ELUCIDATING THE ROLE OF THE DISINTEGRIN METALLOPROTEINASE, ADAM15, IN BREAST AND PROSTATE CANCER PROGRESSION

by

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TABLE OF CONTENTS

ACKNOWLEDGMENTS		ii
LIST OF FIGURES		v
LIST OF TABLES		vii
ABSTRACT		viii
CHAPTER		
1.	INTRODUCTION	1
2.	ADAM15 SUPPORTS PROSTATE CANCER METASTASIS BY MODULATING TUMOR CELL-ENDOTHELIAL INTERACTION	21
3.	THE ECTODOMAIN SHEDDING OF E-CADHERIN BY ADAM15 SUPPORTS ERBB RECEPTOR ACTIVATION	60
4.	THE ROLE OF ADAM15 IN HER2 SIGNALING AND PROSTATE CANCER CELL SURVIVAL	96
5.	SUMMARY	126

LIST OF FIGURES

Figure

1-1.	ADAM protein structure	13
1-2.	Cadherin-dependent adherence junction	14
1-3.	E-cadherin proteolysis	15
2-1.	Knockdown of ADAM15 reduces PC-3 tumorigenicity *in vivo*	46
2-2.	Tumor weight and histology of ADAM15 PC-3 tumors	48
2-3.	Knockdown of ADAM15 attenuates PC-3^{Luc} cell migration and adhesion *in vitro*	49
2-4.	Gold colloid migration assay of PC-3^{Luc} cells	50
2-5.	ADAM15 mediates N-cadherin proteolysis	51
2-6.	Loss of ADAM15 disrupts cell surface receptors levels	52
2-7.	The loss of ADAM15 reduces PC-3-endothelial interaction and transendothelial migration	53
2-8.	Loss of ADAM15 attenuates PC-3 metastasis in an intracardiac dissemination assay	54
3-1	ADAM15 and HER2 expression in breast cancer	83
3-2	ADAM15 cleaves E-cadherin in breast cancer cells	84
3-3	E-cadherin is a substrate for ADAM15	85
3-4	Soluble E-cadherin mediates HER2/HER3 heterodimerization through ErbB receptor binding	87
3-5	Soluble E-cadherin mediates HER2/HER3 phosphorylation and induces ErbB- mediated cell signaling	88

3-6	ADAM15 supports cell migration and proliferation	89
3-7	HER2 stimulation by exogenous soluble E-cadherin	90
3-8	Model for ADAM15-dependent activation of HER2 by soluble E-cadherin	91
4-1	ADAM15 and TIMP profile in prostate cancer	114
4-2	ADAM15 overexpression increases LNCaP tumorigenicity	116
4-3	ADAM15-mediates cleavage of E-cadherin in prostate cancer cells	117
4-4	Binding of sE-cad to HER2 supports receptor activation	119
4-5	ADAM15 supports Akt activation and cell survival	120
4-6	Model for ADAM15-mediated HER2 transactivation in prostate cancer cells	122
5-1.	The role of ADAM15 in cancer progression	137

LIST OF TABLES

Table

1-1 cDNA microarray analysis of ADAM15 in multiple cancers 12

2-1 ADAM15 expression in prostate cancer 45

ABSTRACT

The metastatic progression of cancer requires multiple steps involving tumor cell-stromal interaction, which has been demonstrated to be supported by the matrix metalloproteineases (MMPs). The ADAMs (**a d**isintegrin **a**nd **m**etalloproteinase) are a recently discovered family of proteases related to the MMPs. These zinc-dependent metalloproteinases are involved in a myriad of normal and pathophysiological functions including oocyte fertilization, neurogenesis, inflammation and cancer progression. The domain structure of ADAM family members implicate these enzymes in multiple functions such as cell adhesion, migration, invasion, and signal transduction. A catalytically active member of the ADAM family, ADAM15, has been shown to be upregulated in multiple adenocarcinomas including breast and prostate cancer. It is thought that ADAM15 plays a role in growth factor shedding to mediate cancer cell migration and invasion through its metalloproteinase activity. The ADAM15 disintegrin domain supports microenvironment modulation by mediating extracellular matrix degradation and angiogenesis. In this thesis work, I demonstrate that ADAM15 supports prostate cancer tumorigenesis and metastasis through the regulation of metastatic-associated markers. More importantly and for the first time, ADAM15 was implicated in soluble E-cadherin (sEcad) ectodomain shedding. The sE-cad fragment bound and activated ErbB receptors leading to breast and prostate cancer cell migration, proliferation and survival.

CHAPTER 1

INTRODUCTION

The most diagnosed cancer in men and women in the United States is breast and prostate cancer, accounting for an average of 27% of all cancers recognized (1). These two adenocarcinomas are the second leading cause of cancer related deaths in the male and female population, respectively. It is forecasted that 1 in six men and 1 in 8 women will develop invasive adenocarcinoma of the prostate or breast during their life time. Prostate and breast cancer, as with other cancers, are amenable to treatment when detected early; however, if allowed to progress to metastatic disease, survival drops precipitously. In fact, the five year survival for localized prostate adenocarcinoma is 100%, but metastatic disease has a 33.3% survival rate during the same period of time. Similarly, in breast cancer localized cancer has a five year survival of 88.5% but patients with distant metastasis have only a 26% survival rate. Both prostate and breast cancer have a prevalence to metastasize to the bone, but lymph node, liver and lung have also been shown to be sites exhibiting metastatic involvement (2-4). Cancerous invasion into the aforementioned organs can lead to debilitating complications and death. Despite advances in management of cancer, much needed progress is required for alleviation of metastatic disease. If the developments of novel therapeutics to treat this disease are to succeed, it is critical that the molecular mechanisms that underlie the metastatic process of prostate cancer be elucidated.

Metalloproteinases support metastatic progression

The metastatic cascade includes two essential steps in allowing cancerous spread—cell detachment from the surrounding extracellular matrix and stromal invasion (5). One family of proteins implicated in supporting metastatic progression comprises the zinc-binding matrix metalloproteinases (MMPs). The role of the MMP family in metastasis has been intensely studied. MMP family members, such as stromelysin-1 (MMP-3), have been implicated in inducing cancer cell migration, invasion, and angiogenesis. MMPs contain an active metalloproteinase catalytic domain characterized by the consensus HEXXHXXGXXH sequence (6). The metalloproteinase domain allows these proteinases to degrade extracellular matrix (ECM) proteins such as collagen, laminin and fibronectin. This activity leads to the destruction of the basement membrane allowing cancer cells to invade through the stromal components. MMPs are also able to cleave transmembrane proteins, such as cadherins, on adjacent cells. MMP-3 cleavage of E-cadherin in breast cancer cells was shown to mediate cancer cell detachment and cell migration (6). Because MMP activity underlies fundamental aspects of the metastatic process, extensive effort has been placed on developing anti-cancer therapeutics targeting the metalloproteinase activity of the MMPs. To date this research has led to the discovery of tissue inhibitors of metalloproteinases (TIMPs), and the development of small molecule inhibitors and mimetics (7-9).

The ADAM family

The ADAM (**a** **d**isintegrin **a**nd **m**etalloproteinase) family is composed of 40 family members, of which 13 members are catalytically active, as characterized by the

consensus amino acid sequence HEXXHXXGXXH (9). The ADAM proteinases are type I glycoproteins that are close relatives of the pIII snake venom metalloproteinases. Both family members contain five extracellular domains; a pro-domain, metalloproteinase, disintegrin, and cysteine-rich domains, respectively (Fig. 1-1) (10). A hydrophobic transmembrane domain links the N-terminal extracellular portion of ADAM proteases to their cytoplasmic tails. The c-terminal segment of these disintegrin metalloproteinases possess SH3 and SH2 recognition sequences that function in signal transduction. The ADAMs have been implicated in many biological processes, which include oocyte fertilization, neurogenesis, myogenesis, and growth factor shedding (11-13). More importantly this family of disintegrin metalloproteinases has also been shown to be involved in cancer progression. ADAM 10 is overexpressed in pheochromocytomas and neuroblastomas, while ADAM 12 is overexpressed in both breast and colon cancers (14-16). ADAMs 10,12, and 17 have also been implicated in gastrointestinal carcinoma (17). The multiple domains of these proteinases impart several physiological features, including ECM degradation and shedding of transmembrane growth factors through the active metalloproteinase domain. ADAMs have been shown to cleave EGFR ligands leading to the transactivation of EGFR. ADAM9, 10, 12 and 17 cleave the EGFR ligand HB-EGF, while ADAM17 can also cleave TNF-alpha, TGF-alpha, and amphiregulin (18). Additionally, ADAM15 has been shown to transactivate EGFR by cleaving amphiregulin and TGF-alpha in bladder cancer cells (19). EGFR signaling is implicated in many pathophysiological responses such as cell survival, proliferation, and migration (20). The ability of ADAMs to transactivate EGFR has been found to support their role in neoplastic progression. Similar to the MMPs, the catalytic activity of ADAM family

members is inhibited by both pharmacological inhibitors as well as the endogenous tissue inhibitors of metalloproteinases (TIMPs). ADAM 10 and 17 activity is inhibited by TIMP1 and 3 while TIMP2 inhibited ADAM12 catalytic function (21). TIMP inhibition of ADAM15 has not been empirically elucidated but studies by Joseph et. al. demonstrated supportive evidence for an ADAM15 specific small molecule inhibitor (22).

Another physiological feature of ADAMs utilizes the disintegrin domain, which binds to integrins, allowing for cells to detach from their normal ligand, such as fibronectin or vitronectin, hence supporting cell invasion. In fact, eight members of the ADAM family have been shown to bind integrins with $\alpha_9\beta_1$ serving as a commonly shared ligand (18). The domain structure of this family of disintegrin metalloproteinases impart on it the potential of inducing cell invasion, migration, and proliferation leading to neoplastic progression.

Cadherin Structure-function

The cadherin superfamily is composed of the protocadherins and the classic cadherins, which play a role in a multitude of pathophysiological conditions (23). Members of the classic cadherin family includes E (epithelial)- and N (neuronal)-cadherin are composed of five extracellular domains (EC) that function in calcium-mediated homo- or heterotypic interactions (Fig. 1-2). The first (N-terminal) extracellular domain (EC1) contains a conserved consensus HAV (histadine, alanine, valine) sequence that is critical for cadherin functions (24). Synthetic peptides containing this unique HAV sequence mimic the extracellular domain functions of both E- and N-cadherin,

respectively (25, 26). The extracellular domains are linked to the c-terminal cytoplasmic portions of the cadherin glycoproteins through a transmembrane domain. The c-terminal domain is homologous between different cadherins and contains binding sites for members of the catenin family (Fig. 1-2). Both E- and N-cadherin are able to bind to β-catenin, which in turn interacts with α-catenin to link the cadherin molecules to the actin cytoskeleton (27). These cadherin glycoproteins are also able to interact with the $p120^{ctn}$ which directly links these adhesion proteins to the actin filaments (23). E- and N-cadherin function similarly but in different tissues and their involvement in the cadherin-catenin-actin complex is crucial in maintaining the cadherin-dependent adherence junction (CAJ) in their respective tissue type. E-cadherin is known to maintain epithelial tissue organization, cell polarity, and adhesion and is also involved in wound healing (28, 29), while N-cadherin is essential for neurolation, neuronal growth and survival (30). Dissolution of the CAJ complex through transcriptional or post-translational modification (i.e. cadherin proteolysis, Fig. 1-2) disrupts the biological function that these cadherins perform such as synaptic function and epithelial cell polarity (23, 28).

Epithelial to Mesenchymal Transition in Cancer

The physiological phenomenon of epithelial to mesenchymal transition (EMT) involves the conversion of epithelial cells from a polarized, well differentiated phenotype into a fibroblastoid mesenchymal cell. This transition is critical during the gastrulation phase of development when epithelial cells from the outer ectoderm migrate and differentiate into mesenchymal neuron, glial cells, and melanocyte (31). The conversion from an epithelial phenotype into a mesenchymal state is not limited to development but

has been observed in tumor cell metastasis (32, 33). EMT is characterized by cadherin switching where the epithelial specific cadherin, E-cadherin, is lost and the neuronal specific cadherin, N-cadherin, expression is upregulated.

Re-expression of N-cadherin in cancer cells supports their metastatic potential by aiding tumor-host interaction, stabilizing growth factor receptors and mediating transcription of metastasis-associated genes (34, 35). Outside the actual tumor cell, N-cadherin is expressed in many components of the tumor microenvironment including fibroblasts, endothelial cells and neurons. Tumor cell surface N-cadherin interaction with stromal N-cadherin through heterotypic binding mediate cancer cell invasion through the underlying stroma (36). In prostate cancer, N-cadherin heterotypic ligation supports the perineural invasion of prostate cancer tumor cells (37). Furthermore, N-cadherin can be cleaved at the cell surface to release a soluble 90 kDa N-cadherin fragment (soluble N-cadherin or sN-cad) into the extracellular milieu. The ectodomain shedding of N-cadherin allows for the assembly and dissolution of adherence lamillipodia during tumor cell invasion through the surrounding stroma. The soluble N-cadherin fragment, by anchoring in the extracellular matrix (ECM), has also been found to serve as a substrate for cell migration and invasion through N-cadherin heterotypic binding as well as supporting angiogenesis (38). Derycke et. al. demonstrated that soluble N-cadherin supported endothelial migration and neovascularization through FGFR (fibroblast growth factor receptor) activation and signaling (39). In addition, N-cadherin extracellular shedding reduces β-catenin interaction with N-cadherin at the cell surface and permits β-catenin-mediated transcription of metastatic associated genes such as MMP9, cyclin D1 and c-myc (40, 41).

N-cadherin specific expression also supports cancer cell metastasis through the stabilization of different growth factor receptors. In breast cancer cells, direct N-cadherin binding to FGFR stabilizes receptor expression at the cell surface (35). The N-cadherin-FGFR complex sensitizes the receptor to FGF-2 activation supporting an increase in the amplitude and duration of FGFR signaling which mediates cancer cell invasion through MMP9 activation. N-cadherin-growth factor receptor stabilization is not limited to FGFR but it is also known to stabilize PDGFRβ expression and signaling to support fibrosarcoma cell migration (42).

During EMT, N-cadherin re-expression is accompanied by the downregulation of E-cadherin levels and the loss of E-cadherin portends a poor prognosis in breast, bladder, gastric and prostate carcinoma (32, 33, 43, 44). E-cadherin inactivation occurs on many levels, including chromosomal deletion, promoter hypermethylation, and protein cleavage (43). E-cadherin proteolysis can be extracellular or intracellular and is induced by a myriad of stimuli such as serum deprivation, calcium influx, and phorbol-ester treatment (45-47). The metalloproteinase-mediated extracellular release of the 80 kDa soluble E-cadherin (sE-cad) is upregulated in multiple adenocarcinomas and is known to correlate directly with metastatic prostate cancer (38, 48). Our laboratory demonstrated that a member of the zinc-dependent ADAM (a disintegrin and metalloproteinase) family, ADAM15, mediates the shedding of soluble E-cadherin in breast and prostate cancer (Fig. 1-3). Intracellular proteolysis of E-cadherin is also observed to generate a 97 kDa (E-cad^{97}) or 100 kDa (E-cad^{100}) membrane bound E-cadherin (Fig. 1-3). Kuefer et. al. found that E-cad^{97} correlated directly with localized prostate cancer and may serve as a predictive marker for prostate cancer treatment failure (49). Although a specific protease

has not been identified to be responsible for E-cad^{97} generation, Steinhusen et. al. provided convincing evidence for a role of caspace-3 in E-cad^{97} formation (50). The cysteine protease, calpain, was found to cleave the cytoplasmic tail of E-cadherin to generate E-cad^{100} in response to phorbol-ester treatment (47). Both intracellular and extracellular proteolysis of E-cadherin liberates β-catenin from the cell surface and allows for β-catenin-mediated gene transcription. Moreover, disruption of the cadherin-catenin complex liberates p120^{ctn}, which interacts with the transcriptional repressor Kaiso to inhibit gene expression (Fig. 1-2) (51). The process of EMT permits tumor cells to be more metastatic by two complementary mechanisms initially through the loss of the suppressive E-cadherin-mediated adherence junction and secondly the acquisition of N-cadherin which supports tumor cell migration and invasion through the tumor microenvironment.

The classic cadherin family members are crucial for tissue maintenance and repair. Inactivation of these proteins has many implications due to the mechanisms they regulate. The stabilization of cell surface growth factor receptors, transcription factors, and proteases give this family of adhesion molecules diverse physiological functions. Understanding the role that both full length and the less studied soluble cadherin fragment is crucial in the treatment of disease.

The role of ADAM15 in cancer

An understudied member of the ADAM family, ADAM15, is located at chromosome 1q21, which is amplified in prostate and breast cancer as well as melanoma (52). This transmembrane metalloproteinase is also known in the literature as MDC15

(**m**etalloproteinase/**d**isintegrin/**c**ysteine-rich) or metargidin (a **met**alloproteinase-**RGD**-disintegr**in**). ADAM 15 is a transmembrane glycoprotein that contains five extracellular domains; prodomain; metalloproteinase, disintegrin, cysteine-rich, EGF-like and cytoplasmic domains (Fig. 1-1). The N-terminal prodomain maintains the metalloproteinase activity in an inactive state through a cysteine-switch (53). When this domain is cleaved by a proprotein convertase, i.e. furin, inhibition of ADAM15 metalloproteinase activity is relieved and the proteinase is activated. The inactive precursor form of ADAM15 is a 110 kDa protein, and when matured it is converted into a 90 kDa active protease. ADAM15 catalytic activity degrades both collagen I and IV as well as cleaves the inflammatory cytokine CD23 (54, 55). Moreover, this adamalysin has been implicated in extracellular matrix remodeling within rheumatoid synovial tissue and atherosclerosis (56, 57). Using its disintegrin domain, ADAM15 is able to interact with the integrins $\alpha_V\beta_3$, $\alpha_5\beta_1$, $\alpha_9\beta_1$, and $\alpha_{II}\beta_3$ supporting its involvement in cell invasion (58-60). In fact, ADAM15 plays an important role in glomerular mesangial cell and smooth muscle cell migration (14, 54, 61). Previous work demonstrated that ADAM15 is able to transactivate EGFR by cleaving the EGFR ligands amphiregulin and TGF-α in cancer cell lines (19). Horiuchi et. al. implicated ADAM 15 in neovascularization, which is required for metastatic tumor development, potentially through the shedding of pro-angiogenesis factors by the catalytic activity of ADAM15 (62). The c-terminus of ADAM15 contains 2 SH3 recognition sequences that have been shown to interact with src family members as well as the vascular transport molecules Endophilin 1 and SH3PX1 (63, 64). Interspersed within the cytoplasmic tail are putative kinase recognition sites that act as targets for ADAM15 directed phosphorylation. To this end, ADAM15

has been demonstrated to be a phosphorylation target of the src family member, hck, and this modification supports the interaction of c-abl with ADAM15 (65). The ADAM15 c-terminus may support its targeting in response to different signals. Based on these studies, ADAM15 is thought to serve multiple functions to support cancer progression by mediating growth factor shedding, extracellular matrix degradation and signal transduction.

Scope of dissertation

Previously, our lab found that ADAM15 is significantly upregulated during prostate cancer progression using cDNA and tumor microarrays, respectively (66). ADAM15 was overexpressed in localized prostate cancer compared to normal prostate tissue and its expression was even greater in the metastatic lesions. Within the same study, ADAM15 expression was found to be significantly overexpressed in breast cancer as compared to matched normal tissue. ADAM15 levels were significantly upregulated in invasive carcinoma and metastatic disease. Importantly, ADAM15 expression levels were higher at sites of breast tumor angioinvasion suggesting a role for ADAM15 in cancer metastasis. Using the oncomine database at the University of Michigan (www.oncomine.org), we found that ADAM15 levels were not only upregulated during the metastatic progression of prostate and breast cancer, but also in ten additional cancer types (Table 1-1). Corroborating this evidence was the fact that ADAM family modulated proteins (i.e. $\alpha_V\beta_3$, E-cadherin) were dysregulated during both prostate and breast progression. Furthermore, the domain structure of ADAM15 suggests a strong role for this active protease in tumor cell migration, invasion, proliferation and

neovascularization. To our knowledge, there is no current work that has implicated ADAM15 as a possible mediator of prostate and breast cancer progression. Based on the preliminary data from our lab, my thesis work focused on elucidating the role of ADAM15 in prostate and breast cancer progression. In Chapter 2, I demonstrated that ADAM15 mediates prostate cancer metastasis through the modulation of metastatic cell surface markers. ADAM15 was also implicated in soluble E-cadherin (sE-cad) shedding which in turn binds to and activates ErbB receptors to support breast cancer cell proliferation and migration (Chapter 3). Finally, my thesis project provided compelling evidence for the role of ADAM15 in prostate cancer cell survival (Chapter 4).

Table 1-1. cDNA microarray analysis of ADAM15 in multiple cancers*

Tumor Type	No. of Studies	Status	Range of p-Value
Breast	10	↑	.002 to .041
Prostate	9	↑	5.6E-05 to .039
Lung	9	↑	1.1E-05 to .047
Bladder	8	↑	1.3E-09 to .045
Brain	6	↑	4.2E-05 to .04
Ovarian	5	↑	.028 to .046
Renal	4	↑	1.2E-07 to .03
Colon	3	↑	.01 to .031
Pancreatic	2	↑	2.9E-04 to .006
Endometrial	1	↑	0.025
Liver	1	↑	1.40E-09
Thyroid	1	↑	0.04

*All studies were obtained from the Oncomine database at www.oncomine.org

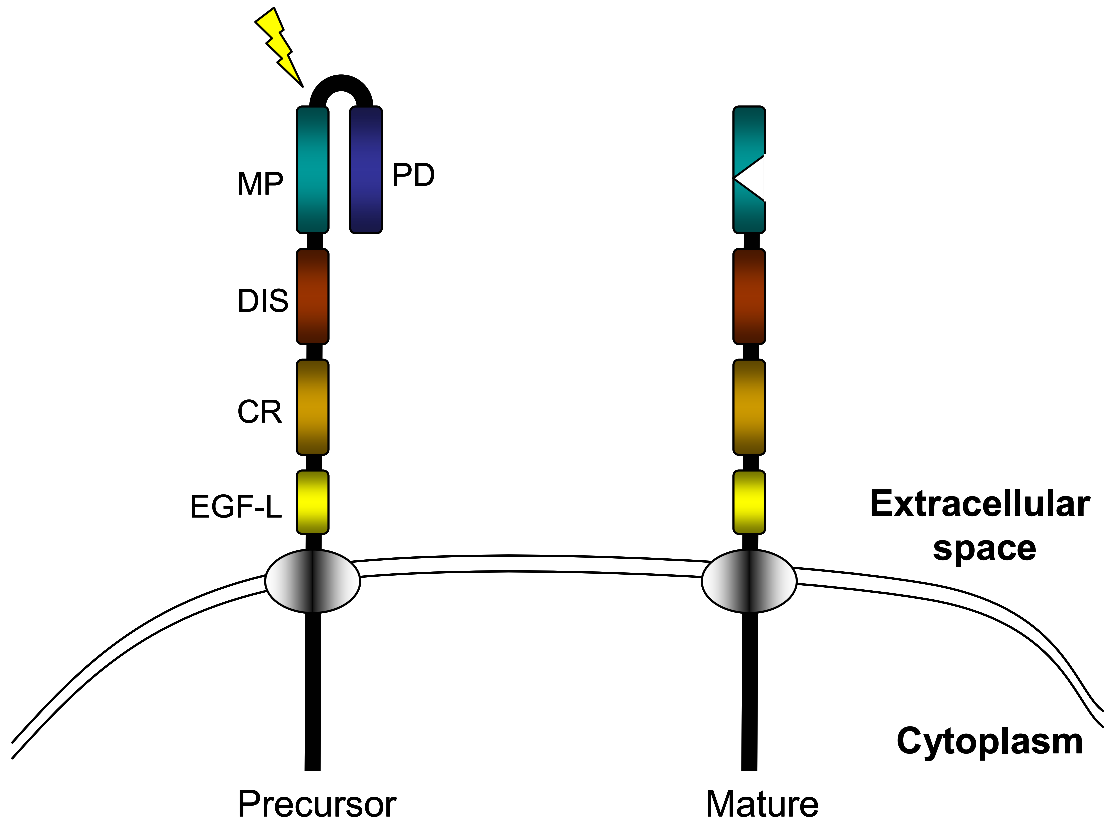

Fig. 1-1. ADAM protein structure. The precursor form of the ADAM proteases contains five extracellular domains. The first domain is an N-terminal prodomain (PD), which maintains the catalytic metalloproteinase (MP) domain inactive through a cysteine-switch. The removal of the prodomain by proprotein convertases (lightening bolt) generates a catalytically active mature protease. The disintegrin domain (Dis) contains integrin binding sequences, which is followed by the cysteine-rich (CR) and EGF-like (EGF-L) domains. The transmembrane domain links the extracellular domains to the C-terminal cytoplasmic tail.

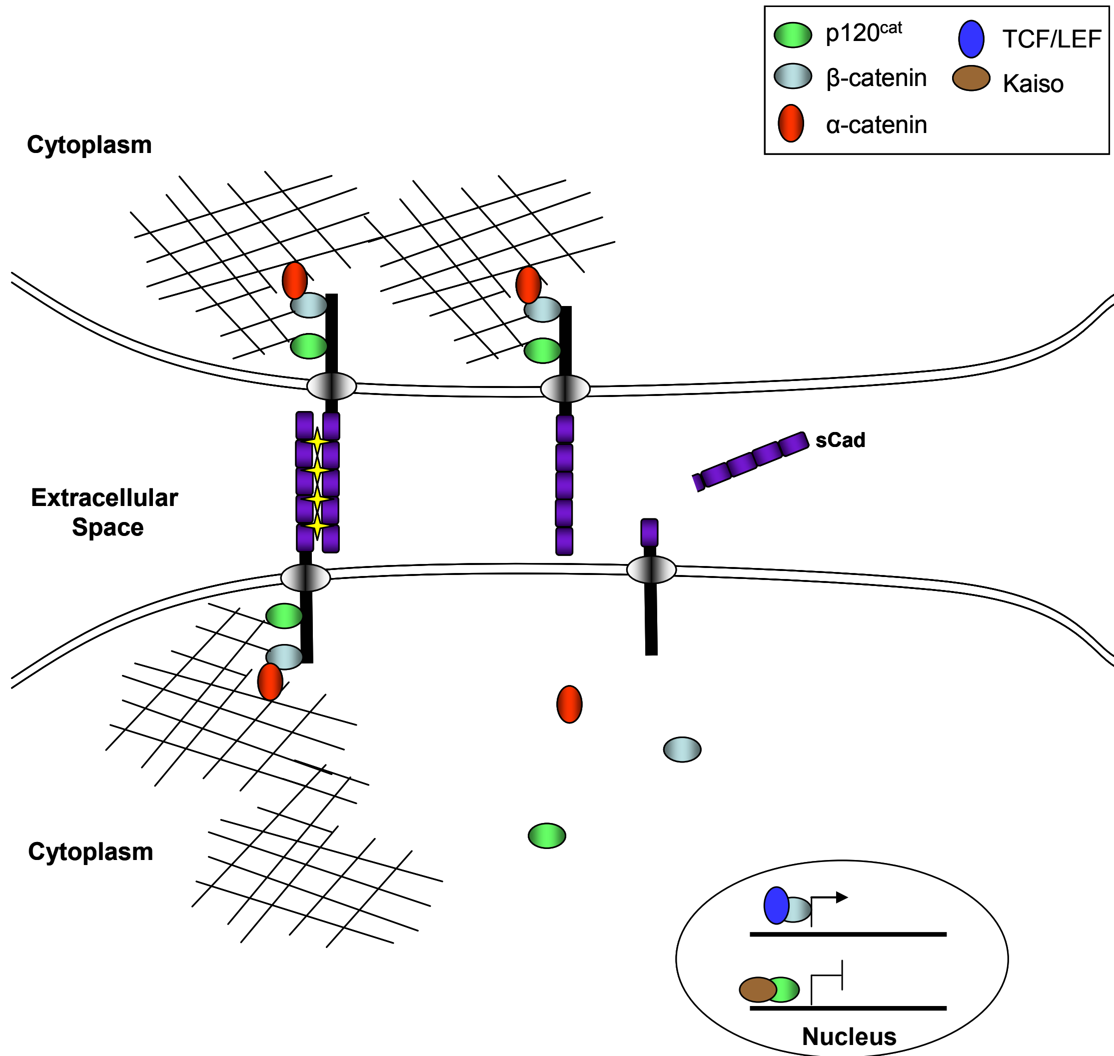

Fig. 1-2. Cadherin-dependent adherence junction. Calcium-mediated homotypic interaction links the extracellular environment of the cell to the actin cytoskeleton through the catenin family members. Disruption of the adherence junction by cadherin shedding releases the membrane-bound catenins to allow for gene expression or repression.

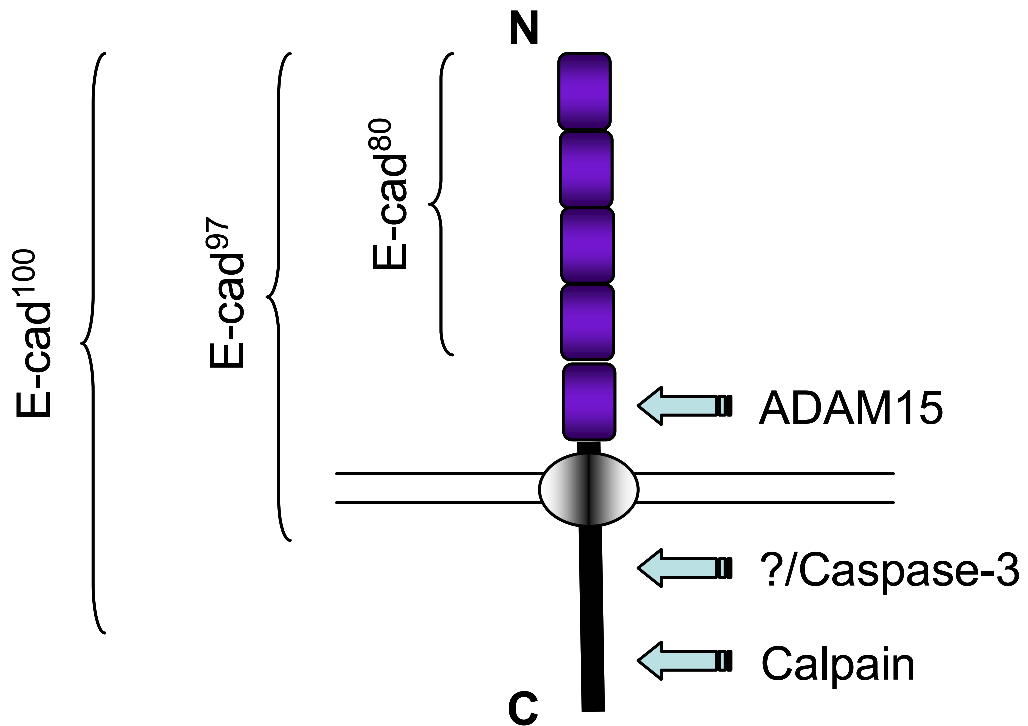

Fig. 1-3. E-cadherin proteolysis. Extracellular shedding by ADAM15 generates a soluble E-cad^{80} fragment. E-cadherin intracellular cleavage is mediated by caspase-3 and calpain to generate E-cad^{97} and E-cad^{100}, respectively.

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CHAPTER 2

ADAM15 SUPPORTS PROSTATE CANCER METASTASIS BY MODULATING TUMOR CELL-ENDOTHELIAL INTERACTION

Abstract

Using human tumor and cDNA microarray technology, we have recently demonstrated that the ADAM15 disintegrin is significantly overexpressed during the metastatic progression of human prostate cancer. In the current study, we utilized lentiviral-based shRNA technology to downregulate ADAM15 in the metastatic prostate cancer cell line, PC-3. ADAM15 downregulation dramatically attenuated many of the malignant characteristics of PC-3 cells *in vitro* and prevented the subcutaneous growth of PC-3 cells in SCID mice. By inhibiting the expression of ADAM15 in PC-3 cells we demonstrated decreased cell migration and adhesion to specific extracellular matrix (ECM) proteins. This was accompanied by a reduction in the cleavage of N-cadherin by ADAM15 at the cell surface. FACS analysis revealed reduced cell surface expression of the metastasis-associated proteins α_v integrin and CD44. Furthermore, MMP9 secretion and activity were abrogated in response to ADAM15 reduction. In an *in vitro* model of vascular invasion, loss of ADAM15 reduced PC-3 adhesion to, and migration through vascular endothelial cell monolayers. Using a SCID mouse model of human prostate cancer metastasis, we found that the loss of ADAM15 significantly attenuated the metastatic spread of PC-3 cells to bone. Taken together, these data strongly support a

functional role for ADAM15 in prostate tumor cell interaction with vascular endothelium and the metastatic progression of human prostate cancer. This manuscript was published in *Cancer Research* 68(4):1092-1099.

Introduction

The dissemination of localized prostate cancer to distant tissues such as the bone, lung and liver represents a prominent healthcare burden in the aging adult male population (1). The underlying mechanisms that promote and support the metastatic spread of prostate cancer remain vague. It is clear that fundamental processes, such as cellular detachment, suppression of apoptosis, vascular intravasation and angiogenesis are essential to the spread of cancer cells and their growth at distant sites (2). One family of proteins supporting malignant progression is the zinc-binding matrix metalloproteinases (MMPs) that degrade components of the extracellular matrix, such as collagen, laminin and fibronectin (3). However, the MMPs are not the only metalloproteinases implicated in human tumorigenesis.

The less studied ADAM (**A** **D**isintegrin **a**nd **M**etalloproteinase) family of membrane metalloproteinases may also support tumor progression by modulating key physiologic cell surface proteins such as membrane anchored growth factors and their complementary receptors, cell adhesion molecules and integrins (4). The ADAM family is composed of 40 members, of which 13 members are catalytically active. These catalytically active members contain a metalloproteinase domain that is implicated in growth factor shedding and ECM degradation. ADAM17 has been reported to process pro-TNF-α, TNF receptors, interleukin-6 receptor and amphiregulin (5, 6) and ADAMs 9, 10, 12 and 17 are believed to cleave the EGFR ligand, HB-EGF, leading to the transactivation of EGFR (7). ADAM family members have also been shown to cleave different cell adhesion molecules such as N-cadherin (8). Expression of N-cadherin in human tumor cells is usually associated with malignant progression (9). Cell surface N-

cadherin supports heterotypic interaction between migrating cancer cells and other cells of the tumor microenvironment such as fibroblasts and endothelial cells to support cancer cell migration, invasion and metastasis.

Another physiological feature of ADAMs utilizes the disintegrin domain to mediate integrin-ECM interactions. Several ADAM family members, including ADAM15, have been shown to bind to specific integrin molecules suggesting roles in cellular adhesion and tumor cell-ECM interactions (10). While the ADAMs have been the focus of rigorous research in many biological processes, such as oocyte fertilization, neurogenesis, myogenesis, and growth factor shedding (11, 12), they have only recently been studied in the context of human tumorigenesis (13-15). For example, ADAM 9 expression has been associated with the incident of low grade prostate cancer formation in mice (16), however, the precise role of the ADAMs in malignant processes remains largely unknown.

A number of proteases including members of the ADAM family are thought to play regulatory roles in the processes of angiogenesis and neovascularization. ADAMs have been implicated in the processing of several key signaling components of inflammation and angiogenesis as well as adhesion molecules that comprise endothelial adherens junctions including vascular endothelial (VE)-cadherin, PCAM-1, and integrins (17). A novel role for ADAM15 in endothelial adhesion was suggested by the observation that ADAM15 co-localized in endothelial cell junctions with VE-cadherin (18). Additionally, Blobel and colleagues provided a compelling argument for the role of ADAM15 in pathological neovascularization using a model of prematurity of retinopathy in mice (19). Further supporting ADAM15 function in endothelial regulation is the

evidence that ADAM15 binds the endothelial integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$ via an RGD motif contained within its disintegrin domain (20, 21). Taken together these studies suggest that ADAM15 is a potential but unexplored component of tumor cell-endothelial cell interaction that may play a role in tumor angiogenesis and/or metastasis.

While a role for ADAM15 in tumor vasculature may be emerging, very little is known concerning the contribution of ADAM15 to tumor initiation and progression. Located on chromosome 1q21, the gene encoding ADAM15, maps to a region of documented amplification associated with the metastatic progression of human cancers including: prostate, breast, ovarian, colon and melanoma (22-24). We have recently completed the first comprehensive expression profile of ADAM15 in human prostate cancer by utilizing both cDNA microarray and multi-tumor array technology. This study demonstrated significant increases of ADAM15 expression in multiple adenocarcinomas and a highly significant correlation with the metastatic progression of human prostate cancer (25). Based on previous work characterizing the catalytic metalloproteinase and disintegrin domains of ADAM15, we anticipated that ADAM15 may serve multiple functions in the metastatic progression of prostate tumors through disruption or degradation of the extracellular matrix, via its disintegrin domain, as well as proteolytic processing of key membrane-bound molecules via the metalloproteinase domain.

To specifically elucidate the role of ADAM15 in prostate cancer progression, we developed a stable shRNA-mediated knockdown of ADAM15 in the highly malignant prostate cancer line PC-3. We have established that the reduction of ADAM15 expression abolishes the malignant characteristics of this cell line *in vitro* and *in vivo* and is associated with alterations of metastatic-associated cell surface proteins. To our

knowledge, this study is the first to provide functional evidence that ADAM15 plays an important role in the metastatic progression of human prostate cancer.

Materials and Methods

Cell lines and cell cultures.

LNCaP and PC-3^{Luc} (26) cells were maintained in RPMI (Bio Whittaker, Walkersville, MD) with either 8% or 5% fetal bovine serum (HyClone, Logan, UT), respectively, and supplemented with 2 mmol/L L-glutamine (Life Technologies, Grand Island, NY), 100 units/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), and 0.25 µg/mL Fungizone (Life Technologies). Cells were incubated at 37°C and subcultured weekly.

Generation of shADAM15 PC-3 and ADAM15 overexpressing LNCaP cell lines.

ADAM15 specific knockdown in PC-3^{Luc} cells and the vector control which features a scrambled sequence that is designed to control for off-target effects were generated as described previously (27). The forward and complementary targeting sequences for ADAM15 were 5'-AACCCAGCTGTCACCCTCGAA-3' and 5'-TTCGAGGGTGACAGCTGGGTT-3'. The shRNA cassette also featured a TTCAAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTTT terminator at the 3' end.

To generate ADAM15 overexpressing LNCaP cells, ADAM15 cDNA was tagged with HA (hemagglutinin) at the C-terminus and transfected into LNCaP cells lines as described previously (25)

Protein isolation and Western blotting.

Cells were harvested by mechanical disruption with cell scrapers followed by gentle centrifugation. Cell pellets were then lysed in appropriate volumes of lysis buffer [50mmol/L Tris (pH 8), 120 mmol/L NaCl, .5% NP40, 1 mmol/L EGTA, 100 μg/mL phenylmethysulfonyl fluoride, 50 μg/mL aprotinin, 50 μg/mL leupeptin, and 1.0 mmol/L sodium orthovanidate] for 1 hour on ice. Tissues were briefly washed in PBS and homogenized in lysis buffer on ice for 20 seconds with a Tissue Tearor. Cellular debris was then pelleted by centrifugation, and supernatants were collected and quantitated using a Bradford protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein were then separated on precast Tris-glycine SDS-polyacrylamide gels (Novex, Carlsbad, CA) and transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then blocked, probed, and developed as previously described (28). Primary antibodies were obtained as follows: actin, luciferase, GFP, and N-cadherin (Sigma, St. Louis, MO); ADAM10, ADAM15 and α_v integrin (Chemicon, Temecula, CA); ADAM17 and CD44 (R&D Systems, Minneapolis, MN).

***In vivo* Tumorigenesis Assay.**

$PC3^{Luc}$ cells were trypsinized, washed twice with PBS, collected and resuspended in PBS; the viability of collected cells was tested by staining with trypan blue. To establish $PC3^{Luc}$ tumor xenografts in mice, 6-week-old C57BL/6 SCID mice were injected subcutaneously in the right flank with 1×10^7 vector or shADAM15 $PC3^{Luc}$ cells in 100 μL of PBS. Tumor volume was monitored weekly by external measurements with a caliper and calculated as $V = (L^2 \times l) / 2$, where L and l represent the smaller and the larger tumor diameter. The observations were ended for ethical reasons after 8 weeks due

to large tumor burden in vector control mice. Animals were maintained under specific pathogen-free conditions with *ad libitum* food and water in the University of Michigan animal housing facilities. At this time, animals were euthanized by CO_2 inhalation followed by induction of a bilateral pneumothorax and tumors were immediately frozen in dry ice or fixed in formalin.

***In vitro* migration assay.**

Cell migration was evaluated by using the scratch wound assay; vector or shADAM15 PC-3^{Luc} cells were cultured for 2–3 days to form a tight cell monolayer. Cells were then serum starved for 16 hours. Following the serum starvation, the cell monolayer was wounded with a 10 μL plastic pipette tip. The remaining cells were washed twice with culture medium to remove cell debris and incubated at 37°C with normal serum-containing culture media. At the indicated times, migrating cells at the wound front were photographed using a Nikon inverted microscope (Thornwood, NY). The cleared area at each time point was measured as a percentage of the cleared area at time 0 hours using NIH Image J software.

Cell adhesion assay.

Collagen I, collagen IV, fibronectin, vitronectin, laminin, or BSA coated 96-well plates were obtained from (Chemicon, Temecula, CA) and adhesion was performed per manufacturer's instructions. Briefly, cells were detached using a cell dissociation buffer (Sigma), washed, and plated at 2.5×10^4 cells/well in culture media. Adhesion was allowed to occur for 1 hour. Nonadherent cells were removed by gentle washing with

warm PBS. Cells were fixed, stained and solubilized for absorbance reading at 550 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA) and Softmax Pro Software.

Fluorescent-activated cell sorting (FACS) analysis.

Cell surface integrin and CD44 receptor expression was monitored by FACS. Briefly, cells were detached using cell dissociation buffer (Sigma) and resuspended in PBS. After washing twice with PBS, cells were incubated with primary antibodies (α_v and CD44) for 1 hour on ice. Cells were washed twice with PBS and incubated with Phar Red-conjugated secondary antibodies (Molecular Probe, Carlsbad, CA) for 1 hour at 4°C. Cells were washed twice with PBS and fixed in 7.5% formaldehyde for 5 minutes at 4°C. Cells were then washed twice and resuspended in PBS and analyzed using a FACSVantage SE three-laser High-Speed Cell Sorter (BD Biosciences, San Jose, CA) with CellQuest Pro Software. Isotype IgG and secondary antibodies alone were used as controls.

Substrate gel electrophoresis.

MMP9 secretion and activity was assessed using gelatin zymography. Vector or shADAM15 PC-3^{Luc} cells were grown up to subconfluency and then serum starved in serum-depleted media for 0, 24, and 48 hours. At each time point, conditioned media was collected by spinning the samples at 2,000 RPM for 15 minutes to pellet any cell debris. The supernatant was collected and 3 mL of it was concentrated using Amicon Ultra concentrators (Millipore, Billerica, MA) according to manufacturer's directions. Equal

volume of the retentate was loaded on a gelatin zymogram and developed according to manufacturer's protocol (Invitrogen, Carlsbad, CA).

Transendothelial interaction and migration.

The endothelial-epithelial interactions assay was performed as previously described (29). Briefly, HDME or HUVE cells were grown to full confluence in 6-well dishes. 2 X 10^5 vector or shADAM15 PC-3^{Luc} epithelial cells were added in 1.8 ml of 50/50 mixture of the EGM-2/RPMI 1640 culture media in triplicate. The unbound fraction were collected at 0, 5 and 20 minutes and cells counted. Data was calculated as the percent of bound epithelial cell fraction by subtracting the unbound fraction from the total cells (at the zero time point calculated for each cell line).

The transendothelial migration (TEM) assay was performed as previously described (30). Briefly, HUVE cells were plated on 3 μm transwells to confluency. 3.8 X 10^4 vector or shADAM15 PC-3^{Luc} cells were added onto the confluent HUVEC monolayer for 4 and 8 hours. Nonadherent PC-3^{Luc} cells and endothelial cells were removed by Q-tip swab from the top filter. The transwell filter was stained with crystal violet, solubilized in 10% acetic acid and quantified by fluorometric analysis using a VersaMax microplate reader (Molecular Devices) and Softmax Pro Software.

N-cadherin proteolysis.

For the *in vitro* proteolysis of N-cadherin, ADAM15 was isolated through immunoprecipitation using an HA specific antibody (Millipore) from LNCaP whole cell lysates. Similarly, N-cadherin was isolated through immunoprecipitation using an N-

cadherin specific antibody (Sigma) from whole cell lysates of N-cadherin expressing PC-3^{Luc} cells. Isolated ADAM15 and N-cadherin were resuspended in equal amounts of ice cold PBS (calcium and magnesium free, Gibco), then mixed together at a 1:1 (volume-to-volume) ratio and incubated for the designated time points at 37°C. At the end of each time point, the reaction was stopped with the addition of reducing loading buffer and boiling. Samples were then loaded on SDS-PAGE for protein analysis. Levels of soluble N-cadherin in conditioned media were monitored via N-cadherin immunoprecipitation from dilute conditioned media samples generated using the MMP9 zymography protocol. Isotype IgG was also used for the immunoprecipitation control.

Intracardiac metastasis model.

Six to seven week old C57BL/6 SCID mice were anesthetized with 1.75% isofluorane/air anesthesia. 2 X 10^5 cells in a volume of 100 µL of sterile PBS were injected into the left ventricle utilizing a 25 gauge needle. The animals were then monitored daily. Mice were imaged utilizing the University of Michigan Small Animal Imaging Resource facility. Images were collected on a cooled CCD IVIS^{TM} system with 50 mm lens, (Xenogen Corp., Alameda, CA). The LivingImage ^{R} software was utilized for analysis of all animals (Xenogen Corp). Prior to imaging, each mouse received an intraperitoneal injection containing 100 µL of a solution containing 40 mg/mL Luciferin dissolved in PBS. These mice were imaged at the indicated time intervals to assess the growth and metastasis of the luciferase-positive cells. At the termination of each experiment, mice were anesthetized, sacrificed and a full necropsy was carried out. Organs of interest were dissected out, weighed and utilized for histological analysis.

Results

Increased expression of ADAM15 correlates with prostate cancer progression. Members of the ADAM family have been suggested to play a role in human angiogenesis and cancer progression. We have focused on one member of this family, ADAM15, which we determined was transcriptionally and translationally upregulated during the progression of several types of adenocarcinoma including metastatic prostate cancer (25). To further evaluate ADAM15 expression in human prostate cancer, we examined the Oncomine database[1] to search published cancer cDNA expression arrays. Table 2-1 is a compilation of six independent and published cDNA microarray studies that revealed significantly increased ADAM15 expression during prostate cancer progression.

shRNA-mediated knockdown of ADAM15 reduces PC-3 tumorigenicity. To assess the role of ADAM15 in prostate cancer progression, we inhibited the expression of ADAM15 using a shRNA-mediated approach in the malignant prostate cancer cell line, PC-3. Three stable ADAM15 shRNA-mediated knockdown lines, shADAM15-1, 2, 3 (Fig. 2-1A), were established using a lentiviral construct to transduce the shRNA into the luciferase-tagged PC-3 cells (PC-3 ^{Luc}). The shRNA construct eliminated both the precursor and mature form of ADAM15 protein in the PC-3^{Luc} shADAM15 cells in comparison to the parental and vector control cells. Off-target effects of the shRNA on other genes, including relatives of ADAM15, ADAM10 and ADAM17, were assess and were shown to be unaltered in the ADAM15 knockdown lines confirming the specificity of the shRNA for ADAM15.

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[1] www.oncomine.com

To investigate whether the loss of ADAM15 reduced the tumorigenic capacity of PC-3 cells, vector or shADAM15 PC-3^{Luc} cells were injected subcutaneously into the flanks of male SCID mice and monitored for 8-weeks in live animals using bioluminescent imaging and caliper measurements (Fig. 2-1B and C). Vector control cells exhibited rapid growth as measured by bioluminescence and tumor volume. In stark contrast, the shADAM15 PC-3^{Luc} cells had a significantly reduced tumor growth rate and its tumor volume plateaued between 5 and 6 weeks. At 8-weeks post injection, mice were euthanized and tumors were extracted for final tumor volume measurements. We found that the vector control mice had an approximate 6-fold increase in tumor volume compared to the shADAM15 PC-3^{Luc} cell line (Fig. 2-1B, C). H&E histology and anti-luciferase immunohistochemistry verified the presence of prostate-derived PC-3^{Luc} cells (Fig. 2-2A and B).

Reduction of ADAM15 in PC-3 cells attenuates cell migration and adhesion. Cellular migration is a characteristic of metastatic tumors. To assess whether ADAM15 downregulation in PC-3^{Luc} cells reduces cell migration, we performed a wound-healing migration assay (31). Vector or shADAM15 PC-3^{Luc} cell monolayers were abraded with a pipette tip, stimulated with serum containing growth media and monitored over 24 hours. Microscopic examination at 3 and 6 hours revealed a significant delay in the wound closure rate of shADAM15 PC-3^{Luc} cells in comparison to the vector control cell line which had completely closed the wound channel by 3 hours (Fig. 2-3A). This finding was corroborated by use of a colloidal gold assay, which demonstrated a decrease in random motility of the shADAM15 PC-3^{Luc} cells compared to the vector control PC-3^{Luc} cells

(Fig. 2-4). Interaction between cell adhesion molecules (CAM) and their complementary ECM targets support cell migration. ADAM15 has been shown to interact with several integrins which in turn bind their complementary ECM substrates to mediate cellular adhesion and migration (20, 21). To assess the role of ADAM15 in cell adhesion to ECM substrates, vector or shADAM15 PC-3^{Luc} cells were plated on plastic coated with collagen I, collagen IV, fibronectin, laminin, and vitronectin. The shADAM15 cell line had a significant reduction in their ability to adhere to fibronectin, laminin, and vitronectin compared to vector control (Fig. 2-3B). Both vector and shADAM15 cells adhered equally unto collagen I and IV (data not shown).

N-cadherin proteolysis is reduced in shADAM15 PC-3 cells. Tumor cell migration, invasion and metastasis require continuous membrane interactions with the surrounding microenvironment through surface adhesion molecules (i.e. N-cadherin) onto and from the underlying stroma. Members of the ADAM family have been shown to mediate proteolysis of several CAMs including N-cadherin, to support cell migration. Since PC-3 cells express high levels of N-cadherin but very little, if any, E-cadherin, we assessed the catalytic activity of ADAM15 in full length N-cadherin processing into the soluble 90 kDa fragment. We first looked at the levels of solubilized N-cadherin in the conditioned media of vector and shADAM15 PC-3^{Luc} cells. Soluble N-cadherin was dramatically decreased in PC-3^{Luc} cells in the shADAM15 cells (Fig. 2-5A). To directly test whether ADAM15 cleaves N-cadherin, we co-incubated purified ADAM15 and N-cadherin to allow for ADAM15-mediated proteolysis over time and found that ADAM15 processed N-cadherin into a soluble fragment (S N-cad) by 6 hours (Fig. 2-5B).

Attenuation of ADAM15 levels decrease CD44 and MMP9 expression. Integrins and CD44 receptors have also been shown to play an important role in facilitating cell migration and adhesion (32, 33). To assess whether these cell surface receptors are affected by reduced ADAM15 expression, we performed FACS and Western blot analysis on vector and shADAM15 PC-3^{Luc} cells. FACS analysis demonstrated that the α_v integrin and the CD44 receptor are significantly downregulated at the cell surface in response to ADAM15 knockdown (Fig. 2-6A). Unlike cell surface integrins, whose expression is difficult to assess by Western blot analysis, CD44 protein expression was nearly depleted in shADAM15 PC-3^{Luc} cells as well as in the shADAM15 derived tumors, shown in Figure 1, in comparison to vector control (Fig. 2-6B). This finding is supported by a previous study showing a correlation between the tumorigenicity of prostate cancer cell lines and CD44 expression (34). Due to CD44 regulation of MMP9 secretion and activity (35), we evaluated the levels of MMP9 in vector and shADAM15 PC-3^{Luc} cells using gelatin zymography and found dramatic reduction in both MMP9 secretion and activity in response to ADAM15 loss (Fig. 2-6C). To rule out the involvement of ADAM15 in gelatin degradation, purified wild type and catalytic-dead ADAM15 were analyzed for gelatinase activity using gelatin zymography. Neither wild type nor the catalytic-dead mutant ADAM15 digested the gelatin substrate (data not shown).

ADAM15 supports epithelial-endothelial interaction and transmigration. In order for cancer cells to metastasize, they must interact with the surrounding microvasculature and

intravasate through the vascular endothelium to gain access to the blood stream. To assess the potential role of ADAM15 in tumor cell-endothelial interaction, we performed a tumor cell-endothelial cell adhesion assay. Vector or shADAM15 PC-3^{Luc} cells were plated on two types of primary human endothelial cell (HUVEC or HDMEC) monolayers for 0, 5, and 20 minutes and bound cells calculated as described in the materials and methods. We observed that shADAM15 PC-3^{Luc} cells had a significantly reduced ability to adhere to the endothelial cell monolayers in comparison to the vector control (Fig. 2-7A). Both vector and shADAM15 epithelial cells attached more effectively on HUVEC monolayer than the HDMEC monolayer. To assess whether ADAM15 reduction would also affect epithelial cell intravasation *in vitro*, we performed a transendothelial migration (TEM) assay. Vector or shADAM15 PC-3^{Luc} cells were seeded on top of a confluent HUVEC monolayer in a transwell chamber and stimulated for 4 or 8 hours. Nearly 3-fold fewer shADAM15 PC-3^{Luc} cells were found to have migrated through the endothelial monolayer at the 8 hour time point in comparison to the vector control (Fig. 2-7B).

Loss of ADAM15 reduced the metastatic spread of PC-3 cells *in vivo*. We have demonstrated that the targeted knockdown of ADAM15 reduces PC-3 cellular motility, ECM binding, endothelial binding, transendothelial migration, and growth *in vivo*. Coupled to the fact that ADAM15 significantly correlates with the metastatic progression of human prostate cancer, these findings strongly support a functional role for ADAM15 in prostate cancer metastasis. To examine this possibility, we injected vector or shADAM15 PC-3^{Luc} cells into the left ventricle of male SCID mice to establish systemic metastasis (26). The growth of metastatic lesions was monitored by bioluminescent

imaging over a 6-week time course in live animals. Bioimaging 24-hours post injection showed equal distribution of both cell lines inside the mice (data not shown). Over the 6-week time course, mice injected with vector control PC-3^{Luc} cells exhibited an increasing accumulation of bioluminescence at disseminated sites indicative of increased metastatic development compared to the shADAM15 PC-3^{Luc} cells, which exhibited weak luminescent signals at distant sites (Fig. 2-8A and B). Animals injected with vector control developed secondary metastasis in the lungs (arrow head), tibia (arrow) and spine (Fig. 2-8A and C). Animals injected with shADAM15 PC-3^{Luc} cells developed few to no metastatic lesions. Tissues exhibiting highly elevated bioluminescence were dissected and prepared for histological analysis. H&E histology and anti-luciferase immunohistochemistry verified the presence of substantial metastatic deposits of vector PC-3^{Luc} cells within the lung, tibia and spine of vector cell injected mice (Fig. 2-8C). Histological analysis of tissues form the shADAM15 PC-3^{Luc} mice revealed micrometastasis only in the lungs (Fig. 2-8D).

Discussion

Alterations in the cellular microenvironment contribute significantly to the progression of human prostate cancer to metastatic disease. In turn, metastatic progression requires multiple steps which involve cell detachment, stromal invasion and intravasation into the vasculature (2). The matrix metalloproteinases (MMPs) have been demonstrated to catalyze the degradation of the extracellular matrix and are implicated in tumor cell migration, invasion, and angiogenesis. However, MMPs are not the only metalloproteinases thought to support human tumorigenesis. Another, less studied family of metalloproteinases, ADAMs, may also support tumor progression through their ability to affect diverse physiologic cell surface proteins such as membrane anchored growth factors and their receptors, ecto-enzymes and cell adhesion molecules, including the cadherins (4). These disintegrin-metalloproteinases contain the conserved modular metalloproteinase motif as well as an integrin-binding domain (disintegrin) in the extracellular region of the molecule and a src-homology (SH3) protein binding domain in the cytosolic portion of the protein. The ADAMs have been implicated in normal biological processes which include oocyte fertilization, neurogenesis, myogenesis, growth factor shedding (11, 12) and have recently been studied in the context of human tumorigenesis (13-15). ADAM9 expression has been associated with the incident of low grade prostate cancer formation in mice (16). Furthermore, ADAM12 expression was elevated in the stroma adjacent to tumor cells in mouse models of breast, colon and prostate cancer (36). However, the precise role of these proteinases in human malignant processes remains largely unknown.

We have recently completed the first comprehensive study of ADAM15 expression in human cancer. Utilizing the Oncomine database, we examined the cDNA

expression arrays of published studies examining normal tissue verses tumor in a number of adenocarcinomas. Using this database and the associated statistical software, we found that ADAM15 was upregulated in multiple adenocarcinomas (25). In addition, we have examined the expression of ADAM15 in 23 independent adenocarcinoma cell lines representing melanoma and tumors of the prostate, breast and colon and in all cases have observed high level expression of activated ADAM15 protein (data not shown). The chromosomal location of ADAM15 on 1q21, a region of specific and high-level amplification in metastatic prostate cancer, makes this disintegrin a strong candidate in the malignant progression of this disease. The current study suggests that aberrant function of ADAM15 supports tumor growth, endothelial interaction and metastasis of prostate cancer cells. Previous research from other laboratories indicated functional roles of ADAM15 in vascular endothelial biology with clear implications in endothelial interaction and angiogenesis (19, 37, 38). However, the specific actions of ADAM15 in tumor epithelium and influences on neighboring endothelial cells in the tumor microenvironment have not, until now, been explored.

To elucidate the function of ADAM15 in prostate cancer, we developed a stable shRNA-mediated ADAM15 knockdown in the highly malignant PC-3 prostate cancer cell line. We demonstrated in this study a dramatic reduction in the malignant capacity of PC-3 cells in the subcutaneous implantation model. In agreement with a tumorigenic role of ADAM15 in PC-3 cells, we have seen that an exogenous overexpression of ADAM15 increased the malignant potential of the minimally invasive LNCaP cells in this same model (date not shown). Taken together, the tumor cell implantation experiments using

two complementary models to alter ADAM15 levels strongly support the role of ADAM15 in the malignant progression of human prostate cancer cells.

ADAM15 is believed to support cellular adhesion and motility in several systems (31, 39). Here, we demonstrated that the loss of ADAM15 in PC-3 cells attenuated the migratory capacity in a wound channel migration assay and decreased PC-3 basal adhesion to fibronectin, laminin and vitronectin. Additionally, we showed that shRNA knockdown of ADAM15 decreased CD44 and α_v integrin surface expression as well as MMP9 secretion and activity. This may have significant ramifications as α_v – containing integrins are known to bind vitronectin while CD44 is considered to encompass laminin and fibronectin binding, respectively (32, 33). Since binding to other substrates, such as collagen I and IV, were unchanged in response to ADAM15 downregulation, we believe that these results are specific to the ADAM15 phenotype. It has also been reported that low $\alpha_v\beta_3$ (40, 41) and CD44 (34, 42) levels portend to a less aggressive prostate cancer cell phenotype. The ECM glycoprotein, osteopontin, using its RGD sequence has been shown to interact with the αvβ3 integrin to regulate cell surface CD44 and MMP9 (35). ADAM15 is the only member of the ADAM family that has an RGD sequence within its disintegrin domain and has been shown to interact directly with the integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (20, 21). Analogous to osteopontin, ADAM15 may regulate CD44, which in turn has been shown to regulate MMP9 expression, through this unique sequence. The interaction between ADAM15 and specific integrins or other transmembrane receptors may coordinate the proteolytic activity of ADAM15 directly to induce ECM degradation and cell migration. Alternatively, ADAM15 could potentially activate downstream substrates such as MMP9 in a hierarchical manner to support cancer cell dissemination.

E (epithelial), N (neuronal), and P (placental)-cadherins function in maintaining cell-cell adhesion through homotypic ligation. The loss of those interactions through a variety of aberrant processes such as cadherin gene mutation, loss of heterozygosity, promoter methylation or proteolytic cleavage can support cancer cell migration and invasion. In aggressive prostate cancer cells, N-cadherin is highly expressed and has been shown to mediate their adhesion and migration (43). The loss of full length N-cadherin reduces cell adhesion and may promote cell migration (44). Furthermore, serum levels of soluble N-cadherin are upregluated in multiple cancers including prostate cancer and correlated directly with an increase in PSA levels (45). Within our current study, we have made the interesting observation that shADAM15 PC-3^{Luc} cells had significantly reduced levels of shed N-cadherin in conditioned media in comparison to vector control. These findings were corroborated by the fact that an N-cadherin expressing melanoma cell line, Mel147, also had reduced soluble N-cadherin levels in its conditioned media in response to ADAM15 downregulation (data not shown). In addition, reconstitution of immunoprecipitated ADAM15 and N-cadherin resulted in the cleavage of N-cadherin into the identical 90 kDa fragment observed in the conditioned media. These observations support two possible scenarios: 1) that ADAM15 cleaves N-cadherin directly or 2) that ADAM15 represents an indirect or upstream event in the shedding of N-cadherin. Regardless of a direct or an indirect role, we believe that N-cadherin solubilization may support the migratory phenotype observed in ADAM15 expressing PC-3 cells by disrupting the homotypic interaction between N-cadherin molecules and increasing N-cadherin cell surface turnover.

The $\alpha_v\beta_3$ integrin has been shown to support endothelial-epithelial adhesion and transendothelial migration (TEM) of tumor cells (29). To assess whether the loss of ADAM15 and the associated reduction of α_v integrin would hinder PC-3 epithelial-endothelial adhesion, we examined the adhesive capacity of PC-3 cells to human endothelial cells and demonstrated that the loss of ADAM15 resulted in a significant reduction in endothelial adhesion. A concomitant reduction in transendothelial migration of shADAM15 PC-3 through HUVEC and HDMEC monolayers, not only supports that ADAM15 mediates endothelial adhesion of tumor cells but coordinates the transmigration of tumor cells through endothelial monolayers.

The CD44 receptor has been implicated in prostate cancer adhesion, migration, invasion and metastasis (34). In this study, we showed that CD44 is downregulated in cell culture and in tumors in response to the loss of ADAM15. To investigate whether ADAM15 contributes to *in vivo* prostate cancer metastasis, we utilized an invasive model of prostate cancer metastasis. We demonstrated that vector PC-3^{Luc} injected mice developed substantial metastatic lesions in the lung, spine and tibia. ADAM15 downregulation within these cells hindered their ability to form distant metastasis. Considering the microarray data, which demonstrates a significant correlation of ADAM15 expression with the metastatic progression of human prostate cancer, results from the mouse metastasis model, suggests that ADAM15 may support the metastatic progression of prostate cancer.

The current study, which has its foundations on the clinical correlation of ADAM15 and the metastatic progression of prostate cancer, provides strong experimental support for this disintegrin in prostate cancer progression. Based on the current findings

we suggest that ADAM15 plays critical regulatory roles in the malignant progression of cancer to metastatic disease.

Table 2-1. ADAM15 expression in prostate cancer.

Author	Class	p-Value	Status	Reference
Dhanasekaran	N/L/M	1.40E-05	↑	46
Glinsky	Gleason6-9	0.002	↑	24
Lapointe	N/L/M	0.004	↑	47
Luo	N/L	0.012	↑	48
Magee	N/L	0.023	↑	49
Yu	N/L/M	2.60E-05	↑	50

Abbreviations: N, normal prostate; L, localized prostate cancer; M, metastatic prostate cancer

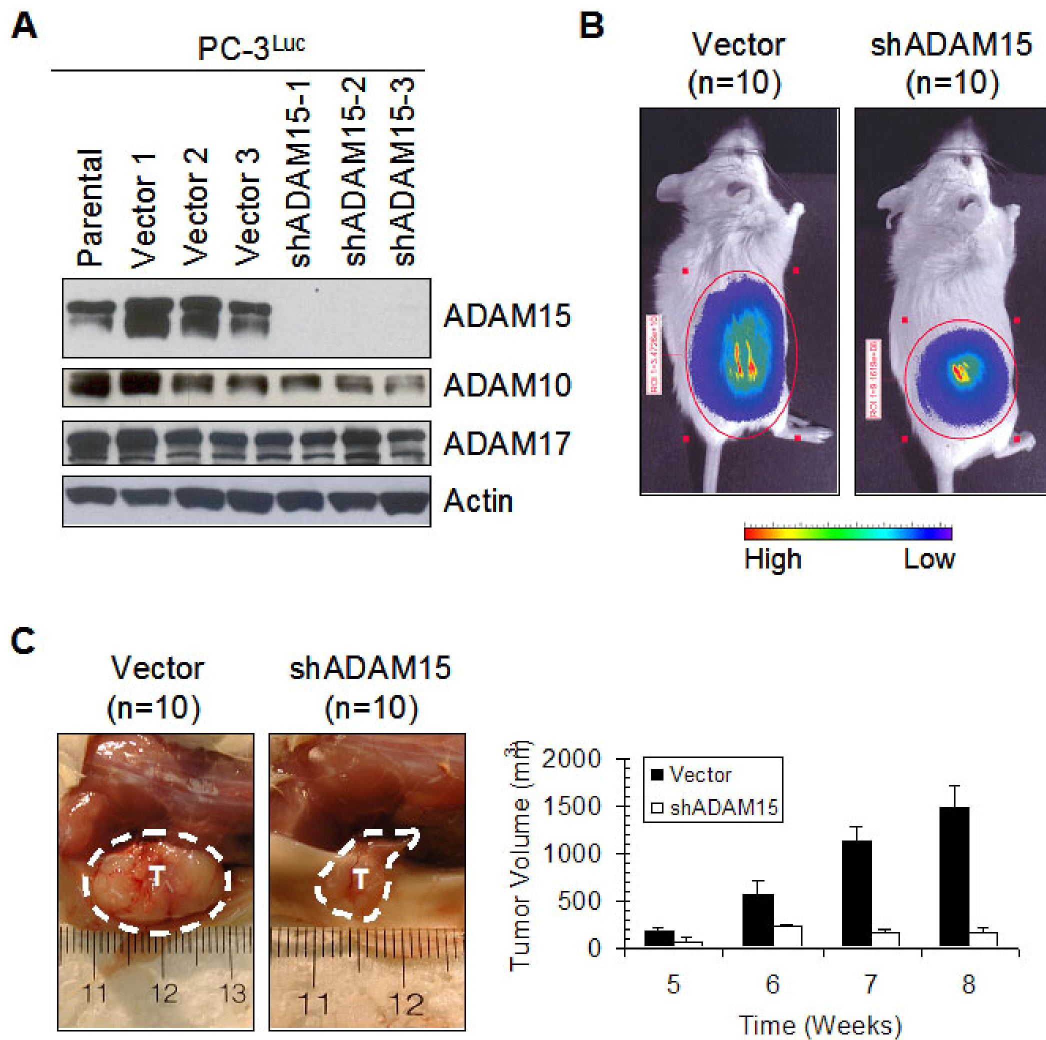

Fig. 2-1. Knockdown of ADAM15 reduces PC-3 tumorigenicity *in vivo*. A) Three separate shRNA ADAM15 PC-3^{Luc} cell lines were established (shADAM15-1-3). Actin was used as loading control. To control for off target effects, ADAM10 and ADAM17 were also examined. B) Vector or shADAM15 PC-3^{Luc} cells injected subcutaneously into male SCID mice were monitored weekly via bioluminescent imaging. The color scale indicates the intensity of photon emissions. C) Gross examination of tumors at 8 weeks revealed significantly larger vector PC-3^{Luc} tumors (T) in comparison to shADAM15 PC-

3^{Luc} tumors. Tumor volume was monitored by caliper measurements weekly for weeks 5-8. Two independent experiments were performed with five animals per cell line that were injected on the right flank and the average tumor volumes were plotted; the *bars* represent the SEM.

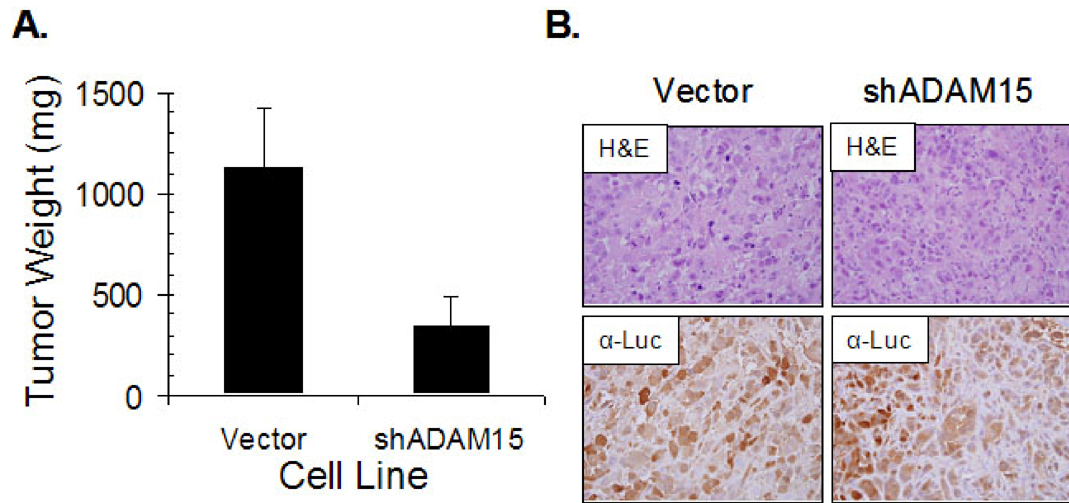

Fig. 2-2 Tumor weight and histology of ADAM15 PC-3 tumors. A) Vector and shADAM15 PC-3^{Luc} tumors were extracted and tumor weight was measure. B) H&E histology and anti-luciferase immunohistochemistry were used to verify the presence of epithelial derived PC-3^{Luc} cells. Magnifications were 400X.

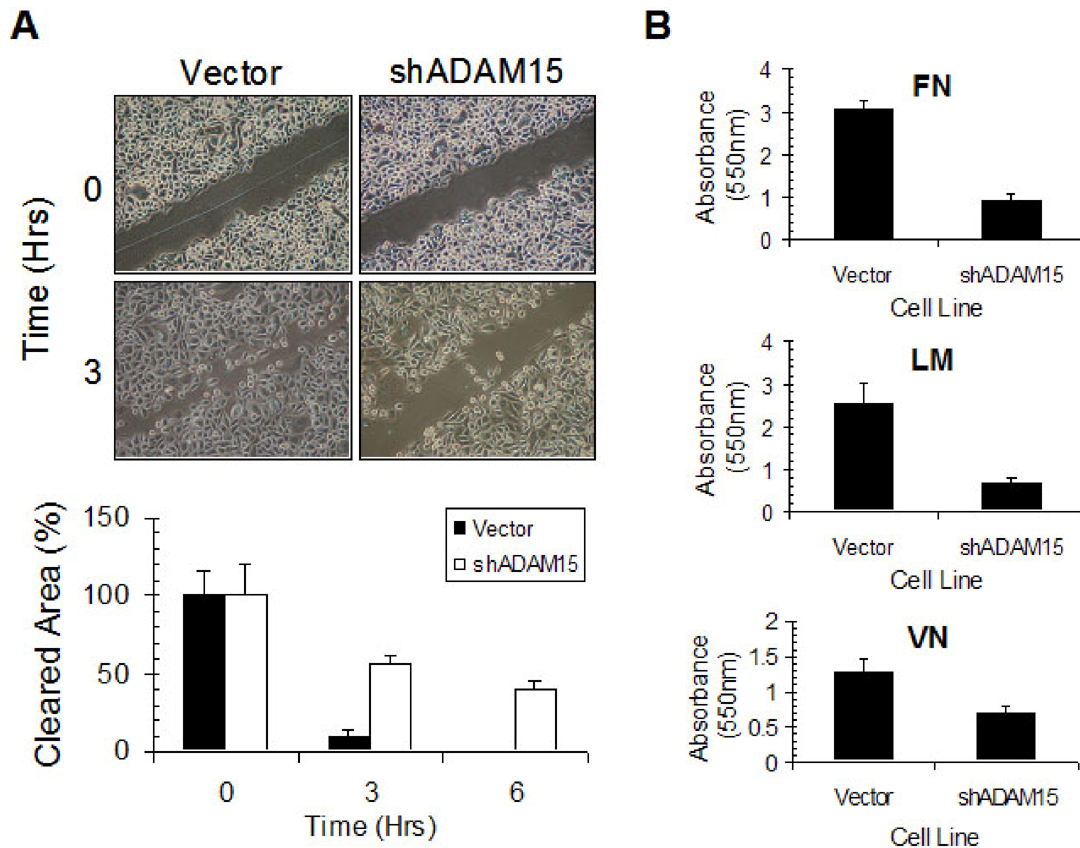

Fig. 2-3. Knockdown of ADAM15 attenuates PC-3^{Luc} cell migration and adhesion *in vitro*. A) Monolayers of vector or shADAM15 PC-3^{Luc} cells were abraded and then monitored at 0, 3, and 6 hours for wound channel closure. The cleared area was measured and plotted as the percentage of the original time point (0 hrs); *bars* represent the SD. Magnifications were 200X. B) Vector or shADAM15 PC-3^{Luc} cells were plated on fibronectin (FN), laminin (LM), or vitronectin (VN) coated plastic dishes and adhesion measured by absorbance at 550 nm. The *columns* represent mean values of three independent experiments measured in sextuplets; the *bars* represent the SD.

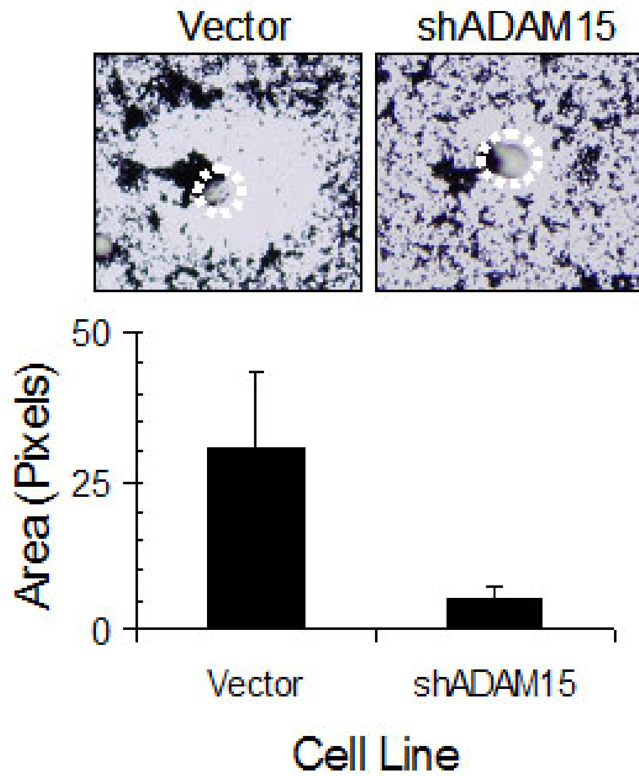

Fig. 2-4 Gold colloid migration assay of PC-3^{Luc} cells. Vector or shADAM15 PC-3^{Luc} cells were plated on gold-coated coverslips and allowed to attach. Cells were then stimulated to migrate with serum-containing media for 48 hours. Photographs of migratory tracks (clear area) around the migrating cell (dashed circle) were collected and analyzed. *Columns* represent the average area of the migratory track; *bars* represent the SD. Magnifications were 200X.

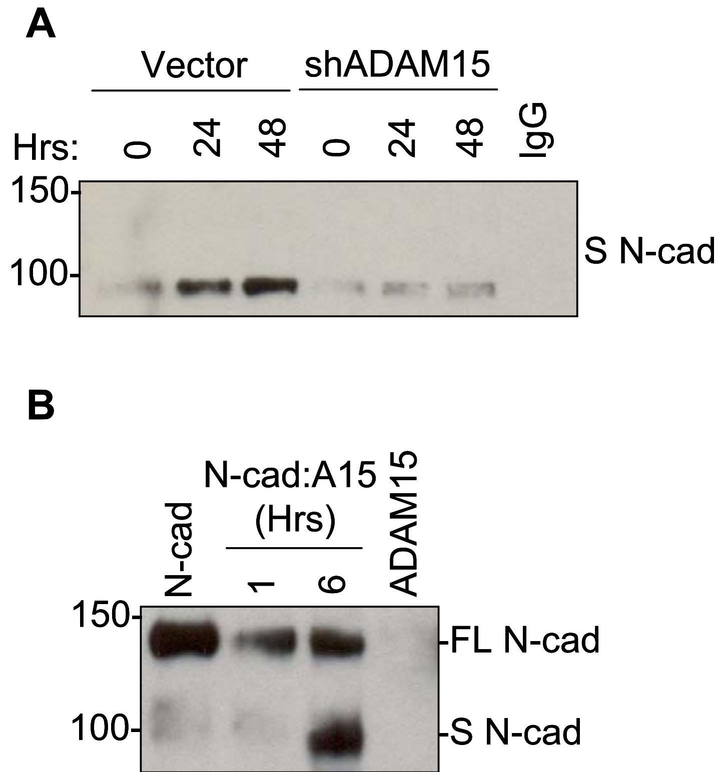

Fig. 2-5. ADAM15 mediates N-cadherin proteolysis. (A) Vector and shADAM15 PC-3[Luc] cells were serum starved for 0, 24, and 48 hours and soluble N-cadherin (S N-cad) was assessed in the conditioned media. (B) ADAM15 was purified and incubated together with purified N-cadherin for 1 or 6 hours. Purified N-cadherin was used as a size control and purified ADAM15 was used as a control for non-specific binding of the soluble N-cadherin fragment. ADAM15 mediated the processing of the 130 kDa full length N-cadherin (FL N-cad) into 90 kDa soluble N-cadherin (S N-cad).

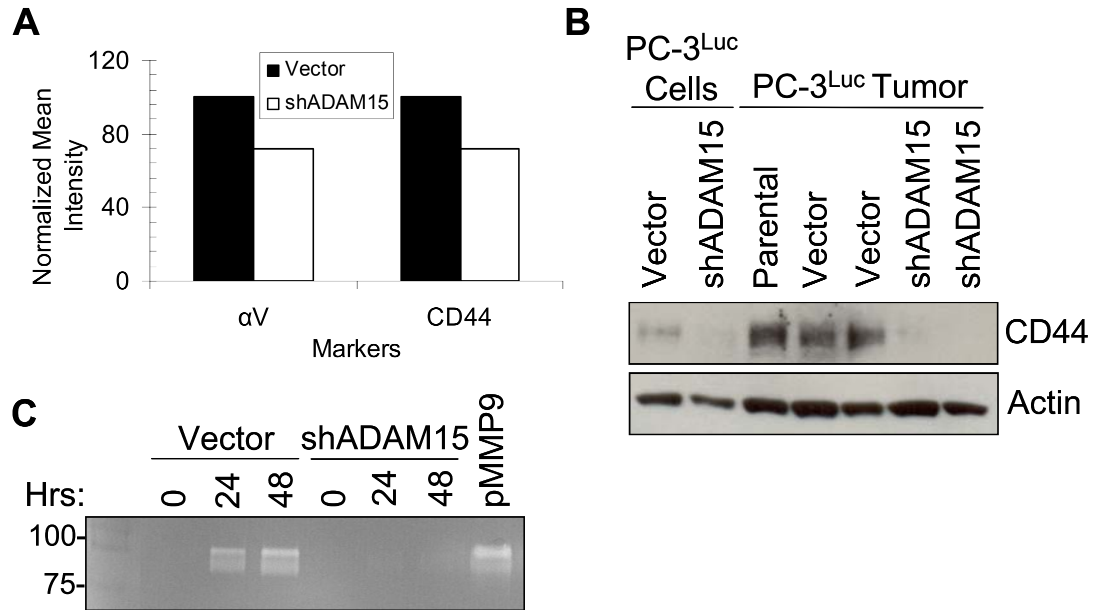

Fig. 2-6. Loss of ADAM15 disrupts cell surface receptors levels. The α_v and CD44 cell surface receptors were assessed via FACS (A) or Western blot (B) analysis in vector and shADAM15 PC-3^{Luc} cells and tumor lysates. (C) Vector and shADAM15 PC-3^{Luc} cells were serum starved for 0, 24, and 48 hours and conditioned media was analyzed for MMP9 activity using a gelatin zymogram. Purified MMP9 (pMMP9) was used as a positive control.

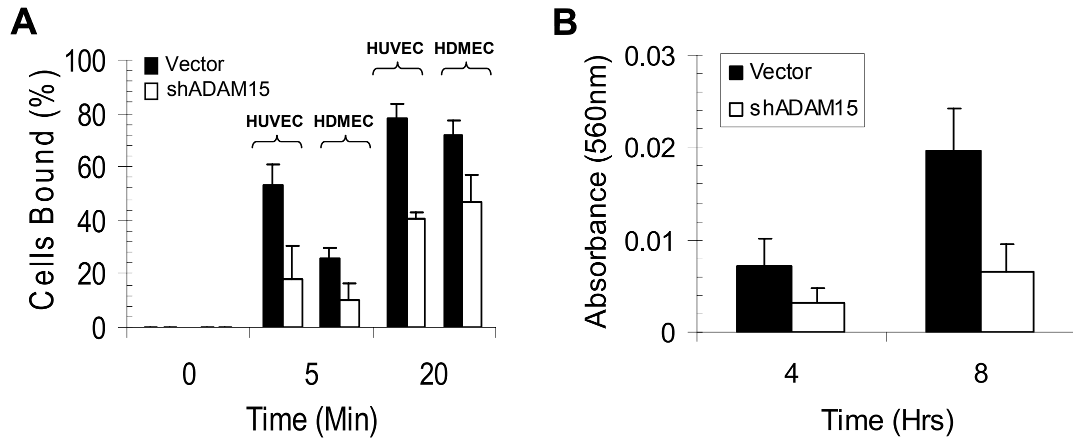

Fig. 2-7. The loss of ADAM15 reduces PC-3-endothelial interaction and transendothelial migration. A) Vector or shADAM15 PC-3^{Luc} cells were incubated on top of confluent HUVEC or HDMEC monolayers. The number of bound cells was determined at 0, 5 and 20 minutes. B) Transendothelial migration of vector or shADAM15 PC-3^{Luc} cells through a confluent endothelial monolayer grown on a 3 µm transwell. The relative migration rate was monitored at 4 and 8 hours via colorimeteric analysis. The *columns* represent mean values of independent experiments measured in duplicates and repeated three times; the *bars* represent the SEM.

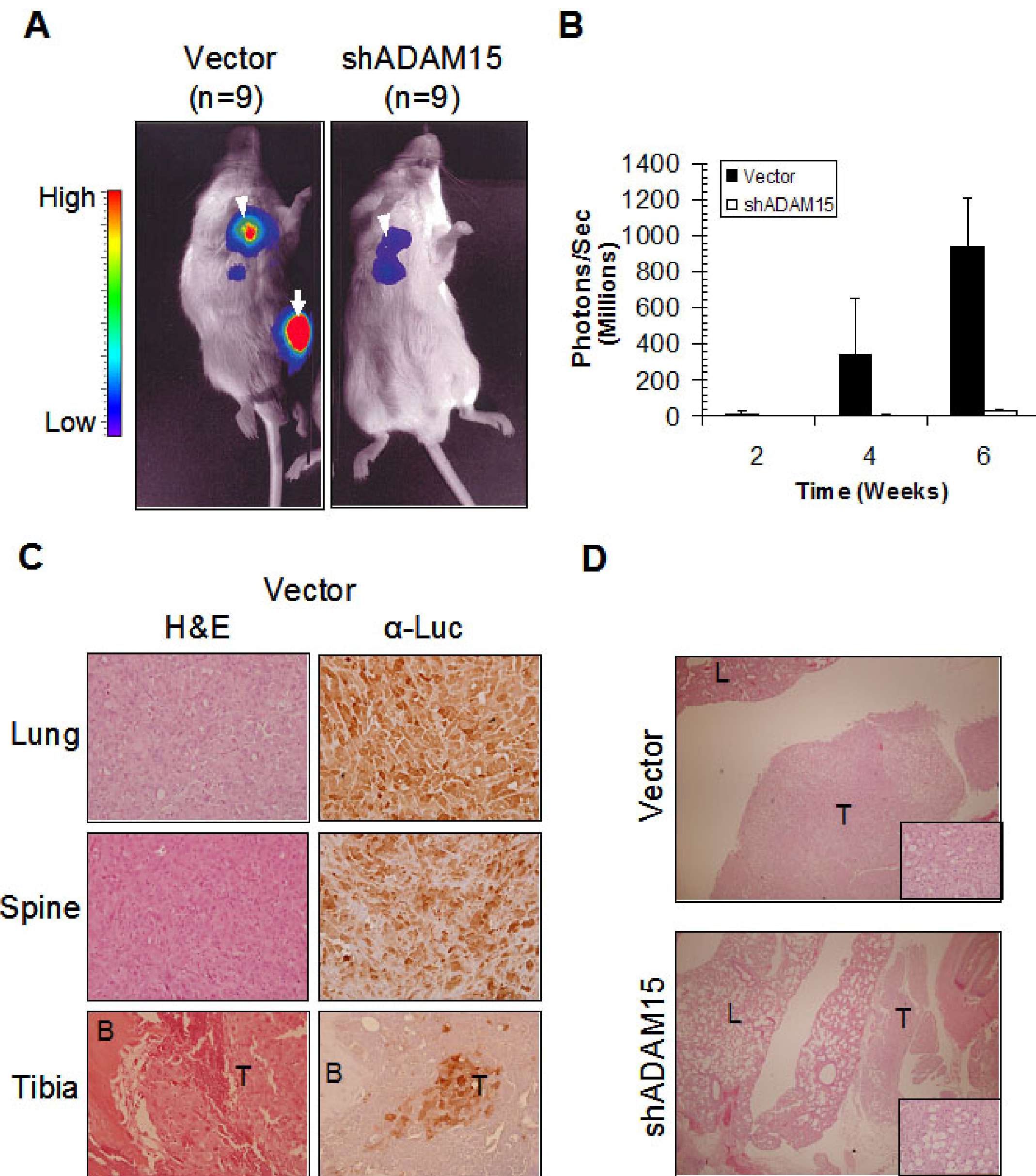

Fig. 2-8. Loss of ADAM15 attenuates PC-3 metastasis in an intracardiac dissemination assay. A) Vector or shADAM15 PC-3^{Luc} cells were injected into the left ventricle of male SCID mice and metastasis monitored via bioluminescent imaging for the duration of the 6-week study. Vector control PC-3^{Luc} cells were visualized in the tibia (arrow) and lung (arrow head) while the shADAM15 PC-3^{Luc} exhibited very low signals in the thoracic

cavity representing a lung micrometastasis (arrow head). B) Total bioluminescence was graphed for both cohorts and the *columns* represent mean values of bioluminescence imaging (photons/sec); the *bars* represent the SEM. C) Metastatic tissues representing sites of strong bioluminescence were extracted for histological evaluation. Lung, tibia and spine metastasis were observed in vector control sites as indicated by H&E histology and anti-luciferase immunohistochemistry. Magnifications were 400X. B, bone. T, tumor. D) shADAM15 PC-3^{Luc} cells formed micrometastatic tumors (T) in the lungs (L) in comparison to the large lung tumors observed in vector control. The inset is a 400X magnification of the 40X tumor image in each animal.

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CHAPTER 3

THE ECTODOMAIN SHEDDING OF E-CADHERIN BY ADAM15 SUPPORTS ERBB RECEPTOR ACTIVATION

Abstract

The zinc-dependent disintegrin metalloproteinases (ADAMs) have been implicated in several disease processes including human cancer. Previously, we demonstrated that the expression of a catalytically active member of the ADAM family, ADAM15, is associated with the progression of prostate and breast cancer. The accumulation of the soluble ectodomain of E-cadherin in human serum has also been associated with the progression of prostate and breast cancer and is thought to be mediated by metalloproteinase shedding. Utilizing two complementary models, overexpression and stable shRNA-mediated knockdown of ADAM15 in breast cancer cells; we demonstrated that ADAM15 cleaves E-cadherin in response to growth factor deprivation. We also demonstrated that the extracellular shedding of E-cadherin was abrogated by a metalloproteinase inhibitor and through the introduction of a catalytically inactive mutation in ADAM15. We have made the novel observation that this soluble E-cadherin fragment was found in complex with the HER2 and HER3 receptors in breast cancer cells. These interactions appeared to stabilize HER2 heterodimerization with HER3 and induced receptor activation and signaling through the Erk pathway supporting both cell migration and proliferation. In this study, we provide evidence that ADAM15 catalyzes the cleavage of E-cadherin to generate a soluble fragment that in turn binds to and

stimulates ErbB receptor signaling. This data is currently in press in *Journal of Biological Chemistry (JBC)*.

Introduction

The classic cadherins, E-(epidermal), N-(neuronal), and P-(placental) cadherins, are type I transmembrane glycoproteins (1). The epidermal specific cadherin, E-cadherin, has five extracellular domain (EC) repeats which are involved in cell binding mediated by E-cadherin homotypic interaction (2). The intracellular domain consists of a conserved sequence that associates with β, γ, and p120–catenins. The interaction of β or γ-catenin with α-catenin links E-cadherin to the cytoskeletal matrix to stabilize the adherens junction mediated by the homotypic E-cadherin complex (3). The involvement of E-cadherin in cell-cell interaction is well established in embryonic development, organ morphogenesis, tissue integrity, and wound healing (4). The disruption of E-cadherin by genetic mutation, promoter hypermethylation, or proteolytic cleavage leads to the loss of cell contact integrity as a consequence of adherens junction dissolution. E-cadherin disruption has been observed in multiple pathophysiological conditions including inflammation and cancer (5). In fact, E-cadherin is considered to function as a metastasis suppressor due to its inhibition of cancer cell migration and invasion (6). Several proteases have been implicated in the extracellular cleavage of E-cadherin, including MMP3, MMP7, MT1-MMP, plasmin, kallikrein 7, and ADAM10. In addition, the cytoplasmic domain of E-cadherin is cleaved by caspace-3 and calpain (7, 8). The ectodomain shedding of a stable 80 kDa soluble E-cadherin (sE-cad) fragment has been shown to increase in the urine and serum of patients with cancers of the bladder, breast, prostate, ovarian, gastric and melanoma and is a marker of poor prognosis (5). At the molecular level, sE-cad is disruptive to cell contact inducing cell scattering and eroding the adherens junction by antagonizing full length E-cadherin (9).

The ADAM (**a** **d**isintegrin **a**nd **m**etalloproteinase) family is composed of 40 members of which thirteen are catalytically active. These zinc-dependent proteases are transmembrane glycoproteins composed of five extracellular domains; prodomain, metalloproteinase, disintegrin, cysteine-rich, and EGF-like domains, respectively. The ADAMs also possess a cytoplasmic C-terminal tail containing SH2 and SH3 recognition sequences that have been shown to interact with different adapter proteins such as Grb2, SH3PX1 and endophilin I, which may play a role in protein localization and signal transduction (10, 11). The catalytic metalloproteinase domain of the ADAM family has a consensus HEXXGXXH sequence and is known to mediate extracellular matrix (ECM) protein degradation as well as ectodomain shedding of growth factors, growth factor receptors, and adhesion molecules (12). Complementing the metalloproteinase domain is the disintegrin domain which has been shown to bind different integrins that may support cell migration, adhesion, and ectodomain shedding (13). The presence of these domains suggests multiple functional roles for the ADAMs in a variety of normal and pathophysiological conditions including cancer progression. To this end, ADAM9 has been demonstrated to support lung cancer invasion and metastasis, while ADAM12 is upregulated in the serum of breast cancer patients and has been shown to mediate breast cancer cell invasion (14, 15). ADAM15 is one of the catalytically active sheddases which has been shown to be upregulated in breast, lung, gastric and prostate adenocarcinoma and is thought to support the metastatic progression of cancer cells by promoting tumor angiogenesis and angioinvasion (16-20). ADAM15 also plays a role in cell migration, neovascularization, and chondrocyte survival (21, 22) possibly through its role in EGFR

transactivation by cleaving the pro-forms of the EGFR ligands TGFα, HB-EGF, and amphiregulin (AREG) (23, 24).

The ErbB family of receptors are composed of four members; epidermal growth factor receptor (EGFR, ErbB1 or HER1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (25). When bound by their respective ligands, these receptors undergo homo- or heterodimerization that activates their inherent receptor kinase domain leading to receptor auto- and trans-phosphorylation and downstream signaling (26). ErbB receptor signaling has been demonstrated to support cancer cell migration, proliferation, and invasion (27). The dysregulation of this family of receptor tyrosine kinases (RTKs) is found in a myriad of pathophysiological conditions including cancer (28). EGFR overexpression and hyperactivity have been implicated in several human cancers including non-small cell lung cancer (NSCLC), ovarian, and breast cancers (29). Similarly, the HER2 receptor is found to be overexpressed in 20-30% of breast cancer and is a marker of poor prognosis (4). Interplay between the ErbB family members and their ligands is necessary to induce a cell response. EGFR has been shown to interact with all of the seven EGFR ligands, while HER3 and HER4 favor binding to the heregulin family of ligands (30). In contrast to the other ErbB family members, HER2 has not yet been demonstrated to bind to a specific ligand.

The ADAM family members, including ADAM15, play an important role in the transactivation of ErbB family members by releasing the latent transmembrane EGFR ligands from their pro-forms on the cell surface (12, 31). Previously, ADAM15 was shown to be upregulated during breast cancer progression using cDNA and tumor microarrays (18). Based on the role of ADAM15 as a membrane sheddase that is

upregulated during breast cancer progression, coupled to the fact that increased sE-cad levels also correlates with breast cancer progression, led us to assess the role of ADAM15 in E-cadherin shedding in breast cancer cells. We report here that ADAM15 is capable of cleaving full length E-cadherin into a soluble, extracellular fragment. We also show that the solubilized E-cadherin fragment, in turn, binds to and stabilizes the ErbB receptor HER2 and HER3 heterodimerization leading to Erk-dependent signaling. To our knowledge, this is the first report demonstrating a potential ligand for the HER2 receptor and a role for soluble E-cadherin in stabilizing ErbB receptor dimerization and signaling.

Materials and Methods:

Cell lines and culture.

LNCaP and SKBr3 cells were maintained in RPMI (Bio Whittaker, Walkersville, MD) with 8% fetal bovine serum (HyClone, Logan, UT). MCF-7^{GFP} cells (which were a kind gift from Dr. Jacque Nör, University of Michigan Dental School) were maintained in DMEM (Gibco, Carlsbad, CA) with 10% fetal bovine serum. All culture media were supplemented with 2 mmol/L L-glutamine (Invitrogen, Carlsbad, CA), 100 units/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 0.25 μg/mL Fungizone (Invitrogen). ADAM15 overexpressing cells were grown under selection with 800μg/mL of G418 (Cellgro, Manassas, VA). Cells were incubated at 37°C and sub-cultured weekly.

Generation of ADAM15 cell lines.

MCF-7^{GFP}cells were infected with ADAM15 specific knockdown oligonucleotides or control oligonucleotides consisting of a scrambled sequence designed to control for off-target effects. The forward and complementary targeting sequences for ADAM15 were 5'-AACCCAGCTGTCACCCTCGAA-3' and 5'-TTCGAGGGTGACAGCTGGGTT-3'. The shRNA cassette also featured a TTCAAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTTT terminator at the 3' end. ADAM15 overexpressing MCF-7cells were generated as described by Kuefer et. al. (18). To generate ADAM15 mutants, first the ADAM15 cDNA was tagged with HA (hemagglutinin) at the C-terminus and transfected into LNCaP cells as described previously (18) to establish wild type ADAM15. Catalytically-dead ADAM15 was

generated by mutating the glutamic acid residue into an alanine at position 350 (E350A) using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). DNA was sequenced to confirm mutations (University of Michigan Sequencing Core).

Microarray analysis.

Expression levels of ADAM15 and HER2 in published breast cancer cDNA arrays were derived using the Oncomine database available at www.oncomine.org. The terms "ADAM15" or "HER2" were used to search the database for differential expression of both of these markers in different breast cancer arrays.

Protein isolation, immunoprecipitation and Western blotting.

Cells were harvested by mechanical disruption with cell scrapers followed by gentle centrifugation at 6000 RPM for 3 minutes. Cell pellets were then lysed in appropriate volumes of lysis buffer [50mmol/L Tris (pH 7.6), 120 mmol/L NaCl, .5% NP40, 1 mmol/L EGTA, 100 μg/mL phenylmethysulfonyl fluoride, 50 μg/mL aprotinin, 50 μg/mL leupeptin, and 1.0 mmol/L sodium orthovanidate] for 1 hour on ice. Cellular debris was then pelleted by centrifugation at 12,000 RPM for 8 minutes, and supernatants were collected and quantitated using a microtiter Bradford protein assay (Bio-Rad, Hercules, CA) with experimental and standard samples were run in triplicate. Equal amounts of protein were then separated on precast Tris-glycine SDS-polyacrylamide gels (Novex, Carlsbad, CA) and transferred to reinforced .2 μm nitrocellulose membrane (Millipore, Temecula, CA). Membranes were then blocked, probed, and developed. Primary antibodies were obtained as follows: actin (Sigma, St. Louis, MO); pErk and Erk

(Cell Signaling Technology, Danver, MA); ADAM15, tubulin, phospho-tyrosine (Millipore); E-cadherin (Zymed, San Francisco, CA); HER2 and HER3 (Lab Vision Corporation, Freemont, CA). All appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from BioRad.

For immunoprecipitation, cell lysates were precleared with an equal amount of a mixture of 2.5% dry milk in TBST/Sephrose Proteins A beads (Zymed) for 30 minutes at room temperature, then beads were spun out. Precleared protein was then immunoprecipitated with the HER2, HER3 (Lab Vision Corporation) antibody or an equal amount of isotype IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 hours at 4°C with end-over-end rotation. This was followed by incubating the immunoprecipitation mixture with 75uL of blocked Sephrose Protein A beads for 90 minutes at 4°C with rotation. The complex was then centrifuged and washed three times with ice-cold PBS. The complex was dissociated from the beads with the addition of 5X sample reducing loading buffer and heated for 5 minutes at 100°C. Samples were then loaded on SDS-PAGE for protein analysis. An ECL system was used to visualize proteins (Millipore).

Immunocytochemistry.

ADAM15-GFP overexpressing cells were grown to sub-confluency in 2-well chambers (BD Falcon, Bedford, MA) then cross linked with 2% formaldehyde in PBS and fixed with 100% ethanol for 5 minutes on ice. Fixed cells were washed three times with ice-cold PBS and blocked with 0.1% milk/FBS solution for 30 minutes. The E-cadherin HECD-1 antibody or an isotype matched IgG were incubated on the slides for 1 hour.

Following the primary antibody incubation, cells were washed three times as mentioned above and incubated for 50 minutes with anti-mouse rhodamine secondary (Invitrogen). Slides were mounted with 1 mm coverslip (Fisher Scientific, Hampton, NH) and photomicrographs were taken utilizing confocal microscopy at the University of Michigan Imaging core.

E-cadherin proteolysis.

For the *in vitro* proteolysis of E-cadherin, ADAM15 was isolated through immunoprecipitation using an HA specific antibody (Millipore) from LNCaP whole cell lysate expressing either wild-type (WT) or catalytic-dead (CD) ADAM15 mutant. E-cadherin was also isolated through immunoprecipitation using an E-cadherin specific HECD-1 antibody (Zymed) from MCF-7 whole cell lysate. Isolated ADAM15 and E-cadherin were mixed together at the designated ratio and incubated for the chosen time points in a 37°C water bath. For the inhibitor assays, 0.05M of 1,10phenanthroline [1,10P] (Sigma) was added to each tube. At the end of each time point, the reactions were stopped by addition of 5X sample reducing loading buffer and heating to 100°C for 5 minutes. Samples were then loaded on SDS-PAGE for protein separation and analysis via Western blot.

Conditioned media analysis.

MCF-7 cells were grown up to sub-confluency (~65-70%) and then serum starved in serum-depleted media for 24 hours. Conditioned media was collected by centrifugation at 2,000 RPM for 15 minutes to pellet any cell debris. Levels of soluble E-cadherin in

conditioned media were monitored via E-cadherin immunoprecipitation using the HECD-1 monoclonal antibody (Zymed).

Cell migration assays.

To assess the affects of endogenous soluble E-cadherin on MCF-7 cell migration, cells were plated in 6-well tissue culture dishes until confluency. Cells were then serum starved for 16 hours then abraded with a 10µL pipette tip. The cells were washed once with warm growth media and incubated in normal growth media. Cell migration was monitored through microscopic imaging at the designated time points. Migration was quantitated as the percent of remaining cleared area by dividing the cleared area at each time point by the original 0 hour time point. Each experiment contained 4 separate samples and performed three times.

Cell proliferation assays.

To assess the affects of endogenous soluble E-cadherin on MCF-7 cell proliferation, $1X10^4$ cells were plated in 96-well plates for 24 hours. Cells were then washed once with warm serum free media, and then incubated for the appropriate amount of time under serum free media. Cell proliferation was assessed using the MTT assay and quantified as fold change over time by dividing the OD readings of each time point by the original time point at 0 hours. Each experiment contained 8 separate samples and performed three times.

To analyze the exogenous effects of E-cadherin, the E-cadherin null cell line SKBr3 was plated at $3X10^6$ cells for four days then washed once with warm serum free

media. Cells were then incubated with either vehicle or 1.5 μg/mL Fc-Ecadherin recombinant protein (R&D Systems) in serum free media for the designated time points. Cell proliferation was measured using the trypan blue exclusion assay (Gibco) and quantified as fold change over time by dividing the number of cells at each time point by the cell number at the original time point at 0 hours. Each experiment was ran in triplicates and performed three times.

Statistical analysis.

All statistical work was performed using a Student's unpaired t-test with a one-tailed distribution. $P<0.05$ was considered statistically significant.

Results

Upregulation of ADAM15 and HER2 during breast cancer progression. The ADAM15 chromosomal locus, 1q21.3, is amplified during the metastatic progression of multiple adenocarcinomas and melanoma (32, 33). We utilized the oncomine database to comprehensively examine ADAM15 expression in published human cDNA microarrays of breast cancer. We observed that ADAM15 was significantly upregulated in eight different cDNA microarray studies. Seven of the aforementioned arrays also demonstrated significant upregulation of the HER2 receptor which is known to be a marker of poor prognosis (Fig. 3-1A). One of these studies is graphically presented in figure 1B to demonstrate the correlative increase in both ADAM15 and HER2 expression in breast cancer tumors over normal tissues (34). Interestingly, ADAM15 expression was downregulated in estrogen receptor (ER)-positive breast cancer tumors (data not shown). Seven of the eight data sets show that ADAM15 and HER2 transcripts are simultaneously and significantly upregulated during breast cancer progression suggesting a role of ADAM15 in breast cancer development (Fig. 3-1B).

ADAM15 cleaves E-cadherin in breast cancer cells. Since ADAM15 was found to be overexpressed in breast cancer and sE-cad levels have been demonstrated to be upregulated during the progression of this disease, we hypothesized that ADAM15 may play a critical role in E-cadherin shedding. To evaluate whether ADAM15 mediates E-cadherin proteolysis, we stably overexpressed an ADAM15-GFP fusion protein in MCF-7 breast cancer cells (ADAM15-GFP cells, Fig. 3-2A). ADAM15-GFP cells exhibited both endogenous and recombinant ADAM15. Two species of endogenous ADAM15

were detected by an ADAM15-specific antibody at 110 kDa (inactive precursor) and 90 kDa (catalytically active). Two species of recombinant ADAM15-GFP were detected at 136kDa and 116 kDa representing the recombinant precursor and active forms, respectively. ADAM15 overexpression exhibited no effects on ADAM15 family relatives, ADAM10 or ADAM17, or other assessed targets (data not shown).

Previously, Damsky et. al. demonstrated that serum deprivation of MCF-7 cells for 24 hours led to the release of sE-cad into the conditioned media of these cells (35). Vector and ADAM15-GFP MCF-7 cells were serum starved for 24 hours and the presence of sE-cad was analyzed in the conditioned media. We found that sE-cad was elevated in the ADAM15 overexpressing cells in comparison to vector control (Fig. 3-2A).

To substantiate the overexpression findings, we stably downregulated ADAM15 in our breast cancer cells using an shRNA construct against ADAM15. Both the precursor and mature forms of ADAM15 were reduced in response to the shADAM15 construct in comparison to the scramble shRNA (shScrm) control cells (Fig. 3-2B). Analysis of the ADAM15 RNA message also demonstrated significant downregulation in response to the shADAM15 inhibitory construct (data not shown). We observed that the ADAM15 shRNA construct was specific to ADAM15 and did not affect ADAM15 relatives, ADAM10 and ADAM17, or other targets (data not shown). Serum starvation of scramble control and shADAM15 MCF-7 cells resulted in decreased shedding sE-cad into the conditioned media in response to ADAM15 downregulation (Fig. 3-2B).

***In vitro* E-cadherin proteolysis by ADAM15.** Immunohistochemistry revealed prominent co-localization of ADAM15 and E-cadherin at the junctional cell membrane (Fig. 3-3A). To directly implicate ADAM15 in E-cadherin cleavage, we isolated ADAM15 and E-cadherin and performed an *in vitro* cleavage analysis. When ADAM15 and E-cadherin were co-incubated at equal ratios, ADAM15 cleaved E-cadherin into the sE-cad fragment in a time-dependent manner (Fig. 3-3B). This fragment migrated at 80 kDa on reducing gels and showed immunoreactivity with extracellular domain specific E-cadherin antibodies. E-cadherin alone was not cleaved and ADAM15 isolation revealed no contamination by E-cadherin. E-cadherin was also cleaved by ADAM15 in a dose-dependent manner (Fig. 3-3C).

To ascertain that E-cadherin proteolysis is mediated by the metalloproteinase activity of ADAM15, we co-incubated isolated ADAM15 and E-cadherin with either vehicle control or the metalloproteinase inhibitor, 1,10 phenanthroline (1,10P), which reduced ADAM15-mediated cleavage of E-cadherin (Fig. 3-3D). Since 1, 10phenanthroline is a broad spectrum inhibitor; we complemented this pharmacologic approach by mutating the metalloproteinase domain of ADAM15 to directly implicate ADAM15 in E-cadherin cleavage. Catalytically-dead (CD) ADAM15 was generated by a single conversion of the glutamic acid residue at position 350 into an alanine (E350A) as previously described (36). Co-incubation of E-cadherin with the catalytic-dead ADAM15 inhibited soluble E-cadherin generation as compared to the wild-type (WT) protease (Fig. 3-3E). Residual sE-cad in the E-cadherin alone lane is a result of non-specific binding of sE-cad during the isolation process.

Solubilized E-cadherin interacts with ErbB receptors. Full length E-cadherin has been shown to interact with EGFR through its extracellular (EC) domain and induce ligand independent signaling in keratinocytes (37, 38). MCF-7 cells are known to express predominantly HER2 and HER3 (data not shown and (39)). We demonstrated in this study that both ADAM15 and HER2 are upregulated during breast cancer progression and ADAM15 functions in the extracellular shedding of sE-cad. Following serum starvation of scramble control or shADAM15 MCF-7 cells to generate sE-cad, we found that HER2 preferentially bound the soluble form of E-cadherin and not the full length E-cadherin as compared to the input control (Fig. 3-4A). Substantially less sE-cad bound to HER2 in the ADAM15 knockdown cells. Interestingly, we also found that HER3 bound s sE-cad in an ADAM15-dependent manner. Furthermore, the sE-cad fragment appeared to enhance the formation of a HER2/HER3 heterodimer as shown by higher intensity HER2 and HER3 bands in the scramble control cells compared to the shADAM15 MCF-7 cells (Fig. 3-4B). The HER2/HER3 heterodimer complex was only observed when we immunoprecipitated with the HER3 antibody. We were not able to detect HER3 with a HER2 immunoprecipitation potentially due to allosteric changes within the HER3 receptor that blocked the antibody from detecting its epitope. These findings were validated within the ADAM15 overexpressing MCF-7 model (data not shown). The sE-cad fragment preferentially bound HER2 over HER3 and the HER2 receptor was observed to bind to a sE-cad doublet whereas HER3 only bound the lower band of this sE-cad doublet.

To confirm that sE-cad interaction with HER2 is HER2-specific, we treated MCF-7 scramble control cells with the humanized HER2 antibody, trastuzumab (Herceptin).

We found that this extracellular-domain specific antibody to HER2 completely eliminated the interaction of sE-cad with HER2 in MCF-7 cells in comparison to vehicle treatment (Fig. 3-4C). In addition, the trastuzumab treatment also abrogated the sE-cad /HER3 complex and the HER2/HER3 heterodimerization mediated by sE-cad within the MCF-7 cells (Fig. 3-4D).

Soluble E-cadherin mediates HER2-dependent signaling. The interaction of EGFR ligands with their complementary receptors leads to receptor phosphorylation on c-terminal tyrosine residues and concomitant receptor activation resulting in downstream signaling (27). To assess whether endogenous sE-cad interaction with HER2 induces receptor phosphorylation, we stimulated E-cadherin shedding by serum starving scramble control or shADAM15 MCF-7 cells. We observed increased phosphorylation of HER2 in scramble control cells; however tyrosine phosphorylation of HER2 in shADAM15 cells was less (Fig. 3-5A). We also monitored HER3 phosphorylation in our MCF7 cells and found that, like HER2, scramble control cells demonstrated more receptor phosphorylation compared to shADAM15 cells in response to serum deprivation (Fig. 3-5B).

The HER2/HER3 dimer has been shown to signal through both the Erk and Akt pathway when activated (28). We assessed Erk signaling in the MCF-7 cells at time points where we observed soluble E-cadherin-mediated HER2 activation. The scramble control cells exhibited increased phosphorylation of Erk in response to serum starvation. In contrast, Erk phosphorylation remained at basal levels in the shADAM15 cells (Fig. 3-

5C). Akt phosphorylation was not detectable in these cells potentially due to a less robust activation of the ErbB receptors signaling cascade by sE-cad (data not shown).

ADAM15 mediates soluble E-cadherin-dependent cell migration and proliferation. Because Erk signaling is known to mediate cell migration and proliferation (28), we assessed whether the MCF-7 scramble control cells possessed a migratory advantage over the shADAM15 cells in response to serum starvation. In a wound channel migration assay, the MCF-7 scramble control cells exhibited more rapid migration than the shADAM15 cells over time (Fig. 3-6A). To analyze if ADAM15 downregulation compromised the proliferative potential of MCF-7 cells we performed proliferation assays on these cells and found that scramble control MCF-7 cells proliferated more than the shADAM15 cells during serum deprivation (Fig. 3-6B).

Exogenous stimulation of HER2 with soluble E-cadherin. Within this study, we demonstrated that ADAM15 mediates endogenous generation of sE-cad, which interacts with ErbB receptors and induces their transactivation. To verify that sE-cad is responsible for HER2 binding and activation, we utilized an extracellular domain of E-cadherin/Fc fusion protein (Fc-Ecad). Experiments with this fusion protein were performed in an E-cadherin negative, HER2 positive cell line to eliminate endogenous soluble E-cadherin background. The breast cancer cell line, SKBr3, which is E-cadherin negative and expresses copious amounts of HER2, was treated with vehicle or the Fc-Ecad fusion protein under serum-free conditions and complex formation between sE-cad and HER2 was assessed. These experiments demonstrated clear Fc-Ecad interaction with HER2 as

compared to vehicle control (Fig. 3-7A), but no Fc-Ecad binding to HER3 was observed. Although the HER3/Fc-Ecad complex was not detected in our experiments, we did observe that Fc-Ecad treatment mediated HER2/HER3 heterodimerization and HER3 receptor phoshorylation (Fig. 3-7B). HER2 phosphorylation was unaffected in response to Fc-Ecad treatment potentially due to constitutive activation as a result of HER2 receptor overexpression (data not shown). We monitored Erk phosphorylation in these cells and observed an increase in its phosphorylation status in response to Fc-Ecad treatment (Fig. 3-7C). Vehicle treated cells exhibited basal Erk phosphorylation, which could be due to HER2 hyperactivation resulting from HER2 overexpression in these cells. In confirmatory experiments, we utilized a human E-cadherin peptide (huEP) and found that Erk phosphorylation was stimulated in response to E-cadherin peptide addition (data not shown). To assess whether Erk activation mediated by exogenous stimuli affected cell growth, we performed proliferation assays using Fc-Ecad and examined the proliferative response in comparison to vehicle control over time (Fig. 3-7D). We demonstrated that the Fc-Ecad fusion peptide induced a significant increase in SKBr3 cell proliferation as compared to vehicle treatment.

Discussion

The cell adhesion molecule, E-cadherin, serves a crucial role in inhibiting tumor cell migration and invasion by maintaining the cell-cell adhesion complex and the inactivation of E-cadherin by gene deletion, promoter hypermethylation or proteolytic cleavage, renders tumor cells prone to a migratory and invasive phenotype due to the loss of cellular contact and polarity (40, 41). Ectodomain cleavage of E-cadherin by several different proteases has been reported to yield an 80 kDa fragment known as soluble E-cadherin (sE-cad). Soluble E-cadherin accumulates in the serum or urine of patients suffering from multiple types of cancers including prostate, breast, bladder and lung cancer (5). Using published cDNA arrays, we report here that a catalytically active member of the ADAM family, ADAM15, is upregulated during the progression of breast adenocarcinoma. Furthermore, ADAM15 expression was elevated in HER2-positive breast cancer tumors and was found to be downregulated in ER-positive breast cancer correlating ADAM15 levels with disease progression. To assess the role of ADAM15 in sE-cad shedding, we overexpressed or knocked down ADAM15 in the MCF-7 breast cancer cell lines and observed an elevation of sE-cad shedding in response to ADAM15 overexpression and a reduction of the sE-cad in ADAM15 knockdown cells. Previously, ADAM10 has been demonstrated to cleave E-cadherin in keratinocytes (42), but in our models ADAM10 levels were unaffected by ADAM15 protein modulation and was constant throughout the analyses. Based on the data presented here, we believe that growth factor deprivation may activate ADAM15 at the cell surface which in turn sheds the ectodomain of E-cadherin into the extracellular milieu (Fig. 3-8).

MT1-MMP has been shown to activate MMP2 and 9 to support cell invasion through ECM degradation (43, 44). To ascertain that ADAM15 is cleaving E-cadherin directly and not activating another protease, we isolated both ADAM15 and E-cadherin, and then co-incubated them together to induce ADAM15 directed proteolysis. We demonstrated that ADAM15 cleaves E-cadherin in a time- and concentration-dependent manner. ADAM15 proteolysis was inhibited by introducing an inactivating mutation in the catalytic domain thus implicating ADAM15 as a direct sheddase of E-cadherin.

Soluble E-cadherin is known to inhibit cell aggregation and induce cell invasion through a yet uncharacterized signaling mechanism (45, 46). These same events have also been shown to be initiated by ligand interaction to the ErbB family which is composed of four members, EGFR (HER1), HER2, HER3 and HER4. When bound to their cognate ligands, these receptors mediate cell proliferation, migration, invasion and differentiation (4). The EGFR ligands are synthesized as inactive transmembrane precursors, which are liberated from their inactive state by metalloproteinases including ADAM family members (12). The activation of the ADAM proteases by a G-protein coupled receptor (GPCR) signal leads to the shedding of EGFR ligands which in turn bind and transactivate their complementary receptors to mediate downstream signaling (31). All of the ErbB family members have a specific ligand except HER2, which functions by forming heterodimers with the other family members potentiating cell signaling (30). ErbB receptor dimerization is accompanied with cross phosphorylation and all the ErbB family members have active kinase domains except HER3 which can only be phosphorylated by its dimerizing partner. Since MCF-7 cells expressed HER2 and HER3 which were upregulated in response to growth factor deprivation, we wanted to assess the

interaction of E-cadherin with these receptors as a potential ligand. We observed that HER2 bound a sE-cad doublet in response to growth factor deprivation in an ADAM15-dependent manner. In addition, HER3 interacted with the lower molecular weight sE-cad and this complex mediated HER2/HER3 heterodimerization. Since we used whole cell lysates for these experiments, the difference in sE-cad banding observed bound to the ErbB receptors maybe due to differential phosphorlyation of this fragment by HER2 as a result of receptor internalization. In addition, sE-cad was found to complex preferentially with HER2 than with HER3 in our assays. The order of sE-cad binding to HER2 and HER3 is yet to be elucidated and is a focus for future wok. Serum deprivation of MCF-7 cells induced ADAM15-dependent phosphorylation of HER2 and the kinase inactive HER3 potentially through the sE-cad -mediated heterodimerization of the kinase active HER2 with HER3 (Fig. 3-8).

The interaction between sE-cad and HER2 or HER3 was inhibited by the HER2 humanized antibody, trastuzumab (Herceptin), suggesting that this complex is HER2 dependent. Trastuzumab treatment also abrogated the sE-cad-mediated HER2/HER3 heterodimerization. Soluble E-cadherin/HER2 complex formation was induced by exogenous addition of a purified extracellular E-cadherin fusion protein (Fc-Ecad), the binding of which led to HER2 activation and downstream signaling through the Erk pathway. Fc-Ecad did not bind to HER3 in our assays potentially due to the abundant amounts of HER2 in SKBr3 cells that competed against HER3 binding. However, we did observe an increase HER2/HER3 dimerization and HER3 phosphorylation in response to Fc-Ecad stimulation suggesting a role for exogenous Fc-Ecad in mediating ErbB receptor transactivation. The HER2/HER3 heterodimer is known to signal through the Erk

signaling pathway, which support cell survival, proliferation and migration (47, 48). In our models, we found that either endogenous shedding of E-cadherin or addition of exogenous soluble E-cadherin fusion proteins or peptides supported cancer cell migration and proliferation possibly through Erk signaling. Previous studies demonstrated that full length cadherin ligation and activation of growth factor receptors activated only Erk signaling (49, 50). In our experimental models Akt activation was not detected which may be a consequence of E-cadherin specific receptor activation.

In this study, we demonstrated that ADAM15 and HER2 are simultaneously upregulated during breast cancer progression. Additionally, overexpression of the ErbB receptor, HER2, and loss of E-cadherin expression is frequently observed in breast cancer and are considered indicators of poor prognosis. However, the functional association between all of these molecules has not been investigated (4, 51). ADAM15 mediated sE-cad shedding which in turn bound to and transactivated both HER2 and HER3. This sE-cad fragment also enhanced Erk activation to support breast cancer cell migration and proliferation. Thus, we have identified a functional interaction between sE-cad and ErbB receptors, although, the structural requirements and fine mapping for these interactions have yet to be elucidated. Further characterization of this signaling axis is warranted and may ultimately lead to novel therapeutic strategies targeting ADAM15, E-cadherin and HER2 in breast cancer.

A

Table 1. ADAM15 and HER2 expression in breast cancer cDNA microarray *

Author	ADAM15 Status	p-Value	HER2 Status	p-Value
Bild_Breast	↑	0.007	↑	2.20E-11
Ginestier_Breast	↑	2.00E-03	↑	3.10E-02
Hess_Breast	↑	0.015	↑	1.50E-14
Minn_Breast_2	↑	0.044	↑	6.00E-04
Perou_Breast	↑	0.019	↑	0.002
Richardson_Breast_2	↑	2.00E-03	↑	2.10E-02
Sotiriou_Breast_3	↑	3.30E-02	↑	8.00E-03

*All studies were obtained from the Oncomine database at www.oncomine.org

B

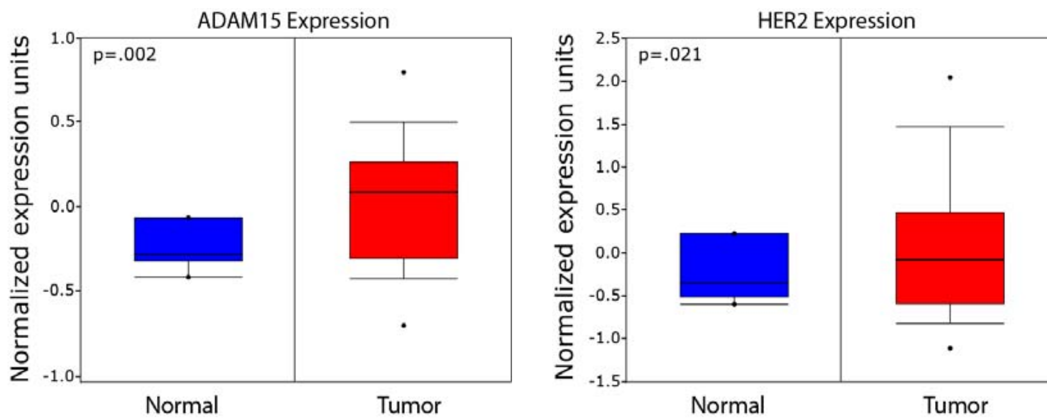

Fig. 3-1. ADAM15 and HER2 expression in breast cancer. (A) ADAM15 and HER2 are simultaneously overexpressed in seven different breast cancer cDNA microarrays. (B) A graphical representation of ADAM15 and HER2 expression in normal and tumor breast tissues using the published Richardson_Breast_2 array available at www.oncomine.org.

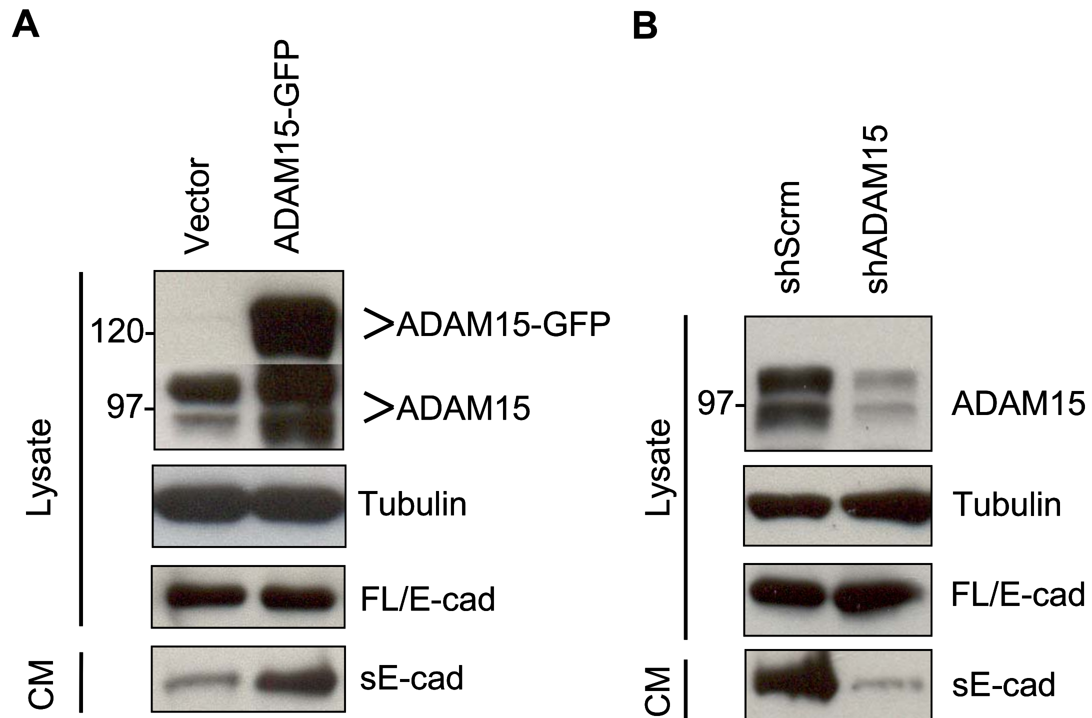

Fig. 3-2. ADAM15 cleaves E-cadherin in breast cancer cells. (A) GFP-tagged and endogenous ADAM15 in MCF-7 cells are indicated (lysate). Due to the intense banding pattern observed in ADAM15 overexpressing (ADAM15-GFP) cells, a lower exposure of the GFP fusion protein was cropped into the ADAM15 panel. Analysis of soluble E-cadherin (sE-cad) in the conditioned media (CM) of ADAM15 overexpressing cells. B) ADAM15 expression was downregulated in MCF-7 cells using a stable shRNA against ADAM15 (lysate) and sE-cad was assessed in the conditioned media (CM). shScrm, scramble shRNA control. Full length E-cadherin (FL/E-cad) remained unchanged in these experiments.

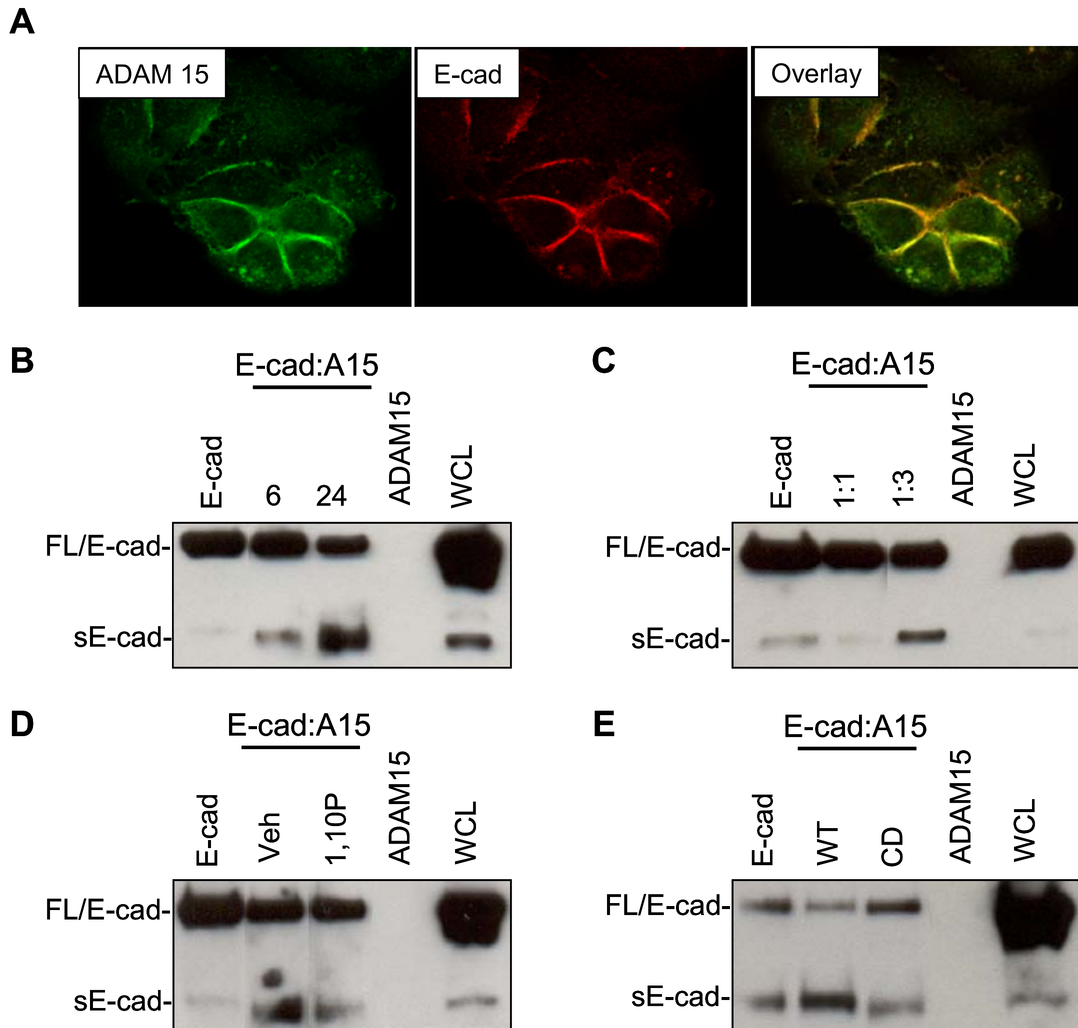

Fig. 3-3. E-cadherin is a substrate for ADAM15. A) Immunocytochemistry analysis of ADAM15 (green) and E-cadherin (red) in MCF-7 cells. Confocal microscopy at 400X. Isolated ADAM15 and E-cadherin were co-incubated. ADAM15 cleaves full length E-cadherin (FL/E-cad) into its soluble fragment (sE-cad) in a time- (B) and concentration-dependent (C) manner. D) E-cadherin and ADAM15 were co-incubated with vehicle (veh) or 1,10 phenanthroline (1, 10P). E) Wild-type (WT) or catalytically-dead (CD)

ADAM15 were co-incubated with isolated E-cadherin. Isolated E-cadherin and ADAM15 alone were loaded as controls on the end lanes. Whole cell lysate (WCL) was used to demarcate E-cadherin banding pattern.

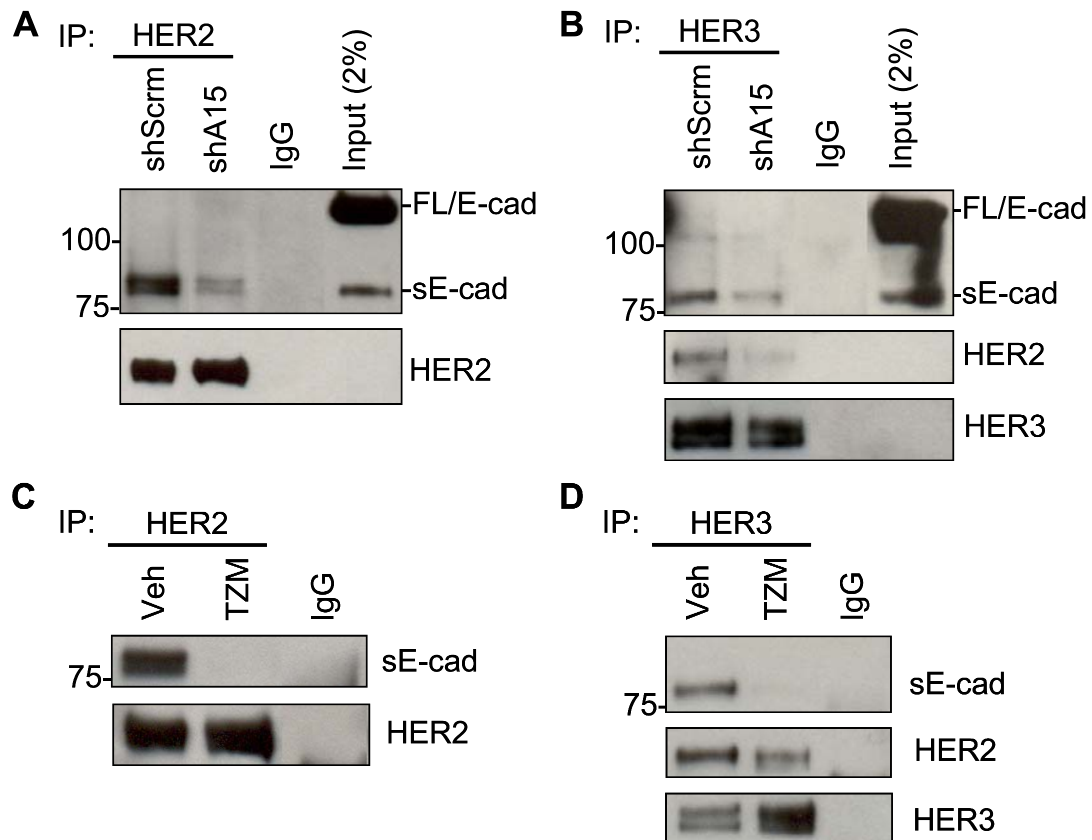

Fig. 3-4. Soluble E-cadherin mediates HER2/HER3 heterodimerization through ErbB receptor binding. Scramble control (shScrm) or ADAM15 knockdown MCF-7 whole cell lysates were immunoprecipitated (IP) with HER2 (A), HER3 (B) or isotype IgG. Scramble control MCF-7 cells were treated with either vehicle (veh) or trastuzumab (TZM) for 24 hours prior to immunoprecipitation either with HER2 (C), HER3 (D) or isotype IgG under growth factor depleted conditions. Immunoblotting with E-cadherin (E-cad), HER2 or HER3 antibodies were used to assess E-cadherin binding to ErbB receptors and receptor dimerization. The input lane (2% the amount of protein used for the IP) was used for E-cadherin banding pattern.

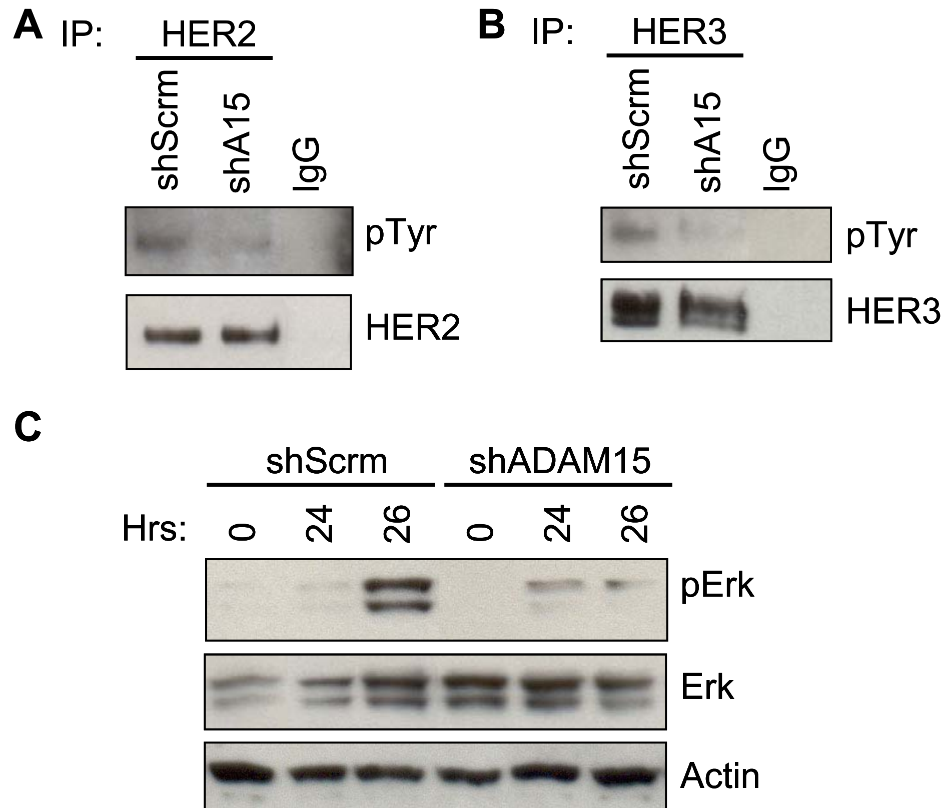

Fig. 3-5. Soluble E-cadherin mediates HER2/HER3 phosphorylation and induces ErbB-mediated cell signaling. Phospho-tyrosine (pTyr) status was assessed in scramble (shScrm) control or shADAM15 (shA15) MCF-7 cells in response to serum starvation. Whole cell lysates were collected and immunoprecipitated with HER2 (A), HER3 (B) or isotype IgG prior to immunoblotting with antibodies specific to pTyr, HER2 or HER3. (C) An increase in Erk activation (pErk) in MCF-7 cells was observed at 24 and 26 hours post growth factor withdrawal.

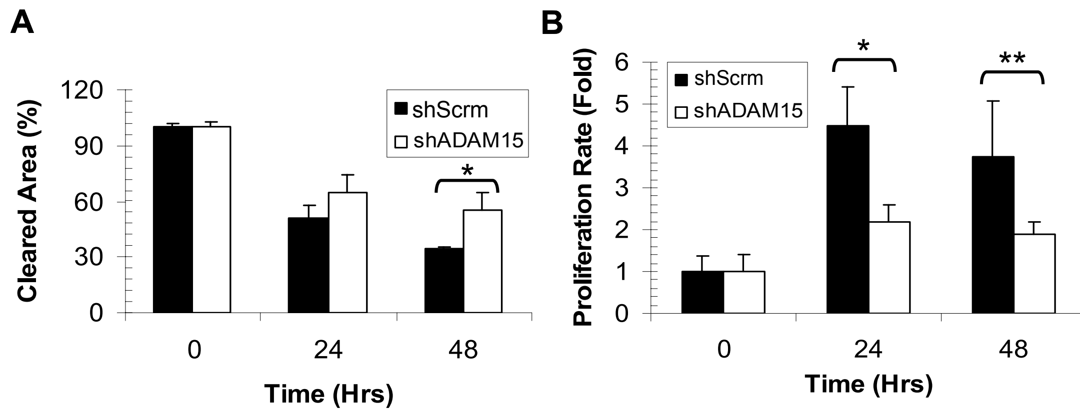

Fig. 3-6. ADAM15 supports cell migration and proliferation. A) Scramble (shScrm) control or shADAM15 MCF-7 cells were abraded with a 10uL pipette tip and wound closure was monitored over time. *Columns* represent the mean of 3 separate experiments quantitated in four different samples. *Bars* are the SD. *P<.04 B) Scramble control or shADAM15 MCF-7 cells were grown under serum depleted conditions and cell proliferation was analyzed as a function of time. *Columns* represent the mean of 3 separate experiments quantitated in eight different samples. *Bars* are the SD. *P<4.6E-05; **P<5.5E-04.

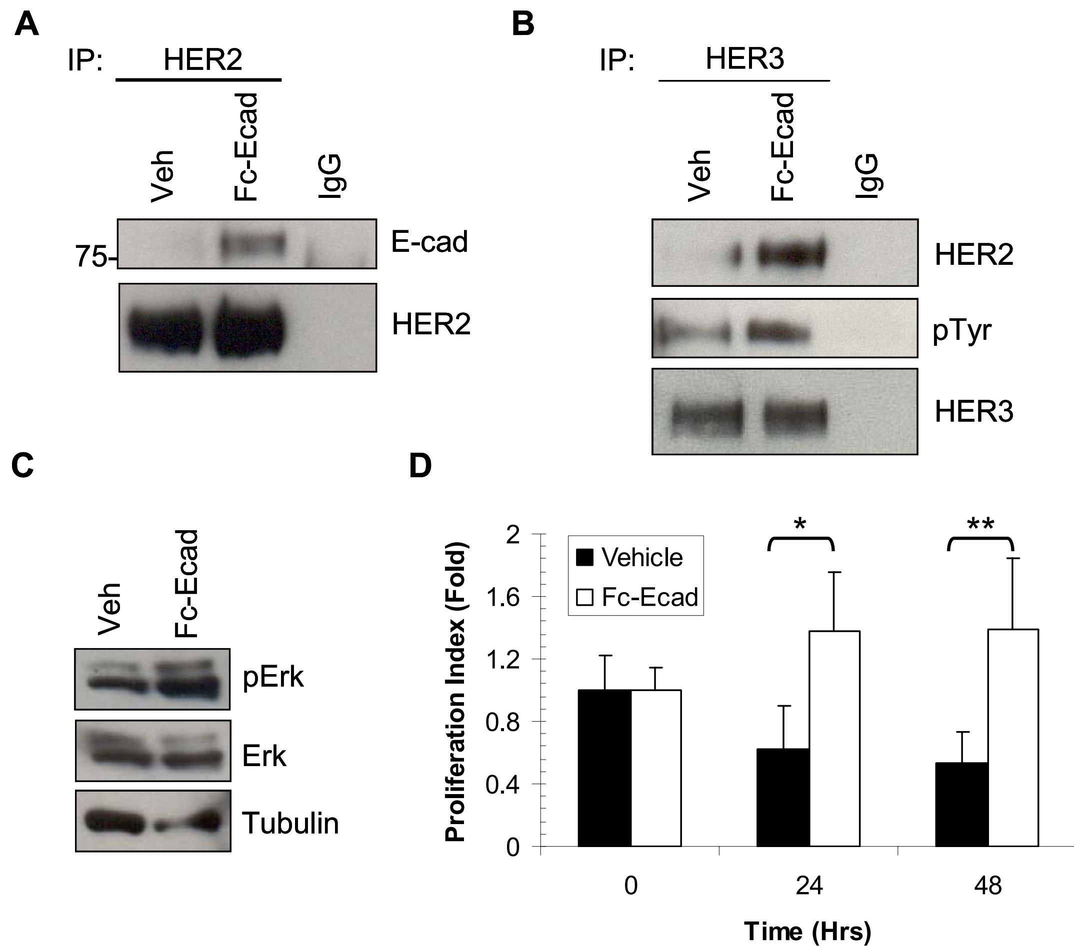

Fig. 3-7. HER2 stimulation by exogenous soluble E-cadherin. A) E-cadherin negative SKBr3 cells were treated with either vehicle (veh) or the extracellular E-cadherin-Fc fusion protein (Fc-Ecad) and lysates were then immunoprecipitated with HER2 or isotype IgG. Immunoblotting with E-cadherin (E-cad) or HER2 was performed to detect Fc-Ecadherin binding. B-D) Fc-Ecadherin fusion protein mediated HER2/HER3 heterodimerization and HER3 phosphorylation as well as increased Erk activation (pErk) and cell proliferation in SKBr3 cells. *Columns* represent the mean of 3 separate experiments quantitated in 8 different samples. *Bars* represent the SD. *P<5E-04; **P<.001.

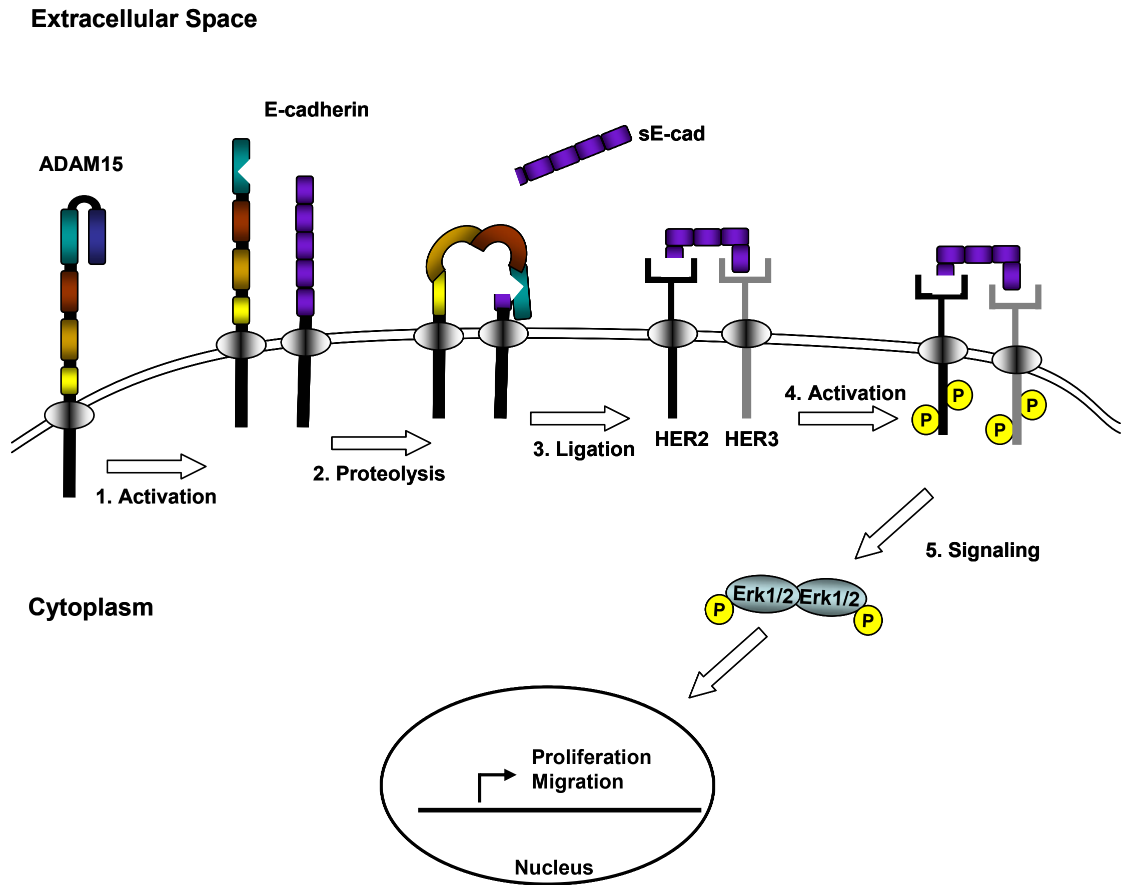

Fig. 3-8. Model for ADAM15-dependent activation of HER2 by soluble E-cadherin. Serum depletion stimulates ADAM15 activation, which in turn cleaves E-cadherin. The liberated E-cadherin fragment (sE-cad) mediates ErbB activation.

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CHAPTER 4

THE ROLE OF ADAM15 IN HER2 SIGNALING AND PROSTATE CANCER CELL SURVIVAL

Abstract

The disruption of the balance between metalloproteinase activity and their inhibitors contribute to cancer cell acquisition of a migratory and invasive phenotype. The active sheddase, ADAM15, is known to support prostate cancer progression through a yet uncharacterized mechanism. In this study we overexpressed ADAM15 in minimally malignant prostate cancer cells and found that ADAM15 upregulation supported prostate cancer tumorigenesis *in vivo*. Furthermore, the catalytic activity of ADAM15 was increased in prostate cancer cells and was accompanied by a downregulation of the metalloproteinase inhibitors TIMP2 and 3. Using overexpression and downregulation technology, we demonstrated that ADAM15 mediated the shedding of full length E-cadherin into the 80kDa soluble E-cadherin (sE-cad) fragment. Importantly, we observed that the shed sE-cad fragment bound the ErbB receptor HER2 and mediated its activation and downstream signaling through the Akt pathway to support prostate cancer cell survival. This report expounds on the role of ADAM15 in ErbB receptor signaling through E-cadherin shedding in prostate cancer. This manuscript is currently in preparation for publication.

Introduction

Prostate cancer is the most diagnosed cancer in men in the United States with more than 218,000 cases in 2007. Of those cases 27,000 succumb to the diseases as a result of metastatic progression into multiple organs (1). Loss of the inhibitory cellular cohesion complex supports cancer cell invasion of the underlying stroma and the eventual dissemination of these cells to distant sites. The cellular adhesion molecule (CAM), E-cadherin, plays a critical role in maintaining epithelial cell polarity, tissue integrity and suppression of tumor metastasis through adherens junction formation (2). This complex involves calcium-mediated cell-cell adhesion supported by the homotypic ligation of E-cadherin molecules through their five extracellular domains. The complex is further strengthened by the interaction of the cytoplasmic domain of E-cadherin with β, γ and p120–catenins which link E-cadherin to the actin cytoskeleton (3). Disruption of this E-cadherin-catenin complex leads to the loss of tissue polarity and differentiation as well as an increase in cell migration and invasion (4). E-cadherin inactivation has been demonstrated in multiple adenocarcinomas including prostate cancer and is associated with a poor prognosis (5, 6). E-cadherin chromosomal deletion, promoter hypermethylation, and metalloproteinase mediated cleavage are different mechanisms by which E-cadherin in inactivated (4). MMP3, 7, plasmin, kallikrein 7 and ADAM10 mediate the ectodomain shedding of full length E-cadherin (120 kDa) into the 80 kDa soluble E-cadherin (sE-cad) (7). Soluble E-cadherin formation is upregulated in multiple adenocarcinomas including gastric, lung, ovarian, and bladder cancer (8). Furthermore, Kuefer et. al. demonstrated that sE-cad shedding was significantly upregulated in prostate cancer and correlated with metastatic disease (9).

The ADAM family is a group of zinc-dependent disintegrin metalloproteinases containing five extracellular domains; prodomain, metalloproteinase, disintegrin, cysteine-rich, and EGF-like. This family is composed of 40 members of which thirteen are catalytically active as a result of the presence of the conserved catalytic consensus sequence HEXXHXXGXXH in their metalloproteinase domain (10). The multiple domains of the ADAM family allow for proteolytic activity, integrin binding, and signal transduction (11). These disintegrin metalloproteinases are implicated in a myriad of normal and pathophysiological functions including oocyte fertilization, neurogenesis, inflammation, and cancer (12). ADAM12 is upregulated in breast cancer and plays a role in supporting cancer survival (13, 14). Furthermore, ADAM10 and 17 are overexpressed in gastric and ovarian cancers (15, 16). Blobel et. al. elegantly demonstrated the role of ADAM9 in prostate cancer development using both knock-in and knock-out models and our laboratory we reported that ADAM15 mediates prostate cancer progression through the modulation of metastatic-associated markers α_V, CD44, and N-cadherin (17, 18). Through their metalloproteinase domain, the catalytically active ADAM family members cleave extracellular matrix (ECM) proteins, cell adhesion molecules, growth factors and growth factor receptors to tumor progression (19). Once activated ADAM family members transactivate different receptors through the release of inactive cell surface growth factors which in turn bind and activate receptor signaling (20). ADAM15 was found to transactivate EGFR signaling and support cancer cell migration and invasion through the shedding of TGFα and amphiregulin (21).

The ErbB family is composed of four members; EGFR (epidermal growth factor receptor, HER1), HER2, HER3 and HER4. EGFR binds to all six EGFR ligands while

HER3 prefers the heregulin family of ligands. HER4 is known to bind only 2 EGFR ligands, HB-EGF and betacellulin, as well as heregulin 1-4 (22). In contrast to other ErbB receptors, no ligand has been identified to bind HER2, but its signaling is mediated by dimerization with other ErbB members. Ligand binding to ErbB receptors mediates homo- and heterodimerization which in turn activates the receptor kinase domain to support auto- and transphosphorylation. Once activated, the ErbB family members signal through a complex downstream network including Erk and Akt to mediate cell proliferation, migration, invasion, and survival (23). ErbB family members are reported to be overexpressed in a variety of cancers including non-small cell lung carcinoma and breast cancer where EGFR is upregulated (24). In prostate cancer, EGFR and its ligands, TGFα and EGF, are upregulated in hormone-refractory cancer to compensate for the loss of androgen signaling (25). Although the role of HER2 in prostate cancer remains controversial, HER2 has been reported to be upregulated during prostate cancer progression (26). HER2-dependent signaling also play a critical role in hormone-refractory prostate cancer by activating androgen receptor signaling through an androgen independent mechanism (27).

Since both ADAM15 and HER2 are upregulated during prostate cancer progression combined with the fact that sE-cad generation is increased during the progression of this disease, we aimed to elucidate the role of ADAM15 in prostate cancer tumorigenesis as well as its catalytic functions in HER2 activation and signaling.

Materials and Methods

Cell lines and culture.

Control sscramble shRNA (shScrm) and shADAM15 LNCaP cells were maintained in RPMI (Bio Whittaker, Walkersville, MD) with 8% fetal bovine serum (HyClone, Logan, UT). ADAM15 overexpressing and control LNCaP cells were grown in RPMI with 5% FBS supplemented with 800μg/mL of G418 (Cellgro, Manassas, VA) for selection. Adam15 +/+ and -/- mouse prostate epithelial cells (msPrEC) and all other prostate cancer cell lines were maintained in 5% fetal bovine serum RPMI media. All culture media were supplemented with 2 mmol/L L-glutamine (Invitrogen, Carlsbad, CA), 100 units/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 0.25 μg/mL Fungizone (Invitrogen). Cells were incubated at 37°C and subcultured weekly.

Generation of ADAM15 overexpressing and shADAM15 LNCaP cell lines.

ADAM15 overexpressing LNCaP and adam15 +/+ and -/- PrE cell lines were generated as described previously (28, 29). ADAM15 specific knockdown in LNCaP cells was generated as described previously (17) using the forward and complementary targeting sequences for ADAM15 were:

5'-AACCCAGCTGTCACCCTCGAA-3' and 5'-TTCGAGGGTGACAGCTGGGTT-3'. The shRNA cassette also featured a TTCAAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTTT terminator at the 3' end. The control construct for the shADAM15 LNCaP cells contained a scramble shRNA sequence which served to control for off-target effects.

cDNA Microarray Analysis. Prostate tissues were obtained through the University of Michigan Rapid Autopsy Program and were used to construct a cDNA microarray as described in Kuefer et. al. (29).

***In vivo* Tumorigenesis Assay.**

LNCaP cells were trypsinized, washed twice with PBS, collected and resuspended in PBS; the viability of collected cells was tested by staining with trypan blue. To generate LNCaP tumor xenografts in mice, 6-week-old C57BL6 SCID mice were injected subcutaneously in the right and left flank with 5×10^5 vector or ADAM15-GFP LNCaP cells in 50 µL of PBS + 50 µL matrigel. Tumor volume was monitored weekly by external measurements with a caliper and calculated as $V = (L^2 \times l) / 2$, where L and l represent the smaller and the larger tumor diameter. The observations were ended for ethical reasons after 8 weeks due to large tumor burden in vector control mice. Animals were maintained under specific pathogen-free conditions with *ad libitum* food and water in the University of Michigan animal housing facilities. At this time, animals were euthanized by CO_2 inhalation followed by induction of a bilateral pneumothorax and tumors were immediately frozen in dry ice or fixed in formalin.

Protein isolation, immunoprecipitation and Western blotting.

Cells were harvested by mechanical disruption and lysed as previously described (17). Cellular debris was then pelleted by centrifugation at 12,000 RPM for 8 minutes, and supernatants were collected and quantitated using a microtiter Bradford protein assay where samples were run in triplicate (Bio-Rad, Hercules, CA). Equal amounts of protein

were then separated on precast Tris-glycine SDS-polyacrylamide gels (Novex, Carlsbad, CA) and transferred to reinforced nitrocellulose membrane (Millipore, Temecula, CA). Membranes were then blocked, probed, and developed. Primary antibodies were obtained as follows: actin (Sigma, St. Louis, MO); pAkt and Akt (Cell Signaling Technology, Danver, MA); ADAM15, phospho-tyrosine, and tubulin (Millipore, Temecula, CA); E-cadherin (Zymed, San Francisco, CA); HER2 (Lab Vision Corporation, Freemont, CA).

For immunoprecipitation; equal amount of protein was precleared with an equal amount of a mixture of 2.5% dry milk in TBST/Sephrose Proteins A beads (Zymed, San Francisco, CA) for 30 minutes at room temperature, then beads were spun out. Precleared protein was then immunoprecipitated with the HER2 (Lab Vision Corporation) antibody or an equal amount isotype IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 hours at 4°C with end-over-end rotation. This was followed by incubating the immunoprecipitation mixture with 75uL of blocked Sephrose Protein A beads for 90 minutes at 4°C with rotation. The complex was then centrifuged and washed three times with ice-cold PBS. The complex was dissociated from the beads with the addition of 5X sample reducing loading buffer and heated for 5 minutes at 100°C. Samples were then loaded on SDS-PAGE for protein analysis. An ECL system was used to visualize proteins (Millipore).

Conditioned media analysis.

LNCaP or PrE cells were grown up to subconfluency and then serum starved in serum-depleted media for 24 hours. Conditioned media was collected by centrifugation at 2,000

RPM for 15 minutes to pellet any cell debris. Levels of soluble E-cadherin in conditioned media were monitored via E-cadherin immunoprecipitation.

Cell viability assay.

Cell viability was performed as described by Rios-Doria et. al. (30). Briefly, 5 X 10^5 cells were plated in 100-mm dishes. Cells were allowed to grow up to 50-60% confluence before treatment with serum-depleted media for the appropriate time points. The attached cells which stained with trypan blue (Gibco) were counted.

To analyze the exogenous effects of E-cadherin, the E-cadherin null cell line SKBr3 was plated at $3X10^6$ cells for four days then washed once with warm serum free media. Cells were then incubated with either vehicle or 1.5 µg/mL of the Fc-Ecadherin recombinant protein per manufacturer's recommendation (R&D Systems, Minneapolis, MN) in serum free media for 8 hours to assess Akt status and for 0, 1 or 2 days to assess apoptosis. Cell viability was measured using the trypan blue exclusion assay (Gibco) and quantified as percent apoptosis by dividing the number of cells at each time point by the cell number at the original time point at 0 hours. Each experiment was ran in triplicates and performed three times. Statistical analyses were performed using a Student's t-test with a one-tailed distribution. $P<0.05$ was considered as statistically significant.

Results

Dysregulation of ADAM15 and the metalloproteinase inhibitors, TIMP2 and 3 in prostate cancer. Previously we reported that ADAM15 is upregulated during prostate cancer progression (29). To corroborate this finding, we assessed ADAM15 expression in a panel of normal and malignant prostate epithelial cells. We found that ADAM15 is upregulated in prostate cancer cell lines in comparison to normal prostate epithelial cells (PrEC) at the messenger and protein levels (Fig. 4-1A). Interestingly as normal prostate cells switch into a malignant phenotype, the precursor form of ADAM15 (110 kDa) is matured into the catalytically active form (90 kDa) (Fig. 4-1A). During cancer progression, disruption of the balance between metalloproteinase activity and tissue inhibitors of metalloproteinases (TIMPs) is observed (31). To examine if TIMPs are dysregulated in prostate cancer, we analyzed their expression levels using a prostate cancer microarray. We demonstrated that both TIMP2 and 3 are downregulated during prostate cancer progression toward metastatic disease while TIMP1 was unaltered in these studies (Fig. 4-1B and data not shown). We observed within this study an upregulation of the active metalloproteinase ADAM15 in prostate cancer progression which is accompanied by a loss of TIMP expression.

Overexpression of ADAM15 increases the tumorigenicity of LNCaP cells *in vivo*. To determine if overexpression of ADAM15 increased malignancy in LNCaP cells, vector control or ADAM15-GFP cells were injected subcutaneously into the flanks of male SCID mice. The ADAM15-GFP cells developed tumors 1-2 weeks earlier than vector control cells (data not shown) and grew faster developing into larger tumors by 7 weeks.

While the ADAM15-GFP tumors grew steadily over seven weeks, the vector control tumors reached their maximum volume at 6 weeks and were smaller at the 7 week time point (Fig. 4-2A and B). Due to the large variation in the volume of the ADAM15-GFP tumors, statistical significance was not reached; however, these tumors were markedly larger than the vector derived tumors. At the end of the experiment the mice were sacrificed and tumors extracted for histological analysis. The ADAM15-GFP tumors were grossly larger than the vector control tumors and histology revealed both tumors were epithelial in origin as indicated by H&E and staining with a human specific anti-E-cadherin antibody (Fig. 4-2A). We monitored sE-cad accumulation in the serum of the injected mice and found that the larger tumors, produced by the ADAM15 overexpressing LNCaP cells, generated more sE-cad in comparison to the vector control cells (data not shown).

Upregulation of ADAM15 increases soluble E-cadherin levels in prostate cancer cells. To assess the role of ADAM15 in prostate cancer progression, we overexpressed ADAM15 in the minimally malignant prostate cancer cell line, LNCaP. The ADAM15 cDNA was tagged at the C-terminus with the green fluorescent protein (GFP) and stably transfected into LNCaP cells. We observed that both E-cadherin and ADAM15 co-localized at the adherens junction (Fig. 4-3A). A15-GFP cells exhibited both endogenous and recombinant ADAM15, where two endogenous species of ADAM15 were detected by an ADAM15-specific antibody at 110 kDa (inactive precursor) and 90 kDa (catalytically active). Two recombinant species of ADAM15-GFP were detected at 136kDa and 116 kDa representing the recombinant precursor and active forms,

respectively (Fig. 4-3B). ADAM15 overexpression did not exhibit off target effects on other genes including the related family member ADAM10 (data no shown).

Previously, we observed that ADAM15 mediated soluble E-cadherin (sE-cad) generation.[2] To evaluate whether ADAM15 mediates E-cadherin proteolysis in prostate cancer cells, we used the ADAM15 overexpressing LNCaP cells described above (Fig. 4-3B). We utilized serum withdrawal to induce sE-cad generation in our model system which has been demonstrated to support sE-cad release into the conditioned media of LNCaP and MCF-7 cells (32). Vector and ADAM15-GFP LNCaP cells were serum starved for 24 hours and the presence of sE-cad was analyzed in the conditioned media. We found that sE-cad was elevated in the ADAM15 overexpressing LNCaP cells in comparison to vector control (Fig. 4-3B). Serum deprivation did not alter full length E-cadherin levels in these analyses.

E-cadherin cleavage is abrogated in response to ADAM15 Loss. To corroborated the overexpression findings, we stably downregulated ADAM15 in LNCaP cells using an shRNA construct against ADAM15. Both the precursor and mature forms of ADAM15 were reduced in response to the shADAM15 construct as compared to control scramble shRNA (shScrm) LNCaP cells (Fig. 4-3C). Off-target affects of the shRNA were analyzed by looking at different genes including the ADAM15 relative, ADAM10, and no affects were observed (data not shown). We serum starved scramble control and shADAM15 LNCaP cells as previously described, and found that sE-cad levels in the conditioned media were decreased in response to ADAM15 downregulation (Fig. 4-3C).

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[2] Najy, A. et. al. (submitted manuscript)

Our lab generated primary prostate epithelial cells (msPrEC) from Adam15+/+ and Adam15-/- mice. We showed that Adam15 is ablated in the Adam15-/- msPrEC in comparison to the Adam15+/+ cells at the protein and RNA levels (Fig. 4-3D and data not shown). Adam15+/+ and Adam15-/- were subjected to serum withdrawal as done with the human cell lines and sE-cad was significantly downregulated in response to Adam15 ablation within the msPrEC (Fig. 4-3D). Expression of full length E-cadherin was assessed via Western blotting and RT-PCR and was shown to be equally expressed in both cell lines independent of Adam15 levels.

The ErbB receptor, HER2, preferentially interacts with soluble E-cadherin.

Although the phenotypic affects of soluble E-cadherin has been elucidated to a certain degree, a signaling cascade mediated by this fragment is yet to be characterized. Previously, full length E-cadherin was shown to interact with EGFR to induce ligand independent signaling in keratinocytes through the extracellular (EC) domain of E-cadherin (33, 34). To assess whether the full length or the soluble E-cadherin interacts with EGFR family members in our models, we first determined the expression profile of EGFR family members in our experiment cell lines. We observed that LNCaP cells expressed EGFR and HER2; we decided to focus on the HER2 receptor for our analysis since it does not have a known ligand. We serum starved scramble control or shADAM15 LNCaP cells to stimulate sE-cad generation, then immunoprecipitated the cell lysates with HER2. We observed that the HER2 receptor preferentially bound sE-cad as indicated by the input control. The interaction between sE-cad and HER2 was reduced in response to ADAM15 downregulation (Fig. 4-4A). To elucidate the interaction further,

we utilized our ADAM15 overexpressing prostate cancer cells. Vector or ADAM15-GFP LNCaP cells were serum starved and lysates were used for HER2 immunoprecipitation. We observed that the vector cells demonstrated sE-cad /HER2 interaction but this complex was significantly enhanced in response to ADAM15 overexpression (Fig. 4-4B).

The interaction of EGFR ligands with their cognate receptors leads to receptor phosphorylation (22). To assess whether endogenous sE-cad interaction with HER2 supports receptor activation, we stimulated sE-cad generation by serum starving scramble control or shADAM15 LNCaP cells. We observed that HER2 was heavily phosphorylated in the scramble control cells but this status was significantly reduced in the shADAM15 cells (Fig. 4-4C). HER2 phosphorylation was also upregulated in response to ADAM15 overexpressing (Fig. 4-4D).

Soluble E-cadherin mediates HER2-dependent signaling. HER2 has been shown to signal through the Akt and Erk pathway when activated (23). Since LNCaP cells do not express Erk, we assessed Akt signaling in these cells at time points where we observed sE-cad-mediated HER2 activation. We demonstrated that scramble control cells showed an increase in Akt phosphorylation in response to serum starvation. In contrast, shADAM15 LNCaP cells did not show Akt stimulation but rather maintained basal activity (Fig. 4-5A). Analysis of ADAM15 overexpressing LNCaP cells demonstrated and increase in Akt phosphorylation when grown under serum-depleted conditions while vector control cells maintained basal Akt phosphorylation levels (Fig. 4-5B).

The Akt signaling cascade mediates cell survival (23). Furthermore, ADAM15 has been demonstrated to support chondrocyte survival under serum depleted conditions

(35). To assess if the LNCaP scramble control cells possess a survival advantage over the shADAM15 cells in response to serum starvation, we performed cell viability assays looking at apoptosing cells post serum withdrawal. We observed that the LNCaP scramble control cells survived longer than the shADAM15 cells under serum depleted conditions, especially as the treatment was prolonged (Fig. 4-5C). Complementing these findings were the results found in ADAM15 overexpressing cells which demonstrated an increase in cell survival as compared to vector control (Fig. 4-5D).

To specifically implicated sE-cad in HER2 signaling, we exogenously stimulated SKBr3 cancer cells with the sE-cad mimetic Fc-Ecad chimera protein, which contains only the ectodomain portion of E-cadherin. We utilized these cells because they are E-cadherin null and HER2 positive to minimize interference of endogenous E-cadherin. Furthermore, previously we have demonstrated that Fc-Ecad binds to and stimulates HER2 signaling.[1] Treatment of SKBr3 cells with Fc-Ecad enhanced Akt phosphorylation (Fig. 4-5E) as well as supported cancer cell survival (Fig. 4-5F). These findings support a role for ADAM15 in mediating HER2 signaling through E-cadherin solubilization.

Discussion

The balance between the metalloproteinases and their natural inhibitors, TIMPs, is important in maintaining tissue homeostasis (36). Disruption of this intricate balance leads to multiple pathological conditions such as inflammation, rheumatoid arthritis, and cancer (37). Overexpression of MMP2 and 9 and the loss of TIMP2 are predictors of poor prognosis in breast and prostate cancer (31). A relative of the matrix metalloproteinases (MMPs), the ADAM family, is also inhibited by TIMP activity. TIMP1 and 3 inhibit the catalytic activity of ADAM10 and 17, while ADAM12 is only inhibited by TIMP3 (38). ADAM10, 12, and 17 have been shown to be upregulated in multiple cancers but the accompanying expression levels of their inhibitors have not been evaluated. A little studied member of the ADAM family, ADAM15, was shown to be upregulated during prostate cancer progression and is known to play a role in supporting the metastatic dissemination of prostate cancer cells (17). In this report, we demonstrated that ADAM15 supported prostate cancer tumorigenesis potentially through its catalytic activity. ADAM15 is synthesized as an inactive 110 kDa metalloproteinase which is matured by proprotein convertase removal of the inhibitory prodomain to generate a catalytically active 90 kDa protease. Using a panel of normal and malignant prostate epithelial cells, we observed that ADAM15 is activated in the cancer cells in comparison to the normal epithelium. Furthermore, we observed that TIMP2 and 3 were downregulated during prostate cancer progression and may likely be novel inhibitors of ADAM15.

ADAM15 has been shown to cleave extracellular matrix components, growth factors, and cellular adhesion molecules to mediate cell migration and proliferation (39, 40). We report here that ADAM15 cleaves the cellular adhesion molecule, E-cadherin, at

the extracellular surface yielding a soluble 80 kDa fragment in response to growth factor deprivation. Full length E-cadherin plays an important role in maintaining epithelial cell polarity and tissue integrity through cell-cell adhesion supported by E-cadherin homotypic dimerization. A loss of this function leads to epithelial cell de-differentiation and an increase in cell migration and invasion (4). In fact loss of E-cadherin portends to a poor prognosis in multiple epithelial-derived malignancies such as breast and prostate adenocarcinomas (8). Although ADAM10 cleaves E-cadherin in human keratinocytes, in our prostate models ADAM10 expression was unaltered supporting the role of ADAM15 in E-cadherin ectodomain shedding in prostate cancer cells.

Although multiple groups have shown sE-cad shedding in their respective models, to date no one completely understands the function of this shed soluble fragment. Damsky et. al. demonstrated that sE-cad disrupts cell-cell adhesion in adult and embryonic tissue acting as a dominant-negative to disrupt the full length E-cadherin homotypic complex (32). Other studies found that soluble E-cadherin mediates downstream signaling through an unknown receptor to support cancer cell invasion (8). In this report, we demonstrated that sE-cad bound to and activated the ErbB receptor HER2 in an ADAM15-dependent manner. EGFR is expressed in our prostate cancer cells, but ADAM15 dependent binding of sE-cad with this receptor was not observed suggesting that sE-cad mediates HER2 homodimerization. Furthermore, sE-cad ligation with HER2 supported downstream Akt signaling. This novel mechanism is not limited to prostate cancer cells; in fact our group demonstrated that sE-cad mediates ErbB transactivation and signaling in breast cancer cells as well.[1]

The ADAM family is known to support growth factor receptor transactivation and ADAM15 was found to cleave cell surface TGFα and amphiregulin to mediate EGFR transactivation (21). In non-small cell lung carcinoma, ADAM-mediated transactivation supported chemotherapy evasion and resistance of these cancer cells through a compensatory mechanism (41). In prostate cancer, treatment of patients with anti-androgens eventually leads to the development of androgen refractory disease. It is believed that these selected cancer cells have adapted to androgen-independent growth by the acquisition of an alternative signaling pathway. Shah et. al. reported that EGFR and its cognate ligands, TGFα and EGF, are upregulated during androgen refractory prostate cancer development (25). In addition, HER2 mediated signaling also supports androgen independent growth of prostate cancer cells (27). Both ADAM15 and HER2 were recently reported to be simultaneously upregulated in prostate cancer supporting the role of this sheddase in mediating the ligation and activation of HER2 signaling by sE-cad (26). This process may serve as an alternative mechanism by which prostate cancer cells can survive androgen depletion. Through a compensatory mechanism, ADAM15 was also reported to support chondrocyte survival under growth factor depletion (35). The HER2/sE-cad complex not only supports a promising potential ligand for the orphan receptor HER2 but it may unveil a new mechanism supporting prostate cancer progression.

We reported here that ADAM15 supports prostate cancer tumorigenesis and E-cadherin shedding. Of importance is the role that this protease plays in mediating HER2 activation and signaling through sE-cad (Fig. 4-6). We believe that the catalytic activity of ADAM15 may serve as a novel target for inhibitor development and study in route to

novel therapeutics.

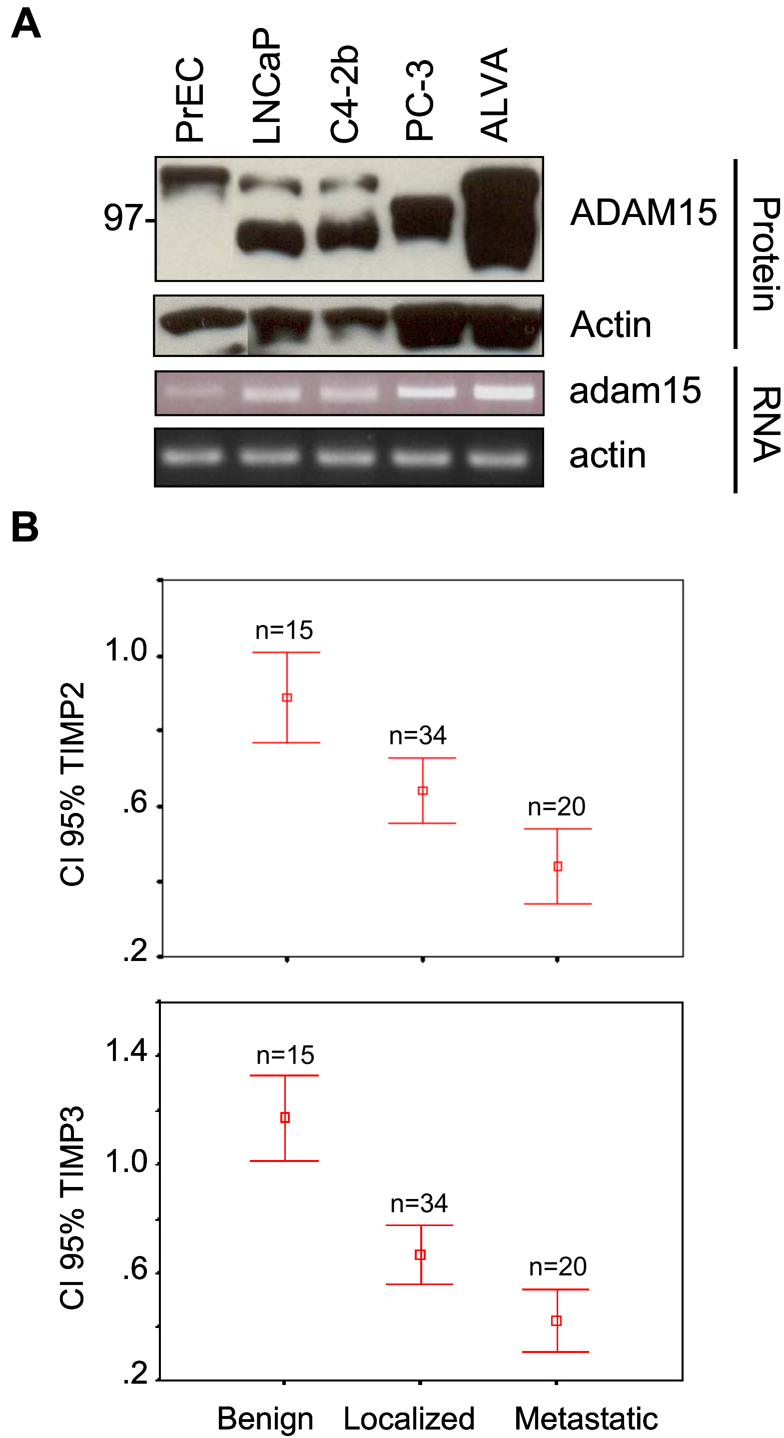

Fig. 4-1. ADAM15 and TIMP profile in prostate cancer. A) A panel of normal (PrEC) and malignant prostate cancer cells were assessed for ADAM15 messenger and protein expression profile. B) A cDNA microarray of benign, localized, and metastatic prostate

cancer tissues was analyzed for tissue inhibitors of metalloproteinases (TIMP)-2 and 3 expression levels.

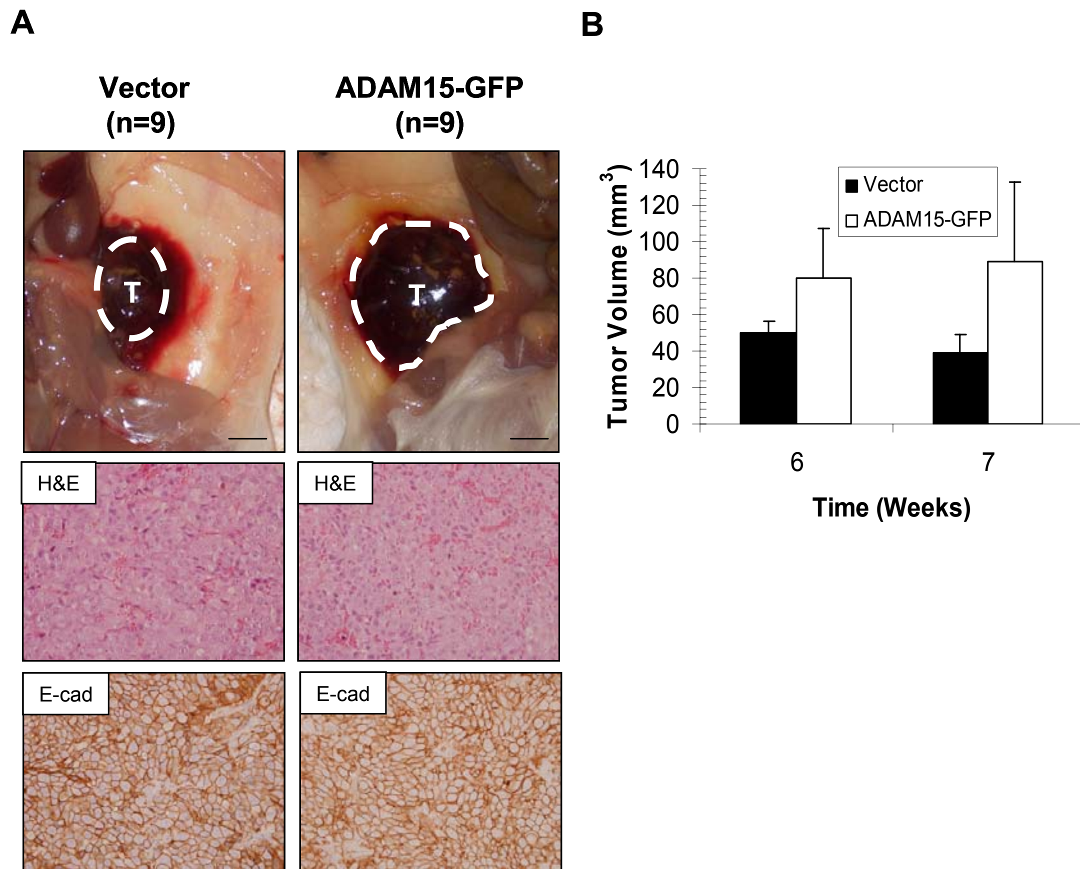

Fig. 4-2. ADAM15 overexpression increases LNCaP tumorigenicity. A) Subcutaneous vector or ADAM15-GFP LNCaP tumors (T) were excised and examined. H&E histology and E-cadherin immunohistochemistry at 400X magnification are indicated. Bar = 2 mm. B) Tumors of vector or ADAM15-GFP LNCaP cells measured at 6 and 7 weeks post-injection. Nine animals per cell line were used and average tumor volume plotted. *Bars* represent the SEM.

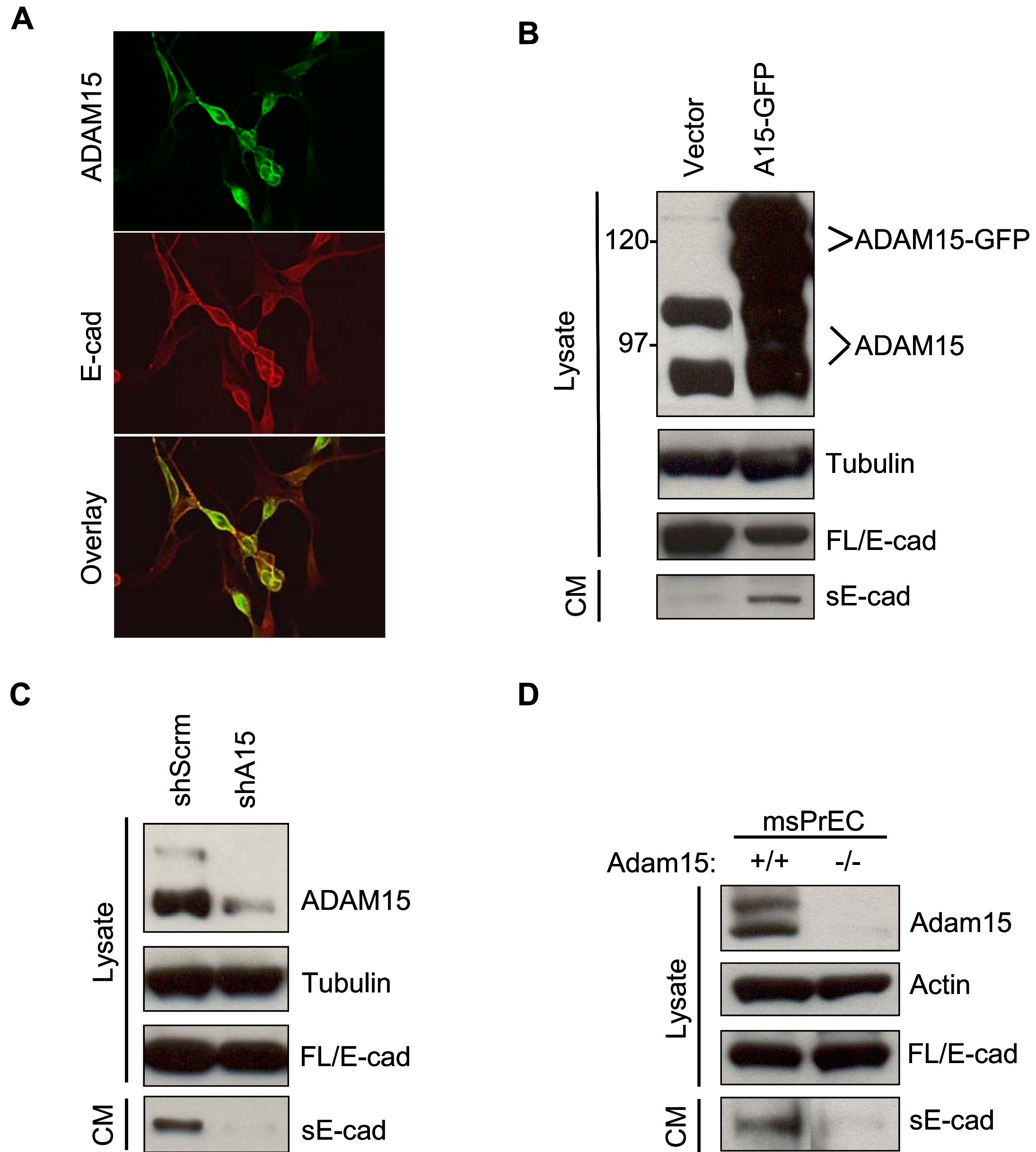

Fig. 4-3. ADAM15-mediates cleavage of E-cadherin in prostate cancer cells. A) ADAM15 (green) and E-cadherin (red) co-localize at the adherens junction (overlay). B) Vector or ADAM15-GFP LNCaP cells were stimulated and analysis of the conditioned media (CM) showed an increase in soluble E-cadherin (sE-cad) in response to ADAM15 upregulation. C) ADAM15 was downregulated in LNCaP cells using a stable shRNA against ADAM15 (lysates). Conditioned media levels of soluble E-cadherin decreased in

ADAM15 knockdown LNCaP cells. shScrm, scramble shRNA control. D) Adam15 +/+ and -/- mouse prostate epithelial cells (msPrEC) were analyzed for E-cadherin shedding. Actin and tubulin were used as loading controls.

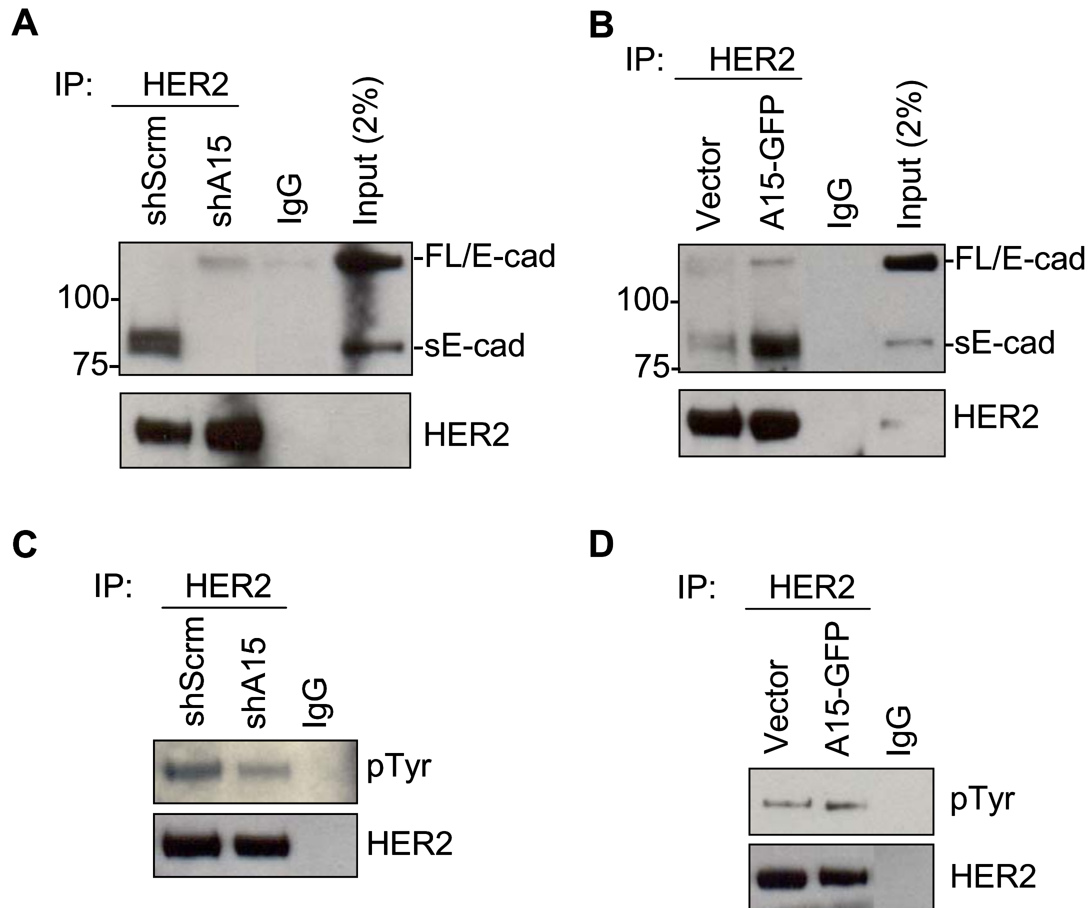

Fig. 4-4. Binding of sE-cad to HER2 supports receptor activation. A) Scrambled control (shScrm) or shADAM15 LNCaP cells were stimulated and lysates were immunoprecipitated (IP) with either HER2 or IgG to assess E-cadherin binding to HER2. B) Overexpression of ADAM15 supported increased sE-cad/HER2 ligation. The input lane was used for E-cadherin banding pattern. sE-cad, soluble E-cadherin. FL/E-cad, full length E-cadherin. HER2 phospho-tyrosine (pTyr) status was assessed in shADAM15 (C) and ADAM15-GFP (D) LNCaP cells in response to serum deprivation.

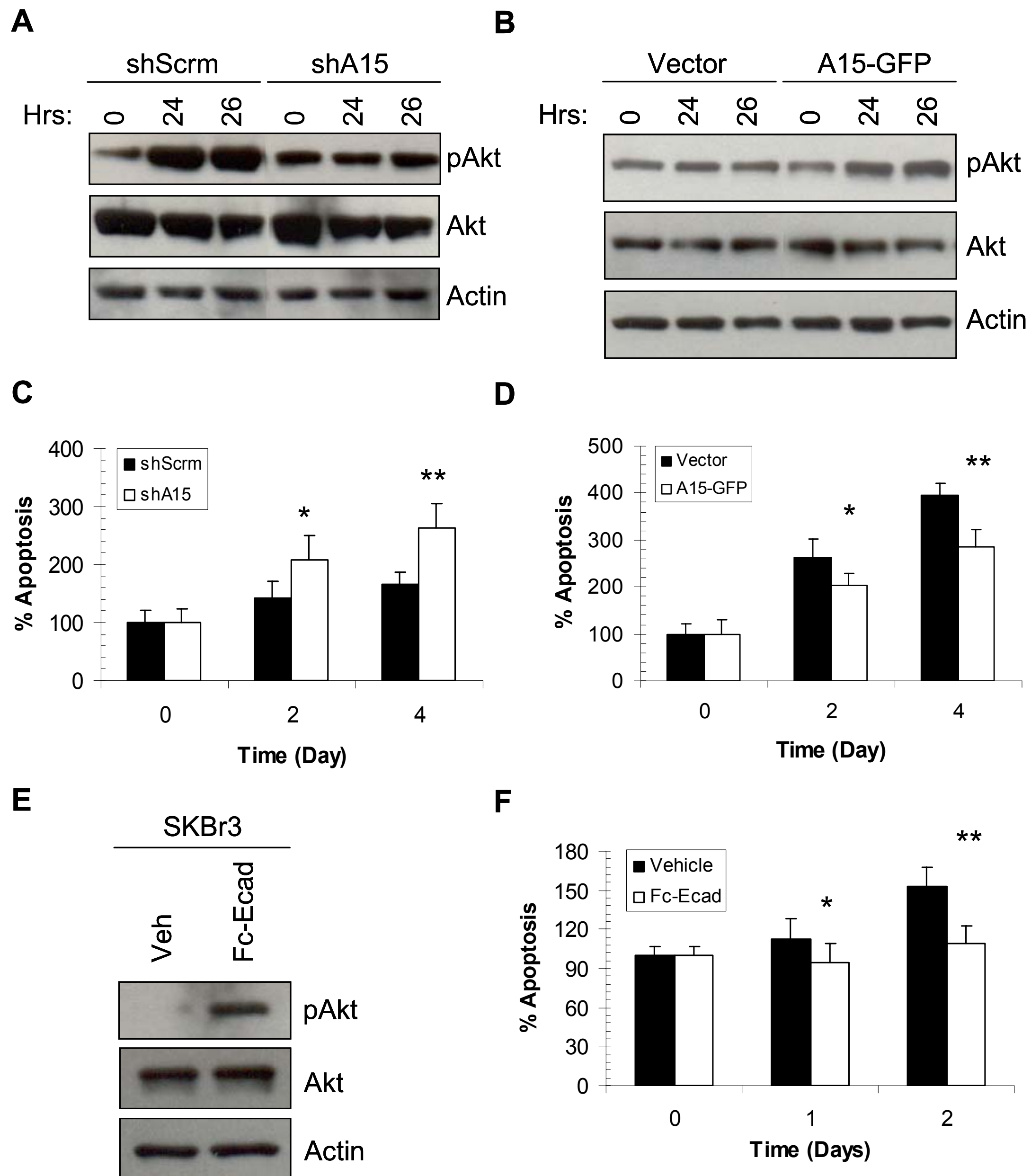

Fig. 4-5. ADAM15 supports Akt activation and cell survival. Control scramble (shScrm) or ADAM15 knockdown (shA15) cells were serum deprived and pAkt (A) and cell apoptosis (B) were assessed. *P<0.006; **P<0.0001. Akt phosphorylation (C) and cell survival (D) were monitored in ADAM15 overexpressing (A15-GFP) cells. *P<0.005;

**P<7.1E-5. SKBr3 cells were stimulated with Fc-Ecad, pAkt (E) and cell survival (F) were analyzed. *P<0.05; **P<1.0E-04.

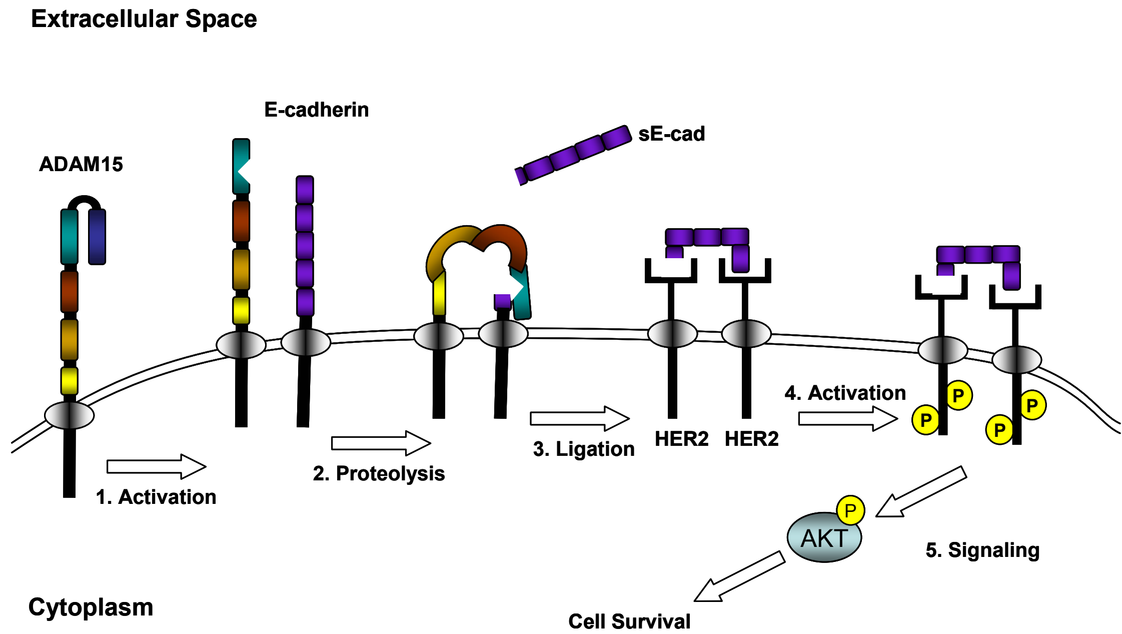

Fig. 4-6. Model for ADAM15-mediated HER2 transactivation in prostate cancer cells. ADAM15 shedding of sE-cad supports HER2 homodimerization and signaling through Akt to enhance cell survival.

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CHAPTER 5

SUMMARY

ADAM family structure-function

The ADAM (**a d**isintegrin **a**nd **m**etalloproteinase) family is part of the metzincins super-family of metalloproteinases which includes the matrix metalloproteinases (MMPs) and is similar to the snake venom metalloproteinases (SVMPs) (1, 2). The ADAMs are expressed in *Drosophila*, *C. elegans*, *Xenopus*, and vertebrates. Forty ADAMs have been demonstrated to be expressed in humans[3] and the majority are expressed in the testes where they are involved in fertilization (3). The disintegrin metalloproteinases are also expressed in somatic tissue where they are involved in different tissue-specific function (see physiological function of the ADAM family). This family of type I transmembrane glycoproteins are also known as the MDC (**m**etalloproteinase, **d**isintegrin, **c**ysteine-rich) family in reference to their domain structure.

The ADAMs are composed of five extracellular domains, a transmembrane domain and a c-terminal cytoplasmic tail (Fig. 1-1). The first of the extracellular domains is the prodomain, which has been shown to be a chaperone for protein folding and stabilization during protein synthesis (4, 5). The prodomain also functions to inhibit the catalytic activity of the adjacent metalloproteinase domain in the precursor form of the protease through a cysteine switch which was observed first in the MMPs (6). The

[3] http://www.uta.fi/%7Eloiika/ADAMs/HADAMs.htm

cleavage of this domain by proprotein convertases (i.e. furin) generates a mature catalytically active protease that functions in targeted degradation of cell surface molecules and extracellular matrix components (7). An understudied member of the ADAM family, ADAM15, is generated as a 110 kDa precursor protein that is matured into a catalytically active 90 kDa protease by furin cleavage (8). The second of the extracellular domains within the ADAM family protein structure is the metalloproteinase domain (Fig. 1-1). Thirteen ADAMs have been shown to contain the metalloproteinase consensus sequence HExxHxxGxxH and are able to target different substrates. Catalytically active ADAMs can cleave growth factors, growth factor receptors, extracellular matrix (ECM) components, and adhesion molecules (9). The catalytically active disintegrin metalloproteinase, ADAM15, sheds TGFα, HB-EGF, and amphiregulin (AREG) in breast and bladder cancer cells (10, 11). ADAM15 also mediates mesangial cell migration in nephropathies through collagen IV and gelatin proteolysis (12). The sheddase activity of the ADAM family is known to support cell migration and invasion in different pathological conditions (13).

Cell adhesion and spreading is also supported by the ADAMs through the disintegrin and cysteine-rich domains. The disintegrin domain contains integrin recognition sequences that allow for ADAM-integrin ligation. ADAM15 is the only ADAM family member that contains an RGD disintegrin sequence involved in RGD-dependent $\alpha v\beta 3$ and $\alpha 5\beta 1$ binding (14, 15). Although cell-cell interaction is supported by integrin-ADAM ligation, the cysteine-rich domain of the ADAM family can also mediate cell adhesion and spreading through syndecans binding (16). The cysteine-rich and the EGF-like domains of the ADAM family have also been proposed to be involved in cell-

cell fusion. The binding of catalytically active ADAMs to integrins or syndecans may target their proteolytic activity for ECM degradation or growth factor shedding (3). The regulation of ADAM targeting may also be through the cytoplasmic tail which contains SH2 and SH3 recognition sequences and sites for serine, threonine, and tyrosine phosphorylation to support inside-out and/or outside-in signaling. ADAM15 interaction with the vascular transport proteins SH3PX1, endophilin I and the EGFR-transactivating mediator Eve1 support a role of this regulatory mechanism in ADAM15 targeting (17, 18). Furthermore, ADAM15 cytoplasmic phosphorylation by the src-family member, Hck, and its interaction with other non-receptor tyrosine kinases suggests a role of ADAM15 in cell signaling (19). The multiple domain structure of the ADAM family implicates this disintegrin metalloproteinase group in cell adhesion, migration, invasion, and cell signaling.

Physiological function of the ADAM family

The first of the ADAM family members, ADAM1 and 2, were discovered in 1992 and were shown to play a critical role in fertilization. Mice null for either *adam1* (also known as fertilin α) or *adam2* (fertilin β) are viable and develop normally but are infertile (1). Further study of these adamalysins revealed that sperm-expressed ADAM2 binds the oocyte integrin $\alpha6\beta1$ to mediate gamete interaction. Gamete fusion is supported by the fusion sequence within the cysteine-rich domain of ADAM1. Cell fusion is also necessary for adipogenesis and myogensis and the fusion sequence within the cysteine-rich domain of ADAM12 is thought to mediate these physiological phenomena (20). In fact, re-expression of *adam12* in the Duchene muscular dystrophy (mdx) mouse models

alleviates the muscular defects due to decreased muscular necrosis (21). ADAM12 is not the only ADAM family member known to ameliorate pathological disorders. *Adam10* gene knock-in alleviated neuronal plaque development in Alzheimer's mouse model. ADAM10 has been found to play a critical role in neuronal development through the delta-notch axis (9). ADAM10 also acts on the amyloid precursor protein (APP) which is involved in neuronal plaque development seen in Alzheimer's patient. Cleavage of APP by the β-secretase, BACE, leads to abnormal plaque development in contrast to α-secretase cleavage by ADAM10 which not only prevents neuronal plaque build up but it alleviates preexisting plaques as is evident by the *adam10* knock-in model (22). Another ADAM family member that is critical for normal embryonic development is ADAM17. *Adam17* null mice are embryonic lethal due to cardiac, lung, and ocular defects. These phenotypes were attributed to the loss of EGF, HB-EGF, and TGFα shedding by ADAM17 (23). Although ADAM15 null mice are fertile and viable, they do exhibit a lower neovascularization potential as demonstrated by the prematurity of retinopathy model (24). The disintegrin metalloproteinases are important for physiological development and their dysregulation is implicated in a myriad of pathological disorders including cancer.

The Role of ADAM15 in Cancer

The metastatic progression of cancer is an intricate multi-step process involving interplay between cell surface molecules and the surrounding stroma. The neoplastic cells at the primary growth site must be able to proliferate and acquire an invasive potential to allow for basement membrane degradation and migration through the underlying stroma.

Once these invasive cells reach stromal blood vessels they must intravasate, survive the shear forces of the blood circulation and then extravasate out of the vasculature to colonize a distant site for metastasis (Fig. 5-1). The ADAM family, specifically ADAM15, has been found to support the metastatic cascade through their versatile domain structure (13).

ADAM15 in Tumorigenesis

The ADAM15 chromosomal locus, 1q21.3, is amplified in multiple adenocarcinomas including breast and prostate cancer (25, 26). Our lab verified these findings by performing cDNA microarray analysis and we found that ADAM15 was significantly upregulated in thirteen different cancer types. Furthermore, this catalytically active adamalysin was overexpressed in nine different prostate and ten breast cDNA microarrays comparing benign, localized, and metastatic disease and these findings were verified using prostate and breast cancer tumor microarrays (TMA) (27). To dissect the role of ADAM15 in prostate cancer progression, we overexpressed this protease in minimally malignant prostate cancer cells (LNCaP) and found that ADAM15 upregulation enhanced the tumorigenic potential of these cells in a mouse xenograft model. Supporting these findings was the knockdown of ADAM15 in highly aggressive prostate cancer cells (PC-3) which demonstrated a significant reduction in their tumorigenicity using the aforementioned xenograft model (discussed in chapters 2 and 4). Supporting the role of ADAM15 in tumorigenesis were the findings of Horiuchi et. al. who reported that subcutaneous xenograft implantation of melanoma cells into *adam15-/-* mice demonstrated decreased tumorigenicity (24). ADAM15 may serve as a crucial

sheddase of different growth factors to support the tumor development by cancer cells *in vivo*.

ADAM15 in cancer cell stromal invasion

In order for cancer cells to spread, they must be able to disengage from the inhibitory cell-cell adherence complex and to acquire a migratory phenotype to traverse through the underlying stromal matrix (Fig. 5-1). The classic E-cadherin molecule serves as a metastatic suppressor by maintaining cell-cell contact through calcium-mediated homotypic binding. Disruption of the E-cadherin molecule by gene mutation, promoter hypermethylation, or protein cleavage is associated with an aggressive phenotype (28). Extracellular cleavage of full length E-cadherin into its soluble form (sE-cadherin or sE-cad) is carried out by multiple catalytically active proteases and serum or urine soluble E-cadherin levels are known to be upregulated during the progression of multiple cancers (29). Our laboratory has shown that serum soluble E-cadherin is upregulated during prostate cancer progression in a pattern mirroring the catalytically active ADAM15 upregulation (30). Furthermore, the top four adenocarcinomas (breast, prostate, bladder, and lung) demonstrating significant ADAM15 upregulation also have been demonstrated to have elevated serum or urine soluble E-cadherin levels. We also have observed that ADAM15 co-localized at the cell-cell adherens junction with E-cadherin. Hence, we hypothesized that ADAM15 may support cancer progression through the cleavage of the E-cadherin molecule. We assessed the serum levels of mice containing ADAM15 overexpressing prostate cancer cells xenografts and found that soluble E-cadherin levels were elevated. This was corroborated by tissue culture findings looking at soluble E-cadherin in the conditioned media of ADAM15 overexpressing or knockdown prostate

cancer cells in response to serum starvation (see chapter 4). Soluble E-cadherin was elevated in response to ADAM15 upregulation and reduced in ADAM15 downregulated cells. Mutation of the catalytic metalloproteinase domain abrogated E-cadherin proteolysis. Breast cancer cells were also analyzed for soluble E-cadherin shedding in response to ADAM15 overexpression or downregulation and we found results that are similar to the prostate cancer cells (see chapter 3). Moreover, breast cancer cells that demonstrated less E-cadherin shedding were less migratory and proliferated less.

E-cadherin is not the only cellular adhesion molecule (CAM) that ADAM15 modulates to support cancer progression. ADAM15, through its disintegrin domain, has been demonstrated to intact with multiple integrins including αvβ3 in an RGD-dependent manner (14, 15). Using our PC-3 model, we demonstrated that cell surface stabilization of the metastatic markers, integrin αv and CD44 is ADAM15-dependent (discussed in chapter 2). ADAM15 expressing PC-3 cells were more migratory and invasion potentially due to the presence of these metastatic associated markers which have been implicated previously in cancer cell migration and invasion. In addition, the CD44 receptor is able to target different metalloproteinases, including MMP9, to the migrating edge of invasive cancer cells (31). Our findings showed that ADAM15 mediated MMP9 secretion and activity likely through CD44 stabilization to support the invasive phenotype of these cells. ADAM15 expressing PC-3 cells also displayed increased N-cadherin shedding which may support the migratory and invasive phenotype observed in these cells through ADAM15-mediated cell adhesion molecule turn over at the cell surface to allow for cell adhesion and release during migration and invasion. Using its

metalloproteinase and disintegrin domain, ADAM15 is able to cleave cell-cell molecules and stabilize cell surface metastatic makers supporting its role in stromal invasion.

ADAM15 and angioinvasion

Epithelial-endothelial cell interaction is necessary to allow for cancer cell intravasation and extravasation. Different cell surface molecules have been implicated in epithelial-endothelial cell interaction including αvβ3 and CD44 (32). Since ADAM15 was shown to be overexpressed in angioinvasive breast cancer (27) and αvβ3 and CD44 were stabilized by ADAM15, we assessed the role of ADAM15 in the process of angioinvasion. We demonstrated that prostate cancer cells were able to adhere to and transmigrate through endothelial cells in an ADAM15-dependent manner (see chapter 2). This process may be carried out through the interaction of epithelial ADAM15 disintegrin domain with the endothelial αvβ3 integrin. The ADAM15 disintegrin domain was also shown to bind to the platelet integrin αIIbβ3 under shear and static conditions (32-34). ADAM15 through its disintegrin domain likely plays multiple roles in mediating intravasation, survival in the blood stream via platelet binding and finally extravasation (Fig. 5-1).

ADAM15 in cancer metastasis

Breast and prostate cancer have a high prevalence to metastasize to the bone (35). It has been hypothesized that cancer cells possess different markers to allow for homing to secondary sites and the stabilization of cell surface receptors by ADAM15 may serve as a homing signal. Using our PC-3 model, we demonstrated that ADAM15 is necessary for prostate cancer cells to metastasize to multiple sites including the spine and tibia using an intracardiac dissemination model (discussed in chapter 2). Although the bone

microenvironment is a harsh environment in comparison to soft tissue, cancer cells have developed different adaptations to survive. Activation of the ErbB family of receptor tyrosine kinases are essential for cancer cell survival and proliferation (36). Previously, the extracellular domain of full length E-cadherin has been shown to support ligand-independent EGFR activation (37, 38). We demonstrated that the ADAM15 shed soluble E-cadherin fragment is able to interact and activate the ErbB family members to mediate cell migration, proliferation and survival (see chapter 3 and 4). Therefore, not only can ADAM15 mediate the metastatic progression of cancer cells to a secondary site but it may also support the propagation of the primary tumor and the colonization of the cells at a distant site. By disrupting the cell-cell adherence junction ADAM15 allows cancer cells to migrate through the basement membrane and traverse the underlying stromal compartment. Furthermore, we believe that ADAM15 plays a novel role in cancer cell angioinvasion and colonization at distant sites (Fig. 5-1).

ADAM15 as a therapeutic target and conclusion

PSA (prostate specific antigen) and HER2 have been great tumor progression markers for prostate and breast cancer, respectively (39), but the need for more precise diagnosis and prognosis markers is still necessary. The ADAM family of disintegrin metalloproteinases has been used as reporting markers in multiple adenocarcinomas. The soluble form of ADAM12 is found to be upregulated during the progression of breast cancer and is a precise predictor of tumor progression (40). Serum levels of ADAM8 have been reported to have potential as markers of renal cell carcinoma and non-small cell lung carcinoma (NSCLC) progression (41, 42). Furthermore, ADAM9 and ADAM11

are found to predict the outcome of tamoxifen in ER-positive breast cancer (43). Our findings that ADAM15 is downregulated in response to neoadjuvant treatment of prostate cancer cases and its expression is upregulated in HER2-positive breast cancer support a growing role for ADAM15 as a marker of tumor progression and therapy response.

The multiple extracellular domains of the ADAM family complement each other to allow for protein function. Recently, phosphorylation of the ADAM17 cytoplasmic domain by PKC in response to phorbol-ester stimulation led to protease activation and HB-EGF shedding (44). Other ADAMs utilize their disintegrin and cysteine-rich domains to bring growth factors into a complex where these mitogens are shed (3). The domains of the ADAM family can serve as targets for therapeutic discovery, especially those of ADAM15. As demonstrated previously, the catalytically active metalloproteinase domain of ADAM15 plays a role in cell-cell disruption through E-cadherin shedding which in turn binds to and activates HER2 to mediate cell proliferation, migration and survival. Previously, So et. al. demonstrated that HER2 can stimulate the androgen receptor (AR) pathway in androgen refractory prostate cancer (45). In addition, HER2 is amplified in 20-30% of breast cancer cases and HER2 status is inversely proportional to estrogen receptor (ER) expression (46, 47). ADAM15-mediated HER2 activation may compensate for a loss of hormone stimulation in hormone refractory prostate and breast cancer. Therefore, the inhibition of the metalloproteinase activity of ADAM15 through small molecule inhibitors or exogenous application of recombinant ADAM15 prodomain may serve as effective inhibitors of ADAM15 proteolytic function.

The disintegrin domain of ADAM15 is the only ADAM family disintegrin that contains an RGD sequence. Purified recombinant ADAM15 disintegrin domain was

shown to inhibit xenograft tumorigenesis and metastasis through the inhibition of tumor angiogenesis (48). In addition, an ADAM15 specific inhibitor demonstrated an abrogation of endothelial cell proliferation, migration, and tube formation *in vitro* (49). Combined with the role that ADAM15 plays in neovascularization (24), these data strongly support a growing role for ADAM15 in angiogenesis. Therapeutics targeting angiogenesis are vital since they can be applied to multiple tumors and they would confine primary tumors to a localized stage where treatment is more efficacious. In addition, an RGD small peptide inhibitor targeting ADAM15 disintegrin activity can specifically inhibit its role in angiogenesis without interrupting the function of other metalloproteinases.

ADAM family function can be modulated by inside-out signaling cascades. Eve1, PKC, and src have been demonstrated to act on the c-terminal tail of different ADAMs, including ADAM15, to mediate catalytic activity specifically growth factor shedding (5, 18, 19). Our findings showed that serum deprivation activates ADAM15 to shed E-cadherin potentially through inside-out signaling. Although the exact activator is yet to be elucidated; an inhibitor to this target would prove valuable in preventing the sheddase activity of ADAM15. The multi-functional domains of ADAM15 support the role it plays within the metastatic cascade as a sheddase, an adhesion molecule, and a signal transducer. These domains present both a challenge to find specific inhibitors but also a great opportunity to develop novel therapeutics due to a multitude of targets.

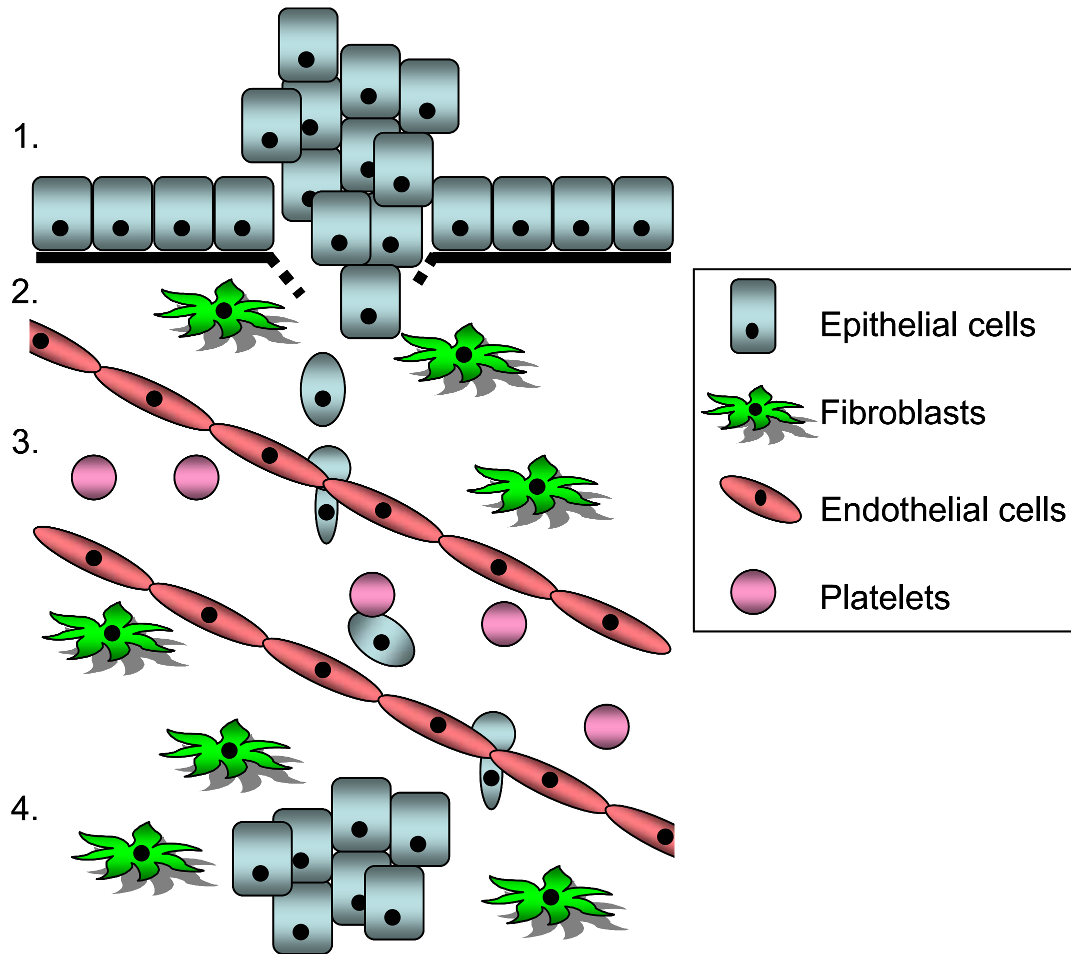

Fig. 5-1. The role of ADAM15 in cancer progression. Cancer metastasis is delineated here in four steps. First, normal epithelial cells acquire genetic alterations that permit for primary tumorigenesis, cell dissociation and basement membrane degradation. Second, invasive epithelial cancer cells traverse the underlying extracellular stroma. These cells then intravasate into the blood stream, adhere onto platelets, and finally extravasate into distant site. Ultimately, the cells colonize the secondary microenvironment to develop metastatic deposits.

Future Studies

Although my thesis work on ADAM15 has contributed greatly to understanding its importance in cancer progression, much work detailing its structure-function is needed. One of the areas that might be of interest is the function of soluble N-cadherin as a potential ligand in aggressive prostate and breast cancer. Previous work demonstrated a role for soluble N-cadherin in promoting angiogenesis through FGFR signaling (50). The fibroblast growth factor receptor (FGFR) is upregulated in prostate and breast cancer and its activity supports cancer cell proliferation and migration (51, 52). One can hypothesize that ADAM15-mediated N-cadherin shedding may lead to FGFR transactivation and signaling. Supporting this hypothesis is our findings in Chapter 3 which demonstrated a role for soluble E-cadherin in the transactivation of the growth receptors HER2/HER3.

The metalloproteinase activity of the ADAM family may be targeted or directed by other domains on these endopeptidases (3). ADAM15 is known to bind to multiple integrins including $\alpha_V\beta_3$ through an RGD-dependent manner potentially coordinating the metalloproteinase activity of ADAM15 (14). Since our laboratory is the first to report physiological substrates for ADAM15, N- and E-cadherin, assessing the role of the metalloproteinase adjacent domains in mediating ADAM15 ligand shedding is amenable. ADAM15 targeting is not limited to the extracellular domains, but may involve the c-terminal tail. The vascular transport proteins, SH3PX1 and endophilin 1, interact with

ADAM15 through its SH3 sequences (17). These trafficking proteins may play a critical role in targeting ADAM15 activity in response to an external stimulus.

Within my thesis work we generated a catalytic-dead ADAM15 mutant as well as demonstrated the sensitivity of ADAM15 to the metalloproteinase inhibitor 1,10phenanthroline (Chapter 3). Other members of the ADAM family are also inhibited by the endogenous tissue inhibitor of metalloproteinases (TIMPs) (13). In our cDNA arrays, TIMP2 and 3 were found to be downregulated during prostate cancer progression and their expression is inversely proportional to ADAM15 levels (Chapter 4). These findings point to a potential function of TIMP2 and 3 as ADAM15 inhibitors. Furthermore, inhibitors of ADAM15 can be design and tested using sE-cad as an experimental output. The University of Michigan biochemistry consortium has a library of inhibitors that may serve as a starting point for designing ADAM15 specific inhibitors. Of course the search may be narrowed utilizing similar protocols used for the development of ADAM10 and 17 inhibitors. Since the prodomain of the ADAM family members function in the inhibition of their metalloproteinase activity, the ADAM15 prodomain could serve as a potent inhibitor to the metalloproteinase activity of ADAM15. In fact, the ADAM prodomain has previously been applied as an inhibitor to both ADAM10 and ADAM17.

Soluble E-cadherin binding to both HER2 and HER3 supported Erk signaling and breast cancer cell migration and proliferation (Chapter 3). The site of E-cadherin and ErbB receptor interaction remains to be elucidated. A HAV (histidine, alanine, valine)-containing 10-mer human E-cadherin peptide, which correspond to a portion of the first extracellular (EC1) E-cadherin domain, mediated a phenotype similar to the soluble 80

kDa E-cadherin fragment. The HAV sequence within the E-cadherin EC-1 domain has been hypothesized to be responsible for the peptide activity (53). These details narrow the search for the area of E-cadherin/receptor interaction. The soluble E-cadherin binding to HER2 can be inhibited by trastuzumab (Chapter 3). This monoclonal antibody is specific for HER2 inhibition and does not act on other ErbB family members. A recently characterized monoclonal antibody, pertuzumab, acts on both HER2 and HER3 (54). Potentially, a new approach may look into the influence of this monoclonal antibody inhibitor on soluble E-cadherin interaction with ErbB family members.

In addition to elucidating the site of E-cadherin/ErbB receptor binding, further characterization of the sE-cad mediated signaling axis is needed. Tyrosine kinase inhibitors (TKIs), such as lapatinib, may serve as great tools in dissecting the signaling cascade. Furthermore, Erk and Akt inhibitors are worthwhile pursuing to clearly elucidate the role of sE-cad in ErbB receptor signaling.

The studies presented within this work delineated a ligand for ADAM15 and uncovered a novel role of ADAM15 in supporting cancer progression as well as a case for future pursuit of ADAM15 targeted therapeutics.

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