Expression of ADAM Metalloproteases during Transforming Growth Factor β-Induced Senescence in Breast Cancer Cells

by

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Abstract

Cellular senescence is a state of irreversible cell cycle arrest in response to non-lethal stress. In cancer cells, senescence can be induced by chemotherapy, radiation, or signals from the tumor microenvironment, such as transforming growth factor β (TGFβ). Senescent cells are metabolically active and have altered gene expression compared to their non-senescent counterparts. Senescent cells release a wide variety of factors, including extracellular domains of transmembrane proteins that require proteolytic cleavage by specific proteases. ADAMs (A Disintegrin and Metalloprotease domain-containing proteins) are enzymes that cleave many transmembrane proteins, such as growth factor precursors or adhesion molecules, and thus may act as sheddases in senescent cells. Here, we investigate ADAM expression levels during TGFβ-induced cellular senescence.

SUM149PT and SUM102PT breast cancer cells were incubated with TGFβ, followed by treatment with high doses of paclitaxel to remove actively proliferating, non-senescent cells. Induction of cellular senescence was examined by evaluating changes in cell size and granularity, and by β-galactosidase staining. *ADAM* mRNA levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Among several *ADAMs* tested, *ADAM12* mRNA was significantly upregulated in senescent cells. In addition, we demonstrated that ADAM12 knock-down leads to decreased activation of epidermal growth factor receptor (EGFR), an important modulator of cancer cell growth, survival, and metastasis. This effect of ADAM12 knock-down was likely due to a diminished release of soluble EGF or EGF-like ligands from cells. Since senescent cells often release increased amounts of these ligands, ADAM12 may modulate the senescence secretome in senescent breast cancer cells.

Table of Contents

List of Figures

List of Tables

List of Abbreviation

ADAMs	A Disintegrin and Metalloproteases
CCN1/CYR61	CYR61, CTGF, NOV/Cysteine-rich angiogenic inducer 61
DDR	DNA damage response
DLL1	Delta-like 1
DNA-SCARS	DNA segments with chromatin alteration reinforcing senescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
FSC	Forward scatter
HB-EGF	Heparin-binding EGF-like growth factor
ICAM-1	Intercellular adhesion molecule
IGFBP	Insulin-like growth factor binding protein
IκB	Inhibitory kappa B
MEF	Mouse embryonic fibroblasts
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor

OPG	Osteoprotegrin
pRb	Phosphorylated retinoblastoma
PRS	Proline-rich sequence
PTX	Paclitaxel
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SA-β-gal	Senescence-associated-β-galactosidase
SAHF	Senescence associated heterochromatin foci
SASP	Senescence associated secretory phenotype
SH3	Src homology 3
SSC	Side scatter
TGFα	Transforming growth factor α
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
TNFR1	Tumor necrosis factor receptor 1
TRAF6	TNF receptor associated factor 6
VEGF	Vascular endothelial growth factor

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Chapter 1 - Literature Review

Cellular Senescence

Cellular senescence is a physiological process that can be induced either by intrinsic or extrinsic factors, such as telomere shortening, signals from the tumor microenvironment, or genomic and epigenomic damage (Sabin & Anderson 2011). Senescence was first described in 1965 as a process that limits cell proliferation. The main feature of senescent cells is that they are irreversibly arrested to prevent the transmission of a cellular damage to daughter cells (Sabin & Anderson 2011). Therefore, senescence is closely linked to aging and tumor suppression (Rodier & Campisi 2011).

Causes of Cellular Senescence

There are many inducers of cellular senescence, such as telomere shortening, signals from the tumor microenvironment, transforming growth factor β (TGFβ), genomic and epigenomic damage, or activation of tumor suppressors using strong mitogenic signals, which is called oncogene induced senescence (OIS) (Campisi 2014; Salminen 2012).

Telomere shortening in eukaryotes is one of the best known mechanisms for induction of cellular senescence. With each cell division, telomeres, which are repetitive DNA sequence that are found at the end of each chromosome, are shortened. This prevents the complete replication of the DNA. As a result, after several cell divisions, the shortening of the telomeres activates the DNA damage response (DDR), which leads to the activation of p53, a tumor suppressor, and growth arrest due to the induction of senescence (Campisi 2014; Fumagalli 2012).

Genomic damage is another way to induce cellular senescence. Ionizing radiation, cytotoxic chemotherapeutic agents, oxidative stress, or other DNA damaging agents can result in genomic damage. These factors produce persistent DDR signaling that leads to the induction of cellular senescence (Salminen 2012; Campisi 2014).

OIS results from applying strong mitogenic signals for an extended period of time on cells. One of the best-known mitogenic signals is the oncogenic protein Ras^{V12}. Serrano *et al.* showed that the expression of Ras^{V12} in human and rodent fibroblasts induces senescent growth arrest as a result of activation of p53 and p16, a cell cycle inhibitor (Serrano 1997). The $BRAF^{E600}$ oncogene is another example of a mitogenic signal whose overexpression in fibroblasts induced cellular senescence (Kuilman 2008). The induction of senescence in response to specific mitogenic stimuli is a mechanism for tumor suppression, which might explain an apparent need to mutate the genes involved in OIS, such as p53 during tumor progression (Serrano 1997).

The focus of the following section will be on TGFβ, which is the agent used in this study to induce cellular senescence.

Induction of Senescence by TGFβ

TGFβ is a member of the cytokine family that has important implications in cell proliferation, differentiation, and apoptosis (Mu 2012). TGFβ mediates cellular processes via canonical, Smad-dependent, or non-canonical, Smad-independent pathways. In the Smad-dependent pathway, TGFβ binds to TGFβR2, which recruits and phosphorylates TGFβR1. Then, TGFβR1 phosphorylates R-Smads that bind to Smad4 and translocate into the nucleus. In the

nucleus, R-Smad/Smad4 complexes act as transcription factors to regulate the expression of various target genes (Mu 2012).

Multiple Smad-independent pathways have been identified; one of the examples of those pathways is the TGFβ-TAK1-NF-κB pathway. This pathway can also modulate the induction of cellular senescence (see ***The SASP Regulation*** section) (Figure 1.1). It has been shown that TGFβ induces growth arrest and cellular senescence in different cells, such as hepatocellular carcinoma cells (Senturk 2010), mouse embryonic fibroblast (MEF) (Pérez-Rivero 2008), or mouse keratinocytes (Vijayachandra 2009). Moreover, many reports showed that TGFβ induces the expression of different cell cycle inhibitors, such as p15, p21, p57, or p16, which leads to growth arrest (Senturk 2010; Vijayachandra 2009). Finally, TGFβ and its receptors were induced by the oxidative stressor H_2O_2 in human fibroblasts, which led to the induction of cellular senescence (Dumont 2001).

Hallmarks of Cellular Senescence

Senescent cells have many characteristics, such as irreversible growth arrest and increased β-galactosidase enzyme activity; however, no single marker is exclusive to senescent cells. Moreover, it is not necessary for senescent cells to express all known features of senescence. Senescent cells should express a group of markers that collectively define their state (Rodier & Campisi 2011; Campisi 2014; Ohtani & Hara 2013).

The following are some of the main features of senescent cells:

1) Unlike cells in a quiescent state, which is a state of reversible cell cycle arrest, senescent cells undergo a permanent, irreversible growth arrest. This means that even if a growth

stimulus is provided to senescent cells, the cells will not re-enter the cell cycle (Rodier & Campisi 2011; Campisi 2014; Sabin & Anderson 2011).

2) Senescent cells undergo various morphological changes; along with being approximately two-fold larger than their non-senescent counterparts, senescent cells also have a flattened morphology under tissue culture conditions (Rodier & Campisi 2011; Sabin & Anderson 2011; Campisi 2014).

3) Senescent cells are characterized by increased activity and expression of the lysosomal senescence-associated-β-galactosidase (SA-β-gal) enzyme, which is detectable at near-neutral pH (Rodier & Campisi 2011; Campisi 2014).

4) Senescent cells have an increase in the expression of one or more of the following cell cycle regulatory proteins: p16, p19, p21, or p53 (Rodier & Campisi 2011; Campisi 2014; Sabin & Anderson 2011).

5) Senescent cells have different types of nuclear foci, such as DNA segments with chromatin alteration reinforcing senescence (DNA-SCARS), which are sites that contain different DDR proteins. Senescence associated heterochromatin foci (SAHF) are other examples of foci found in senescent cells. SAHF are formed as a result of p16 activation. This type of foci contain silenced pro-proliferative genes (Campisi 2014; Rodier & Campisi 2011).

6) Senescent cells produce and secrete different factors such as cytokines, chemokines, and matrix remodeling factors. Collectively, this phenotype is called senescence associated secretory phenotype (SASP). The effects and the identities of SASP vary, depending on the cell type and the method of induction of senescence. SASP is a feature that potentially

could explain the role of senescent cells in aging and several pathological conditions like cancer (Rodier & Campisi 2011; Campisi 2014).

SASP components can be divided into three groups:

1) Soluble factors. This group includes interleukins, such as IL-6, 7 and 13, various chemokines, such as eotaxin, and growth factors, such as NGF, and many others.
2) Shed receptors or ligands, which include ICAM-1 and EGF. These factors need specific sheddases in order to be cleaved and released from the cell surface of senescent cells.
3) Insoluble factors, which include fibronectin, collagens, and laminin.

Collectively, SASP factors can affect the surrounding cells by acting on specific cell-surface receptors, resulting in the activation or inhibition of different signaling pathways. Changes in the activities of these pathways may lead to different pathological conditions, such as cancer (Coppé 2010).

Different Roles of Cellular Senescence

Cellular senescence plays an important role in different processes, including wound healing, tissue repair, tumor suppression, and tumor progression (Rodier & Campisi 2011; Sabin & Anderson 2011).

In cutaneous wound healing, the matricellular protein CCN1/CYR61, which is a highly expressed at the sites of tissue repair, induces cellular senescence in fibroblasts. CCN1/CYR61 increases the accumulation of reactive oxygen species (ROS), resulting in the induction of DNA damage response and activation of the p53 tumor suppressor gene (Jun & Lau 2010). Activation

of DNA damage response and p53 in turn leads to the activation of the $p16^{INK4a}$ cell cycle inhibitor and causes fibroblasts to become senescent. Senescent fibroblasts express antifibrotic genes that limit fibrosis during the wound healing process (Jun & Lau 2010; Sabin & Anderson 2011). Similarly, it has been found that senescent cells that are derived from activated hepatic stellate cells produce matrix metalloproteinases to resolve fibrosis resulting from the production of the extracellular matrix during liver injury (Krizhanovsky 2008).

In cancer, cellular senescence has long been considered a mechanism of tumor suppression for several reasons. First, senescent cells undergo growth arrest, whereas tumor cells are highly proliferative, which is one of the hallmarks of cancer (Siddiqa & Marciniak 2008). Second, senescent cells are activated via two important tumor suppressor pathways, the tumor suppressor p53 pathway and the pRB/p16 pathway (Campisi & d'Adda di Fagagna 2007; Chen 2005; Rodier 2007). These pathways are highly mutated in most cancers in human and mice (Rodier & Campisi 2011). Moreover, some of the SASP components have been shown to maintain the senescence state. For example, secretion of the proinflammatory cytokines IL-6 and IL-8 is increased upon induction of senescence by the $BRAF^{E600}$ oncogene. This increase in IL-6 and IL-8 maintains the senescent state. Furthermore, knocking-down IL-8 reduces the number of senescent cells (Acosta 2008; Kuilman 2008).

In contrast, cellular senescence can be also considered a driving force for cancer, mainly due to the function of different SASP components. For example, MMP3, also called stromelysin, and amphiregulin, which are SASP components, are able to stimulate the tumor cell growth (Bavik 2006; Liu & Hornsby 2007). Liu *et al*. showed that when senescent fibroblasts were co-transplanted with cancer cells into immunocompromised mice, the proliferation of cancer cells was increased (Liu & Hornsby 2007). Moreover, it has been shown that different SASP

components induce the Epithelial-to-Mesenchymal transition (EMT), a critical process in metastatic tumors (Laberge 2012). Finally, it is known that senescent cells secrete factors that increase the resistance of non-senescent cells to specific chemotherapeutic agents, such as doxorubicin (Gilbert & Hemann 2010).

Regulation of SASP

Several positive and negative regulators of SASP have been identified. Among those regulators, nuclear factor kappa B (NF-κB) is the major inducer of SASP components (Salminen 2012; Rovillain 2011). NF-κB is a transcription factor, which in its inactive state is found in the cytoplasm and interacts with inhibitory kappa B (IκB) protein. Upon activation, IκB is phosphorylated, poly-ubiquitinated and degraded, which allows NF-κB to translocate to the nucleus, dimerize, and regulate the expression of several genes, such as IL-1, 6, and 8, cell adhesion molecules, and different cell cycle regulators. NF-κB has been implicated in different pathological conditions including diabetes, atherosclerosis, asthma, and cancer, as well as other biological processes, such as the adaptive and innate immunity (Salminen 2012; Serasanambati & Chilakapati 2016; Sun 2004).

Chien *et al.* showed that p65, an NF-κB subunit, was enriched in the chromatin of senescent cells. Moreover, Rouvillain *et al*. showed that the inhibition of NF-κB could overcome senescence induced by p53/p21 signaling in immortalized human fibroblasts (Rovillain 2011). This agrees with results of another study, in which it was shown that the suppression of NF-κB in a mouse lymphoma model prevented the induction of cellular senescence upon treatment with etoposide, a chemotherapeutic drug (Chien 2011).

Different potential signaling pathways were identified to regulate NF-κB (Figure 1.2). It has been shown that TGFβ receptor activates TGFβ-activated kinase 1 (TAK1), which leads to the activation of IκB kinase. IκB kinase phosphorylates IκB, which results in its poly-ubiquitination and degradation by the 26S proteasome. As mentioned previously, this allows to NF-κB to translocate into the nucleus and activate the transcription of different genes (Serasanambati & Chilakapati 2016; Sun 2004).

The ADAM Metalloproteases

A Disintegrin and Metalloprotease-containing proteins (ADAMs) are a family of membrane-anchored proteases (Giebeler & Zigrino 2016). ADAMs regulate important cellular processes, such as cell adhesion, migration, and differentiation. ADAMs are mainly involved in the proteolytic ectodomain release or shedding of various transmembrane proteins, such as growth factors, cytokines, different receptors, or cell adhesion molecules (Reiss & Saftig 2009).

In the human genome, twenty-two *ADAMs* have been described. Among them, twelve genes encode proteins that express a typical metalloprotease Zn-binding active site; those are *ADAM8*, *9*, *10*, *12*, *15*, *17*, *19*, *20*, *21*, *28*, *30, and 33* (Jones, Rustagi, & Dempsey 2016). ADAMs are multidomain transmembrane proteins. The domain structure includes a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic domain (Figure 1.3). In addition to ADAM domains, ADAMs have an N-terminal signal sequence that directs them into the secretory pathway as type 1 transmembrane proteins (Weber & Saftig 2012). The signal sequence is removed in the endoplasmic reticulum, then ADAMs are folded, glycosylated, and trafficked to the Golgi

apparatus. In the trans-Golgi network, ADAMs are activated by cleaving off their prodomain via pro-protein convertases. Finally, the mature, active form of an ADAM protein is usually trafficked to the cell surface (Gomis-Ruth 2009; Wong 2015).

The prodomain, which is located at the N-terminal portion of ADAMs, has two functions; first, it serves as an autoinhibitory domain of the catalytic metalloprotease domain, and second, it acts as an intramolecular chaperone to assure the correct folding of the ADAM protein (Wong 2015).

Downstream of the prodomain, there is a metalloprotease domain that contains the catalytic HEXGHXXHD motif. The three histidine residues bind with a zinc atom and form the catalytic center (Jones, Rustagi, & Dempsey 2016; Gomis-Ruth 2009). Since several ADAMs (ADAM18, ADAM22, ADAM23, ADAM29, and ADAM32) are catalytically inactive, it has been suggested that ADAMs might also function in protein-protein interaction.

Downstream of the disintegrin domain, there is a cysteine-rich domain, which plays a role in protein-protein interactions and determines the substrate specificity (Jones, Rustagi, & Dempsey 2016). It has been suggested that this domain is important in regulating the catalytic activity of ADAMs. Takeda *et al*. crystalized the vascular apoptosis-inducing factor-1, which is a snake venom toxin that contains Metalloproteinase/Disintegrin/Cysteine-rich (MDC) domains similar to those present in ADAMs (Takeda 2006). The results showed that the cysteine-rich domain faces the metalloprotease domain at ~4 nm, which suggests that this domain may capture specific substrates in order to be processed by the metalloprotease domain (Takeda 2006). The EGF-like domain may mediate multimerization of ADAM17 (Lorenzen 2011). The cytoplasmic domain contains one or more proline-rich sequence (PRS), which potentially enables the interaction with Src homology 3 (SH3) domain that is found in many signaling molecules. For

example, ADAM10 has two PRSs (Lettau 2014), ADAM12 contains multiple SH3 binding motifs which are known to interact with Src kinases and the p85α subunit of phosphatidylinositol 3 kinase (PI3K) (Li 2013; Kang 2001).

Patterns of ADAMs Expression

In general, ADAMs are expressed at different levels in different tissues. For example, approximately one third of human ADAMs, including ADAM2, 7, 18, 20, 21, 29, 30, are expressed in the testis and/or its associated structures (Cho Chughee 2012; Edwards 2009; Seals & Courtneidge 2003). In the following sections, the other two groups of ADAMs, hematopoietic and globally expressed ADAMs, will be reviewed. Only the catalytically active ADAMs will be discussed in each group.

Hematopoietic ADAMs

The hematopoietic ADAMs include ADAM8 and ADAM28 (Edwards 2009). These two ADAMs are catalytically active and able to autocleave their respective prodomains (Romagnoli 2014; Edwards 2009). In other words, they do not need pro-protein convertases to cleave their prodomain in order to become active.

ADAM8, also called CD156a, is expressed mainly in immune cells, such as granulocytes, monocytes, myeloid cells and neutrophils (Yeh & Klesius 2009; Romagnoli 2014). In neutrophils, ADAM8 is activated and participates in the neutrophil inflammatory response (Yeh & Klesius 2009). ADAM8 has been also implicated in different pathological conditions, such as allergy and brain, prostate, and lung cancers (Yeh & Klesius 2009; Romagnoli 2014). ADAM8

can cleave different transmembrane proteins, such as L-Selectin, a cell adhesion protein expressed in neutrophils (Gomez-Gaviro 2007; Fourie 2003). However, despite of the link between ADAM8 and various ailments, a mouse knock-out of *ADAM8* is viable and has no pathological phenotype (Kelly 2005).

ADAM28, which is also known as the lymphocyte metalloprotease MDC-L, is mainly expressed in lymphocytes, monocytes, and neutrophils; however, it is also expressed in the lung, spleen, and epididymis (Bridges 2002; Nuttall 2004). Moreover, Mitsui *et al*. showed that ADAM28 is overexpressed in human breast cancer cells and its expression increases the proliferation of cells (Mitsui 2006). ADAM28 has different functions; for example, it has been shown that the disintegrin domain of ADAM28 interacts with α4β1 integrin that is found in leukocytes. This raises a possibility that ADAM28 plays a role in lymphocyte-leukocyte interactions (Bridges 2002). Also, ADAM28 cleaves insulin-like growth factor binding protein-3 (IGFBP-3) into two major fragments. The cleavage of IGFBP-3 results in releasing of IGF-I from the IGF-I/IGFBP-3 complex, which leads to increase in proliferation of the breast carcinoma cells (Fourie 2003; Mochizuki 2004; Mitsui 2006).

Globally Expressed ADAMs

This group contains the largest number and the most diverse ADAMs. Catalytically active ADAMs from this group include ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, and ADAM33. Catalytically inactive, globally expressed ADAMs, like ADAM11, ADAM22, ADAM23, and ADAM32, will not be discussed in this review.

ADAM9 expression is increased in different pathological conditions such as breast, prostate and intestinal cancers (Edwards 2009). *ADAM9* knock-out mice do not exhibit a defect in the growth or development (Sahin 2004). ADAM9 cleaves different transmembrane proteins, for example, pro-EGF and pro-HB-EGF (Reiss & Saftig 2009; Izumi 1998; Mohan 2002).

ADAM10 has been extensively studied because of its broad substrate specificity. Moreover, knock-out of the *ADAM10* gene in mice showed embryonic lethality as a result of cardiovascular and central nervous system defects (Hartmann 2002). ADAM10 is mainly expressed in stem cells, placenta, bladder, and blood myeloid cells (Edwards 2009). Also, ADAM10 is upregulated in prostate cancer and it has an important role in Alzheimer's disease (Edwards 2009). Chen *et al.* showed that ADAM10 and ADAM17 shed the anti-aging transmembrane protein klotho in COS-7 cells and rat kidney; cotransfection of klotho with ADAM10 or ADAM17 small interference RNA decreased the secretion of klotho (Chen 2007). Furthermore, ADAM10 cleaves a variety of substrates such as EGF, E-cadherin, and IL-6 receptor (Reiss & Saftig 2009).

ADAM12 is mainly expressed in stem cells and the placenta during pregnancy (Edwards 2009). *ADAM12* knock-out mice studies showed 30 % embryonic lethality and abnormalities in the brown fat tissues (Kurisaki 2003). ADAM12 was shown to be upregulated in breast and bladder cancer (Lendeckel 2005; Narita 2012). It has been shown that the overexpression of ADAM12 in breast cancer cells and in a mouse model of breast cancer, mouse mammary tumor virus-polyoma middle T antigen (MMTV-PyMT), promoted tumor growth. In a complementary approach, knock-down of ADAM12 in the same MMTV-PyMT mouse model reduced tumor progression (Roy 2011; Fröhlich 2011). Those data suggest that ADAM12 is an important modulator of breast cancer progression.

ADAM12 cleaves many transmembrane proteins (Edwards 2009). Asakura *et al.* showed that ADAM12 increases the secretion of HB-EGF in cardiomyocytes (Asakura 2002). Delta-like 1 ligand (DLL1) is another protein that is cleaved by ADAM12, and other ADAMs, such as ADAM9 and ADAM17 (Edwards 2009; Dyczynska 2007). DLL1, which is an integral membrane protein, interacts with Notch receptor on the neighboring cells in a *trans* manner, and as a result, the Notch signaling pathway is activated (Kopan 2012). The Notch signaling pathway is conserved between species and it plays a key role in cell proliferation, differentiation, and death (Kopan 2012). The cleavage of DLL1 via ADAMs inhibits Notch signaling since the ligand becomes less available at the cell surface to interact in *trans* with Notch receptor. Moreover, it has been shown that ADAM12 degrades different extracellular matrix (ECM) proteins, such as gelatin, type IV collagen, and fibronectin, which suggests that ADAM12 might have an important role in ECM remodeling (Roy 2004).

ADAM15 and ADAM19 are expressed in a wide range of tissues (Edwards 2009). *ADAM15* knock-out mice showed normal development, however, these mice developed osteoarthritis later in life (Böhm 2005). *ADAM19* knock-out mice showed 80% lethality due to cardiovascular defects (Reiss & Saftig 2009). ADAM15 cleaves different transmembrane proteins, including CD23, HB-EGF, and E-cadherin (Reiss & Saftig 2009). ADAM19 cleaves other transmembrane proteins, for example, neuregulin β1 in the Golgi apparatus of sensory neurons (Yokozeki 2007).

Similar to ADAM10, ADAM17 is an extensively studied ADAM, since it acts upon a wide variety of substrates (Edwards 2009). Knock-out of the *ADAM17* gene in mice is lethal due to heart defects (Jackson 2003; Edwards 2009). ADAM17 is expressed in many tissues, with the highest expression being observed in the lymphatic system (Edwards 2009; Reiss & Saftig

2009). Moreover, ADAM17 is upregulated under many pathological conditions, such as rheumatoid arthritis, multiple sclerosis, diabetes, and breast cancer (Edwards 2009). ADAM17 overexpression in breast cancer cell lines increased cell proliferation, invasion, and tube formation. Downregulation of ADAM17 expression by ADAM17 siRNA or inhibition of ADAM17 activity caused opposite effects using the same metrics (Zheng 2009). In addition, knock-down of ADAM17 resulted in a decrease in secretion of transforming growth factor α (TGFα) and vascular endothelial growth factor (VEGF), two important factors that increase the proliferation of normal or neoplastic epithelial cells and formation of new blood vessels, respectively (Zheng 2009). ADAM17 is also called tumor necrosis factor α (TNFα) converting enzyme since it is the main enzyme responsible for cleaving TNFα, a pro-inflammatory cytokine in inflammation (Zelová & Hošek 2013). Beatriz *et al.* showed that ADAM17 expression at the mRNA and protein levels was increased in senescent p95HER2-exppressing MCF7 cells. HER2 is an oncogene and its overexpression induces cellular senescence in certain cell lines, such as MCF7. Moreover, the results of the same study indicated that ADAM17 sheds different transmembrane proteins, such as amphiregulin, an EGFR ligand, upon induction of cellular senescence (Morancho 2015). However, another study showed by Western blotting and by a cell surface biotinylation assay that ADAM17 protein expression does not increase in senescent PC3 prostate cancer cells (Effenberger 2014). In that study, cellular senescence was induced by a different approach, namely by using a chemotherapeutic drug doxorubicin. In contrast, ADAM17 activity was increased upon induction of senescence due to the increase of phosphorylation of Thr735, one of the well-characterized posttranslational modifications that increase the activity of ADAM17. Finally, it was shown that ADAM17 increases the release of amphiregulin and tumor necrosis factor receptor 1 (TNFR1) (Effenberger 2014).

ADAM33 is expressed in different systems in humans, including the respiratory, urogenital, gastrointestinal, and endocrine system (Reiss & Saftig 2009; Edwards 2009). Although ADAM33 is expressed in many tissues, ADAM33 knock-out in mice showed no pathological phenotype (Reiss & Saftig 2009; Edwards 2009). ADAM33 sheds a transmembrane protein CD23 (Reiss & Saftig 2009).

EGFR Signaling

Epidermal growth factor receptor (HER) family is an important modulator of cancer cell growth, survival, and metastasis (Kataoka 2009; Foley 2011). The HER family consists of four tyrosine kinase receptors, EGFR /HER1, HER2, HER3, and HER4 (Kataoka 2009; Foley 2011). These four receptors are activated by different transmembrane ligands. Eleven epidermal growth factor receptor (EGFR) ligands have been identified; those are EGF, TGFα, amphiregulin, HB-EGF, betacellulin, epiregulin, epigen, and neureglulin-1, 2, 3 and 4 (Foley 2011). Each of these ligands binds with one or more receptors of the HER family. A characteristic feature of these ligands is that they are transmembrane proteins that require cleavage from the cell surface. Upon their cleavage, the ligands are released and bind to the corresponding receptor. As a result, a homo- or hetero-dimer is formed with another HER family member; this leads to the activation of the tyrosine kinase activity. This allows multiple tyrosine residues in the cytoplasmic tail of the receptor to be phosphorylated, thereby forming docking sites for different proteins that further modulate intracellular signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, or the phosphoinositide-3 kinase (PI3K) pathway (Kataoka 2009; Foley 2011; Montemurro 2013).

EGFR, HER3, and HER4 need to bind a ligand in order to become active. HER2 does not require ligand binding to be activated. Overexpression of HER2 in cancer leads to constitutive activation of the downstream signaling pathways, and as a result, sustained stimulation of cell proliferation (Montemurro 2013). HER3 lacks the intrinsic tyrosine kinase activity necessary for the downstream signaling cascade to occur (Kataoka 2009; Foley 2011). HER4 requires EGFR ligands in order to be activated, however, it's activation leads to reduced proliferation and promotion of apoptosis in breast cancer (Koutras 2010).

In the absence of HER2 overexpression, estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative (or triple negative) breast cancer (TNBC) cells depend largely on EGFR to convey the growth signals (Wilson 2009; Foley 2011).

As mentioned above, EGFR ligands are transmembrane proteins that need to be cleaved to interact with and activate their corresponding receptors from the HER family. The cleavage of specific EGFR ligand is mediated by one or more ADAMs, such as ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, or ADAM19 (Kataoka 2009).

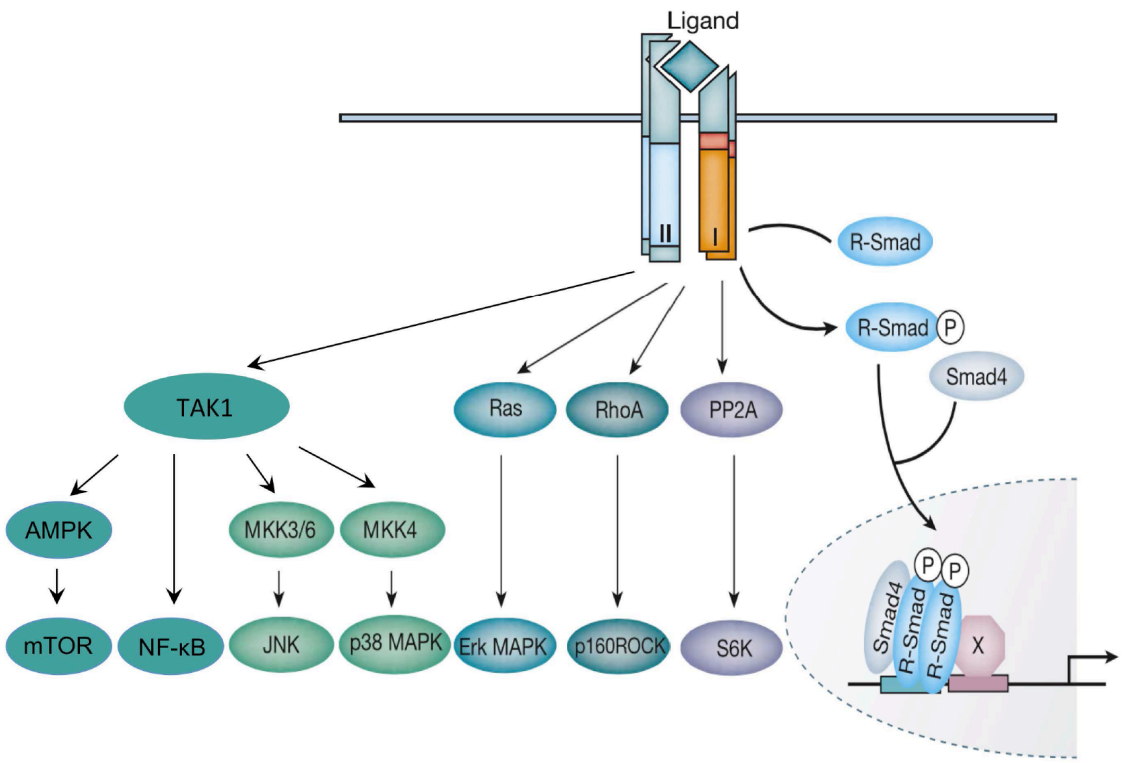

Figure 1.1 TGFβ signaling pathways are mediated either through the canonical, Smad-dependent or non-canonical, Smad-independent pathways.

Based on Figure 6 from (Derynck & Zhang 2003), with permission from Nature Publishing Group.

TAK, TGFβ-activated kinase; AMPK, AMP activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, Nuclear factor kappa B; MKK, dual specificity mitogen activated protein kinase kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase; Erk, extracellular signal regulated kinase; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated protein kinase; PP2A, protein phosphatase 2A; S6K, ribosomal protein S6 kinase.

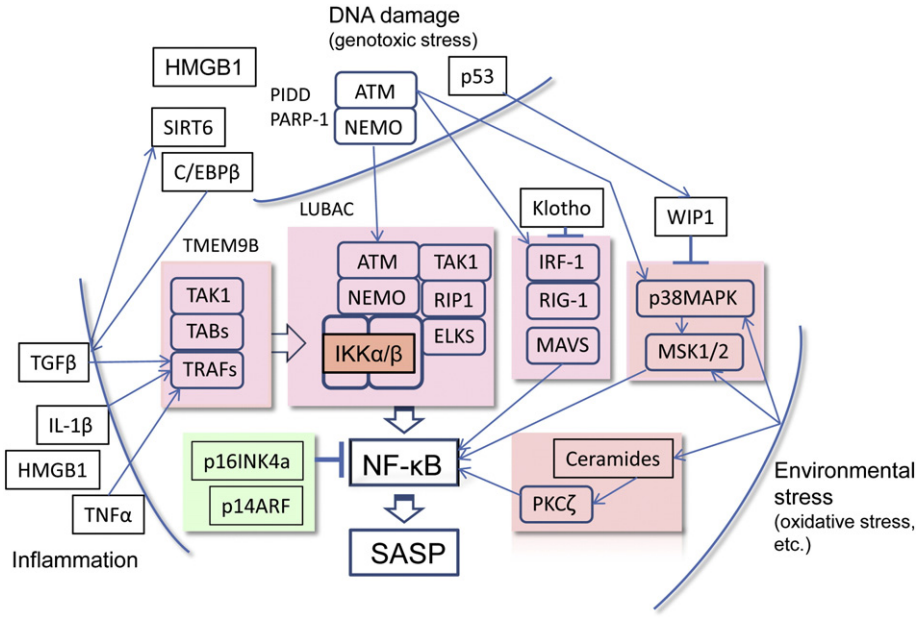

Figure 1.2 Different signaling pathways can regulate NF-κB.

Based on Figure 1 from (Salminen 2012)**,** no changes were made to the figure.

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ATM, Ataxia telangiectasia mutated; C/EBPβ, CCAAT/enhancer binding protein β; ELKS, a protein rich in the following amino acids: glutamic acid, leucine, lysine, and serine; HMGB1, high mobility group box 1; IKKα/β, IκB kinase α and β; IL-β, interlukin-1β; IRF-1, interferon regulatory factor-1; LUBAC, linear upiquitin chain assembly complex; p38MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral-signaling protein; MSK1/2, mitogen- and stress-activated protein kinases 1 and 2; NEMO, NF-κB essential modifier; p14ARF, alternate reading frame product of p16INK4a; p16INK4a, cyclin dependent kinase inhibitor 2A; PARP-1, poly (ADP-ribose) polymerase-1; PIDD, p53-induced protein with a death domain;

PKCζ, protein kinase Cζ; RIG-1, retinoic acid inducible gene-1; RIP1, receptor interacting protein 1; SIRT6, silent information regulator 6; TAB, TAK1-binding protein; TMEM9B, transmembrane protein 9B; TNFα, tumor necrosis factor α; TRAFs, TNF-receptor-associated factors; WIP1, protein phosphatase 2Cδ.

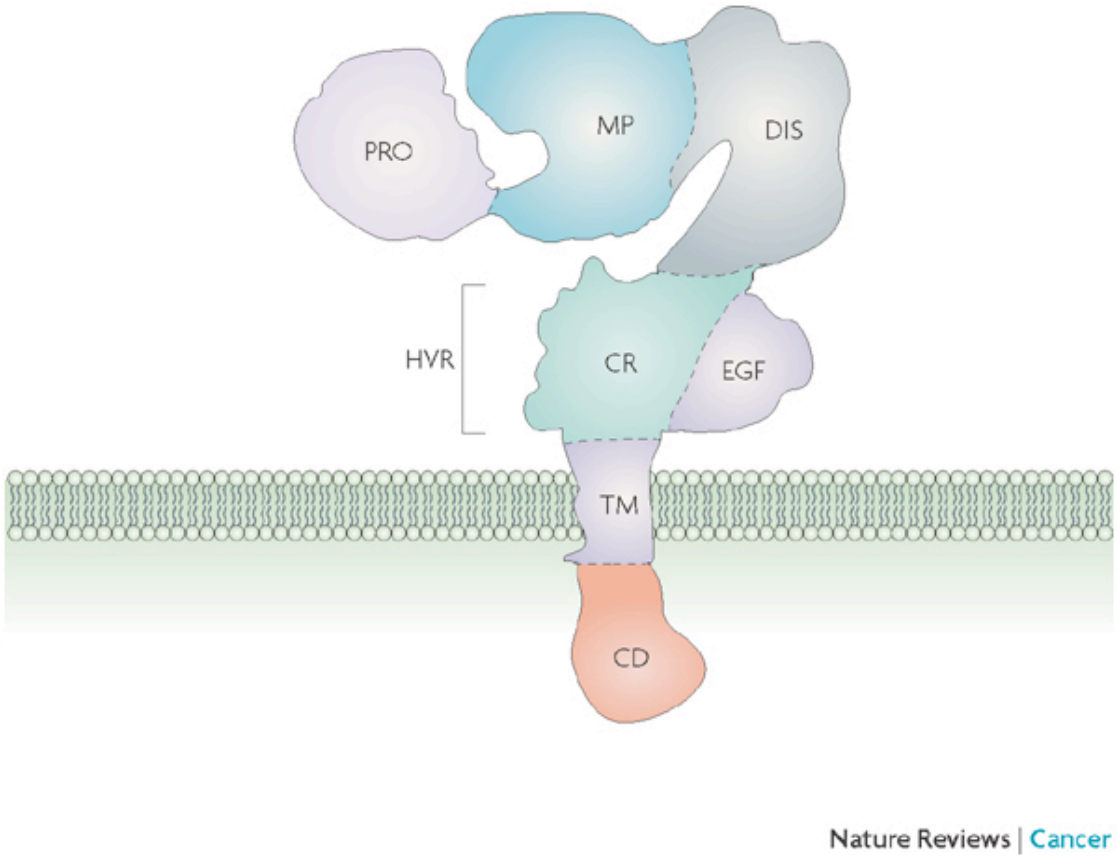

Figure 1.3 Domain organizations of ADAM metalloproteases.

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PRO, prodomain; MP, metalloprotease; DIS, disintegrin; CR, cysteine-rich; EGF, EGF-like; TM, transmembrane; CD, cytoplasmic domain, HVR, hypervariable region.

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Chapter 2 - Expression of ADAM Metalloproteases during Transforming Growth Factor β-Induced Senescence in Breast Cancer Cells

Abstract

Cellular senescence is a state of irreversible cell cycle arrest in response to different inducers. Many inducers, intrinsic and extrinsic, have been identified to cause senescent growth arrest; for example, transforming growth factor β (TGFβ). Senescent cells secrete different factors, such as inflammatory cytokines, chemokines, or matrix remodeling factors. Those factors affect neighboring cells by increasing their proliferation, inducing epithelial-mesenchymal transition (EMT), or causing other effects. Some of the secreted factors are transmembrane proteins that require cleavage from the surface of senescent cells in order to be released and to affect neighboring cells. A Disintegrin and Metalloprotease (ADAM) family proteins are sheddases for many transmembrane proteins.

In the present study, we tried to induce cellular senescence in two breast cancer cell lines, SUM102PT and SUM149PT cells, using TGFβ, followed by treating the cells with a high dose of paclitaxel to remove the actively proliferating, non-senescent cells. The induction of cellular senescence was tested using senescence associate β galactosidase (SA-β-gal) staining and assessment of cell size and granularity. The induction of cellular senescence was confirmed in SUM102PT cells. The mRNA expression levels of different catalytically active *ADAMs; ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM 19, and ADAM28*, were investigated. Among these ADAMs, the mRNA level of *ADAM12* was significantly upregulated

in SUM102PT cells upon induction of senescence. Further investigations are needed to test ADAM12 protein expression upon induction of cellular senescence.

Introduction

Cellular senescence represents a response of cells to non-lethal stress (Shay & Roninson 2004; Rodier & Campisi 2011; Campisi 2014). Senescent cells undergo an irreversible cell cycle arrest to prevent the transmission of the damage to daughter cells and to prevent neoplastic transformation (Campisi 2014; Sabin & Anderson 2011).

Senescent cells have different characteristic features: 1) Unlike quiescent cells, senescent cells are not able to re-enter the cell cycle upon proliferative stimuli. 2) Senescent cells are 2-fold larger than their non-senescent counterparts. 3) Senescent cells have profound changes in their chromatin organization and have different types of nuclear foci, such as DNA-SCARS and SAHF foci. 4) Senescent cells have increased activity of the SA-β-gal enzyme. 5) Senescent cells are metabolically active and secrete different factors, such as cytokines, chemokines, matrix remodeling enzymes, and growth factors. This secretory phenotype is called senescence associated secretory phenotype (SASP). Collectively, those hallmarks define the senescent state (Rodier & Campisi 2011; Campisi 2014; Shay & Roninson 2004; Campisi & d'Adda di Fagagna 2007).

SASP components have both beneficial and deleterious effects. In cancer, some SASP components maintain the senescent state. On the other hand, other SASP components promote tumor growth by increasing cell proliferation or inducing EMT. Therefore it is important to

understand the mechanisms that lead to the release of SASP factors from senescent cells (Rodier & Campisi 2011; Coppé 2010).

Different agents or treatments can induce cellular senescence. TGFβ is an example of such agents (Salminen 2012; Campisi 2014). Besides induction of cellular senescence, TGFβ induces and modulates other processes in the cells. For example, TGFβ induces EMT, a developmental process that occurs in normal tissues and in tumors. Moreover, TGFβ regulates cell growth, differentiation, and apoptosis (Derynck & Zhang 2003; Vijayachandra 2009; Heldin 1997).

A disintegrin and metalloproteases (ADAMs) are able to proteolytically cleave many transmembrane proteins, which have been found to be integral parts of SASP (Coppé 2010; Reiss & Saftig 2009). Moreover, expression of several ADAMs is upregulated in different cancers including breast cancer, which reflects the relevance of those ADAMs for carcinogenesis (Edwards et al. 2009).

The goal of this study was to test the expression levels of different catalytically active ADAMs upon induction of cellular senescence in breast cancer cell lines. We identified *ADAM12* as a novel metalloprotease that is upregulated at the mRNA level upon induction of cellular senescence in SUM102PT cells. ADAM12 protein expression at the cell surface was also examined by flow cytometry. However, since senescent cells are significantly larger than their non-senescent counterparts, we were not able to definitely establish whether the slight increase in the anti-ADAM12 staining in senescent cells was as a result of upregulation of ADAM12 or increased non-specific binding of anti-ADAM12 antibody. Additional investigations are needed to test ADAM12 protein at the cell surface of senescent cells.

If ADAM12 is indeed upregulated in senescent cells, it may play a role in releasing certain SASP components. Thus, targeting ADAM12 could be used as a strategy to block the release of these SASP components.

Materials and Methods

Reagents and antibodies

Senescence β-Galactosidase Staining (SA-β-Gal staining) Kit (#9860) was from Cell Signaling Technology. Antibodies used for flow cytometry were: anti-ADAM12 (clone 632525) and IgG1 isotype control (clone 11711), both from R&D Systems. Allophycocyanin (APC)-conjugated anti-mouse IgG antibody was from Jackson ImmunoResearch. Other reagents used include paclitaxel (Sigma-Aldrich), TGFβ (R&D) and propidium iodide (BD Biosciences).

Cell culture

SUM149PT cell line was purchased from Asterand. SUM102PT cells were a gift from Dr. Fariba Behbod (University of Kansas Medical Center). SUM149PT 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. SUM102PT cells were cultured in Ham's F-12 medium, which was supplemented with 5% FBS, 1 μg/ml hydrocortisone, and 5 μg/ml insulin. Cells were maintained at 37°C under humidified atmosphere containing 5% CO_2.

Flow cytometry

ADAM12 staining: SUM102PT cells were treated with or without 2 ng/ml TGFβ for 10 days. At day 6, cells were treated with or without 200 nM paclitaxel for 4 days. Cells were trypsinized, washed, and prepared as a single cell suspension at 10^6 cells/100 µl in DPBS with 3% BSA. Cells were incubated with either anti-ADAM12 or IgG1 isotype control antibody at 1:10 dilution (25 µg/ml) for 30 minutes on ice. Then, cells were washed three times with DPBS and incubated with APC-conjugated anti-mouse IgG antibody at 1:50 dilution for 30 minutes on ice. Finally, cells were washed with DPBS and suspended in 500 µl DPBS containing 3% BSA. Cells were analyzed with a BD FACS Calibur cytometer. All data were analyzed with the FCS Express 4 software (DeNovo Software).

Cell survival assay

SUM149PT or SUM102PT cells were treated with or without 2 ng/ml of TGFβ for 10 days. At day 6, cells were detached, counted by a cellometer, and seeded into a 96-well plate at 3000-5000 cells/well. Cells were then treated with various doses of paclitaxel until day 10. At day 10, viable cells were evaluated using CellTiter-Glo Luminescent Cell Viability Assay (Promega) and Synergy H1 microplate reader (BioTek); at least 3 wells per each cell type were measured for each dose of paclitaxel. The dose response for paclitaxel was calculated using the GraphPad Prism software.

β-Galactosidase staining

SUM149PT or SUM102PT cells were incubated with or without 2 ng/ml TGFβ for 10 days. At day 6, cells were treated with or without 100 nM (SUM149PT cells) or 200 nM

(SUM102PT cells) paclitaxel for additional 4 days. At day 10, cells were fixed for 15 minutes, followed by applying β-Galactosidase staining solution and incubation overnight at 37°C. Cells were examined by light microscopy for the development of blue color and several images were acquired using a 4X objective.

Quantitative real-time PCR

Total RNA was extracted using the Qiagen RNeasy kit and subjected to on-column digestion with deoxyribonuclease I. One microgram of total RNA was reverse-transcribed using the SuperScript III First Strand Synthesis system (Invitrogen). Quantitative real time-PCR (qRT-PCR) was performed on a BioRad CFX96 instrument. At the end of each run, a melt curve analysis was performed to ensure that a single product had been synthesized. The relative expression of each gene, normalized to actin, was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 2.1 Primer sequences.

Type of primers		Primer sequence
ADAM8	F	5'-AGG CTC AGC TGC TGT TCT AA-3'
	R	5'-CAC CTG GAG ACA CGT ACA CA-3'
ADAM9	F	5'-GAC TCA GAG GAT TGC TGC AT-3'
	R	5'-GAG GCT CTT CTT CTT CAT CC-3'
ADAM10	F	5'-AGC AAC ATC TGG GGA CAA AC-3'
	R	5'-CCC AGG TTT CAG TTT GCA TT-3'
ADAM12	F	5'-AGC CAC ACC AGG ATA GAG AC-3'
	R	5'-CGC CTT GAG TGA CAC TAC AG-3'
ADAM15	F	5'-CTT CAG GGT CCT CCC ATT AT-3'
	R	5'-ATC AGC CAC AAT CAC CAA CT-3'

ADAM17	F	5'-CTG TGG TGC AAA AGC AGA AA-3'
	R	5'-TGC CAA ATG CCT CAT ATT CA-3'
ADAM19	F	5'-CTG ATT ACG GTG AGC AGC AA-3'
	R	5'-TCG CTT CTT GGT CTG TTG TG-3'
ADAM28	F	5'-ATG CTG TGT GTG ACC ATG AG-3'
	R	5'-CGG ATT ACC ATA GCA ACC AC-3'
β- Actin	F	5'-TTG CCG ACA GGA TGC AGA A-3'
	R	5'-GCC GAT CCA CAC GGA GTA CT-3'

Results

TGFβ-induced senescence in breast cancer cell lines

To examine the expression levels of different ADAM metalloproteases during senescence, we used TGFβ to induce cellular senescence in two breast cancer cell lines, SUM102PT and SUM149PT. Since TGFβ may induce different phenotypes, in addition to cellular senescence (Figure 2.1a), cells were treated with paclitaxel (PTX), a chemotherapeutic drug to remove actively proliferating cells and enrich for senescent cell populations. PTX is an antimitotic agent that binds to microtubules during mitoses and stabilizes them and, as a result, causes an arrest at the G2/M phase, followed by apoptosis (Fan 1999). Senescent cells are resistant to PTX because they are not proliferative.

First, we performed a dose response to PTX in SUM102PT or SUM149PT cells that were either treated or untreated with 2 ng/ml TGFβ for 6 days. At day 6, TGFβ treatment continued and cells were treated with different doses of PTX for additional 4 days. We observed that there was a small population of cells that were resistant to high doses of PTX in both cell lines,

SUM102PT and SUM149PT (Figure 2.1 b and c). We didn't observe a similar effect in SUM159PT cells (data are not shown), another breast cancer cell line. These results suggested that TGFβ might act as an inducer of cellular senescence in SUM102PT and SUM149PT, but not in SUM159PT cells.

To further examine the properties of SUM102PT and SUM149PT cells treated with TGFβ and PTX, we used flow cytometry to measure the forward scatter (FSC) and the side scatter (SSC). FCS and SSC are indicative of the size and complexity of the cells, respectively (Shinohara 2000; Hégaret 2003). Cells treated with TGFβ and with 200 nM (in the case of SUM102PT) or 100 nM (in the case of SUM149PT) PTX were significantly larger and more complex than untreated cells (Figure 2.2 a and b, respectively).

Next, we performed a β-Galactosidase staining assay for either untreated cells or cells treated with TGFβ and PTX in both SUM102PT and SUM149PT cells (Figure 2.2 c and d, respectively). Many SUM102PT cells were stained blue after they were treated with TGFβ and PTX. However, we detected very few β-Gal-positive SUM149 cells after treatment with TGFβ and PTX. Moreover, after 4 days of recovery, SUM102PT cells appeared positive for β-Gal and didn't resume proliferation. SUM149PT cells, in contrast, didn't appear to be positive for β-Galactosidase, although they were not able to proliferate during the 4-day recovery period.

The increases in size, complexity, upregulation of β-Galactosidase expression, and the lack of the proliferation during the recovery period, were consistent with the features of senescent cells. We concluded that TGFβ treatment, followed by PTX-mediated elimination of proliferating cells, resulted in senescence in SUM102PT cells. The induction of senescence in SUM149PT cells was less clear.

Survey of ADAM expression in senescent cells

To answer the question of which ADAMs are upregulated upon induction of cellular senescence, we measured the mRNA levels of *ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM 19, and ADAM28* in SUM102PT senescent cells (Figure 2.3). The same *ADAM* were also tested in SUM149PT cells (Figure 2.4). These eight ADAMs are catalytically active and broadly expressed. The results showed that the mRNA expression of *ADAM12* was significantly increased upon induction of cellular senescence in SUM102PT cells. Interestingly, the mRNA expression of *ADAM28* was also increased in senescent cells. mRNA expression of tested *ADAM* followed the same trend in SUM149PT cells, although the increase *ADAM12* and *ADAM28* was not significant.

Cell surface expression of ADAM12 in senescent cells

Finally, we examined ADAM12 protein levels by flow cytometry. We observed a small increase in ADAM12 staining in senescent cells compared to non-senescent cells (Figure 2.4 a and b). ADAM12 expression was also increased in SUM149PT cells after TGFβ and PTX treatment (Figure 2.4 c and d). However, flow cytometry experiments had important limitations. As mentioned previously, senescent cells are larger than their non-senescent counterparts and they have high level of non-specific staining. Therefore, it is possible that increased ADAM12 staining observed in senescent cells was caused by a higher level of background non-specific staining. Clearly, more investigations are needed to evaluate ADAM12 protein expression in senescent cells.

Discussion

Cellular senescence is a state of irreversible cell cycle arrest in response to a variety of non-lethal stress conditions (Coppé 2010; Freund 2010). For a long time, this process was considered inhibitory during tumorigenesis. However, it was later discovered that senescent cells secrete various proteins, such as cytokines, chemokines, growth factors, and matrix remodeling enzymes. This feature has been termed SASP. SASP components may promote carcinogenesis by increasing cell proliferation and/or inducing EMT, among others. A group of SASP components are expressed at the surface of senescent cells as transmembrane proteins; those proteins need to be cleaved in order to act on their prospective targets (Rodier & Campisi 2011; Freund 2010; Coppé 2010). Since the catalytically active ADAMs act as sheddases for different transmembrane proteins that are SASP components, we tested the expression levels of different ADAMs upon induction of cellular senescence.

In this report, we examined the induction of cellular senescence in two breast cancer cell lines, SUM102PT and SUM149PT. We used TGFβ as an inducer of cellular senescence (Salminen 2012). While senescence was confirmed in SUM102PT cells, unfortunately, we were not able to detect β-Galactosidase staining in SUM149PT cells after a 4-day recovery period, which suggested that those cells might not be senescent. While TGFβ- and PTX- treated SUM149PT cells had increased size and complexity and they didn't resume proliferation after a recovery period, those features are not exclusive to senescent cells and they should not be treated as a strong evidence of senescence.

The expression levels of several catalytically active ADAMs, namely *ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM 19, and ADAM28*, were tested. *ADAM12* mRNA expression level was significantly increased upon induction of senescence in SUM102PT

cells. One of the identified intracellular pathways that increases *ADAM12* expression is the NF-κB pathway (Ray et al. 2010). Ray *et al.* identified NF-κB responsive elements in the *ADAM12* promoter and demonstrated that NF-κB increases *ADAM12* expression. This could be a possible reason for the upregulation of *ADAM12* expression during senescence, since TGFβ induces cellular senescence and SASP via the activation of the NF-κB transcription factor.

Another important observation in this report is that the expression level of *ADAM17* didn't change upon induction of cellular senescence. These results do not agree with the results published by Morancho *et al*., who showed that *ADAM17* mRNA levels in MCF7 cells were increased upon induction of cellular senescence via the expression of p95HER2 (Morancho 2015). On the other hand, our results agree, indirectly, with the results published by Effenberger *et al.*, who showed that although ADAM17 expression didn't change upon induction of cellular senescence in PC3 cells, the activity of ADAM17 protease was increased (Effenberger 2014). The possible explanation for this discrepancy may lie in using different cell lines in each study, or using different approaches to induce cellular senescence, or both.

In summary, our results demonstrate that *ADAM12* mRNA expression was increased in senescent cells. This upregulation of *ADAM12* mRNA may have important implications, if it is indeed accompanied by an increase in ADAM12 protein level. ADAM12 protease might release specific SASP components that could have an impact on the tumor microenvironment. Further investigations are needed to test ADAM12 protein expression. Cell surface biotinylation assays could be used as an alternative technique to evaluate ADAM12 expression at the cell surface and to overcome the limitations of flow cytometry experiments. If ADAM12 expression is upregulated in senescent cells and is associated with releasing SASP components, then this work may have important implications in cancer therapy. Targeting ADAM12 to block senescence-

associated ectodomain shedding might be a more efficient approach than targeting individual SASP components.

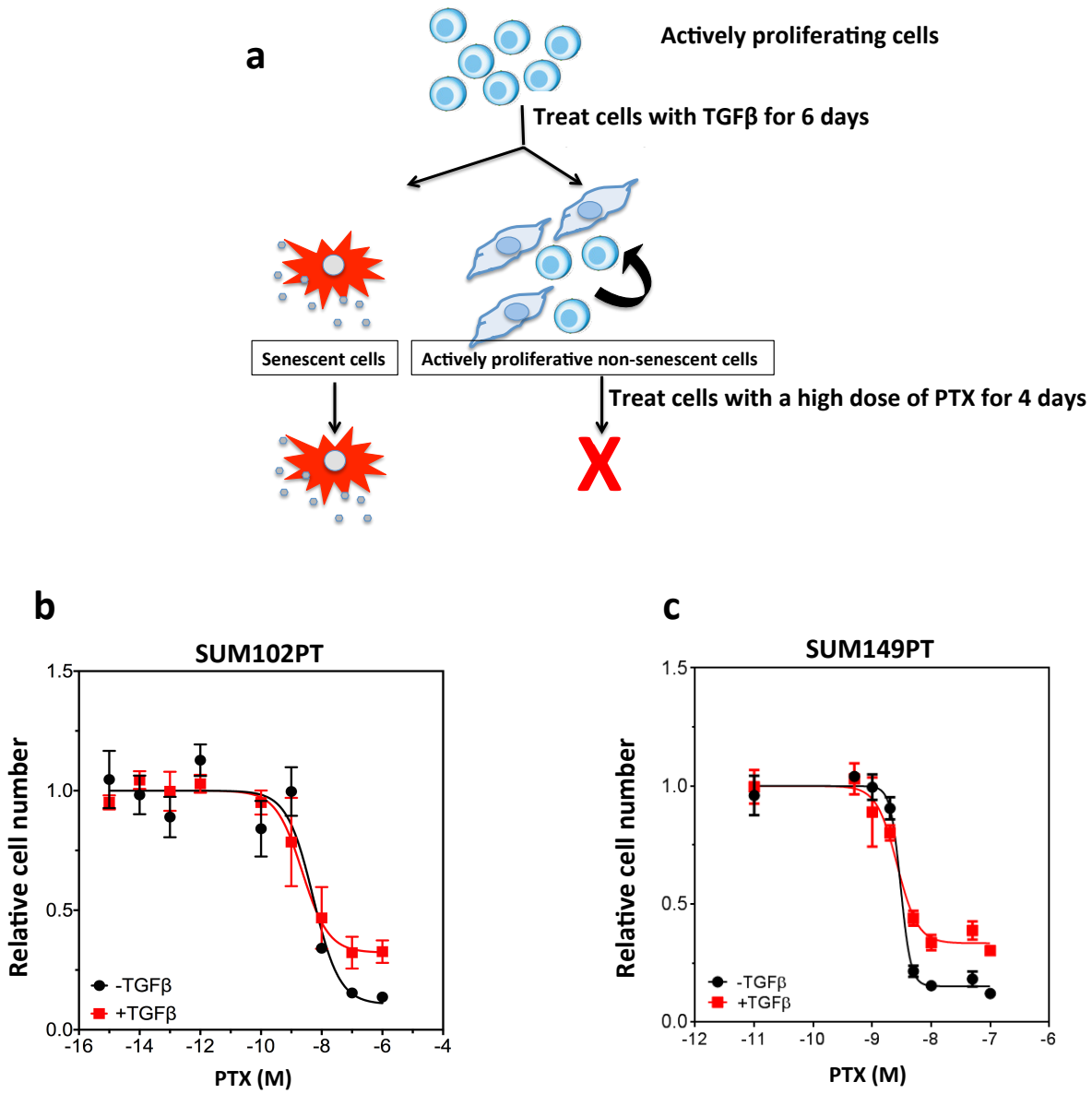

Figure 2.1 Small populations of cells are resistant to high doses of paclitaxel after TGFβ treatment in SUM102PT and SUM149PT cell lines.

(Panel c was performed by Yue Qi.)

(a) A cartoon showing the effect of TGFβ on proliferating cell. Treatment with high doses of paclitaxel targets actively proliferating cells. (**b**) SUM102PT cells and (**c**) SUM149PT cells were treated with or without 2 ng/ml TGFβ for 10 days. At day 6, cells were treated

with different concentrations of paclitaxel for 4 days. CellTiter-Glo assay was used to assess cell viability. Sigmoidal dose response curves were fitted using the GraphPad Prism software. Values were calculated from triplicate wells for each dose of paclitaxel.

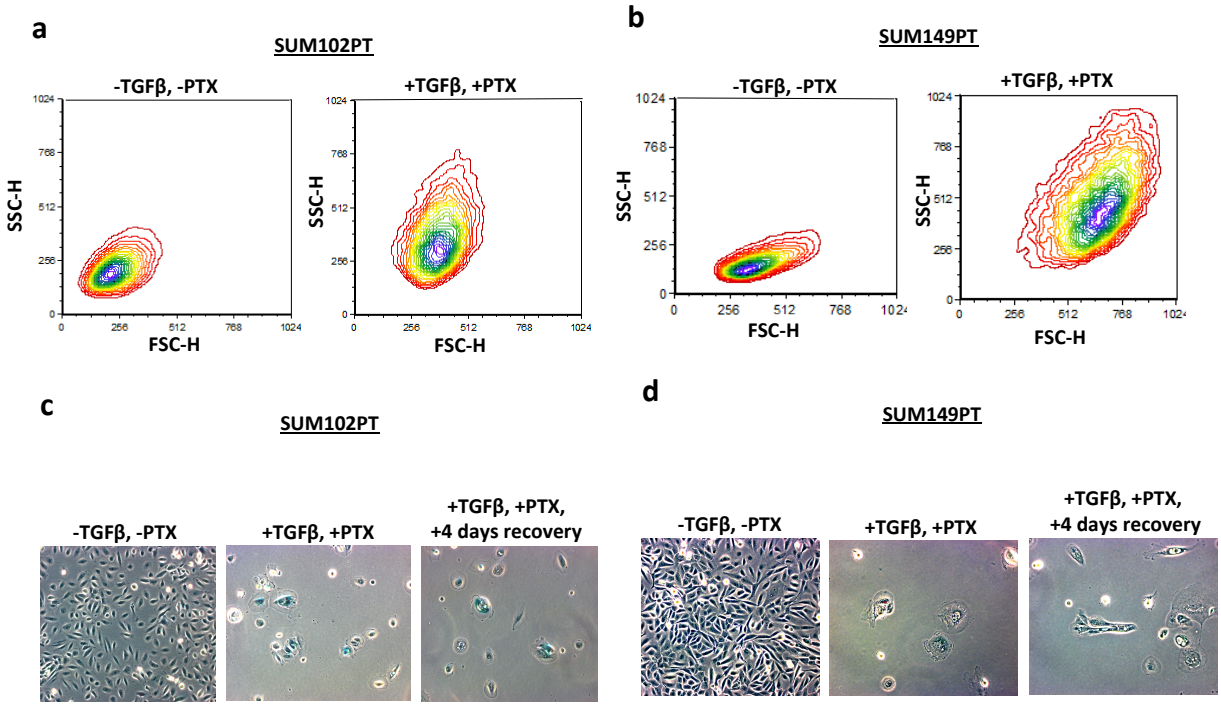

Figure 2.2 TGFβ treatment induces cellular senescence in SUM02PT cells.

(**a-b**) Representative flow cytometry measurement of the forward scatter (FSC) and side scatter (SSC) of SUM102PT cells (**a**) and SUM149PT cells (**b**). Cells were either untreated or treated with 2ng/ml TGFβ for 10 days and 100 nM (SUM149PT cells, n=3) or 200nM (SUM102PT cells, n=2) paclitaxel from day 6 to day 10. (**c-d**) SA-β-Gal staining. Representative phase-contrast images of SUM102PT cells (**c**) or SUM149PT cells (**d**) that were either untreated or treated with 2 ng/ml TGFβ for 10 days, including 100 nM (SUM149PT cells) or 200 nM (SUM102PT cells) paclitaxel from day 6 to day 10, and then allowed to recover for 4 days (n=1-2).

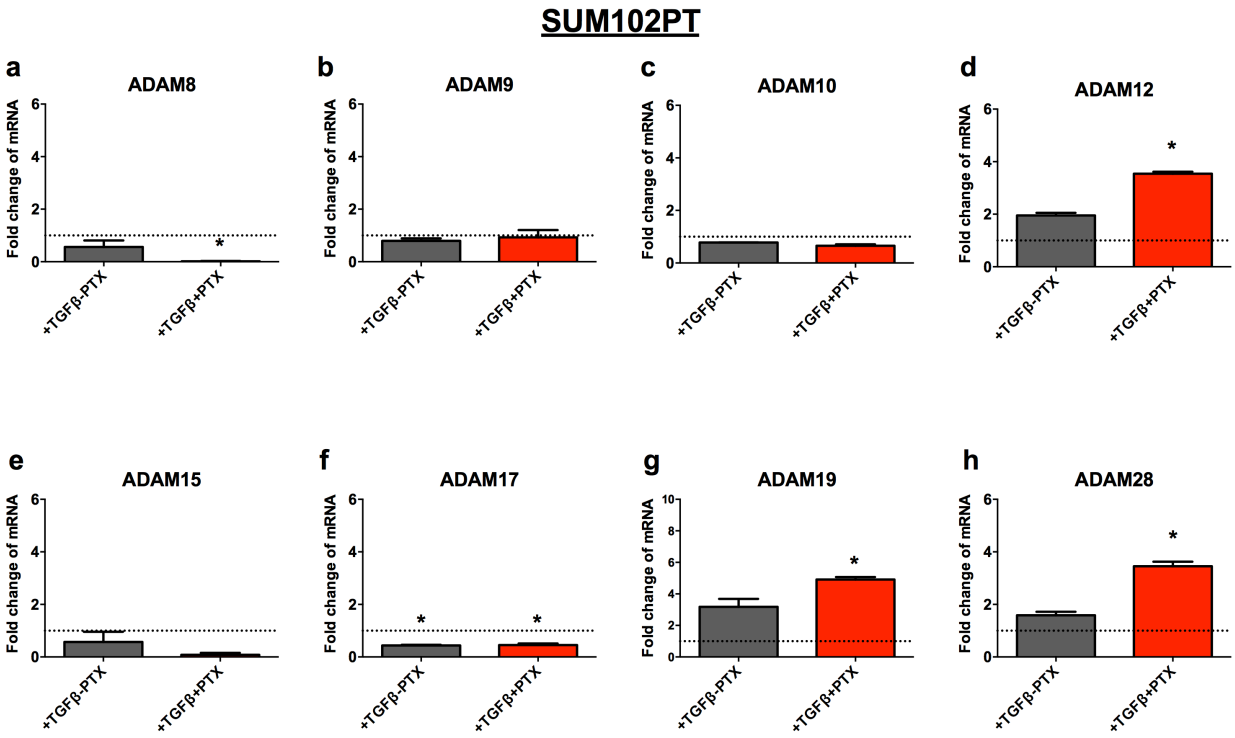

Figure 2.3 Survey of *ADAM* expression in senescent SUM102PT cells.

(**a-h**) SUM102PT cells were treated with or without 2 ng/ml TGFβ for 10 days. At day 6, cells were treated with or without 200 nM paclitaxel. *ADAM8* (**a**), *9* (**b**), *10* (**c**), *12* (**d**), *15* (**e**), *17* (**f**), *19* (**g**), and *28* (**h**) mRNA levels were measured by qRT-PCR and normalized to *β-ACTIN*. Fold change in TGFβ-treated or TGFβ- and paclitaxel-treated cells versus untreated cells were shown as the average values ± SEM, (n=2 for ADAM8, ADAM9, ADAM10, and ADAM15; n=3 for ADAM12, ADAM19, and ADAM28; one sample *t* test, * $p<0.05$).

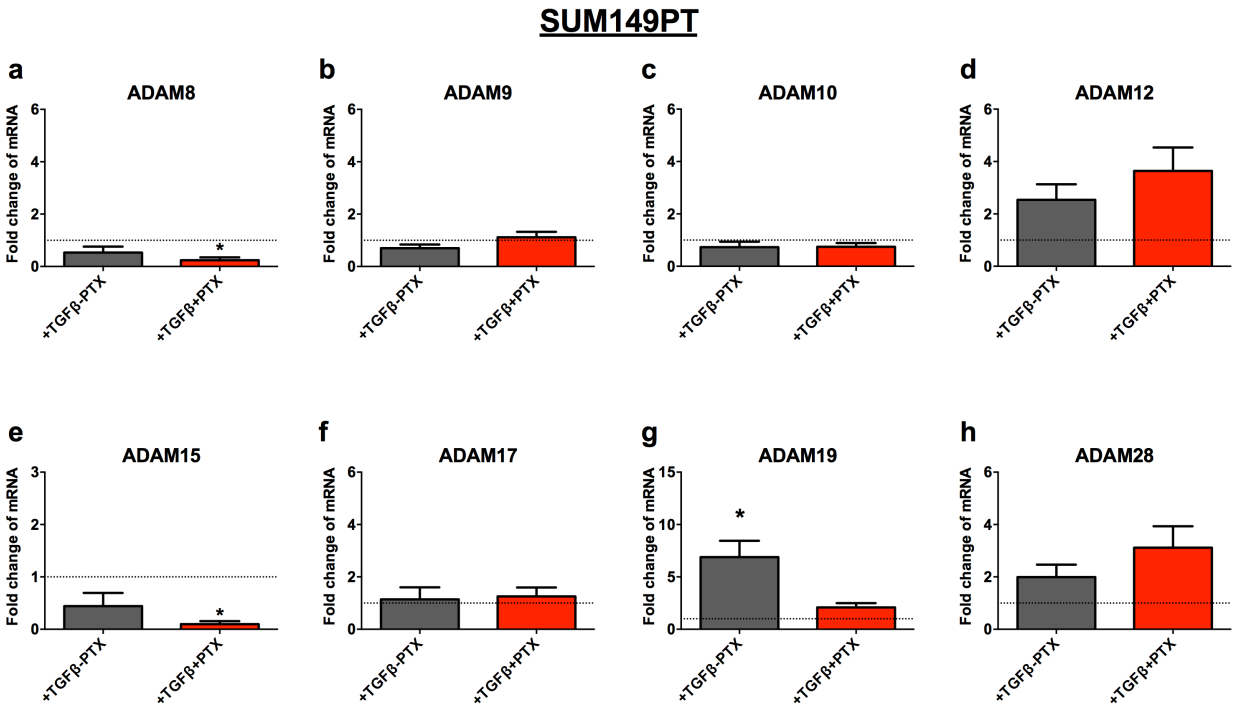

Figure 2.4 Survey of *ADAM* mRNA expression in senescent SUM149PT cells.

(**a-h**) SUM149PT cells were treated with or without 2 ng/ml TGFβ for 10 days. At day 6, cells were treated with or without 100 nM paclitaxel. *ADAM8* (**a**), *9* (**b**), *10* (**c**), *12* (**d**), *15* (**e**), *17* (**f**), *19* (**g**), and *28* (**h**) mRNA levels were measured by qRT-PCR and normalized to *β-ACTIN*. Fold change in TGFβ-treated or TGFβ- and paclitaxel-treated cells versus untreated cells were shown as the average values ± SEM, (n=3 for ADAM8, ADAM9, ADAM10, and ADAM15, n=4 for ADAM12, ADAM19, and ADAM28; one sample *t* test, * $p<0.05$).

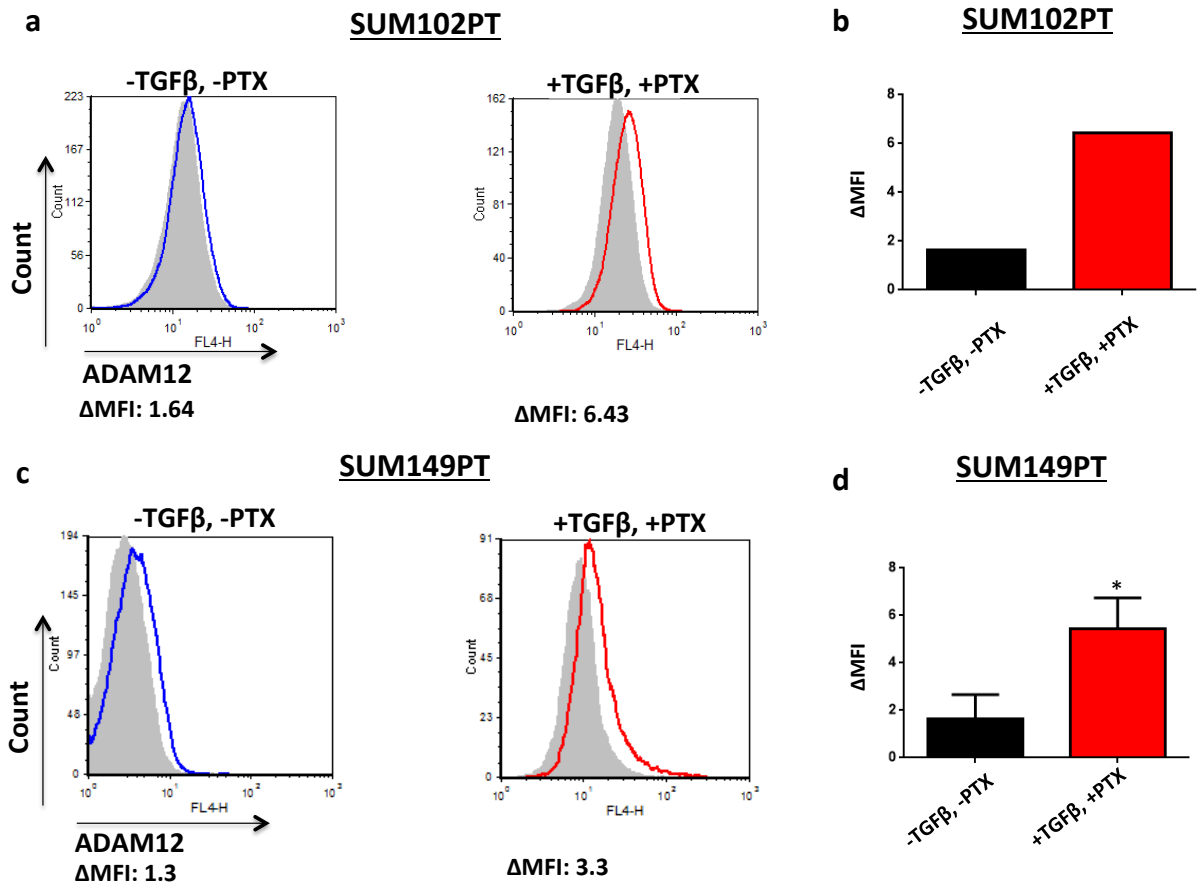

Figure 2.5 ADAM12 protein expression at the cell surface upon induction of senescence in SUM102PT cells.

(**a**, **c)** ADAM12 protein expression at the cell surface was evaluated by flow cytometry in SUM149PT cells (**a**) and SUM102PT cells (**c**). Untreated cells or cells treated with 2 ng/ml TGFβ for 10 days and 100 nM (SUM149PT cells) or 200 nM (SUM102PT cells) paclitaxel from day 6 to day 10 were stained with an anti-ADAM12 (red) or IgG1 isotype control (grey). The cells were then incubated with APC-conjugated anti-mouse IgG antibody. (**b, d**) The difference in median fluorescence intensity (ΔMFI) in SUM102PT cells (**b**) and SUM149PT cells (**d**) were

determined in one experiment in (**b**) and three independent experiments in (**d**). The results are shown as means ± SEM, *, $p<0.05$.

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Chapter 3 - ADAM12 Activates EGFR Signaling through the Cleavage of EGFR Ligands

Abstract

Epidermal growth factor receptor (EGFR), also called HER1 or ErbB1, is a member of the ErbB family. EGFR requires a ligand binding to dimerize, autophosphorylate, and stimulate its tyrosine kinase activity. EGFR signaling plays an important role in cell proliferation, survival, and tumor metastasis in triple negative breast cancer (TNBC). Soluble EGFR ligands are derived from their transmembrane precursors by ADAM metalloproteases, however, the specific role of ADAM12 in activating EGFR in breast cancer cells has not been explored.

Here, we demonstrate that ADAM12 activates the EGFR pathway via releasing EGFR ligands rather than acting upon EGFR itself. Using the SUM159PT breast cancer cell line, we detected basal activation of EGFR by measuring the phosphorylation of a tyrosine residue in the cytoplasmic tail of EGFR, pY1068, using Western blotting. This phosphorylation was increased in cells treated with EGF, an EGFR activator, and decreased in cells treated with erlotinib, an EGFR inhibitor. The basal activation of EGFR resulted from the endogenously produced EGFR ligands, and ADAM12 knock-down decreased the level of pY1068. Batimastat, a metalloprotease inhibitor, and exogenous EGF blocked the effect of ADAM12 knockdown on pY1068. Moreover, the phosphorylation of Y1068 was not changed in MCF10A cells stably expressing ADAM12 compared to the control cells treated with different doses of exogenous hEGF for 10 or 30 minutes. Finally, co-immunoprecipitation was performed to test a direct

physical interaction between ADAM12 and EGFR; no interaction between these two proteins was detected. These results indicate that ADAM12 activates EGFR and this activation occurs most likely through the release of EGFR ligands.

Introduction

EGFR/HER1/ErbB1 is a member of the ErbB family. The ErbB family is composed of four receptors: Epidermal growth factor receptor (EGFR/HER1/ErbB1), HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 (Kataoka 2009; Hynes & MacDonald 2009). EGFR is activated upon its binding to different EGFR ligands. As a result of ligand binding, autophosphorylation and activation of the tyrosine kinase domain takes place. HER2 is constitutively active and does not require ligand binding for activation. On the other hand, HER3 requires ligand binding but it is a kinase-defective receptor and it is phosphorylated only when dimerized with another HER family receptor. Signaling by EGFR, HER2, and HER3 stimulates breast cancer cell proliferation, survival, and tumor metastasis (Foley 2011). HER4 is a unique member in the ErbB family; despite the fact that HER4 requires EGFR ligands for activation and has a tyrosine kinase activity, it elicits an antiproliferative response in mammary epithelium (Muraoka-Cook 2008).

Ligand-mediated EGFR activation is essential to promote tumor growth and progression in triple negative breast cancer (TNBC), in the absence of HER2 overexpression (Kataoka 2009; Wilson 2009).

Eleven EGFR ligands have been identified: epidermal growth factor (EGF), tumor growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin,

epigen, epiregulin, betacellulin, and neureglulin-1, 2, 3 and 4 (Foley 2011). These ligands are synthesized as transmembrane proteins that require shedding from the membrane in order to be released, to bind, and to activate target receptor/s. Ligand shedding is mediated predominantly via members of the ADAM (a disintegrin and metalloprotease) family. Different ADAMs are capable of generating soluble EGFR ligands (Kataoka 2009). ADAM12 is one of the catalytically active ADAMs that is strongly upregulated in breast tumors (Li et al. 2012a; Fröhlich 2011; Roy 2011). It has been shown that ADAM12 is capable of cleaving HB-EGF (Asakura, 2002). Moreover, RNA sequencing experiments, which were performed in our laboratory by Dr. Sara Duhachek-Muggy, showed that the gene expression changes upon ADAM12 knock-down were similar to changes observed after inhibition of EGFR signaling (Duhachek-Muggy and Qi 2017). These results led us to hypothesize that ADAM12 might modulate the EGFR signaling pathway.

The goal of this study was to examine whether ADAM12 is capable to activate EGFR in breast cancer cells and whether this activation is achieved by increasing the release of EGFR ligands or by direct interactions with EGFR. Our results suggest that ADAM12 activates EGFR signaling through the release of EGFR ligands.

Materials and Methods

Reagents and antibodies

ADAM12 siRNA#1 (D-005118-01), ADAM12 siRNA#2 (D-005118-02), ON-TARGETplus non-targeting siRNA pool (D-001810-10), and DharmaFECT1 transfection reagent were obtained from GE Dharmacon. Batimastat (BB-94) was from EMD Millipore,

erlotinib was from Cell Signaling, and human recombinant EGF was from Life Technologies. Antibodies used for Western blotting were: rabbit monoclonal anti-pY1068 EGFR (clone D7A5), anti-total EGFR (clone D38B1), and anti-total MEK 1/2 (clone D1A5), all from Cell Signaling. Anti-α-tubulin (clone DM1A) was from Sigma-Aldrich.

Cell culture

SUM159PT cell line was purchased from Asterand. MCF10A cells were purchased from American Type Culture Collection. SUM159PT 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. MCF10A cells were cultured in DMEM/F-12 medium supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml human EGF, and 100 ng/ml cholera toxin. Cells were maintained at 37°C under humidified atmosphere containing 5% CO_2.

Western Blotting

Cells were lysed using sample buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH (7.4), 0.5 % sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 5 mM EDTA, protease inhibitors (1 mM AEBSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 10 mM 1,10-phenathroline, and 5 μg/ml aprotinin), and phosphatase inhibitors (50mM NaF, 2mM Na_3VO_4, and 10 mM $Na_4P_2O_7$). All lysates were freshly prepared and resolved by SDS-PAGE (8% gel), and then Western blotting was performed. The following primary antibodies were used: anti-ADAM12 antibody (Ab# 3394 (Li et al. 2012b)) raised against the cytoplasmic tail of human ADAM12 and used at 1:10,000 dilution, rabbit monoclonal anti-pY1068 EGFR at 1:5,000

dilution, anti-total EGFR at 1:5,000 dilution, anti-total MEK 1/2 at 1:5,000 dilution, and anti-α-tubulin at 1:200,000 dilution. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody were used as a secondary antibody at 1:5,000 dilution. The signal was detected using SuperSignal West Pico chemiluminescence detection kit (Pierce) and an Azure c500 digital imaging system.

Immunoprecipitation assay

MCF10A.ADAM12 or MCF10A.EV cells, which were established as previously described (Li et al. 2013), were lysed, and 400 µl cell lysate was incubated with either anti-ADAM12 antibody (clone 632525) or IgG1 control antibody (clone 11711), both from R&D Systems, at 10 µg/ml concentration overnight at 4°C. On the following day, 40 µl of protein G-Sepharose (GE Healthcare, 1:1 slurry) was added to each sample and incubated for 1 hour. Beads were washed three times with lysis buffer. Proteins were eluted in SDS sample buffer, fractionated by SDS-PAGE (8% gel), and Western blotting was performed. The following primary antibodies were used: anti-ADAM12 antibody (Ab# 3394 (Li et al. 2012)) at 1:10,000 dilution, and anti-total EGFR (clone D38B1) at 1:5,000 dilution. Horseradish peroxidase-conjugated anti-rabbit IgG antibody were used as a secondary antibody at 1:5,000 dilution. The signal was detected using SuperSignal West Pico chemiluminescence detection kit (Pierce).

Results

The basal activation of EGFR is mediated by endogenously expressed ligands in SUM159PT cells

We first examined the basal activation level of EGFR in SUM159PT cells, a TNBC cell line, by measuring the phosphorylation at Tyr 1068, pY1068. This Tyr residue is one of the major autophosphorylation sites in EGFR and often serves as a read-out for the activation status of the receptor. The level of pY1068 was decreased upon treating cells with 1 μM erlotinib, an EGFR inhibitor, and increased when cells were treated with hEGF, an EGFR activator. This confirmed that EGFR receptor was fully functional in SUM159PT cells (Figure 3.1 a).

Next, we asked whether the basal activation of EGFR was mediated by the endogenously expressed EGFR ligands, which require ADAM proteases in order to be released from cells, or by EGF-like factors that might be present in the serum-supplemented culture media. SUM159PT cells were incubated with 0, 10, or 50 μM batimastat (BB-94), a metalloprotease inhibitor, in the presence or absence of serum in the culture media for 24 hours (Figure 3.1 b). The phosphorylation of Y1068 was decreased in cells treated with BB-94, regardless of the presence or absence of serum. These results suggest that the basal activation of EGFR is achieved through the endogenously expressed EGFR ligands that require ADAM-mediated cleavage, rather than exogenous EGF that might be present in the serum.

ADAM12 knock-down does not change EGFR responsiveness to EGF

To explore the role of ADAM12 in the activation of EGFR, SUM159PT cells were transfected with either control siRNA or two individual siRNAs targeting ADAM12, siA12#1 and #2. Transfected cells were incubated in serum-free medium or in complete medium that contained serum. Down-regulation of ADAM12 reduced the level of pY1068 regardless of the presence or absence of serum (Figure 3.2 a). The effect of knock-down of ADAM12 on EGFR phosphorylation was eliminated when cells were incubated with 10 μM BB-94 or 20 ng/ml hEGF (Figure 3.2 b). Moreover, the rate of the phosphorylation of Y1068 was not changed in MCF10A cells stably expressing ADAM12, MCF10A.ADAM12, compared to the control cells treated with 0, 0.5, 2, 8, 24, and 96 ng/ml exogenous hEGF for 10 or 30 minutes (Figure 3.3 a and b). Finally, no physical interaction was detected between ADAM12 and EGFR when co-immunoprecipitation experiment was performed (Figure 3.3 c). Collectively, those results suggest that ADAM12 activates EGFR, most likely by mediating the release of endogenous EGFR ligands.

Discussion

EGFR plays an essential role in tumor progression in TNBC where ER, PR, and HER2 are absent. Therefore, it is not surprising that 60-70% of the TNBCs show increased expression of EGFR (Corkery 2009; Shien 2005; Viale 2009). As a result, anti-EGFR therapies in TNBC have been tested in several clinical trials (Carey 2011). EGFR activation depends on ligands that are first synthesized as transmembrane precursors and need to be released from the surface of cells by ADAM sheddases (Kataoka 2009). Depending on the individual contribution of specific ADAMs to releasing EGFR ligands and modulating the activation of the EGFR signaling pathway, ADAMs might be promising targets in treatment of TNBC patients. ADAM12 is one of

the catalytically active ADAMs and its expression is strongly upregulated in breast cancer (Li 2012; Fröhlich 2011; Roy 2011). Moreover, it has been shown that ADAM12 acts as a sheddase for HB-EGF, an EGFR ligand, during cardiac hypertrophy (Asakura 2002). Also, our RNA sequencing results showed that ADAM12 knock-down induces similar gene expression changes as inhibition of EGFR signaling (Duhachek-Muggy and Qi 2017). Thus, ADAM12 might play a critical role in the activation of EGFR in breast cancer cells.

In this report, we identified ADAM12 as a modulator of the EGFR pathway in SUM159PT TNBC cell line. We analyzed the basal level of pY1068, one of the major autophosphorylation sites in EGFR. The phosphorylation of Y1068 was increased when cells were treated with hEGF and decreased upon treating cells with erlotinib, which confirmed the functionality of the receptor and the specificity of the antibody. Also, we showed that the activation of EGFR in SUM159PT cells depends mainly on the endogenously expressed ligands that need to be cleaved from the cell surface by ADAM sheddases. The main purpose of this study was to investigate the role of ADAM12 in the activation of the EGFR pathway; knocking-down ADAM12 decreased the basal activation level of EGFR, most likely by diminishing the release of EGFR ligands.

In the scientific literature, ADAM10 and ADAM17 are considered to be the main sheddases releasing EGFR ligands in many cell types (Jones, Rustagi, & Dempsey 2016). In breast cancer, it has been postulated that ADAM17 is the main ADAM responsible for the cleavage of EGFR ligands and activating EGFR signaling (Giricz 2013). Our data indicates that ADAM12 is also capable of activating EGFR in breast cancer cells. This finding may have many important implications in developing a future treatment for TNBC. First, since TNBC is non-responsive to anti-estrogen and anti-HER2 therapies, the only remaining the therapeutic option is

chemotherapy, which usually targets not only cancer cells but also normal dividing cells. Therefore, developing specific therapies that target ADAM12 to limit the release of EGFR ligands might serve as an alternative approach to treat TNBC patients. Second, knowing the major sheddases responsible for the high activity of EGFR in TNBC could help identify patients who might be the best candidates for anti-EGFR therapies.

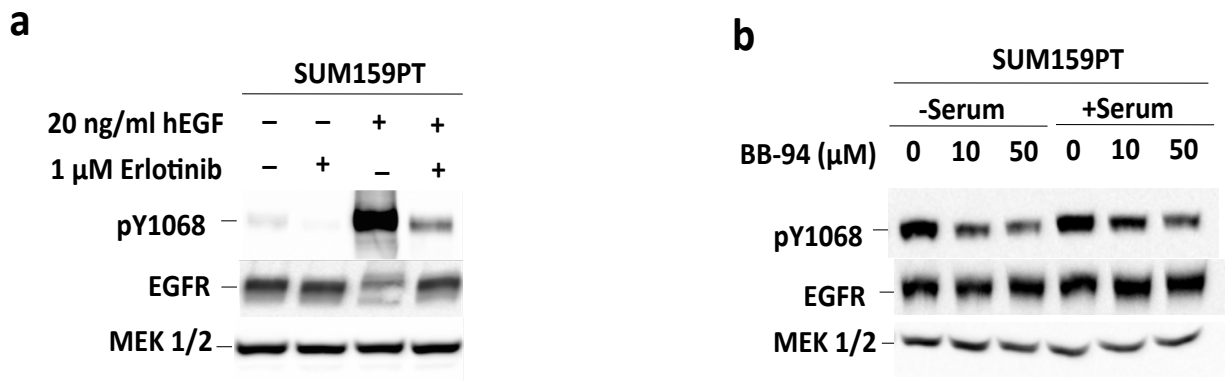

Figure 3.1 The basal activation of EGFR in SUM159PT cells is mediated by endogenously expressed ligands.

(**a**) Basal activation of EGFR in SUM159PT cells. Total cell lysates from untreated cells, cells treated for 24 hours with 1 μM erlotinib, an EGFR inhibitor, cells treated for 30 minutes with 20 ng/ml human recombinant EGF (hEGF), or both, were analyzed by Western blotting using anti-pY1068 EGFR, anti-EGFR and anti-MEK 1/2 antibodies. MEK 1/2 was used as a gel-loading control. (**b**) The effect of BB-94 on the basal level of EGFR activation. SUM159PT cells were treated with 0, 10, or 50 μM of BB-94, a metalloprotease inhibitor, for 24 hours. Protein levels of pY1068 EGFR, EGFR and MEK 1/2 were analyzed by Western blotting.

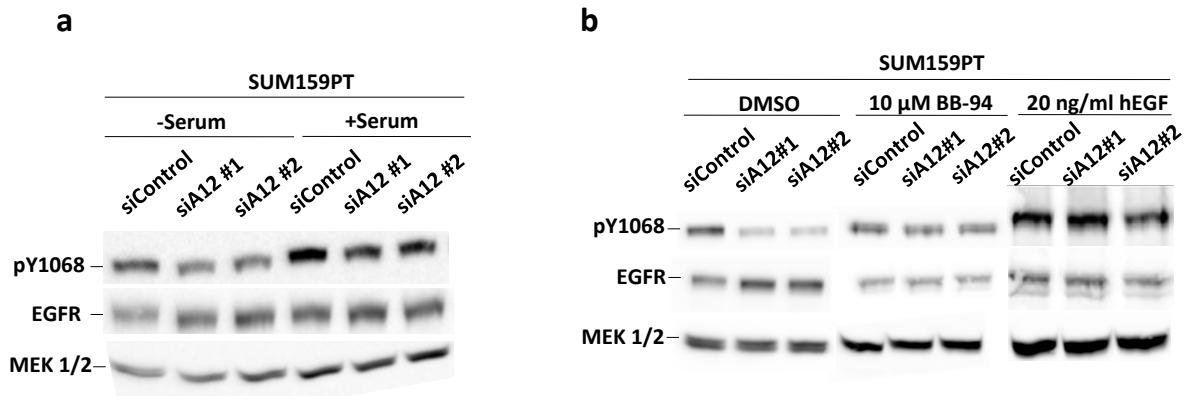

Figure 3.2 ADAM12 knock-down decreases the basal activation of EGFR.

(**a**) SUM159PT cells were transfected with a pool of four control siRNAs (siControl) or two different ADAM12 siRNAs (siA12 #1 and siA12 #2, respectively). Cells were incubated with either complete medium or serum-free medium for 24 hours before collecting total lysates 72 hours after transfection. Representative blots are shown (n=2). (**b**) SUM159PT cells were transfected with a pool of four control siRNAs (siControl) and two different ADAM12 siRNAs (siA12 #1 and siA12 #2, respectively). Cells were either untreated or treated with 10 μM BB-94 for 24 hours or with 20 ng/ml hEGF for 30 minutes before the analysis.

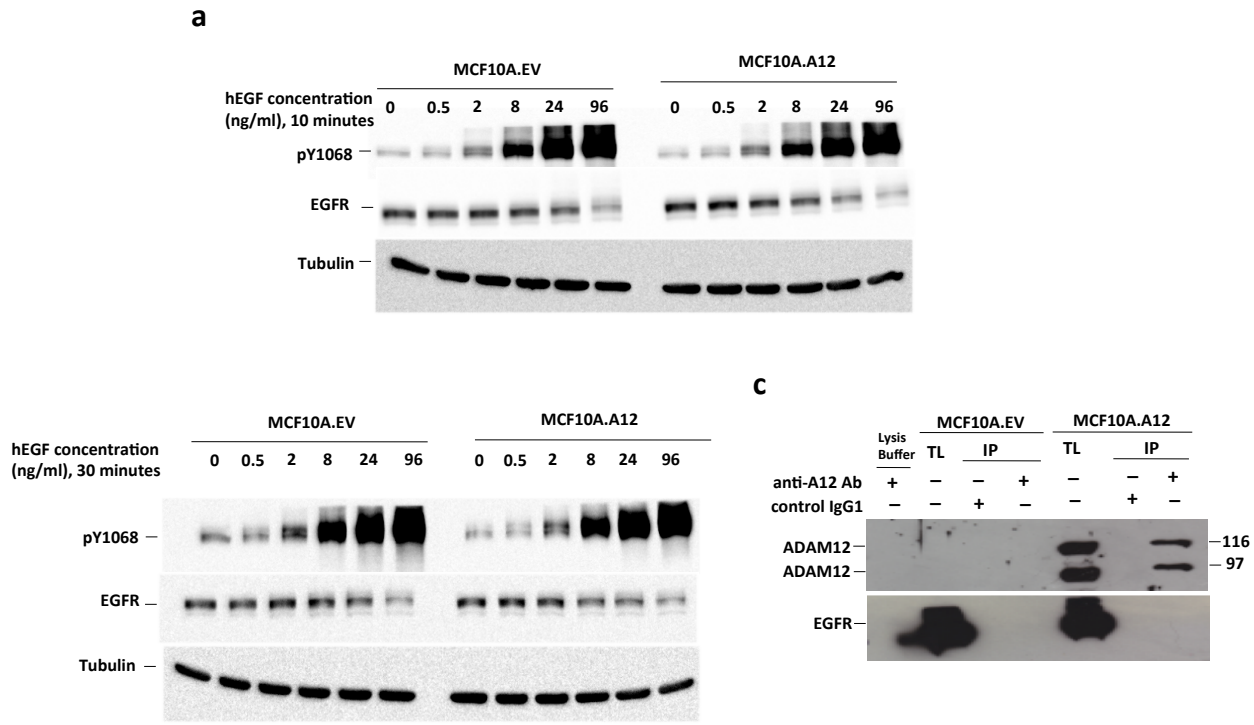

Figure 3.3 ADAM12 does not alter the EGFR responsiveness to EGF.

(**a**, **b**) EGFR response of MCF10A.A12 and MCF10A.EV cells to exogenous hEGF after 10 (**b**) and 30 (**c**) minute treatment. MCF10A cells with stable over expression of ADAM12 or control empty vector (EV) were treated with the indicated doses of hEGF. Total cell lysates were analyzed by Western blotting using an antibody specific for pY1068 EGFR or total EGFR. Tubulin was used as a gel-loading control. (**c**) Lack of co-immunoprecipitation of ADAM12 and EGFR. MCF10A.A12 or MCF10A.EV cell lysates were incubated with anti-ADAM12 or control IgG1 antibody, and the immunoprecipitates were immunoblotted with antibodies against ADAM12 or EGFR. The band at 116 kDa represents the nascent, full-length and catalytically inactive form of ADAM12, the band at 97 kDa represents the mature, processed and catalytically active form of ADAM12.

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Chapter 4- Final conclusions

ADAMs function as sheddases when they are catalytically active (Edwards 2009; Seals & Courtneidge 2003; Mochizuki & Okada 2007). Sheddases are proteins that cleave ectodomains of specific transmembrane proteins, resulting in the release of their ectodomains to the surrounding space (Mochizuki & Okada 2007). These transmembrane proteins might be growth factor precursors, membrane-anchored ligands or receptors. The sheddase activity results in the activation or inhibition of various signaling pathways that might modulate specific cellular processes in the cell or might lead to or be associated with a particular pathological condition (Seals & Courtneidge 2003; Murphy 2008; Mochizuki & Okada 2007). In humans, twenty-two different ADAMs have been identified, and not all of the identified ADAMs are catalytically active. In addition, ADAMs show different patterns of expression, for example, one third of human ADAMs are expressed in the testis or its associated structures. Moreover, ADAM expression is upregulated in different pathological conditions (Edwards 2009; Reiss & Saftig 2009). Among the identified human ADAMs, ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, and ADAM28 are catalytically active (Jones, Rustagi, & Dempsey 2016; Reiss & Saftig 2009). ADAM17 is the most extensively studied ADAM since it has a wide variety of substrates. In breast cancer, it has been shown that ADAM12, ADAM17, and ADAM28 promote cancer cell proliferation by shedding different transmembrane proteins, such as HB-EGF (Mochizuki & Okada 2007; Jones, Rustagi, & Dempsey 2016).

As mentioned previously, shedding of different transmembrane proteins has important consequences for many signaling pathways or biological processes. For example, activation of the EGFR signaling pathway depends on shedding its ligands, such as EGF, HB-EGF, and others, from the cell surface (Kataoka 2009). Moreover, SASP components that are expressed as

transmembrane proteins need to be shed from the cell surface to affect the neighboring cells (Coppé 2010; van Deursen 2014). ADAM metalloproteases are good candidates to modulate those processes.

In chapter 2, the expression levels of catalytically active ADAMs, ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, and ADAM28, were tested after induction of senescence in SUM102PT breast cancer cell line using TGFβ as an inducer of senescence. *ADAM12* mRNA levels were significantly increased upon induction of cellular senescence. A future question that needs to be answered is: is ADAM12 protein also upregulated at the surface of senescent cells? If ADAM12 protein is upregulated, what is the function of ADAM12 in cellular senescence? Does ADAM12 regulate the cleavage of critical SASP components that might have an effect on the neighboring cells and, as a result, modulate tumor progression? Exploring the identities of SASP components that might require ADAM12 for cleavage is also important. The proteomic analyses could be used to answer this question, followed by performing knock-down experiments toward understanding the functional role of ADAM12. Modulating cell proliferation might be one of the functions of ADAM12, since we found in chapter 3 that ADAM12 activates EGFR signaling. Another interesting question that needs to be answered is whether ADAM12 expression is upregulated regardless of the approach that is used to induce cellular senescence. If the answer is yes, could ADAM12-targeting therapy provide a valuable approach to control the release of SASP components that increase tumor growth and progression of cancer? Induction of cellular senescence by different approaches and testing ADAM12 expression is a good strategy to answer those questions.

Another important observation in chapter 2 is that *ADAM17* mRNA expression did not increase upon induction of cellular senescence, however, this does not exclude the possibility

that ADAM17 protein expression or its activity is increased. Effenberger *et al* showed that ADAM17 activity, but not its protein level, increased upon induction of cellular senescence (Effenberger 2014); this could be the case in TGFβ-induced senescence as well.

Finally, *ADAM28* expression was upregulated upon induction of cellular senescence. Previously, ADAM28 was found to be upregulated in breast cancer cells (Mitsui 2006). It would be interesting to study the effect of combined knock-down of ADAM12 and ADAM28 on SASP.

In chapter 3, we showed that ADAM12 modulates EGFR activation. These results were a part of a bigger study that showed that ADAM12-mediated activation of EGFR leads to enrichment for cancer stem cells (CSCs) populations. CSCs are resistant to cancer therapies and play important roles during metastasis (Ghaffari 2011). Inhibition of ADAM12 might in the future represent an anti-CSC therapy, as well as an approach to diminish SASP.

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