquire the resistance to chemotherapies (Ikushima *et al*., 2009; Bhola *et al*., 2013). I believe that an in-depth analysis of ADAM12 function in TGFβ signaling will not only increase our knowledge of ADAM12 in the pathology of breast cancer but, more importantly, it will lay a solid foundation for the development of ADAM12 targeting therapies in the future.

In Chapter 4, I identified the biochemical characteristics and the biological outcomes of six different somatic mutations in the ADAM12 gene in human breast cancers. My data indicates that the correct proteolytical processing is critical for the surface localization and proper function of ADAM12. Importantly, ADAM12 harboring D301H, G479E, G668A, and T596A mutations and showing impaired protease activity do not appear in breast cancers with the triple-negative status. This observation reveals an apparent relationship between the intact activity of ADAM12

and the triple-negative subtype of breast cancer, though the correlation appears border-line significant (P =0.0667, Fisher's exact test) due to the small sample size (n = 6). This finding again supports the notion that ADAM12 with active metalloprotease function might be required for the progression of triple-negative breast cancers.

To summarize, all these results indicate that ADAM12 may be a potential therapeutic target in triple-negative breast cancer. Specific antibodies targeting the protease domain of ADAM12 or a recombinant protein mimic the pro-domain of ADAM12 (Miller *et al*., 2015) would be a good targeting strategy, when the protease activity of ADAM12 is required for the tumor development in breast cancer patients. In contrast, the protease inhibition method may not be needed if the function of ADAM12 is mediated via the interaction of its cytoplasmic domain with other molecules. In such a case, ADAM12 targeting antibody could be potentially conjugated with certain cytoplastic chemicals or ADAM12 targeting siRNAs. The thorough understanding of the roles of ADAM12 in breast cancers will empower us to design ADAM12-targeting therapies, with much more efficient and precise outcomes.

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