The role of A Disintegrin and Metalloproteinase with Thrombospondin Motifs-15 (ADAMTS-15) in Breast Cancer

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Acknowledgements

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Dedicated to the memory of my grandfather

John Vincent Kelwick

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Abstract

Breast cancer is the most common cancer in women and in 2008 accounted for 8% of UK cancer related deaths. A poor prognosis is particularly conferred upon individuals with evidence of metastatic breast cancer. With some studies noting that at least 70% of patients dying with breast cancer have evidence of metastatic disease. In order to develop novel therapeutic strategies a greater understanding of breast cancer tumourigenesis and metastasis is required.

Metalloproteinases were implicated as key drivers of metastasis through their ability to degrade the components of the extracellular matrix. This perspective is now superseded with evidence highlighting the involvement of metalloproteinases in an array of biological roles, from maintaining tissue homeostasis to angiogenesis, and importantly these roles can have tumour suppressive effects. Several metalloproteinases from the A Disintegrin and Metalloproteinase with thrombospondin motifs (ADAMTS) family are candidate tumour suppressors, including ADAMTS-15. In the context of breast cancer relatively high levels of ADAMTS-15 expression had previously been associated with increased relapse free survival. However the functional consequences of ADAMTS-15 expression in breast cancer are unknown and are the focus of this thesis.

ADAMTS-15 reduced the migration of MDA-MB-231 and MCF-7 cells, in a metalloproteinase-independent manner. This anti-migratory effect likely involves syndecan-4, since modulation of syndecan-4 expression and signalling attenuated this effect. In contrast to its effects on cell migration, only *wildtype* ADAMTS-15 exhibited an anti-angiogenic effect in *in vitro* and *ex vivo* models of angiogenesis. In experimental metastasis assays, both ADAMTS-15 and E362A (metalloproteinase inactive form of ADAMTS-15) reduced metastasis of MDA-MB-231 cells to the liver, though paradoxically, ADAMTS-15 but not E362A enhanced lung colonisation. Taken together these studies demonstrate for the first time that extracellular ADAMTS-15 has multiple tissue context-dependent actions on breast tumour pathophysiology, some of which require its proteolytic activity whereas others do not.

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Abbreviations

(v/v), volume per volume

(w/v), weight per volume

2D, 2 dimensional

3D, 3 dimensional

ADAM, a disintegrin and metalloproteinase

ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs

ADAMTS-15 (TS15), wild-type ADAMTS-15

ADAMTS-15 E362A (E362A), metalloproteinase inactive ADAMTS-15

ADH, atypical ductal hyperplasia

ALH, atypical lobular hyperplasia

CD1 nu/nu, CD1 nude mouse

DCIS, ductal carcinoma in situ

DMEM, dulbecco's modified eagle's medium

DNA, deoxyribonucleic acid

ECL, enhanced chemiluminescence

ECM, extracellular matrix

EDS type VIIc, ehler-danlos syndrome type 7c

EGF, epidermal growth factor

EMT, epithelial to mesenchymal transition

EV, empty vector control pcDNA3.2

FCS, foetal calf serum

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

HELU, hyperplastic enlarged lobular units

HRP, horseradish peroxidase

HUVEC, human umbilical vascular endothelial cell

IBC, invasive breast cancers

IgG, immunoglobulin

IV, intravenous

Kb, kilo base

kDa, kilo dalton

LCIS, lobular carcinoma in situ

MDA-MB-231, m.d.anderson metastatic-breast 231.

MCF-7, michigan cancer foundation 7.

MMP, matrix metalloproteinase

MMPI, matrix metalloproteinase inhibitors

mRNA, messenger RNA

MT-MMP, membrane type matrix metalloproteinase

NGS, nottingham grading system

PBS, phosphate buffered saline

pcDNA3.2, plasmid vector

qRT-PCR, quantitative reverse transcription polymerase chain reaction

RNA, ribonucleic acid

RR, relative risk of invasive breast cancer

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM, standard error of mean

siRNA, small interfering RNA

SNP, single nucleotide polymorphism

TBS, tris buffered saline

TDLU, terminal duct lobular units

TGF-β, transforming growth factor beta

TIMP, tissue inhibitor of metalloproteinases

TNF-α, tumour necrosis factor alpha

TTP, thrombotic thrombocytopenic purpura

VEGF, vascular endothelial growth factor

WB, western blot

Chapter One: Introduction

1.1 Cancer

Cancer is a group of diseases associated with uncontrolled cellular proliferation that occurs when cells lose key regulatory mechanisms associated with the control of cell growth, genome stability and cell survival (Figure 1.1; Brown *et al.* 2009; Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Cancer aetiology and progression are the result of complex processes. It was originally thought that a single, as of yet undiscovered event was at the heart of cancer initiation (Foulds, 1958; Klein, 1998). In 1958 Leslie Foulds changed the carcinogenesis paradigm when his research proposed that cancer development was a progressive process in which tissue abnormalities associated with cancer were acquired over time (Foulds, 1958; Klein, 1998). Ideas emerged which complemented Fould's concept with somatic mutation theory, whereby it was the acquisition of genetic mutations and epigenetic alterations which over time equipped cancer cells with the capacity for uncontrolled proliferation and survival (Klein, 2009; Sonnenschein and Soto, 2008).

Cancer cells that are able to escape the local tumour microenvironment and establish distant secondary tumours (metastasis) are of the greatest clinical concern, with some indications that metastasis may account for up to 90% of cancer deaths (Croft and Olson, 2008; Klein, 2009; Nguyen *et al.* 2009 Pagani *et al.* 2010). For metastasis, cancer cells must invade into the surrounding tissues, intravasate into the circulatory or lymphatic system, extravasate into

distant tissues and then proliferate to form a novel tumour (Kopfstein and Christofori, 2006; Nguyen *et al.* 2009).

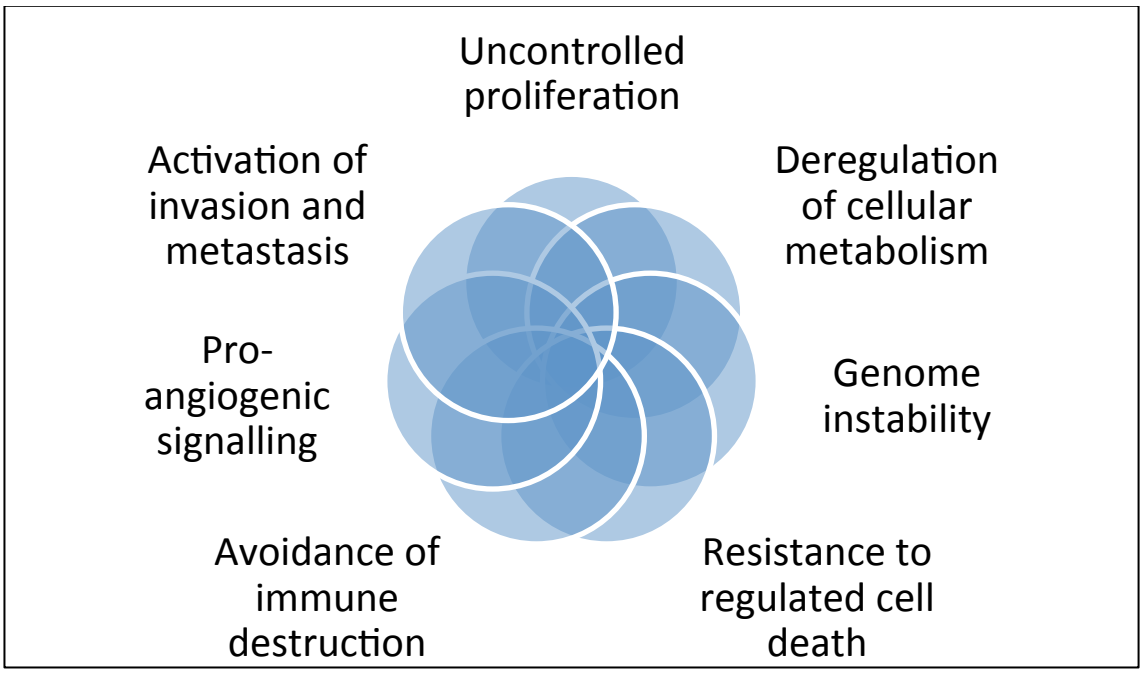


Figure 1.1: A summary of the fundamental characteristics that tumour cells acquire. Figure based upon figures 1 and 3 in Hanahan and Weinberg, 2011.

1.2 Breast Cancer

Breast cancer is the most common cancer in women and in 2008 accounted for 8% of UK cancer related deaths (Cancer Res. UK, 2009; Quinn *et al.* 2008) Although screening and therapeutic strategies have contributed to a 35% decline in UK mortality since 1989 levels, the annual incidence of breast cancer continues to rise (Autier *et al.* 2010; Quinn *et al.* 2008). In recent years the lifetime risk for women developing breast cancer in the UK has risen from 1in9 to 1in8 (Cancer Research UK, 2013). A poor prognosis is particularly conferred upon individuals with evidence of metastatic breast cancer, with some studies noting that at least 70% of patients dying with breast cancer have evidence of metastatic disease (Buijs and van der Pluijm, 2009; Rubens, 1998).

Breast cancer progression from healthy tissue towards an invasive carcinoma is complex and was originally classified through the emergence of histo-morphological changes in which normal breast architecture is aberrantly disrupted (Wellings and Jensen, 1973). Mammary glands in healthy breast tissue form into a layered structure consisting of luminal epithelial cells, which are responsible for the secretion of milk into the adjacent lumen (Figure 1.2; Adriance *et al.* 2005). Underneath luminal epithelia, myoepithelial cells form a continuous layer interfacing between the luminal epithelial cells and the basement membrane (Figure 1.2; Adriance *et al.* 2005). A highly fibrous connective stroma and adipose tissue surrounds the glandular structures of the mammary ductal tree (Adriance *et al.* 2005).

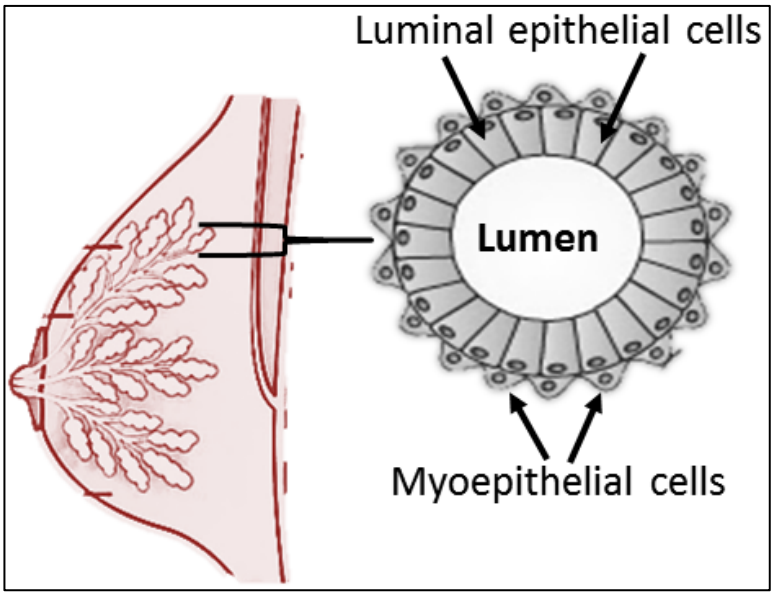


Figure 1.2: Schematic of human breast tissue architecture. The diagram is a representation of the general features of human breast tissue architecture. A cross section of a breast duct is shown as are two important cell types that are responsible for breast tissue homeostasis and function. The luminal epithelial cells, which line the lumen of the duct, are responsible for the generation of milk proteins. The myopepithelial cells are phenotypically a combination of muscle and epithelial like features. Myoepithelial cells are involved in the generation of the contractile forces needed to secrete the milk proteins into the lumen during lactation. This figure is based upon information that was adapted from *Butcher et al.* 2009 and Gordon *et al.* 2003.

During lactation these glandular structures, termed terminal duct lobular units (TDLUs), are under mechanical pressure, due to the stresses exerted with the build-up of milk (Adriance *et al.* 2005; Butcher *et al.* 2009; Cichon *et al.* 2010). Myoepithelial cells, a combination of muscle (myo) and epithelial-like character, must contract to secrete luminal milk to alleviate these pressures and restore tissue homeostasis (Adriance *et al.* 2005; Butcher *et al.* 2009). Myoepithelial and luminal epithelial cells seek clues about the environmental tensile forces from cell-cell (e.g. E-Cadherin), desmosomal (e.g. DSg3) and hemidesmosomal (e.g. integrin $\alpha 6 \beta 4$) signalling interactions (Butcher *et al.* 2009; Gordon *et al.* 2003). *In vitro* studies demonstrate that signalling interactions between the

epithelial cell layer and the basement membrane are essential for apical-basal polarity and differentiation for the generation of milk proteins (Barcellos-Hoff *et al.* 1989; Butcher *et al.* 2009). Desensitisation of these signalling elements through constant exposure to tensile stress can lead to a loss of tissue integrity invoking extensive tissue remodelling (Butcher *et al.* 2009). Luminal epithelial-like cells and in rarer cases myoepithelial-like cells, which have acquired the capacity for unrestrained proliferation, gain a selective advantage during this remodelling process and potentially initiate tumourigenesis (Adriance *et al.* 2005; Gordon *et al.* 2003; Polyak and Hu, 2005).

Unrestrained proliferation of malignant cells in the TDLUs results in the emergence of tissue abnormalities (Figure 1.3; Allred *et al.* 2001; Wellings and Jensen, 1973). The most common tissue abnormalities that develop during disease progression have been extensively characterised and histologically modelled (Allred *et al.* 2001; Cichon *et al.* 2010; Wellings and Jensen, 1973). According to histological models of breast cancer, hyper-proliferation of the TDLU epithelium leads to the development of extended tissue structures, such as cysts and unfolded lobules at the proliferative disease without atypia (PDWA) and hyperplastic enlarged lobular units (HELU) stage (Figure 1.3; Cichon *et al.* 2010; Lee *et al.* 2006). Over time alterations in cell adhesion, polarity and other cellular abnormalities develop resulting in the distension of acini (Allred *et al.* 2008; Cichon *et al.* 2010). These changes are characteristic of the transition into the atypical hyperplasia stage, which can manifest itself in ductal (atypical ductal hyperplasia, ADH) or lobular (atypical lobular hyperplasia, ALH) forms (Allred *et al.* 2008; Cichon *et al.* 2010). Differences between ductal and lobular

forms are largely descriptive and not necessarily representative of the lesions' site of origin (Cichon *et al.* 2010).

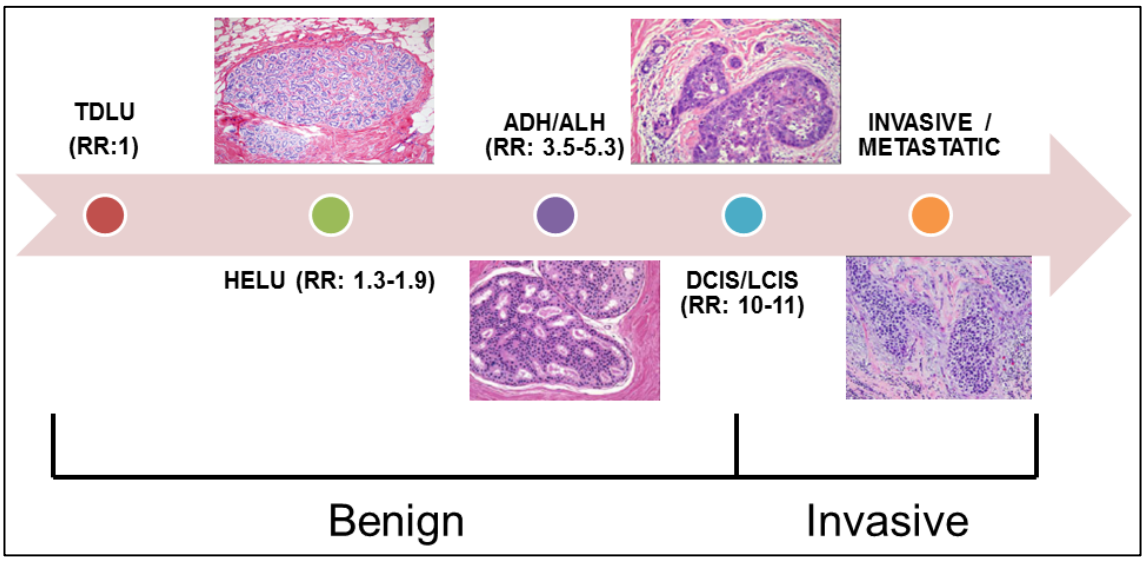


Figure 1.3: Schematic of the histological model of breast cancer progression. Abbreviations: TDLU, terminal duct lobular units; HELU, hyperplastic enlarged lobular units; ADH, atypical ductal hyperplasia; ALH, atypical lobular hyperplasia; DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ; IBC, invasive breast cancers; RR, relative risk of invasive breast cancer. This figure is based upon data that was adapted from Allred *et al.* 2001; Cichon *et al.* 2010; Wellings and Jensen, 1973.

Hyperplasias can transition into either ductal or lobular forms of carcinoma in situ, DCIS or LCIS respectively (Allred *et al.* 2008; Bombonati and Sgroi, 2011). Carcinoma *in situ* is a descriptor for the last stage of benign breast neoplasia and DCIS in particular may account for up to 20% of all detected breast cancers (Ernster *et al.* 2000; Jones, 2006). DCIS is a broad descriptive term, which is frequently sub-divided into three groups, depending upon the appearance of cytonuclear features (Jones, 2006; NHSBSP Guidelines, 2005). At the *in situ* stage the myoepithelial and basement membrane interface is intact, essentially maintaining the rapidly proliferating neoplastic tissue within the ducts or lobules of the breast (Adriance *et al.* 2005; Cichon *et al.* 2010).

Invasive disease often follows DCIS, with up to 50% of DCIS lesions reported to progress to invasive disease (Jones, 2006; Sanders *et al.* 2005). Transition to invasive and/or metastatic disease typically requires the breakdown of the myoepithelial-basement membrane interface, an event that is likely to occur post-DCIS, in part due to the accumulation of myoepithelial cell genetic abnormalities (Adriance *et al.* 2005; Hsiao *et al.* 2011; Jones, 2006; Polyak and Hu, 2005). Once basement membrane integrity is compromised tumour cells are able to spread to distant tissues and organs. For breast tumours metastatic spread is typically to the lymph nodes, lungs, liver, bone and brain (Buijs and van der Pluijm, 2009; Nguyen, Bos, and Massagué, 2009)

The descriptive histological characteristics of invasive disease are based upon observed growth patterns and diverging resemblance from healthy epithelial cells (differentiation) (Rakha *et al.* 2010). The Nottingham Grading System (NGS) describes the criteria for a semi-quantitative analysis and grading (I, II, III) of breast tumour patho-morphology which in combination with Lymph node (LN) status has prognostic value (Rakha *et al.* 2010). Despite the prognostic and therapeutic utility of histo-pathological models of breast cancer, they are arguably an over-simplistic representation of breast cancer disease progression.

Critical appraisal of these models highlights several limitations, which refinements in descriptive histological terminology have not managed to overcome, but a molecular analysis may yet address (Bombonati and Sgroi, 2011; Cichon *et al.* 2010; Griffith and Gray, 2013; Ma *et al.* 2003; Rakha *et al.* 2010). Not all patients display clearly progressive histological features and even when they do the progression duration varies greatly, taking in some cases

decades (Allred *et al.* 2008; Jones, 2006; Sanders *et al.* 2005). Prediction of progression timescales from benign disease towards invasive disease is difficult from a purely histological analysis. For instance several DCIS studies have revealed that not all DCIS have invasive potential, or at least not in the patients' lifetime (Emery *et al.* 2009; Jones, 2006; Sanders *et al.* 2005). This has an impact on therapeutic strategy. It is perhaps unfortunate for patients to undergo aggressive therapies with harmful side effects, when their form of cancer may not progress to a threatening stage within their lifetime (Jones, 2006). Perhaps more importantly these studies allude to the hidden diversity of breast cancer origins, progression and invasive potential, which can be masked in a purely histological analysis.

It is argued that a molecular stratification of breast cancer is needed to fully understand the complexity of breast cancer progression and to usher in truly personalised therapeutic strategies (Bombonati and Sgroi, 2011; Curtis *et al.* 2012; Prat *et al.* 2010; Rakha *et al.* 2010; Stephens *et al.* 2009; Swanton *et al.* 2011). Breast cancer molecular subtypes are broadly categorised by the expression of Oestrogen Receptor (ER), Progesterone Receptor (PgR) and Human Epidermal Growth Factor Receptor 2 (HER2) respectively (Table 1.1). Luminal subtypes are the most frequently clinically presented of the breast tumour subtypes and since they are positive for ER, these cancers respond well to anti-endocrine therapies. However prolonged direct (Tamoxifen) or indirect (aromatase inhibitors, AIs) therapeutic modulation of ER signalling often leads to endocrine resistance and disease recurrence (Leary and Dowsett, 2006).

Only 25% of breast tumours over-express the epithelial growth factor receptor (EGFR), Her-2 (Table 1.1; Dean-Colomb and Esteva, 2008). Yet for those

patients that have HER2+ tumours, treatment with the anti-HER2 monoclonal antibody Trastuzumab (Herceptin) has proven particularly effective (Dean-Colomb and Esteva, 2008; Pero *et al.* 2007). Arguably the HER-2 subtype is currently the best example of how an examination of the molecular subtypes can inform a highly personalised and effective therapeutic strategy. In contrast to this, basal and claudin-low subtypes do not respond well to either anti-endocrine or HER based therapies and are associated with a poor prognosis (Kennecke *et al.* 2010; Prat *et al.* 2010).

	Normal-like	Luminal A	Luminal B	HER-2	Claudin-Low	Basal
ER status	+	+	+	-	-	-
PgR status	+	+	+	-	-	-
ErbB2 (Her2/neu) status	+	-	+	+	-	-
% of triple negative tumours	0%	3-4%	4-9%	14-22%	61-71%	73-80%

Table 1.1: The hormone status of breast cancer subtypes. The percentage of these tumour subtypes which display a triple negative hormone status was derived from three patient data sets; UNC337, NKI295 and MDACC133 respectively. Table based upon Prat *et al.* 2010.

Breast cancer subtypes also display differential capacities for metastatic spread, with basal tumours being particularly metastatic (Kennecke *et al.* 2010). These capacities are not just intrinsic to the tumour cells themselves but also involve co-operation with the tumour microenvironment. In other words only

tumour cells, which acquire the capacity to co-opt the tissue microenvironment are capable of successful metastatic spread (Klein, 2004; Klein, 2009; Langley and Fidler 2011; Mueller and Fusenig, 2004; Paget, 1889).

1.3 The tumour microenvironment

Tumour cells do not reside in isolation but are in fact surrounded by multiple cell types and an extracellular matrix (ECM), which in combination form the constituents of the tumour microenvironment (stroma) (Figure 1.4). The extracellular matrix includes the structural proteins such as collagens, laminins, fibronectin, as well as proteoglycans such as, hyaluronan, aggrecan and versican, (Butcher *et al.* 2009; Huxley-Jones *et al.* 2007; Mao and Schwarzbauer, 2005). The ECM influences tumour cell behaviour either directly through interaction with cellular receptors leading to cell signalling or mechano-transduction of force, or indirectly via the proteolytic release of matricryptic fragments that in turn elicit cell signalling (Butcher *et al.* 2009; Hojilla, Wood and Khokha, 2008; Xu, Boudreau and Bissell, 2009).

Within the context of breast tissues stromal cells include fibroblasts, adipocytes and the multiple cell types of the tumour-associated vasculature. The vasculature includes the pericytes and the endothelial cells that form the vessels themselves, as well as the natural killer cells, neutrophils, macrophages and other circulatory cells of the immune system. Tumour cells interact with and co-opt stromal cells to produce growth factors, cytokines and proteases that in combination create a pro-inflammatory environment which is conducive to tumourigenesis (Allen and Jones, 2011; Kessenbrock, Plaks, and Werb, 2010). Of these factors proteases are particularly important since they mediate several

tumorigenic processes including an acceleration of tumour growth and the induction of the epithelial to mesenchymal transition (EMT), a process in which cancer cells become malignant (Edwards *et al.* 2009; Kempen, de Visserc and Coussens, 2006; Lopez-Otin and Matrisian, 2007; Ota *et al.* 2009). Originally, however, proteases were considered for their ability to degrade the ECM and as a result were thought to clear a pathway in which tumour cells could grow, invade and spread to distant tissues (Lopez-Otin and Matrisian, 2007).

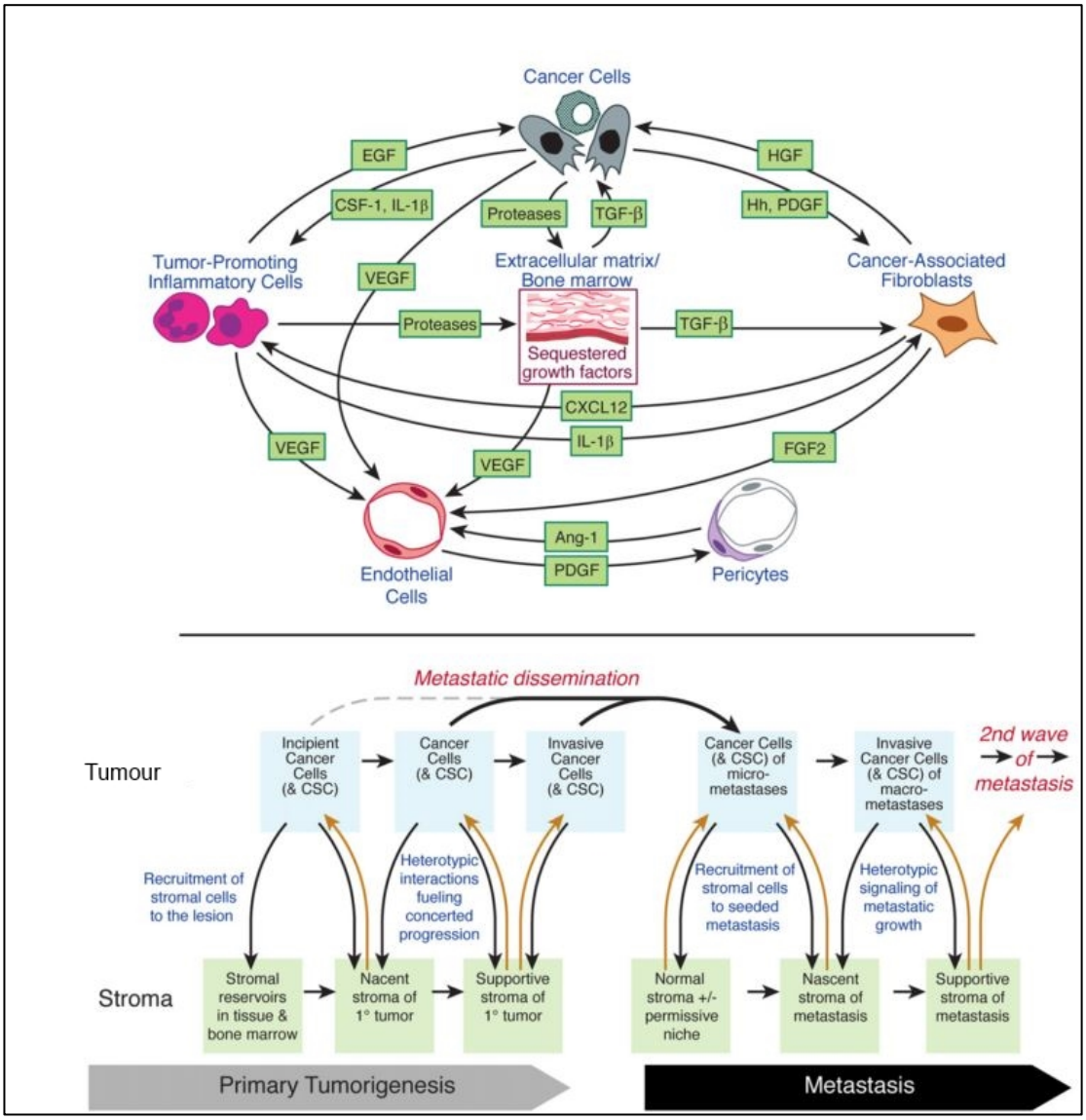


Figure 1.4: Tumour-stroma signalling interactions during tumourigenesis and metastasis. (**Upper**) The assembly and collective contributions of the assorted cell types constituting the tumour microenvironment (stroma) are orchestrated and maintained by reciprocal heterotypic signalling interactions, of which only a few are illustrated. (**Lower**) The intercellular signalling depicted in the upper panel is reciprocal and highly dynamic. These signalling interactions drive the increasingly aggressive phenotypes that underlie growth, invasion, and metastatic dissemination. (Figure 5 from Hanahan and Weinberg, 2011. Permission granted via RightsLink Licence Number: 3167020799762).

1.4 Proteases in tumour biology

Fisher (1946) proposed that the proteolytic activity of tumours actively facilitated the degradation of the ECM, permitting tumour growth, invasion and metastasis. This provided the basis for implicating proteases in cancer progression (Liotta *et al.* 1980; Lopez-Otin and Bond, 2008; Lopez-Otin and Matrisian, 2007; Overall and Kleifeld, 2006). Protease research continued and by the 1970s individual proteases were identified and later classified into five distinct classes: Cysteine-, Serine-, Aspartic-, Threonine- and Metallo- proteases (Lopez-Otin and Bond, 2008; Neurath and Walsh, 1976).

The matrix metalloproteinases (MMPs) emerged from amongst the five classes of proteases as distinctly important in ECM turnover (Brinckerhoff and Matrisian, 2002; Page-McCaw *et al.* 2007). A rationale evolved which placed the activities of MMPs as key drivers of tumour mediated ECM degradation and subsequent metastatic action (Egeblad and Werb, 2002). Evidence demonstrating the propensity for tumours in advanced disease stages to express high levels of MMPs strengthened arguments in favour of MMP inhibition (Baker *et al.* 2002; Brown, 1998; Greenwald, 1999; Sternlicht and Werb, 2001). An array of small-drug MMP inhibitors (MMPIs) were developed in the 1980s and 1990s (McCullagh *et al.* 1984; Low *et al.* 1996; Zucker *et al.* 2000). However the proposed benefits associated with broad action MMP inhibition did not translate into the clinic. Phase III clinical trials with MMPIs did not increase the survival prospects of patients with advanced cancer (Coussens *et al.* 2002; Pavlaki and Zucker, 2003; Zucker *et al.* 2000).

Between 1962, which saw the discovery of the first MMP, and 1984, which marks the patenting of a first generation MMPI, only a handful of the 23 human MMPs had been discovered (Gross and Lapiere, 1962; Kessenbrock *et al.* 2010; McCullagh *et al.* 1984). Perhaps then with hindsight it was not too surprising that first generation MMPIs, designed upon incomplete knowledge of the MMP family did not increase the survival prospects of patients with advanced cancer (Pavlaki and Zucker, 2003; Zucker *et al.* 2000). Critics have also argued that the design of early Phase III first generation MMPI clinical trials was also a major contributing factor to the failure of MMPIs. (Zucker *et al.* 2000). The selection of advanced stage patients placed the focus on treating mature tumours and in doing so the roles of MMPs in earlier stages of tumour development were ignored. This focus also overlooked the major source of MMPs, the tumour stroma (Table 1.2), thus downplaying the importance of the interactions between the tumour and the tumour microenvironment.

Breast architecture is frequently disturbed by the stresses of normal breast physiology (Butcher *et al.* 2008). In the healthy breast MMPs are major players in the tissue remodelling processes needed to maintain breast tissue homeostasis and to facilitate the architectural changes required for mammary gland growth, lactation and involution. To prevent aberrant remodelling processes the MMPs are tightly regulated at the level of transcription, activation of the pro-enzyme, and through the action of 4 endogenous MMP inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (Baker *et al.* 2002; Clark *et al.* 2008). In tumourigenesis however, tumour cells are able to disrupt and aberrantly modulate the MMP:TIMP axis needed for the maintenance of breast tissue homeostasis. In doing so tumour cells are able to co-opt the surrounding

stroma into the production of yet more MMPs to aid an array of disease progression processes (Table 1.2; Kessenbrock *et al.* 2010; Page-McCaw *et al.* 2007). These cancer disease progression processes of which MMPs are implicated include the epithelial-to-mesenchymal transition (EMT) of tumour cells to a more invasive phenotype (Ota *et al.* 2009), inflammatory responses (Hojilla *et al.* 2008), tumour angiogenesis (Handsley and Edwards, 2005) and mediation of the pre-metastatic niche (Kaplan *et al.* 2005).

Stromal Cell	MMPs / TIMPs
Neutrophils	MMP-8 and MMP-9. TIMP-1
Macrophages	MMP-1, -2, -7, -9, -12 and -14. TIMP -1, -2 and -3
Mast Cells	MMP-2 and MMP-9. TIMP-1
Fibroblasts	MMP-1, -2, -3, -9, -11, -13, -14 and -19. TIMP-1, -2 and -3
Lymphocytes	MMP-3 and MMP-9. TIMP-1

Table 1.2: Stromal sources of MMPs and TIMPs. Table based upon Kessenbrock *et al.* 2010.

In contrast to other matrix metalloproteinases, MMP-8 (Collagenase-2), an MMP that is involved with inflammatory and tissue remodelling processes may be tumour suppressive (Balbin *et al.* 2003; Gutiérrez-Fernández *et al.* 2007; Thirkettle *et al.* 2013). Male mice experimentally deficient of MMP-8 (MMP-8 -/-) demonstrated an increased susceptibility to skin cancer, marked by an increase

in skin tumour incidence (Balbin *et al.* 2003). *In vitro* studies where MMP-8 was over expressed in melanoma cell lines, showed reduced invasive capacity as expected for a tumour suppressor gene and breast cancer patients which show relatively high MMP-8 expression have a good prognosis (Gutiérrez-Fernández *et al.* 2008).

In light of these studies the failure of first generation MMPIs can now be attributed in part to the broad-spectrum inhibition of MMPs, many of which had not yet been discovered and whose roles in cancer are still not fully understood. The unintentional inhibition of cancer anti-targets, such as the tumour suppressor MMP-8 was also a contributing factor and may even explain the poorer outcome of some MMPI treated patients (Devy *et al.* 2009; Gutierrez-Fernandez *et al.* 2008; Overall and Kleifeld, 2006; Pavlaki and Zucker, 2003; Zucker *et al.* 2000). Additionally the failure of early generation MMPIs may also have been due to unforeseen off-target effects, most significantly that MMPIs demonstrate both direct and indirect inhibition of members of the related zinc moiety containing protease families, a disintegrin-like and metalloproteinase (ADAM) and a disintegrin-like and metalloproteinase with thrombospondin type 1 motif (ADAMTS) (Figure 1.5; Figure1.6; Butler *et al.* 2008; Fridman *et al.* 2007; Pavlaki and Zucker, 2003; Porter *et al.* 2005).

The ADAMS and ADAMTSs have complex roles in health and disease. Atypical expression, regulation or function of these metalloproteinases can lead to inherited genetic disorders, or contribute to aberrant patho-physiological processes in arthritis, cardiovascular disease and cancer. Whilst the ADAMs are generally pro-tumourigenic, interestingly members of the ADAMTS family

exhibit tumour suppressor functions in some contexts and are therefore of great interest (Kelwick and Edwards, 2014).

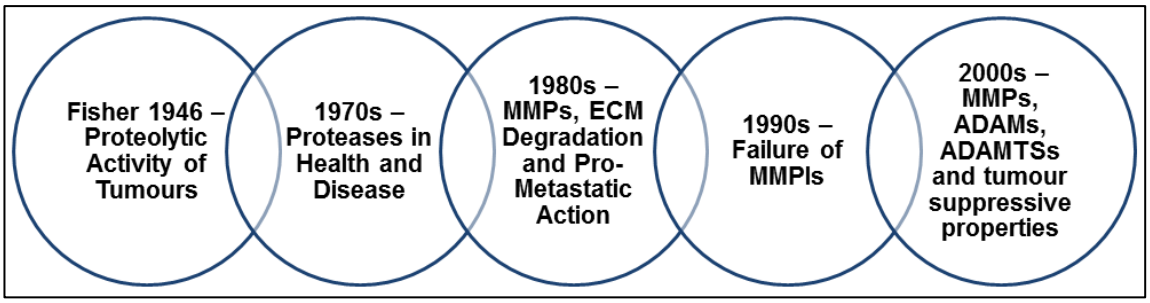


Figure 1.5: Historical overview of MMP cancer research. Proteases implicated in metastasis (Fisher, 1946; LopezOtin and Bond, 2008) through to the failure of MMPIs (Pavlaki and Zucker, 2003), the discovery of tumour suppressive MMPs (Gutierrez Fernandez 2008) and the related ADAMs (Edwards *et al.* 2008), ADAMTS (Kelwick and Edwards, 2014; Porter *et al.* 2005; Wagstaff *et al.* 2011).

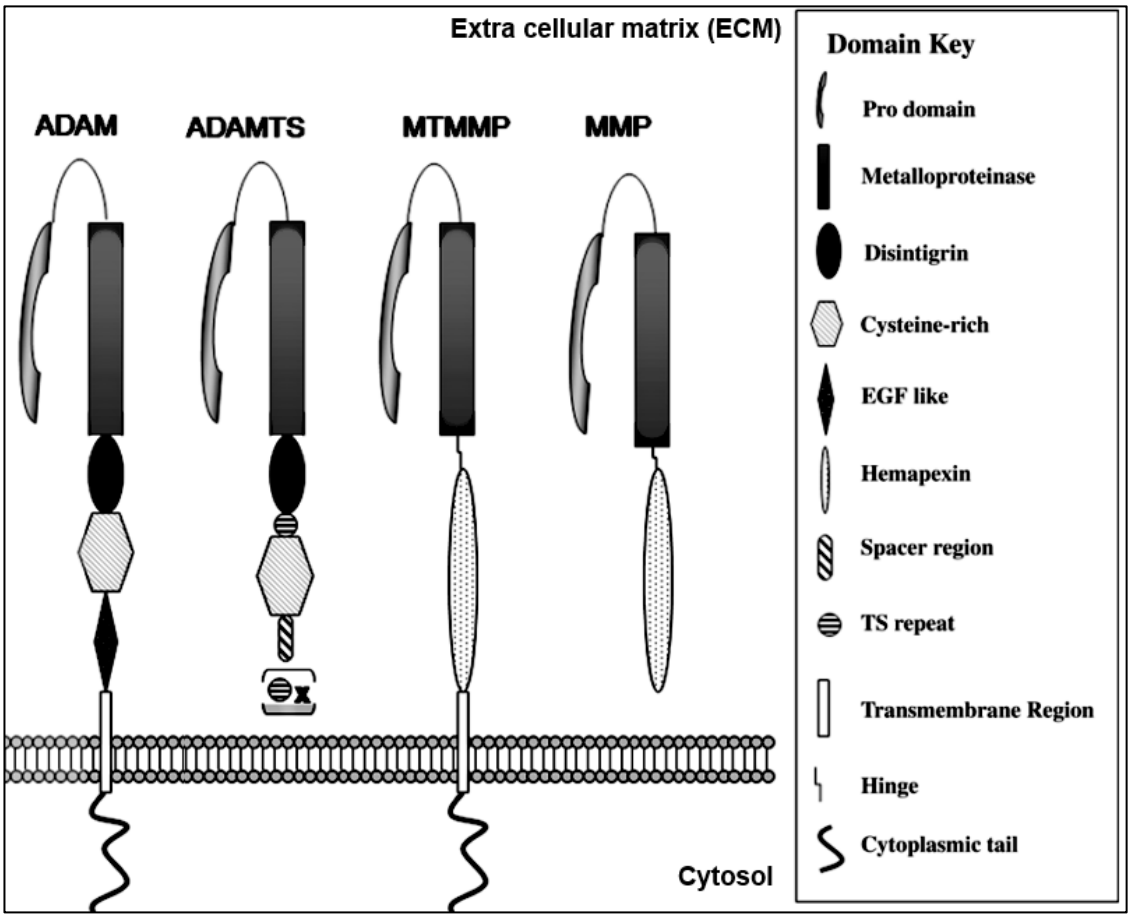


Figure 1.6: General domain organisation of several members of the metzincin superfamily. ECM associated MMPs and membrane type (MT-MMPs) are depicted along with the ADAMs and ADAMTSs. The defining characteristics of these related families includes the HEXXH Zn-coordinating catalytic core and the presence of a Met residue downstream that sets up the topology of the active site cleft; a feature exploited by MMPIs. Figure adapted from Edwards *et al.* 2008.

1.5 The ADAMTS Family

The human ADAMTSs (A Disintegrin and Metalloproteinase with Thrombospondin motifs) are a family of 19 secreted, matrix-associated zinc metallo-endopeptidases. The founding member of the family, ADAMTS-1 was discovered by Kuno *et al.* 1997. Subsequent research has identified a total of 19 human ADAMTSs that can be sub-grouped based on phylogenetic analysis and according to their known substrate specificities (Figure 1.7; Table 1.3; Appendix A1; A3).

The ADAMTSs have diverse roles in the regulation of tissue morphogenesis, patho-physiological tissue remodelling, inflammation, neural plasticity and angiogenesis (Kelwick and Edwards, 2014; Porter *et al.* 2005; Wagstaff *et al.* 2011). Whilst the loss of individual ADAMTSs may in some cases be phenotypically mild, mutations or changes in ADAMTSs expression, localisation or function are associated with disease. Mutations in *ADAMTS-2* lead to Ehler-Danlos syndrome type VIIc, a connective tissue disorder and mutations in *ADAMTS-13* drive an inherited form of thrombotic thrombocytopenic purpura (TTP), a disease associated with the accumulation of platelet thrombi that can obstruct the cardiovascular system (Li *et al.* 2001; Pimanda *et al.* 2004; Prockop *et al.* 1998). Broad changes in ADAMTS expression, function or localisation are also associated with an array of arthritis and cancer patho-physiological processes (Kelwick and Edwards, 2014; Kevorkian *et al.* 2004; Lin and Liu 2010; Porter *et al.* 2004; Wagstaff *et al.* 2011).

The basic structure of the ADAMTSs is broadly organised into a protease domain and an ancillary domain. The proteinase domain comprises of the

signal, pro, metalloproteinase and disintegrin-like domains. The ancillary domain varies greatly between different family members and is composed of one or more Thrombospondin type 1 Sequence Repeats (TSR), a Cysteine-rich domain and a spacer domain. Several ADAMTSs also contain one or more specialist domains as part of their ancillary domain, which regulate enzyme function in terms of substrate preferences, localisation, secretion and proteolytic activity (Figure 1.7). Beyond intrinsic structural features the ADAMTSs are also regulated at the level of transcription, via post-translational modifications and via endogenous inhibition (TIMPs).

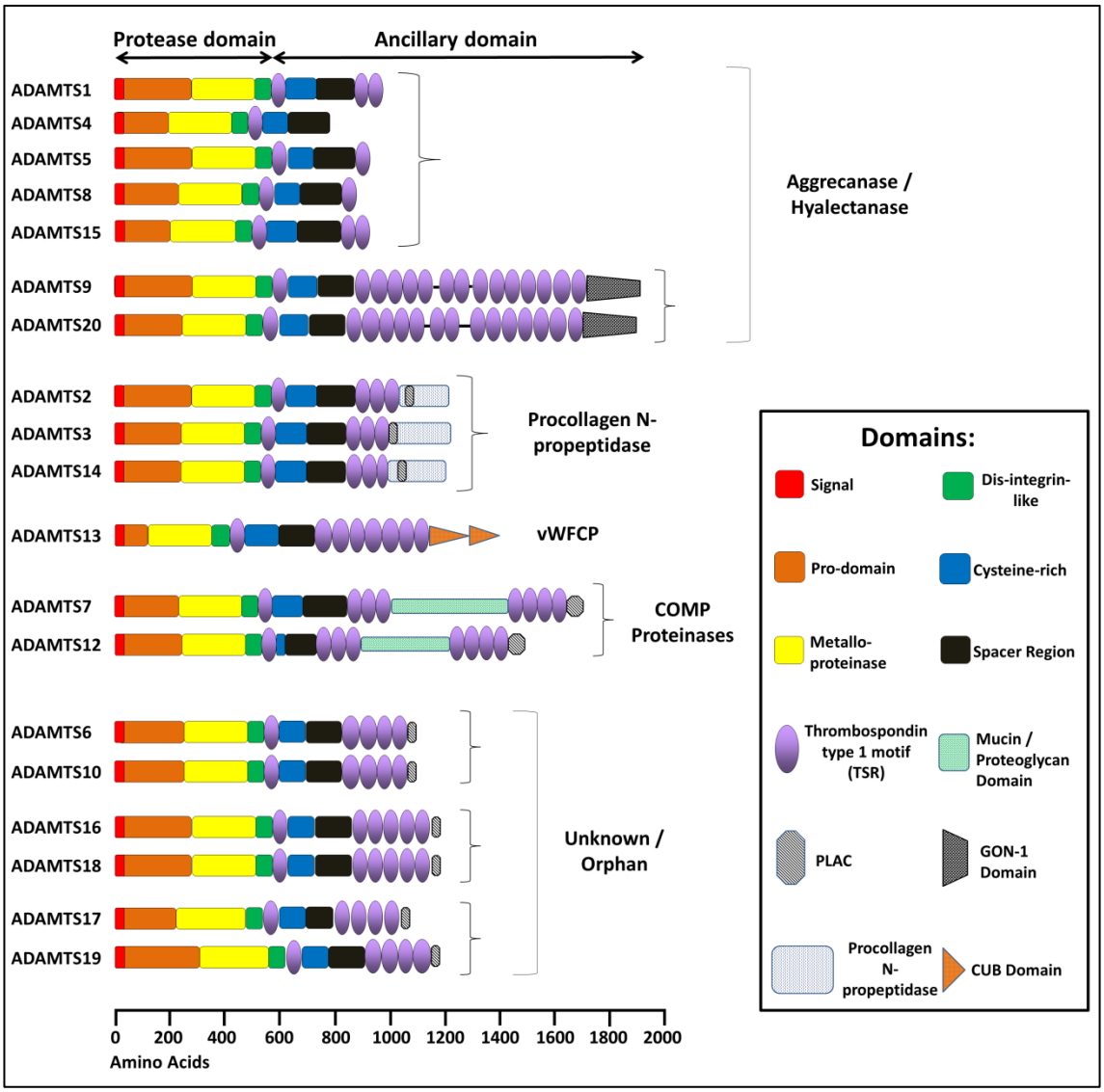


Figure 1.7: The A Disintegrin And Metalloproteinase with ThromboSpondin motifs (ADAMTS) family. The figure illustrates the basic domain organisation of the 19 human ADAMTS family members and their major functional groups. Structurally the ADAMTS members are broadly organised into a proteinase domain and an ancillary domain. The diagram is drawn to scale. Adapted from Kelwick and Edwards, 2014.

1.6 ADAMTS functional subgroups

The major functional ADAMTS sub-groups include, the pro-collagen N-pro-peptidases (ADAMTS-2,-3 and -14), the cartilage oligomeric matrix protein (COMP) cleaving proteases (ADAMTS-7 and -20), von-Willebrand Factor proteinase (ADAMTS-13) and the largest sub group, the hyalectanases / aggrecanases (ADAMTS-1,-4,-5,-8,-9,-15 and -20). There are also several orphan proteases, with no known substrates (ADAMTS-6,-10,-16 to -19) (Table1.3). These subgroups will be examined in the coming sections.

However it should also be noted that beyond the currently known sub-groups, proteomics based approaches are identifying novel ADAMTS substrates and by inference a greater understanding of the biological functions of the ADAMTS family (Canals *et al.* 2006; Esselens *et al.* 2010; Lopez-Otin and Overall, 2002). These types of degradomic approaches are highlighting that the ADAMTSs have functions beyond the current sub-group descriptors, which may be biologically relevant to breast cancer tumourigenesis and metastasis (Esselens *et al.* 2010; Rodríguez-Manzaneque *et al.* 2009).

Gene	Substrates
	Hyalectanases
ADAMTS-1	Aggrecan, Versican, Dystroglycan, Syndecan-4, TFPI-2, Semaphorin 3C, Nidogen-1, -2, Desmocollin-3, Mac-2, Gelatin (Collagen type I).
ADAMTS-4	Aggrecan, Versican, Neurocan, Biglycan, Brevican, Reelin, Matrilin-3, α2-Macroglobulin, COMP.
ADAMTS-5	Aggrecan, Versican, Biglycan, Brevican, Reelin, Matrilin-4, α2-Macroglobulin.
ADAMTS-8	Aggrecan.
ADAMTS-9	Aggrecan, Versican.
ADAMTS-15	Aggrecan, Versican.
ADAMTS-20	Versican.
	Pro-collagen peptidases
ADAMTS-2	Fibrillar pro-collagens type I-III and V.
ADAMTS-3	Fibrillar pro-collagen type II, Biglycan.
ADAMTS-14	Fibrillar pro-collagen type I (pNα1 and pNα2 chains).
	Cartilage oligomeric matrix protein (COMP) proteinases
ADAMTS-7	COMP.
ADAMTS-12	COMP.
	von Willebrand factor (vWF) proteinase
ADAMTS-13	vWF.
	Orphan ADAMTSs
ADAMTS-6	-
ADAMTS-10	Fibrillin-1.
ADAMTS-16	-
ADAMTS-18	-
ADAMTS-17	-
ADAMTS-19	-

Table 1.3: Known ADAMTS substrates. Adapted from Kelwick and Edwards, 2014

1.6.1 Pro-collagen peptidases (ADAMTS-2, -3 and -14)

Ehler-Danlos syndrome (EDS) type VIIc is an autosomal recessive disorder caused by a reduction in the cleavage and maturation of pro-collagen N-pro-peptides (Colige *et al.* 2004). Maturation of collagen fibrils is essential to ensure their tensile strength and to ensure that appropriate tissue morphologies are formed (Cabral *et al.* 2005; Colige *et al.* 2004). Patients with Ehler–Danlos syndrome (EDS) type VIIc present several phenotypes including severe skin fragility, depressed nasal bridges, micrognathia and short stature (Cabral *et al.* 2005; Colige *et al.* 2004). The major proteases involved in collagen fibril maturation have been identified as ADAMTS-2, -3 and -14 (Cabral *et al.* 2005; Cal *et al.* 2002; Colige *et al.* 2002; Colige *et al.* 2004; Colige *et al.* 2005; Fernandes *et al.* 2001). ADAMTS-2 can process the removal of the N-terminal pro-peptides from pro-collagen types I, II and III, whereas ADAMTS-3 is only associated with the processing of type II and ADAMTS-14 can process type I (pN α 1 and pN α 2). (Table 1.5; Cal *et al.* 2002; Cabral *et al.* 2005; Colige *et al.* 2002; Colige *et al.* 2004; Colige *et al.* 2005; Fernandes *et al.* 2001; Wang *et al.* 2006).

In the context of EDS type VIIc ADAMTS-2 is the most important pro-collagen peptidase. Several mutations in ADAMTS-2 have been described and are associated with EDS type VIIc (Colige *et al.* 2004). In addition to this ADAMTS-2 null mice demonstrate that ADAMTS-3 and -14 are not sufficient to compensate for the loss of ADAMTS-2 within the skin, though ADAMTS-3 and -14 may be physiologically important in other contexts such as cartilage (Colige *et al.* 2002; Fernandes *et al.* 2001; Li *et al.* 2001).

1.6.2 COMP proteinases (ADAMTS -7 and -12)

Cartilage oligomeric matrix protein (COMP) is a non-collagenous component of cartilage formed from five ~110 kDa subunits, held together by disulphide bonds (Liu 2006a,2006b; Oldberg *et.* al 1992). COMP only accounts for around 1% of the weight of articular tissue, though interest in this glycoprotein has increased since the discovery that COMP degradation is common in post-traumatic and arthritic joints (Di Cesare, 1996; Liu *et al.* 2006a, 2006b; Lohmander, 1994). COMP fragments can be found at higher levels in rheumatoid and osteoarthritic cartilage and may serve as a useful biomarker (Di Cesare, 1996; Liu *et al.* 2006a, 2006b; Lohmander, 1994).

ADAMTS-12 has been reported as the major protease responsible for COMP degradation. Increased ADAMTS-12 expression is common in osteoarthritic joints, with reports also showing that ADAMTS-12 is capable of binding to and proteolytically degrading COMP (Liu *et al.* 2006a, 2006b). ADAMTS-12 is not the only known protease that is able to degrade COMP. ADAMTS-7 which shares the same general structure as ADAMTS-12 is also able to degrade COMP (Figure 1.4; Liu *et al.* 2006a, 2006b). The binding of COMP to both ADAMTS-7 or-12 involves an interaction between their four C-terminal TSRs and the EGF-like repeat domain found in COMP (Figure 1.4; Liu *et al.* 2006a, 2006b). COMP shares sequence homology with the Thrombospondins, which are known to reduce pathophysiological inflammatory and angiogenic processes in rheumatoid arthritis (Park *et al.* 2004). Therapeutic targeting of ADAMTS-7 and -12 may become of interest particularly if they are also shown to degrade the Thrombospondins.

1.6.3 vWF cleaving protease (ADAMTS-13)

The processing of Von-Willebrand Factor (vWF) proteins from large multimeric vWF precursor proteins into smaller cleaved vWF proteins is important to ensure proper blood coagulation (Dong *et al.* 2002; Fujikawa *et al.* 2001). This is apparent in thrombotic thrombocytopenic purpura (TTP), a thrombotic disorder in which ultra large (UL) vWF multimers coagulate with platelets that in combination ultimately occlude circulation (Dong *et al.* 2002; Fujikawa *et al.* 2001; Metcalf *et al.* 2008). Several studies have associated mutations in ADAMTS-13 with the inherited form of TTP. An analysis of a patient with TTP revealed the presence of a frameshift mutation (4143-4144InsA) in the *ADAMTS-13* gene (Pimanda, 2004). This mutation caused a truncated form of ADAMTS-13 to be expressed which lacked the final 49 amino acids in the second CUB domain (Pimanda, 2004).

Whilst ADAMTS-13 has been confirmed to be capable of processing von-Willebrand Factor (vWF) and ADAMTS-13 mutations are associated with TTP there are still some unanswered questions (Akiyama *et al.* 2009; Dong *et al.* 2002; Pimanda *et al.* 2004). When a mutant form of ADAMTS-13 (4143-4144InsA), which was discovered in a TTP patient, was expressed in COS-7 cells, ADAMTS-13 displayed normal vWF protease activity (Pimanda, 2004). In another study ADAMTS-13 deficient mice did not display any apparent thrombocytopenia, however there was evidence of the presence of unusually large vWF multimers (Banno *et al.* 2006). Taken together it is clear that ADAMTS-13 is a physiologically important vWF cleaving protease, though more research is needed to fully understand TTP (Banno *et al.* 2006; Pimanda, 2004).

1.6.4 Hyalectanases (ADAMTS-1, -4, -5, -8, -15, -9 and -20)

The Hyalectanases were originally termed the aggrecanase sub-group largely due to efforts from researchers that were interested in understanding aggrecan cleavage during osteoarthritis (OA) (Troeberg and Nagase, 2012). The proteoglycan aggrecan is a member of the hyalectans and is a major component of the extracellular matrix (Troeberg and Nagase, 2012). Aggrecan structure is augmented with several keratin sulphate (KS) and CS GAG chains (Figure 1.8). The propensity for these structural features to attract water, enables aggrecan to provide a swelling pressure that contributes to the load bearing properties of tissues such cartilage and tendon (Chandran and Horkay, 2012). In osteoarthritis (OA) aggrecan is progressively degraded and is lost from the cartilage (Echtermeyer *et al.* 2009; Fosang and Little, 2008; Jones and Riley, 2005; Kelwick and Edwards, 2014; Lin and Liu, 2010). For this reason the proteases responsible for aggrecanase activity became of interest to the arthritis field.

ADAMTS-4 (AKA aggrecanase-1) and in particular ADAMTS-5 (AKA aggrecanase-2) were identified as the major physiologically relevant aggrecanases in OA (Abbaszade *et al.* 1999; Arner *et al.* 1999; Fosang and Little, 2008; Song *et al.* 2007; Troeberg and Hideaki Nagase 2012). ADAMTS -1, -8 and -15 display negligible expression in osteoarthritic and normal cartilage (Kevorkian *et al.* 2004; Naito *et al.* 2007). Subsequent studies have identified at least limited aggrecanase activity amongst ADAMTS-1, -8, -9, and -15, though their physiological importance as aggrecanases is perhaps not related to cartilage (Collins-Racie *et al.* 2004; Kelwick and Edwards, 2014; Kuno *et al.* 2000; Rodríguez-Manzaneque *et al.* 2002; Somerville *et al.* 2003; Yamaji *et al.*

2000). For instance an ADAMTS-1 knock-out mouse model showed that ADAMTS-1 is an important aggrecanase in the developing kidney (Lee *et al.* 2005).

Because the aggrecanases can also cleave other hyalectans including versican, brevican and neurocan it has been argued that it is more appropriate for this sub-group to be termed the hyalectanases (Table 1.3; Figure 1.7; Kelwick and Edwards, 2014; Kuno *et al.* 2000; Stanton *et al.* 2011; Stupka *et al.* 2013; Tauchi *et al.* 2012; Zeng *et al.* 2006). Versican cleavage can be carried out by several hyalectanases including ADAMTS-1, -4, -5, -9, -15 and -20 (Figure 1.6; Table 1.5; Kintakas and McCulloch 2011; Russell *et al.* 2003; Sandy *et al.* 2001; Somerville *et al.* 2003; Stupka *et al.* 2013). Versican-null mice die during development primarily due to cardiac defects, though defects in versican expression or cleavage can affect limb morphogenesis, palate formation, melanoblast development and myogenesis (Dupuis *et al.* 2011; Kern *et al.* 2010; McCulloch *et al.* 2009; Mjaatvedt *et al.* 1998; Silver *et al.* 2008; Stupka *et al.* 2013). The cleavage of versican is critical during developmental processes for several reasons. Versican rich matrices provide a temporary structural support which must be removed during morphogenesis and the cleavage of versican can release versican fragments that are bioactive (McCulloch *et al.* 2009; Stupka *et al.* 2013). In combination these two mechanisms can influence tissue architecture, cell adhesion, migration and proliferation (Dupuis *et al.* 2011; Kern *et al.* 2010; McCulloch *et al.* 2009; Mjaatvedt *et al.* 1998; Silver *et al.* 2008; Stupka *et al.* 2013).

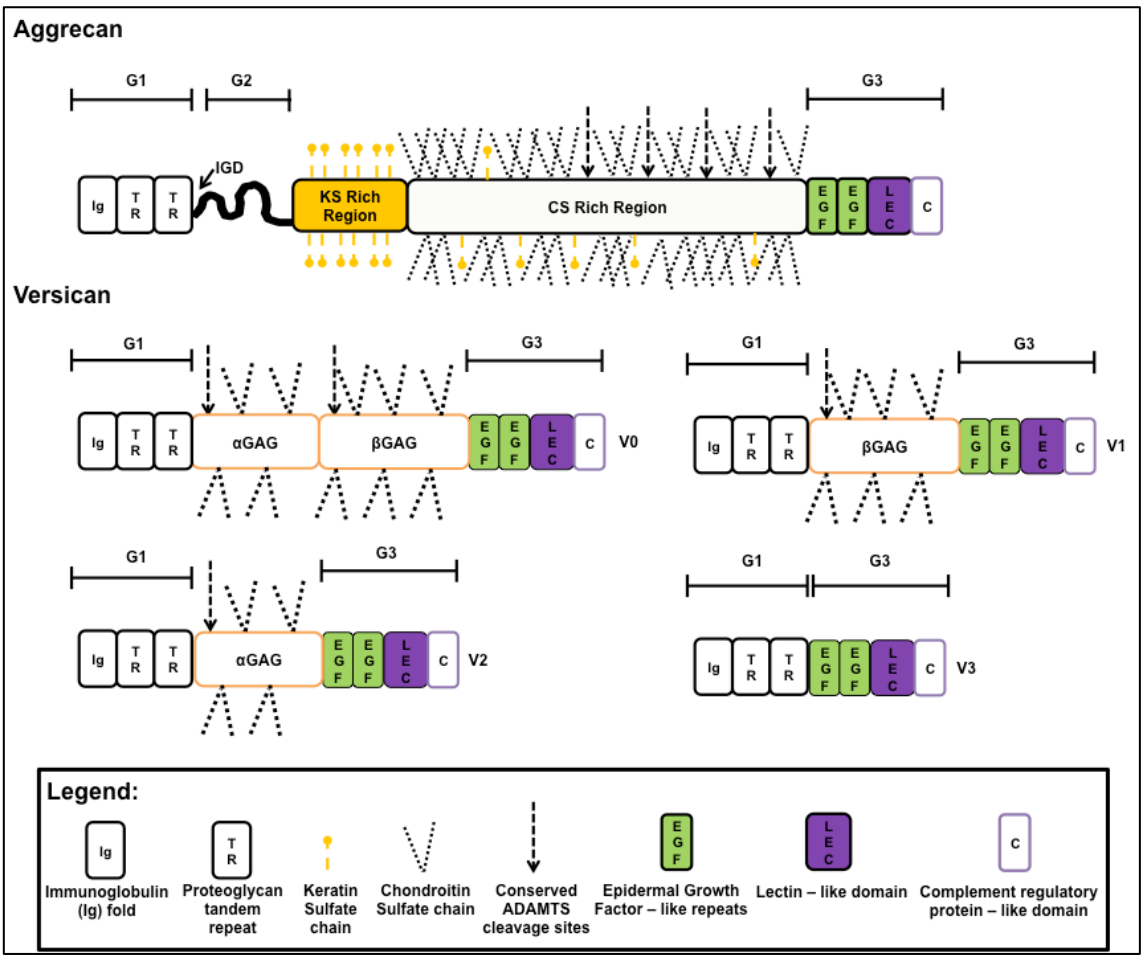


Figure 1.8: Aggrecan and Versican. The figure shows a schematic of the major structural features of Aggrecan and Versican. This figure is based upon Kelwick and Edwards, 2014; Stanton *et al.* 2011; Stupka *et al.* 2013; Wight, 2002.

ADAMTS-15 along with ADAMTS-5 are emerging as important versicanases within the context of muscle development (Stupka *et al.* 2013). Several hyalectanases (ADAMTS-1, -4, -5 and -15) are expressed in developing embryonic muscle and in an *in vitro* myogenesis model which utilises differentiating C2C12 cells (Stupka *et al.* 2013). Knockdown of ADAMTS-5 in differentiating C2C12 myoblasts impaired their ability to fuse together to form multinucleated myotubes (Stupka *et al.* 2013). For myoblast fusion to occur versican must be cleared from the pericellular matrix to allow the myoblasts to make direct cell-cell contact and subsequent fusion (Stupka *et al.* 2013). This phenotype was rescued through the addition of catalytically active forms of ADAMTS-5 or ADAMTS-15, highlighting these hyalectanases as important versicanases within this process (Stupka *et al.* 2013). In other studies involving either the combinatorial knockout of ADAMTS-4 and -5 or a combinatorial deletion of ADAMTS-5, -9 and -20 there were no signs of significant skeletal muscle myopathy (Rogerson *et al.* 2008; Stupka *et al.* 2013). It has been suggested that the lack of any skeletal muscle myopathy in these knock-out mice was due to functional compensation from ADAMTS-15 (Rogerson *et al.* 2008; Stupka *et al.* 2013). Ultimately it may be therapeutically useful to know the expression of ADAMTS-15 in patients which might be treated with ADAMTS-5 proteolytic inhibitors, such as those with arthritis. Whilst inhibition of ADAMTS-5 proteolytic activity might be therapeutically beneficial in terms of reducing aggrecan loss in OA, loss of ADAMTS-5 versicanase activity without compensation from ADAMTS-15 may lead to detrimental muscle wasting and/or impaired regeneration (Cudic *et al.* 2005; Rogerson *et al.* 2008; Stupka *et al.* 2013; Vankemmelbeke *et al.* 2003).

The hyalectanases have also been noted for their ability to modulate angiogenic processes and are sometimes referred to as an angioinhibitory sub-group (Wagstaff *et al.* 2011). Several ADAMTSs, particularly the hyalectanases exert their pro and anti-tumorigenic effects through a regulation of angiogenesis.

1.7 ADAMTSs in cancer

The ADAMTSs influence an array of biological processes including tissue remodelling, cell migration, inflammation and angiogenesis (Esselens *et al.* 2010; Kelwick and Edwards, 2014; Porter *et al.* 2005; Rodríguez-Manzaneque *et al.* 2009; Wagstaff *et al.* 2011). During tumourigenesis and metastasis many of these processes are aberrantly regulated and emerge as the hallmarks of cancer (Hanahan and Weinberg, 2011). Therefore it is unsurprising that the expression profiles of the ADAMTSs show significant alterations in cancer. In breast cancer ADAMTS-1, -3, -5, -8, -9, -10 and -18 are all downregulated compared to normal tissue, whereas ADAMTS-4, -6 and -14 are up-regulated (Porter *et al.* 2004). Interestingly ADAMTS-15 expression is not significantly different between normal tissues and cancerous mammary tumours. However ADAMTS-15 expression does inversely correlate with tumour grade, with expression significantly reduced in grade III breast tumours compared to grade I or II tumours (Porter *et al.* 2004). ADAMTS-15 expression has also been shown to inversely correlate with tumour grade in colorectal cancer (Viloria *et al.* 2009).

The loss of expression of genes in a cancer context may be indicative of a tumour suppressive function (Hanahan and Weinberg, 2011). However determining whether an ADAMTS might be a tumour suppressor based upon expression level is complicated for several reasons. Firstly the pattern of

ADAMTS expression (down or up-regulated) can differ between cancer type and stage. For instance ADAMTS-1 expression is down-regulated in primary head and neck carcinomas, breast cancer and around 85% of colorectal cancer cell lines (Demircan *et al.* 2009; Lind *et al.* 2006; Porter *et al.* 2004). Yet in contrast to this ADAMTS-1 is up-regulated in metastatic head and neck carcinomas and also non-small cell lung cancer (NSCLC) (Demircan *et al.* 2009; Rocks *et al.* 2006). Secondly the mechanism underpinning the inactivation of the ADAMTS differs, where some ADAMTSs are epigenetically silenced and others are inactivated by mutation (Table 1.4). For example whilst ADAMTS-15 is inactivated by mutation in colorectal cancer, in breast cancer ADAMTS-15 is epigenetically silenced (Table 1.4; Porter *et al.* 2005*;* Porter *et al.* 2006; Viloria *et al.* 2009).

Epigenetically Silenced	Inactivated via Mutation	Unknown
ADAMTS-1(*)	ADAMTS-15(*)	ADAMTS-3
ADAMTS-8(*)		ADAMTS-5(*)
ADAMTS-9(*)		ADAMTS-10
ADAMTS-12		
ADAMTS-15(*)		
ADAMTS-18		

Table 1.4: Members of the ADAMTS family that are down-regulated in cancer. (*) denotes that these genes have also been shown to be up-regulated in some cancers. Table adapted from Dunn *et al.* 2006; Jungers *et al.* 2005; Kelwick and Edwards, 2014; Koo *et al.* 2010; Kutz *et al.* 2008; Li *et al.* 2009; Llamazares *et al.* 2007; Lopez-Otin and Matrisian, 2007; Porter *et al.* 2005; Porter *et al.* 2006; Viloria *et al.* 2009; Stanton *et al.* 2004.

These seemingly ambiguous patterns of ADAMTS expression in cancer are further confounded by the activities of the ADAMTSs which can be anti- or pro-tumorigenic in a context dependent manner. These have been extensively covered in several reviews (Edwards *et al.* 2005; Kelwick and Edwards, 2014; Wagstaff *et al.* 2011), however an overview of ADAMTS-1 will be given since ADAMTS-1 is the archetype of these complexities (de Arao Tan, Ricciardelli, and Russell, 2013).

The catalytic activities of ADAMTS-1 have been shown to be pro-tumourigenic in several cancers. Expression of catalytically active ADAMTS-1 enhances the metastatic propensity of TA3 mammary carcinoma and Lewis Lung Carcinoma cells *in vivo* (Liu *et al.* 2006). In other contexts, including breast cancer, ADAMTS-1 shedding of syndecan 4 or semaphorin 3C enhances the migratory behaviour of cancer cell lines (Esselens *et al.* 2010; Rodríguez-Manzaneque *et al.* 2009).

Conversely ADAMTS-1 proteolytic activity can also be anti-metastatic. ADAMTS-1 proteolytic cleavage of thrombospondins -1 and -2 releases anti-angiogenic fragments (Lee *et al.* 2006). ADAMTS-1 also displays auto-catalytic behaviour, at a cleavage site within its own spacer region (Liu *et al.* 2006). This autocatalytic event releases N-terminal and C-terminal anti-metastatic fragments, that primarily act as anti-angiogenic factors (Liu *et al.* 2006). ADAMTS-1 can also elicit anti-tumorigenic effects in a metalloproteinase independent manner, through the binding of VEGF. The binding of VEGF sequesters both pro-angiogenic and pro-migratory signalling (Freitas *et al.* 2013; Luque *et al.* 2003).

1.8 Cell Migration

Cell migration is a highly dynamic and tightly regulated process which is critical to the development and function of living systems. Perturbations of these processes contribute to pathological forms of migration such as tumour cell invasion and metastasis. To this end an understanding of the processes and factors, which influence cell migration, may elucidate druggable targets that will inhibit metastasis.

Several migration types have been identified, and include mesenchymal and amoeboid forms (Ashby and Zijelstra, 2012; Even-Ram and Yamada, 2005; Germena and Hirsch, 2013; Holmes and Edelstein-Keshet, 2012; Petrie *et al.* 2012). These forms of single cell migration are occasionally controversial since invading 'mesenchymal-like' tumour cells may not need to release proteases in order to clear a path for migration if they adopt an ameboid configuration to squeeze through the ECM (Ashby and Zijelstra, 2012; Even-Ram and Yamada; 2005; Mierke *et al.* 2008). Cells can also collectively migrate as part of complex and highly dynamic multi-cellular structures (Friedl *et al.* 2012; Rørth, 2012). In the context of cancer, tumour cells may collectively invade and endothelial cells may collectively migrate to vascularise the tumour (Friedl and Wolf, 2003; Nguyen-Ngoc *et al.* 2012).

The factors which influence cell migration are varied and include those which are intrinsic (e.g. regulation of cytoskeleton, adhesion receptors, proteases), extrinsic (e.g. cell-cell interactions, soluble signalling factors) and matrix associated (e.g. composition, structure, stiffness) (Ashby and Zijelstra, 2012; Even-Ram and Yamada, 2005; Holmes and Edelstein-Keshet, 2012; Schiller *et*

al. 2013). Whilst the full complexities of cell migration processes have not yet been fully characterised a general model of the migration cycle is fairly well established.

1.8.1 Migration cycle

Cell migration is often conceptualised as a cyclical model in which four general stages occur, as summarised in Figure 1.9. Initially cells become polarised, in which a migration direction is determined. Cellular polarisation is primarily regulated by the Rho GTPase, Cdc42, which is active at the leading edge of a migrating cell (Mullins and Hansen, 2013; Petrie, Doyle and Yamada, 2009; Reymond *et al.* 2012). Cdc42 acts to localise and reorient the microtubule-organising centre and the Golgi apparatus towards the leading edge (Hehnly *et al.* 2010; Raftopoulou and Hall, 2004; Ridley *et al.* 2003). The leading edge is visibly defined through the extension of the cell membrane either as 'finger like' filopodia or as 'broad ruffle like' lamellipodia projections (Mullins and Hansen, 2013; Raftopoulou and Hall, 2004; Ridley *et al.* 2003). These transient extensions are broadly driven by the forces elicited by the dynamic assembly and disassembly of actin bundles (actin polymerisation) (Carlsson, 2010; Mullins and Hansen, 2013). Actin polymerisation dynamics are themselves regulated by Cdc42 acting in co-operation with the Rho-GTPase, Rac1 (Bass *et al.* 2007; Choi *et al.* 2011; Keum *et al.* 2004; Riddley *et al.* 1992). However Cdc42 and Rac1 do not directly bind to actin bundles, instead they act via members of the WASp/SCAR/WAVE family of scaffold proteins (Mullins and Hansen, 2013; Raftopoulou and Hall, 2004; Ridley *et al.* 2003). Cdc42 acts via the direct activation of WASp (Lamalice, Le Boeuf and Huot, 2007; Raftopoulou and Hall, 2004; Rohatgi, Ho and Kirschner, 2000; Ridley *et al.* 2003). Rac1 on

the other hand acts via an indirect pathway in that it activates members of the Scar/WAVE family of scaffold proteins via the Nck-adapter complex (Raftopoulou and Hall, 2004; Ridley *et al.* 2003; Rohatgi *et al.* 2001). Activated WASp/SCAR/WAVE proteins subsequently go on to activate the Arp2/3 complex, which initiate *de novo* actin polymerisation (Mullins and Hansen, 2013). Cdc42 regulated actin polymerisation tends to favour filopodia membrane protrusions, though Rac1 regulated actin polymerisation leads to the formation of lamellipodia (Mullins and Hansen, 2013; Ridley *et al.* 1992; Ridley *et al.* 2003).

Cdc42 and in particular Rac1 also signal to recruit transient adhesion complexes (Mullins and Hansen, 2013; Reymond *et al.* 2012; Ridley *et al.* 2003). Transient adhesion complexes include integrins and co-receptors such as syndecans (Beauvais and Rapraeger, 2004; Reymond *et al.* 2012; Schiller *et al.* 2013). These complexes maintain localised Rac1 and Cdc42 signalling at the leading edge and as a result assist in the stabilisation of the lamellipodia (Beauvais and Rapraeger, 2004; Petrie, Doyle and Yamada, 2009; Schiller *et al.* 2013). It is thought that these adhesion complexes also serve as regions in which cells can dynamically 'sample' the rigidity and composition of the extracellular matrix in order to seek out an optimal migration pathway (Beauvais and Rapraeger, 2004; Petrie, Doyle and Yamada, 2009; Schiller *et al.* 2013).

However in order to migrate, cells require a secure attachment site from which enough force can be generated to facilitate cell migration (Lui, Lee and Nelson, 2012; Lai, Hsieh and Chang, 2003). The GTPase RhoA induces the formation of stress fibres and stable focal adhesions that provide the cytoskeletal tension required for cell contractility (Brew *et al.* 2009; Mullins and Hansen, 2013;

Tilghman *et al.* 2010). RhoA acts via several downstream effectors including the Rho-associated kinases, ROCK (Bhadriraju *et al.* 2007; Brew *et al.* 2009; Tilghman *et al.* 2010). Rock activated myosin II promotes the stabilisation of actin-myosin filament bundles which provide the cellular tension needed for cell contractility (Bhadriraju *et al.* 2007; Brew *et al.* 2009; Mullins and Hansen, 2013; Tilghman *et al.* 2010). Tension is transduced via mechanosensitive integrins which elicits further activation of RhoA (Ross *et al.* 2013; Schiller *et al.* 2013; Streuli and Akhtar, 2009). This positive feedback network increases focal adhesion size and induces the formation of stress fibres via increased RhoA activation of mDia (Doherty and McMahon, 2008; Ridley *et al.* 2003; Schiller *et al.* 2013; Spiering and Hodgson, 2011). Thus RhoA signalling acts synergistically to alter cytoskeletal dynamics in order to generate the contractile forces needed for cell migration.

The force of cell migration is largely transmitted to the leading and trailing edges of the cell (Beningo *et al.* 2001; Holmes and Edelstein, 2012; Mierke *et al.* 2008; Ridley *et al.* 2003). In response, adhesion complexes must be temporally co-ordinated to disassemble at both edges of the cell. At the leading edge cell adhesion complexes must disassemble and then reform as the cell membrane rolls forward (tread milling effect) (Carlsson, 2010; Friedl and Wolf, 2003 Germena and Hirsch, 2013; Ridley *et al.* 2003). These newly formed adhesion complexes reinforce Cdc42 and Rac1 signalling at the leading edge (Friedl and Wolf, 2003; Ridley *et al.* 2003). Rac1 and RhoA are mutual inhibitors of their activities (Caswell, Vadrevu and Norman, 2009; Germena and Hirsch, 2013; Ridley *et al.* 2003). As a consequence Rac1 activity is restricted to the leading edge and RhoA activity is highest at the periphery and trailing edges (Bass *et*

al. 2007; Friedl and Wolf, 2003; Ridley *et al.* 2003). This maintains migration polarity whilst also serving to co-ordinate the detachment of adhesion complexes at the trailing edge, which is a RhoA and myosin-dependent process (Schiller *et al.* 2013; Terry *et al.* 2012). However it should be noted that multiple signalling pathways are also implicated in focal adhesion turnover such as focal adhesion kinase (FAK), ERK and Src (Bass *et al.* 2007; Beningo *et al.* 2001; Ross *et al.* 2012; Streuli and Akhtar, 2009).

RhoA also stabilises microtubules at the trailing edge (Fonseca *et al.* 2010; Fonseca and Corbeil, 2011; Heck *et al.* 2012). As noted earlier Cdc42 and Rac1 also regulate microtubule dynamics, such that Cdc42 orientates microtubules towards the direction of migration and Rac1 enables the elongation of microtubules into the newly forming lamellipodia (Hehnly *et al.* 2010). In co-ordination these GTPases provide a platform from which adhesion receptor complexes can be recycled and transported along the microtubules towards the leading edge (Caswell, Vadrevu and Norman, 2009). Thus the migration cycle comes full circle.

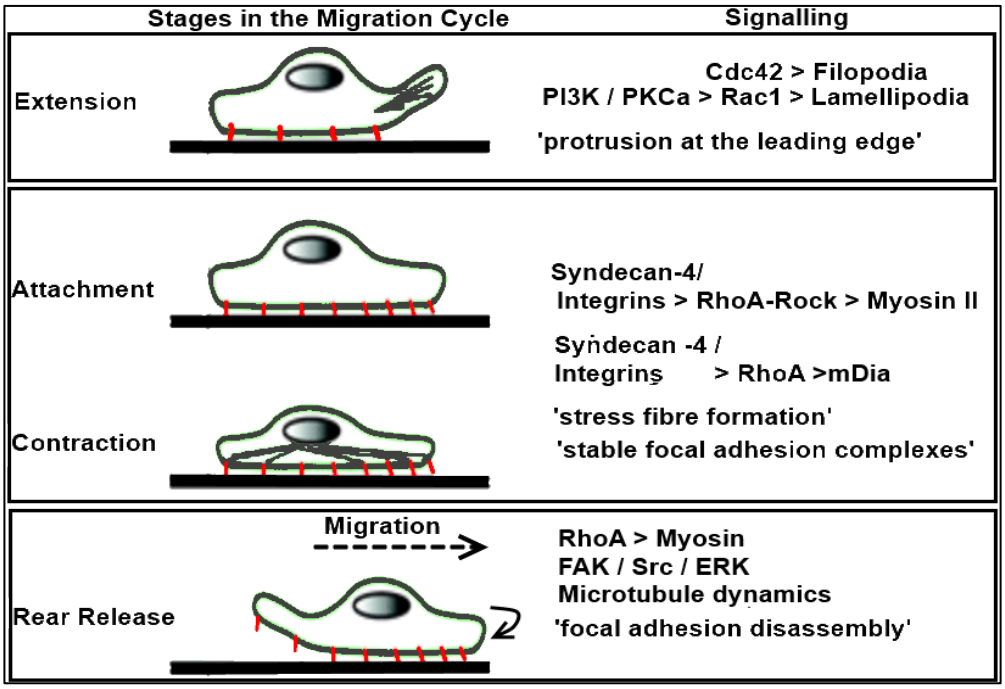


Figure 1.9: Cell migration cycle. The 4 main stages of the cell migration cycle are depicted alongside several key signalling pathways. Cells may become polarised in response to a pro-migratory stimulus such as a chemo-attractant gradient or matrix topology (stiffness etc.) (**1**) Polarisation of the cell and the formation of lamellipodia from which a migration direction is established. (**2**) The formation of more stable focal adhesion sites and stress fibres that enable the cell to generate enough force to (**3**) contract. (**4**) The rear of the cell is released and migration signalling complexes are reorganised around the newly formed leading edge. This figure is based upon information from Lamalice, Le Boeuf and Huot, 2007.

1.8.2 Integrins and cell migration

Integrins are the principal family of cell adhesion receptors in vertebrates (Humphries *et al.* 2006; Hynes, 2002; Hynes, 1987). Integrins form into one of 24 known mammalian heterodimers that consist of an α and a β subunit (Figure 1.10; Humphries *et al.* 2006; Hynes, 2002). Integrin heterodimers, span the cell membrane such that their ectodomains directly bind to the matrix and their intracellular domains directly interact with the cytoskeleton and the cell signalling machinery, including: Rac1, RhoA and Cdc42 (Figure 1.10, 1.11; Beauvais and Rapraeger, 2004; Beningo *et al.* 2001; Schiller *et al.* 2013; Wertheimer *et al.* 2012). Ligand binding is thought to cause a conformational change in integrin structure, which subsequently alters the propensity for membrane receptors and intracellular signalling proteins to form signalling clusters around activated integrins (Campbell and Humphries, 2011). Thus integrins are able to modify cellular behaviour through direct 'outside-in' signalling pathways.

Within the context of cell migration, integrins play several important roles that have been extensively reviewed, but are not yet fully understood (Huttenlocher and Horwitz, 2011; Hynes, 2002; Plotnikov and Waterman, 2013). Principally, integrins enable cells to adhere to the extracellular matrix and thus are a major component of the adhesive structures required to generate the traction needed for cell migration (Huttenlocher and Horwitz, 2011; Plotnikov and Waterman, 2013). Additionally, integrin heterodimers are matrix-ligand specific, thus a layer of specificity is added to these interactions, which provide context to the regulation of downstream signalling (Table 1.5; Caswell *et al.* 2009; Humphries *et al.* 2006; Hynes, 2002; Streuli and Akhtar, 2009).

Matrix component	Integrin heterodimers
Collagen	α10β1, α2β1, α1β1, α11β1
Laminin	α3β1, α6β1, α6β4, α7β1, α1β1, α2β1, α10β1
Fibronectin	αVβ3, αVβ6, αVβ1, α5β1, α8β1

Table 1.5: Integrin heterodimers that bind to major components of the extracellular matrix. This table is based upon Humphries, Byron and Humphries, 2006.

Due to the specificity of integrin-ligand binding, changes in the expression of integrins can drastically alter the adhesive, migratory and/or invasive capacity of a cell. For instance, an increase in the expression of integrin α6β4 is generally associated with an increase in the invasive capacity of breast tumour cells (Chen *et al.* 2008; Gabarra *et al.* 2010; Gordon *et al.* 2003). Yet interestingly, it is generally understood that overexpression of integrins may ultimately inhibit cell migration, since cells may become 'anchored' to the matrix (Huttenlocher and Horwitz, 2011). Therefore, a balance in the expression and turnover of integrins is required to ensure persistent cell migration (Huttenlocher and Horwitz, 2011). Indeed, integrin-containing adhesion complexes, of which focal adhesions are the most characterized, are largely transient (Beauvais and Rapraeger, 2004; Petrie, Doyle and Yamada, 2009; Schiller *et al.* 2013). The transient nature of focal adhesion complexes is also of paramount importance because they are sites at which cells can dynamically enact integrin-mediated sampling of the rigidity and composition of the ECM (Beauvais and Rapraeger,

2004; Petrie, Doyle and Yamada, 2009; Schiller *et al.* 2013). Integrin signalling is co-ordinated with an array of signaling and adhesion receptors, such as the syndecans, that together influence cell adhesion and migration pathways (Beauvais and Rapraeger, 2004; Huttenlocher and Horwitz, 2011; Plotnikov and Waterman, 2013).

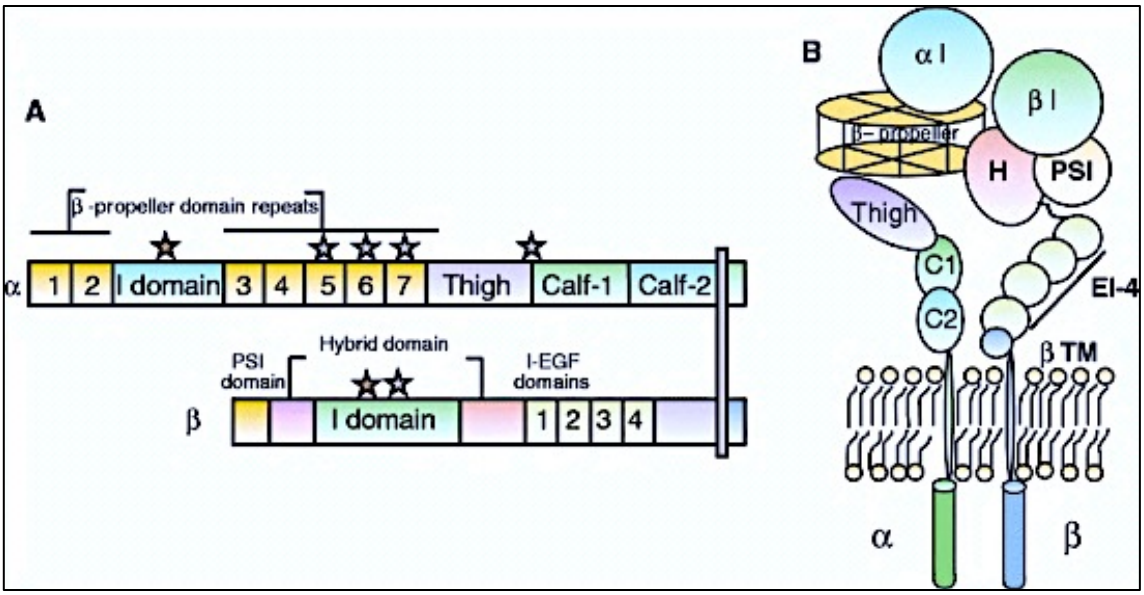


Figure 1.10: Typical integrin structure. (A) The schematic displays a typical integrin heterodimer. **(B)** The schematic displays the structure of a typical integrin as it sits within the membrane. Adated from Barczyk *et al.* 2010.

1.8.3 Syndecans and cell migration

The syndecans are a family of four heparan sulfate proteoglycans that sit within the cell membrane as type I transmembrane receptors (Choi *et al.* 2011). Syndecans are functionally diverse and have been shown to partake in the regulation of cell adhesion and migration signalling (Choi *et al.* 2011; Couchman and Woods, 1999; Morgan *et al.*, 2007). There seems to be an intimate connection between the syndecans and the ADAMTSs, which co-ordinate with other metalloproteinases to regulate cell-ECM interactions. In transfected human chondrosarcoma cells, the MMP-17 processed form of ADAMTS-4, termed p53, directly associates with syndecan-1 (Gao *et al.* 2004). Since ADAMTS-4 does not undergo autolytic processing within this context it is possible that the association of ADAMTS-4 within the MMP:ADAMTS:syndecan complex favours proteolytic processing, rather than autolytic processing. (Gao *et al.* 2004). This is functionally important since proteolytic and autolytic processing can alter ADAMTS localisation, substrate preferences and proteolytic activity.

In the case of ADAMTS-5, its association with syndecan-4 within an osteoarthritic context enhances ADAMTS-5 aggrecanase activity (Echtermeyer, *et al.* 2009). Syndecan-4 enhances ADAMTS-5 aggrecanase activity in part through their direct interaction and also through the syndecan-4 enhanced expression of matrix metalloproteinase-3 (MMP-3) (Echtermeyer, *et al.* 2009). Thus these complexes may serve as a regulatory mechanism from which to modulate the activities of secreted ADAMTSs.

Metalloproteinases can also feedback into these complexes since it has been demonstrated that TIMP-3 responsive metalloproteinases are responsible for the shedding of syndecan-1 and -4 (Fitzgerald *et al.* 2000). More specifically ADAMTS-1, and -4 are major syndecan-4 ectodomain sheddases and MMP-7, and MT1-MMP are major syndecan-1 sheddases (Rodríguez-Manzaneque *et al.* 2009). MMP-9 and ADAM-17 may also shed syndecan -1 and -4 in some contexts (Brule *et al.* 2006; Pruessmeyer *et al.* 2010). Critically these shedding activities have functional consequences, particularly with regards to the regulation of cell adhesion and migration (Rodríguez-Manzaneque *et al.* 2009).

Syndecan-4 can mediate cell adhesion to fibronectin either directly or as a co-receptor with Integrin α5β1 as part of a focal adhesion complex (Figure 1.11; Choi *et al.* 2011; Rodríguez-Manzaneque *et al.* 2009). Upon engagement with fibronectin, the cytoplasmic domain of syndecan-4 recruits, stabilises and activates PKCα at the plasma membrane (Keum *et al.* 2004; Woods and Couchman, 1997). Subsequent downstream Rac1 signalling is thus localised in a PKCα-dependent manner to a region of the cell in which syndecan-4 and other co-receptors are engaged with the extracellular matrix (Bass *et al.* 2007; Bass, Morgan and Humphries, 2007). Rac1 activity promotes the spreading and ruffling of the cell membrane into actin-rich lamellipodia (Riddley *et al.* 1992). PKCα also activates RhoA signalling which is required to generate stable focal adhesion complexes (Avalos *et al.* 2009; Brew *et al.* 2009; Car *et al.* 2013) In combination these structures provide a leading edge from which cell polarity and a stable migration direction can be established (Bass *et al.* 2007; Choi *et al.* 2011; Keum *et al.* 2004; Riddley *et al.* 1992). Thus syndecan-4 promotes persistent migration (directionality) by maintaining Rac1 localisation at the

leading edge (Bass *et al.* 2007). ADAMTS-1 shedding of syndecan-4 from the cell surface results in a functional loss of cell adhesion and promotes a pro-migratory phenotype with a concomitant loss of directionality (Bass *et al.* 2007; Choi *et al.* 2011; Rodríguez-Manzaneque *et al.* 2009). Shedding of syndecan-4 also results in a functional loss of cell adhesion and an altered cytoskeletal topology, such that actin stress fibres are redistributed to the periphery (Rodríguez-Manzaneque *et al.* 2009). Therefore it is likely that the ADAMTSs are functionally linked with the syndecans within the context of regulating the ability of a cell to adhere and migrate in response to a highly dynamic ECM.

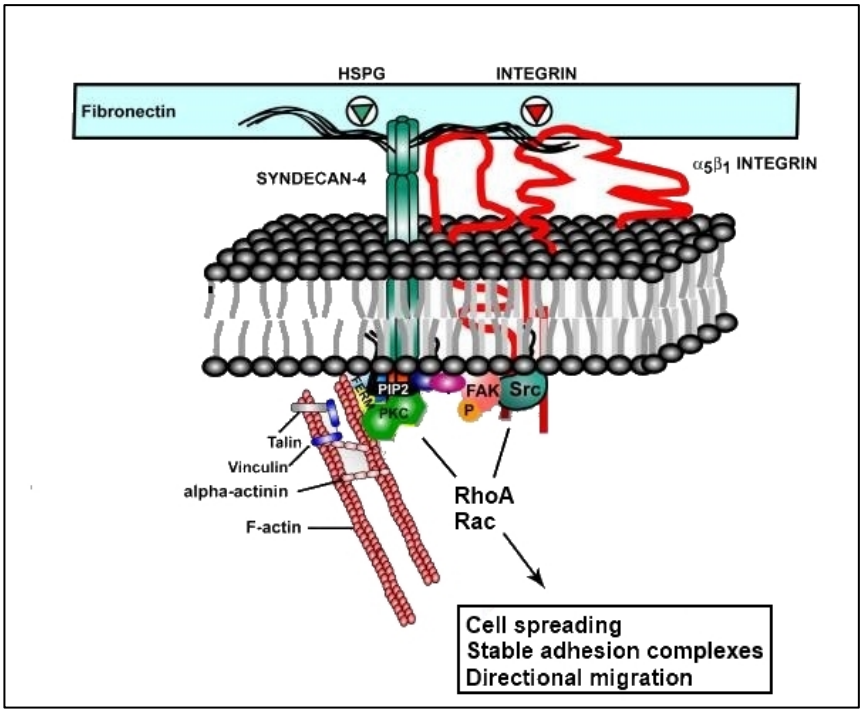


Figure 1.11: Syndecan-4 in co-operation with α5β1 provides a mechanical link between the extracellular matrix and the actin cytoskeleton. An adapted version of the figure from Beauvais and Rapraeger, 2004. © 2004 Beauvais and Rapraeger; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL: http://www.rbej.com/content/2/1/3

1.9 Project hypothesis and thesis aims

Hypothesis: ADAMTS-15 is a tumour suppressor gene within the context of breast cancer.

An array of *in vitro* and *in vivo* approaches will be utilised to determine the functional consequences of the expression of ADAMTS-15 within the context of breast cancer disease progression.

1. Characterisation of appropriate myoepithelial and epithelial breast cancer cell lines to establish *in vitro* models for the investigation of ADAMTS-15 in breast cancer.
2. Investigate the effects of ADAMTS-15 expression on breast cancer cell behaviour in an array of *in vitro* cell assays, including: aggrecanase, proliferation, adhesion and migration assays.
    a. Determine the mechanism(s) underpinning any observed ADAMTS-15 tumour suppressive effects.
    b. Discern whether ADAMTS-15 metalloproteinase activity is required for the tumour suppressive actions of ADAMTS-15 in breast cancer.
3. Determine if ADAMTS-15 modulates angiogenic processes.
4. Investigate the consequences of ADAMTS-15 expression on *in vivo* tumour development and metastasis.

Chapter Two: Materials and Methods

2.1 Buffers

Buffer	Contents
Aggrecanase Assay Buffer	50mM Tris-HCl (pH 7.5), 150 mM NaCl; 10mM $CaCl_2$; 0.05% (v/v) Brij-35
Blocking / Antibody Buffer	5% (w/v) half-fat milk or 5% (w/v) BSA in PBS (or TBS) with or without 0.02% (v/v) Tween-20
Coating Buffer	0.1M $NaCO_3$, (pH 9.6)
Coomassie Blue	45% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.25% (w/v) Coomassie Brilliant Blue G-250 (Fluka), dH_2O
Coomassie Blue (De-stain)	20% (v/v) methanol, 5% (v/v) glacial acetic acid, 75% (v/v) dH_2O.
Lysis Buffer	50mM Tris-HEPES pH 7.8, 150mM NaCl and 1% (v/v) Triton X-100 detergent supplemented with protease inhibitor (Roche, Hertfordshire, UK; #11836170001)
Methylene Blue	1% (w/v) Methylene Blue, 10mM borate buffer (pH8.5), 50% (v/v) Methanol.
Methylene Blue (De-stain)	50% Ethanol, 50% 0.1M HCl
PBS (1x)	Dissolve in 1 litre of dH_2O: 8g of NaCl, 0.2g of KCl, 1.15g of Na_2HPO_4, 0.2g of KH_2PO_4, (pH 7.4)
Ponceau S Stain	5% (w/v) Acetic acid, 0.1% (w/v) Ponceau S in dH_20
TAE (Tris/Acetate/EDTA)	50x Stock: 2M Tris-HCl, 1M glacial acetic acid, 100ml 0.5M EDTA, made up to 1L with dH_2O. TAE (1x): 1:50 dilution of stock in dH_2O.
Tris-buffered saline (TBS) (1x)	50mM Tris-Cl (pH 7.5), 150mM NaCl
trypsin-EDTA (TE)	10mM Tris-HCl, 1mM EDTA, (pH 8.0)

Washing Buffer	1x PBS, 0.1% (v/v) Tween-20
Western Blotting Sample Buffer	125mM TRIS-HCl, 5% SDS, 15% Glycerol, Bromophenol Blue, (pH 6.8)

Table 2.1: List of buffers and their contents

2.2 Reagents

Reagent	Description	Catalogue No.
ADAMTS-15 siRNA	ON-TARGET plus SMART pool	Thermo scientific #L-005766-00-0005
BCA	BCA protein assay kit	Thermo scientific #PN23227
Collagen Type I	Rat tail type I, sterile solution 100ml for gels.	First Link UK Ltd. #60-30-810
Collagen Type I	Collagen, Type I solution from rat tail.	Sigma #C3867
Control siRNA	ON-TARGET plus Non-targeting siRNA	Thermo scientific #D-001810-01-05
ECL	Pierce ECL WB Substrate	Thermo scientific #32106
EGF	epidermal growth factor 1mg	Sigma #E4127
FGF-2	Basic fibroblast growth factor	PeproTech
Fibronectin	Fibronectin from bovine plasma	Sigma #F1141
HyClone Water	HyPure Cell Culture Grade Water	Thermo Scientific #SH30529.02
Insulin	insulin 10mg	Sigma #10516
Laminin	Laminin from human placenta sterile filtered	Sigma Aldrich #L6274-.5MG
LipoD293	DNA In Vitro Transfection Reagent	SignaGen Laboratories #SL100668
Lipofectamine	RNAiMAX Reagent	Life technologies #13778-100
Matrigel	BD Matrigel Basement membrane Matrix	BD Biosciences #356234
Milk	Blotting grade milk	Bio Rad #170-6404

MM	TaqMan master mix universal 5x5ml	Applied Biosystems #4440048
NEAA	Non-essential amino acids	Sigma #M7145
Oligofectamine	Transfection reagent	Life technologies #12252-011
OPTIMEM	with Glutamax	Life technologies #51985
ProtoGel	Acrylamide 30% w/v stock solution 37.5:1	National Diagnostics #EC-890
RNAzol	RNAzol B	Amsbio #CS105B
ROCK inhibitor	Y-27632	Sigma #Y0503-1MG
Sodium Pyruvate	TC supplement	Sigma #S8636
SuperScript II	Reverse Transcription	Life technologies #18064-022
Syndecan-4 siRNA	ON-TARGET plus SMART pool	Thermo scientific #L-003706-00-0005
Tween-20	Tween 20 sigma ultra	Sigma #P7949-500ML
VEGF	vascular endothelial growth factor	PeproTech
Phalloidin	Alexa Fluor 568 Phalloidin	Life technologies #A12380
SV Total RNA Isolation Kit	RNA extraction kit.	Promega #Z3105

Table 2.2: List of experimental reagents

2.3 Cell culture

Cells were generally cultured at 37^{o}C and 5% CO_2 in T75 tissue culture flasks (Nunc) containing 12ml of culture media as described below. Cells were passaged upon reaching 80% confluency and were routinely tested for mycoplasma. Cell lines were also validated via 'SNP barcodes' (Demichelis *et al.* 2008) to confirm that the correct cell types were being used.

Cell Line	Description	Culture Media
MDA-MB-231	Epithelial (more mesenchymal-like) human breast adenocarcinoma with an invasive phenotype (Lacroix and Leclercq, 2004).	DMEM (Invitrogen, Paisley, UK, #21885) supplemented with 10% FCS and non-essential amino acids.
MDA-MB-231 **EV / TS15 / E362A**	MDA-MB-231 cells constitutively expressing empty vector (EV), wild type ADAMTS-15 (TS15) or metalloproteinase inactive ADAMTS-15 (E362A).	As above plus selection antibiotic G418 (500µg/ml of culture media).
MCF7	Epithelial-like human breast adenocarcinoma (Lacroix and Leclercq, 2004)	DMEM (Invitrogen #21885) supplemented with 10% FCS and non-essential amino acids.
MCF7 **EV / TS15 / E362A**	MCF7 cells constitutively expressing empty vector (EV), wild type ADAMTS-15 (TS15) or metalloproteinase inactive ADAMTS-15 (E362A).	As above plus selection antibiotic G418 (400µg/ml of culture media).
HCT-116	Epithelial -like human colorectal carcinoma (Viloria *et al.* 2009).	McCoy's 5A (Invitrogen #36600-021) + 10% FCS
1089-β4 / 1089-β6	Myoepithelial –like (Jones *et al.* 2009)	Ham's F12 Plus media (Sigma #N4888) with 10% FCS, L-Glutamine (2mM), Hydrocortisone (1µg/ml), insulin (1µg/ml) and EGF (100µg/ml).
HUVEC (Primary Cells)	Primary human umbilical vein endothelial cells (HUVECs) (Cooley *et al.* 2010).	Large Vessel Endothelial Cell Basal Medium (TCS Cell Works #ZHM-2951) +2% FCS

Table 2.3: Cells and culture conditions.

2.4 ADAMTS-15 constructs

To investigate the role of ADAMTS-15 in *in vitro* models of breast cancer several ADAMTS-15 constructs were generated by Dr. Laura Wagstaff. In addition to full length ADAMTS-15, a metalloproteinase inactive form of ADAMTS-15 (E362A) and several truncated ADAMTS-15 constructs (ΔA, ΔB, and ΔC) which lack C-terminal domains were also generated. These constructs were PCR cloned into pcDNA 3.2 plasmid (Invitrogen #K2440-20). A FLAG tag was also cloned in-frame into the C-terminal sequence of all ADAMTS-15 constructs to enable purification and detection of ADAMTS-15 protein.

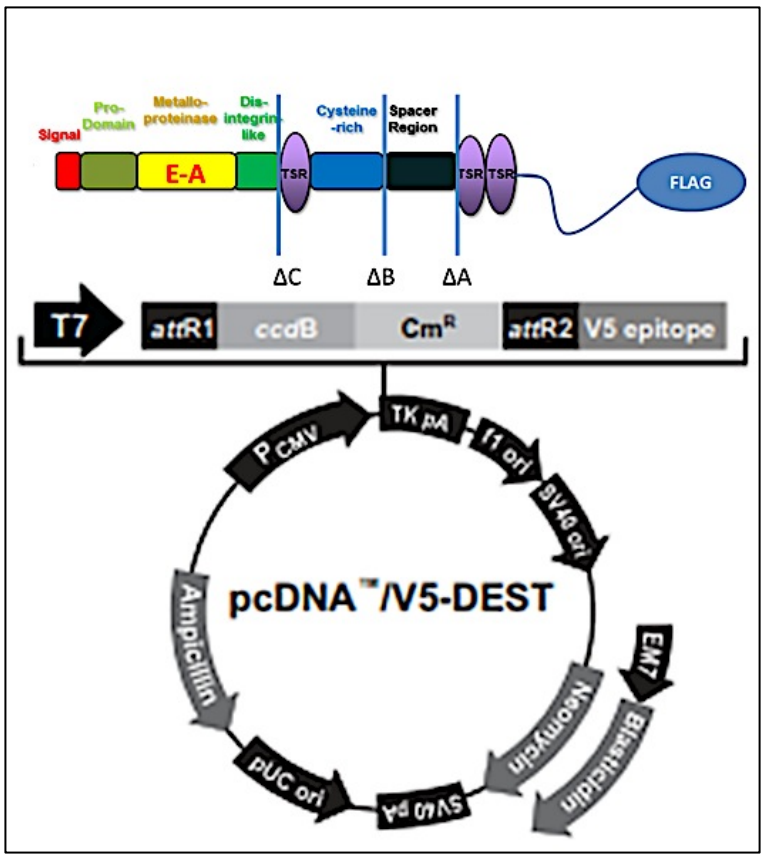


Figure 2.1: ADAMTS-15 constructs and pcDNA3.2 plasmid map. In total six constructs were generated: *wildtype* ADAMTS-15 (TS15), a metalloproteinase inactive form of ADAMTS-15 (E362A) and three truncation constructs (ΔA, B, C) that lack the c-terminal domains to the right of the indicated blue line. A FLAG tag was cloned in frame into the C-terminal sequence of all ADAMTS-15 constructs to enable purification and detection of ADAMTS-15 protein.

2.4.1 Constitutive expression

ADAMTS-15 constructs were established in several breast cancer cell lines; MDA-MB-231 cells and MCF-7 cells. The constructs were cloned into the pcDNA3.2 backbone and were transfected according to manufacturer's guidelines (Invitrogen #12489-019) into the cell lines for stable constitutive expression by Dr. Laura Wagstaff. Polyclonal pools were used in all experiments and antibiotic selection (G418) was utilised to select for overexpressing cells.

2.5 Cell growth assay

MDA-MB-231 EV / TS15 / E362A cells were seeded into 24 well plates at a density of 10,000 cells / well in 1ml of serum containing media. Cells were left overnight at 37^{o}C / 5% CO_2 to adhere. The next day the wells were washed with DPBS and changed to either 1ml of serum containing media (10% FCS) or 1ml of serum free media (0% FCS). At days 2, 3 and 4 post seeding cells were trypsinised, suspended and counted using a haemocytometer to determine total cell number. Four wells were counted for each cell line at each time point. The data was averaged to determine cell growth curves.

2.6 Cell viability: MTT assay

The MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was utilised to determine cell viability (van Meerloo *et al.* 2011). Cells were seeded into 96 well plates and cultured for three days. MTT treated cells (0.5mg/ml final concentration) were cultured for 4 hours at 37^{o}C before Absorbance of the DMSO solubilised formazan product was measured at 550nm with a plate reader (ThermoMAX, Molecular Device). Cell number was

optimised for each cell line used and wells containing MTT substrate only were used as a negative (background signal) control.

2.7 Aggrecanase assay

ADAMTS-15 and E362A were partially purified from serum-free conditioned media of heparan-treated (100 µg/ml) MDA-MB-231 TS15 and E362A cultures by affinity chromatography on anti-FLAG Sepharose. Bovine Aggrecan (0.5 mg/ml) was incubated with either purified ADAMTS-5 (2nM; domain deletion mutant consisting of catalytic, disintegrin and thrombospondin 1 domains), ADAMTS-15 (~0.6 nM) or E362A mutant ADAMTS-15 (~0.6 nM) in 700µl of aggrecanase assay buffer (Table 2.1) with or without N-TIMP-3 (200 nM) for the indicated time at 37 °C. Assay samples were de-glycosylated using chondroitinase ABC and keratanase (each 0.01 U / 10 µg of aggrecan) at 37°C overnight as previously described (Kashiwagi *et al.* 2004). De-glycosylated samples were analysed by SDS PAGE and western blotted using aggrecan neo-epitope antibody (anti-AGEG), which recognizes the N-terminal AGEG generated by aggrecanase cleavage of bovine aggrecan at Glu1771-Ala1772 (Troeberg *et al.* 2008).

2.8 Plasmid DNA transient transfection

Cells were seeded into tissue culture treated 6 well plates and grown to 90% confluency. LipoD293 transfection reagent was utilised in all plasmid DNA transient transfection experiments. Transfection mixes containing 50µl serum free media and 3µl LipoD293 and DNA mixes containing 50µl serum free DMEM and 1µg plasmid DNA for each well were prepared. Transfection and DNA mixes were pipetted into the same tube and then mixed by tapping the

tube. The transfection/DNA mix was incubated for 15 minutes at room temperature. During the incubation steps cells were washed twice with DPBS and 900µl serum free media. 100µl transfection/DNA mix was added to each well. Media was replaced 6 hours post transfection with either 1ml serum free media or 1ml serum containing media.

2.9 siRNA transfection

MDA-MB-231 cells were seeded into 6 well plates at a density of 200,000 cells / well in 1ml of serum containing media. Cells were left overnight to adhere at 37^{o}C / 5% CO_2. The next day media was removed from each well and then washed with OPTIMEM. 800µl of OPTIMEM was then added to each well. Transfection master mixes were then created containing 5µl Oligofectamine, 194µl OPTIMEM and 1µl ADAMTS-15 siRNA (10nM final concentration) per well. 200µl of transfection master mix was added to relevant wells. At 4 hours post siRNA transfection 500µl / well of serum containing media was added.

2.10 Adhesion assay

Tissue culture treated 96 well plates were coated with either Collagen type I, Laminin or Fibronectin at a final concentration of 5µg/ml. Matrix components were diluted to the final concentration in coating buffer (Table 2.1) and left overnight at 4ºC.

Matrix coated wells were then washed twice with DPBS (1x) and blocked in 1% bovine serum albumin/DPBS (w/v) for 30 min at 37ºC. MDA-MB-231 cells were seeded at a density of 60,000 cells / well and MCF7 cells were seeded at a density of 100,000 cells / well in 100µl of serum free DMEM.

Seeded plates were spun at 193 x g for 1 min and then incubated for 30 min at 37ºC and 5% CO_2. Media was aspirated off and wells were carefully washed with 100µl DPBS to remove any non-adherent cells. Adhered cells were fixed and stained for 30 minutes at room temperature with a Methanol/Methylene Blue solution (Table 2.1). Plates were rinsed with dH_2O and then 100µl Methylene Blue de-stain (Table 2.1) was added to each well. 10 minutes post-addition of de-stain, plates were read in a micro-plate reader (ThermoMAX, Molecular Device). The absorbance of stained adhered cells was determined at 630 nm. These assays are based upon Oliver *et al.* 1989.

2.11 2D Cell migration assays

MDA-MB-231 or MCF7 cells were seeded into 24 well plates at a density of 10,000 cells/well with serum containing media and left overnight to adhere at 37^{o}C / 5% CO_2. Then each well was washed with DPBS and replaced with 1ml serum free media. For some assays cells were treated with EGF (0-100 ng/ml), Phorbol myristate acetate (PMA) (0-100nM) or bis-indolylmaleimide I (BIM I) (0-1µM). 2D cell migration was analysed as described in section 2.11.4. 2D cell migration assay methods reviewed by Ashby and Zijlstra, 2012.

2.11.1 Matrix migration assay

Tissue culture treated 24 well plates were coated with either Collagen type I, Laminin or Fibronectin at a final concentration of 5µg/ml. Matrix components were diluted to the final concentration in coating buffer (Table 2.1) and left overnight at 4ºC. Matrix coated wells were then washed with DPBS (1x) and blocked in 1% bovine serum albumin/DPBS (w/v) for 30 min at 37ºC.

Wells were washed again with DPBS and 10,000 MDA-MB-231 cells were seeded in1ml of serum free media per well. Seeded plates were spun down in a centrifuge at 193 x g for 1 minute. Time-lapse microscopy 2D Migration was tracked as described in section 2.11.4.

Time-lapse images, taken at 4 hours post seeding were also utilised to determine cell spreading on each matrix. The polygon tool in Image J (http://rsbweb.nih.gov/ij/) was utilised to determine the spreading area (pixel area) of individual cells.

2.11.2 Conditioned matrix assay

100,000 MDA-MB-231 EV / TS15 / E362A cells were seeded into 12 well plates with serum containing media and were cultured for 3 days to reach 100% confluency. Wells were washed with DPBS and cells were removed with EDTA/EGTA (5mM) to preserve the underlying conditioned matrices. 20,000 MDA-MB-231 naïve EV cells were then seeded onto each conditioned matrix and migration was tracked via time-lapse microscopy for 24 hours.

2.11.3 siRNA treated migration assay

MDA-MB-231 cells were seeded into 6 well plates at a density of 25,000 cells / well in 1ml of serum containing media. Cells were left overnight to adhere at 37^{o}C / 5% CO_2. The next day media was removed from each well and then washed with OPTIMEM. 800µl of OPTIMEM was then added to each well. Transfection master mixes were then created containing 5µl Oligofectamine (Invitrogen #12252-011), 194µl OPTIMEM and 1µl siRNA (10nM final concentration) per well. 200µl of transfection master mix was added to relevant wells. At 4 hours post siRNA transfection 500µl / well of serum containing media

was added immediately prior to the start of time-lapse microscopy. 2D Migration was tracked as described below.

2.11.4 Time-lapse microscopy and analysis

2D and 3D migration was tracked with a Zeiss Inverted CCD microscope at 10x magnification and phase contrast illumination. Images were automatically taken every 10min for 24 hours to create time-lapse microscopy videos. Image J and two plugins (Manual Tracking and Chemotaxis Migration Tool) were utilised to manually track individual cells in order to determine average cell velocity (μm/min) and cell directionality.

2.12 3D migration assay

MDA-MB-231EV / TS15 / E362A cells were seeded into 12 well plates at a density of 87,500 cells / well. The cells were seeded within 150μl / well of Matrigel or Collagen I. Gels were set for 30 min at 37^{o}C. Once gels were set 250μl serum free media was added per well. The plates were incubated for an additional 60 min at 37^{o}C / 5% CO_2 to allow cells to adhere. Migration was tracked as described above, except multiple Z planes for each XY position were also tracked.

2.13 Scratch assay

MDA-MB-231 cells were seeded at high density (450,000 cells / well) into 12 well plates with 1ml serum containing media and left overnight to adhere. The next day scratches were generated with a pipette tip. Wells were then washed with 1ml DPBS before the addition of 1ml / well of serum containing media. Scratch recovery was tracked via time-lapse microscopy, with images automatically taken every 10min for 24 hours on a Zeiss Inverted CCD

microscope. Image J software was utilised to determine scratch wound closure (% of initial wound area that has recovered) at 12h and 24h post scratch. Assay reviewed in Ashby and Zijlstra, 2012.

2.14 RNA extraction

Cells were cultured to 80%-90% confluency in a T75 Flask. Media was removed; cells were washed twice with DPBS and then scrapped into 5ml DPBS using a cell scrapper (Fisher #FB55199). Cells were suspended into DPBS and then centrifuged in a 15ml tube (193 x g, 5min). PBS was removed and then the cell pellet washed in PBS. Cells were then centrifuged (193 x g, 5min); PBS removed and re-suspended in 1ml RNA BEE (Amsbio #CS105B). RNA was extracted using SV Total RNA Isolation Kit. A Nano-drop (NR-1000, Labtech) was utilised to determine sample RNA concentration. RNA absorbance was measured at 260nm and the absorbance ratios of 260nm/280nm (typically used to identify contaminants that absorb at 280nm, such as proteins or phenols) and 260nm/230nm (typically utilised to identify contaminants that absorb at 230nm, such as carbohydrates and phenols) were utilised to determine the purity of the RNA samples. RNA samples were stored at -80^{o}C.

2.15 Reverse transcription

RNA samples were reverse transcribed to cDNA according to the Invitrogen superscript II protocol. Typically 1µg of RNA was reverse transcribed per reaction. RNase OUT Inhibitor (40 U/ µl) was added into the reaction mix. Transcribed samples were stored at -20^{o}C.

2.16 Quantitative real time PCR

To determine relative gene expression using mRNA, Quantitative Real Time PCR (qRT-PCR) was utilised. Primers and probes were designed to be specific for each gene. Reaction volumes were 20µl and contained 1-5ng of sample cDNA, 50% 2 x Fast master mix (v/v), 200 nM of primer and 100 nM of Universal Probe. Reaction cycles were programmed to follow: 2 minutes at 50^{o}C, 10 minutes at 95^{o}C then 40 cycles of 95^{o}C for 15 seconds / 60^{o}C for 1 minute. Data was analysed using 7500 SDS Software (Applied Biosystems).

The Comparative CT method (Schmittgen and Livak, 2008) of relative quantitation was utilised to determine the results. All samples were normalised to 18S.

2.17 Collection of cell lysates

Cells were washed and scrapped into 5ml DPBS before being centrifuged at 2000 x g for five minutes. Cell pellets were lysed with lysis buffer (Table 2.1).

2.18 Collection of cell derived matrix

MDA-MB-231 EV / TS15 / E362A cells were seeded into 6-well tissue culture plates. Cells were cultured in 1ml serum free media/well until cell confluency reached 80%. The cell media was removed and then the cell layer was washed and detached with 1ml/well of 5mM EDTA / 5mM EGTA in DPBS. Matrices were scrapped into sample buffer and analysed via SDS-PAGE gel electrophoresis and western blotting to detect ADAMTS-15.

2.19 TCA precipitation of conditioned media samples

MDA-MB-231 EV / TS15 / E362A cells cultured in 6-well tissue culture plates were grown to 80% confluency in 1ml of serum free media to generate conditioned media. Samples were stored at -20^{o}C. To concentrate protein content, samples were precipitated with 10% (trichloroacetic acid) TCA/dH_2O (v/v) at a ratio of 1:3 (sample: 10%TCA) on ice for 4 minutes. Samples were then centrifuged at 10 000 g, for 4 minutes at 4^{0}C. Pellets were washed twice with ice-cold acetone and centrifuged again at 10 000 g, for 4 minutes at 4^{0}C. Supernatant was removed, pellets were then air dried and re-suspended in sample buffer (Table 2.1). Samples were then used for SDS-PAGE gel electrophoresis and western blotting to detect ADAMTS-15.

2.20 Determination of total protein

The bicinchoninic acid (BCA) assay was utilised to determine the total protein concentration of cell lysates. The assay was carried out according to manufacturers' guidelines. Protein samples were then analysed via SDS PAGE and Western Blot analysis.

2.21 SDS PAGE gel electrophoresis

Typically protein samples (30µg) in loading buffer were reduced with ß-mercaptoethanol (β-ME) and loaded into 10% Sodium dodecyl sulfate polyacrylamide (SDS) gels as described according to Cleveland *et al.* 1977. Protein samples were separated for one hour at 200v, alongside a protein molecular mass marker (dual colour).

2.22 Antibodies

Target / Description	Species / Application	Company	Dilution / Dilution Buffers
Primary Antibodies			
Anti-FLAG M2	Mouse / WB	Stratagene 200472-21	1:500 / PBST-Milk
Anti-MMP-14, catalytic domain, clone LEM-2/15.8	Mouse / FC	Millipore MAB3328	1µg / PBS-BSA
Cytokeratin 14	Mouse / WB	Abcam ab9220	1:1000 / PBST-Milk
Cytokeratin 18	Mouse / WB	Cell Signaling 4548	1:2000 / PBST-Milk
DYKDDDDK Anti-Flag	Rabbit / IF	Cell Signaling 2368P	1:800 / PBST-BSA
E-Cadherin	Rabbit / WB	Cell Signalling 4065	1:1000 / PBST-Milk
GAPDH – HRP Conjugated	Rabbit / WB	Cell Signalling 3683	1:2000 / PBST-Milk
Integrin α6	Rabbit / WB	(Clone U21) Gift from Professor Uli Mayer	1:1000 / PBST-BSA
Integrin αV	Rabbit / WB	Millipore AB1930	1:5000 / PBST-Milk
Integrin β1	Mouse / WB	AbD Serotec MCA1189	1:1000 / PBST-Milk
Integrin β1 (12G10)	Mouse / IF	Ab cam ab30394	1:500 / PBST-BSA
Integrin β1 (APC Conjugated)	Mouse / FC	BD 559883	20ul / / PBS-BSA
Integrin β4	Rabbit / WB	Cell Signaling 4707	1:1000 / TBST-BSA
Integrin β4	Mouse / FC	Millipore MAB1964	1:500 / PBS-BSA
p44/42 MAPK (Erk1/2)	Rabbit / WB	Cell Signaling 9102	1:1000 / TBST-BSA

Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit / WB	Cell Signaling 9101	1:1000 / TBST-BSA
Syndecan-4 (5G9)	Mouse / FC	Santa Cruz sc-12766	1µg / PBS-BSA
Vimentin	Rabbit / WB	Cell Signalling 3932	1:1000 / PBST-Milk
β-Catenin	Rabbit / WB	Cell Signalling 9587	1:1000 / PBST-Milk
Secondary Antibodies			
Donkey Anti-Mouse Alexa 488	/ FC	Invitrogen A21202	1µg / PBS-BSA
Allophycocyanin Goat Anti-Mouse	/ FC	Invitrogen A-865	1µg / PBS-BSA
Mouse IgG1	/ FC	Invitrogen MG100	1µg / PBS-BSA
Mouse IgG2a	/ FC	Invitrogen MG200	1µg / PBS-BSA
Goat anti-Rabbit (FITC)	/ IF	Sigma	1µg / ml PBS-BSA
Rabbit anti-Mouse Alexa Fluor 488	/ IF	Invitrogen	2µg / ml PBS-BSA
Rabbit anti-Mouse (HRP)	/ WB	Dako P0260	1:1000 / PBST or TBST-Milk
Goat anti-rabbit (HRP)	/ WB	DAKO P0448	1:1000 / PBST or TBST-Milk

Table 2.4: Antibodies used for Western Blot, Immunofluorescence and Flow Cytometry experiments.

2.23 Western blot

Protein samples were transferred by the semi-dry method from the gels onto a nitrocellulose membrane at 100mA for one hour. Membranes were then Ponceau S stained (Table 2.1) to confirm that the protein samples had successfully transferred. Membranes were then blocked for one hour in the appropriate blocking buffer (Table 2.1). Application of the primary antibody (Table 2.4), typically diluted 1:1000 into antibody dilution buffer (Table 2.1) was either for one hour at room temperature or sixteen hours overnight at 4^{o}C. Membranes were washed with washing buffer (Table 2.1) three times for ten minutes on a shaker before application of the secondary antibodies. Application of the secondary antibody (Table 2.4) typically diluted (1:1000) into antibody dilution buffer was for one hour at room temperature. Membranes were then washed with washing buffer three times for ten minutes on a shaker and ECL was applied according to manufactures instructions. Protein bands were imaged and detected using a Fuji Film (LAS-3000) system. Method reviewed by Bolt and Mahoney, 1997.

2.24 R&D phosphokinase array

MDA-MB-231 EV/ TS15 / E362A cells were grown to 80% confluency in a T75 (nunc) tissue culture flask. Cells were washed and scrapped into 5ml DPBS before being centrifuged at 2000g for five minutes. Cell pellets were lysed with the lysis buffer supplied with the R&D kit (R&D Systems #ARY003) according to manufactures guidelines. Cell lysate samples were BCA analysed to determine the total protein concentration of each extract as described in methods section 2.20.

100µg of cell lysate per sample was utilised in the R&D Phosphokinase array and the array was carried out according to manufacturers' guidelines (R&D). Membranes were imaged with Fuji Film imager (LAS-3000) and images were analysed with Image J according to manufacturers' guidelines.

2.25 Immunohistochemistry

300,000 cells were seeded onto coverslips, placed into tissue culture treated 6-well plates. Cells were incubated overnight in 1ml of serum containing media at 37^{o}C and 5% $CO_{2.}$ The next day coverslips were washed twice in DPBS at 37ºC prior to fixation. Methods reviewed Odell and Cook, 2013.

2.25.1 Fixation and antibody staining

Cells on coverslips were fixed with 4% paraformaldehyde/DPBS (w/v) at room temperature for 10 min. Once fixed cells were washed twice with DPBS and then blocked with immunofluorescence blocking buffer (1% bovine serum albumin BSA in DPBS (w/v)) for 30 minutes at room temperature. Cells were then incubated with primary antibodies (Table 2.4) diluted in 1% BSA/DPBS (w/v) for 60 minutes at room temperature. Cells were then washed three times with blocking buffer before 60 minute incubation in the dark with secondary antibody (Table 2.4) diluted in 1% BSA/DPBS (w/v). Cells were then washed twice with blocking buffer (as described above). Coverslips were mounted onto slides with ProLong Gold-DAPI.

2.25.2 Phalloidin staining

Cells were seeded and fixed with 4% paraformaldehyde/DPBS (w/v) as described above. Once fixed cells were washed twice with DPBS and then blocked with blocking buffer (1% bovine serum albumin BSA in DPBS (w/v)) for

30 minutes at room temperature. Cells were washed twice with DPBS and stained in the dark with 0.2 U Phalloidin in DPBS for 20 minutes at room temperature. Cells were then washed twice with blocking buffer and Coverslips were mounted onto slides with ProLong Gold-DAPI.

2.25.3 Imaging

Images were taken using either a charge-coupled device (CCD) upright microscope or a LSM Confocal Microscope (Carl Zeiss). Images were analysed and fluorescence intensity determined with Axiovision 4.7.1 software or Zeiss LSM Examiner 4.0 software.

2.26 Flow Cytometry

All flow cytometry experiments were carried out using an Acruri C6 (BD Biosciences) and analysed with BD Acuri C6 Software or FlowJo. A gating strategy was utilised to identify events which correspond to single live cells (doublet discrimination, FL2A:FL2W; Wersto *et al.* 2001).

2.26.1 Cell cycle analysis

50,000 MDA-MB-231 EV / TS15 / E362A cells were seeded into tissue culture treated 6 well plates and cultured for three days in serum containing media at 37^{o}C and 5% $CO_{2.}$ Media was removed and wells were washed with DPBS. Cells were trypsinised and suspended in 600µl of serum containing media. Cells were then spun down at 2000g for 5 minutes and the cell pellet was washed with DPBS. Cells were then fixed with 70% ethanol in DPBS (v/v) and incubated for 2 hours at 4^{o}C. Cells were washed with DPBS and spun down at 2000g for 5 minutes. Cell pellets were re-suspended and stained with propidium iodide solution (5µg PI, 10µg RNAase A in 1ml DPBS). Samples were analysed with a

flow cytometer. Events were gated as described above and readings were taken with FL2-A. Cell cycle analysis was performed using FlowJo and the Watson Pragmatic Model (Watson *et al.* 1987).

2.26.2 Cell surface protein detection

MDA-MB-231 EV / TS15 / E362A cells were scrapped into ice cold DPBS. Cells were centrifuged at 2000g for five minutes and then cell pellets were re-suspended and fixed in 4% PFA for 10 minutes at room temperature. Cells were then washed twice via centrifugation and re-suspended with 100µl antibody blocking buffer and incubated for ten minutes at room temperature. Primary or isotype control antibodies (Table 2.4) were added at the appropriate dilution and incubated for one hour. Cells were washed twice as described before and re-suspended in 100µl antibody blocking buffer (Table 2.1). Secondary antibody (Table 2.4) was applied at the appropriate dilution and incubated for 30 minutes. Cells were washed via centrifugation as described before and re-suspended in DPBS. Samples were analysed with a flow cytometer. Events were gated as described above and the appropriate filter for each fluorochrome-conjugated secondary antibody was used. Assay based upon protocols described by Beauvais *et al.* 2004.

2.27 Angiogenesis Assays

2.27.1 Generation of ADAMTS-15 conditioned media

300,000 MDA-MB-231 cells constitutively expressing either empty vector (EV), wild type ADAMTS-15 (TS15) or metalloproteinase inactive ADAMTS-15 (E362A) were seeded into T75 tissue culture treated flasks (nunc) with serum containing media. The next day cells were washed and HUVEC media with 2%

FCS was applied. HUVEC media was conditioned for 48 hours before being used in HUVEC 3D tube formation assays.

2.27.2 HUVEC 3D tube formation assay

Primary human umbilical vein endothelial cells (HUVECs) when cultured in 3D Collagen Type I gels spontaneously form tube like structures (Cooley *et al.* 2010). This and similar assays are used as *in vitro* models of angiogenesis.

HUVECs were routinely grown on flasks coated with 60µg/ml Collagen type I and were used before reaching the sixth passage for all experiments. For the 3D tube formation assay HUVECs ($1.25x10^6$ cells/ml) were suspended into Collagen gels (1.6 mg/ml) and seeded into 24-well plates. Collagen gels were prepared using rat-tail Collagen Type I solution (BD Biosciences) mixed with 10× 199 medium (Sigma), VEGF (25ng/ml) and FGF-2 (25ng/ml). Collagen gels were then polymerised by adjusting the pH to 7.5 using sodium hydroxide (1N). The process of polymerisation was carried out at 37°C and 5% (v/v) CO_2 for 30 minutes. ADAMTS-15 conditioned media (MDA-MB-231 EV / TS15/ E362A) was added to appropriate wells. Positive control wells were cultured with serum containing HUVEC media supplemented with VEGF (25ng/ml) and FGF-2 (25ng/ml). At 24 hours post seeding into collagen gels, wells were washed with DPBS and fixed with 4% PFA. Gels were then stained with Phalloidin and DAPI. Images were taken using a charge-coupled device (CCD) upright microscope (Zeiss). Images were focused at multiple Z-axis focal plane using DAPI staining as a guide. Phalloidin staining of HUVEC F-actin cytoskeleton was used to identify HUVEC tube like structures. Fluorescence from background signals and non-tube like structures (e.g. cell debris) was removed from the analysis.

2.27.3 Aortic ring assay

Thoracic aortae were isolated from 6 to 8 week-old mice and prepared for embedding in collagen type I matrix as described in Baker *et al.* 2012. Aortic rings were treated with MDA-MB-231 EV, TS15 or E362A conditioned media as described in 2.27.1 and VEGF (30ng/ml). Six days later, rings were fixed and the number of micro vessels were quantified under phase contrast.

2.28 Experimental Metastasis: Tail Vein Assay

To determine the effect of ADAMTS-15 expression on the metastatic potential on human breast cancer cells an experimental model of metastasis was utilised. The tail vein assay as described by Elkin and Vlodavsky, 2001 involves the intravenous injection of tumour cells into immune-compromised mice (e.g. CD1 nu/nu mice), enabling circulating tumour cells to spread to the lungs, liver and other organs or tissues.

Prior to the experiment all cell lines were tagged by retroviral transduction with a β-galactosidase marker (lacZ tagged) as previously described in (Krüger *et al.* 1994). $1x10^6$ MDA-MB-231 (EV, TS15 or E362A) cells were suspended into 200µl of DPBS and injected intravenously into the tail vein of CD1 nu/nu mice. Mice were culled 35 days post injection. Lungs, Liver, Bone and Spleen were excised and stained for analysis of micro-metastasis.

2.29 Statistical analysis

In all experiments ADAMTS-15 and E362A transfected breast cancer cell lines were compared with EV (control cells containing empty pcDNA3.2 plasmid). Experimental procedures were repeated independently at least three times and statistical analysis was performed using two-tailed, unpaired t-test. Aortic ring

assay statistics were based upon Mann-Whitney non-parametric μ test. Data bars represent the mean value with error bars indicating s.e.m. Values of *p≤0.05 were considered to be statistically significant.

Chapter Three: Characterisation of ADAMTS-15 Expression Systems

3.1 Introduction

Previous studies have demonstrated that ADAMTS-15 expression inversely correlates with tumour grade in breast cancer, with expression significantly reduced in grade III breast tumours compared to grade I or II tumours (Porter *et al.* 2004). ADAMTS-15 expression also inversely correlates with tumour grade in colorectal cancer (Viloria *et al.* 2009). Another study involving a cohort of 229 Dutch patients showed that relatively high levels of ADAMTS-15 expression were associated with a good prognosis in breast cancer (Porter *et al.* 2006).

Taken together these studies indicate that ADAMTS-15 is protective in breast and colorectal cancer and that an examination of ADAMTS-15 expression may be a clinically useful independent predictor of survival (Porter *et al.* 2004; Porter *et al.* 2006; Viloria *et al.* 2009). However the functional consequences of ADAMTS-15 expression in breast cancer are unknown. To this end, this thesis set out to explore the effects of ADAMTS-15 on human mammary cell behaviour. This chapter outlines the characterisation of human mammary cell lines, and the ADAMTS-15 expression systems that were used in this study.

3.2 Characterisation of human mammary cell culture models

3.2.1 Myoepithelial cells

In healthy adult breast tissue ADAMTS-15 is expressed in myoepithelial cells, which sit in a continuous layer between the luminal epithelial cells and the basement membrane of the ductal lumen (Figure 1.8; Table 2.3; Porter *et al.* 2004). Myoepithelial cells are a combination of muscle (myo) and epithelial-like character that function to secrete luminal milk and to maintain breast tissue architecture (Adriance *et al.* 2005; Butcher *et al.* 2009; Gordon *et al.* 2003). To counteract the tensile forces that breast tissues experience during lactation, myoepithelial cells mediate ECM remodelling processes (Butcher *et al.* 2009; Gordon *et al.* 2003). As part of these remodelling processes myoepithelial cells express an array of factors that also help to maintain luminal epithelial cell apical-basal polarity (Barcellos-Hoff *et al.* 1989; Butcher *et al.* 2009). Several of these factors are potentially tumour suppressive, including MMP-8 and ADAMTS-15 (Decock *et al.* 2008; Gordon *et al.* 2003; Hsiao *et al.* 2011; Jones *et al.* 2003; Polyak and Hu, 2005; Thirkettle *et al.* 2013; Xiao *et al.* 1999).

The role of myoepithelial cells in maintaining breast tissue architecture is particularly important to understand as a subset of aggressive breast cancer tumours express myoepithelial markers such as (α6β4 integrin) and therefore may be of myoepithelial cell-like origin (Gordon *et al.* 2003; Jones *et al.* 2004; Polyak and Hu, 2005; Tan and Ellis, 2013). Even in the majority of breast cancers which are thought to originate from luminal epithelial cells, an absence of a myoepithelial cell population is observed during the progression to invasive disease (Gordon *et al.* 2003; Hsiao *et al.* 2011; Porter *et al.* 2004). Thus the

loss of *wildtype* myoepithelial cells and/or the loss of the protective factors expressed by these cells contribute to the transformation of *in situ* tumour cells into invasive disease (Gordon *et al.* 2003; Hsiao *et al.* 2011; Porter *et al.* 2004).

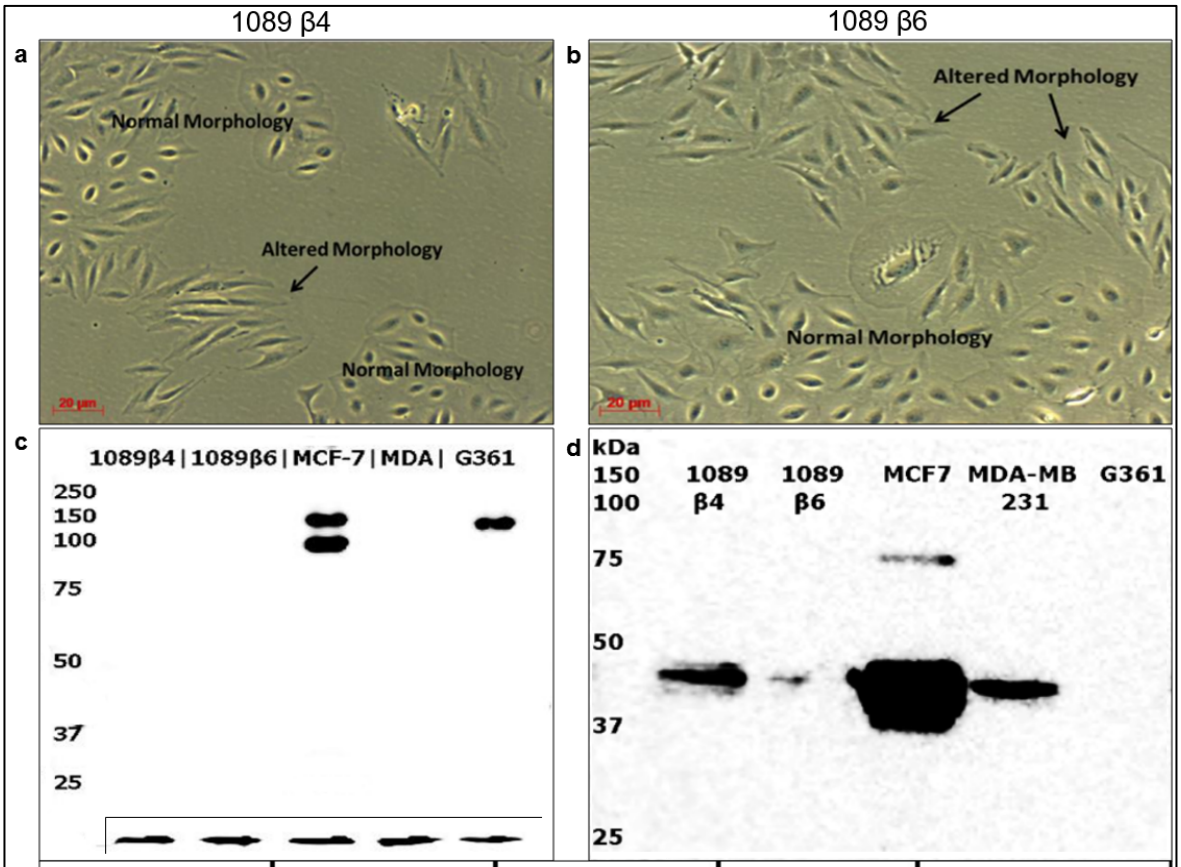
To investigate the functional consequences of ADAMTS-15 expression within a myoepithelial cell population, two cell lines 1089 β4 and 1089 β6 were acquired. These cell lines were kindly gifted to our lab from Professor Louise Jones, Queen Mary University of London. 1089 β4 and β6 cells are immortalised myoepithelial cell lines and both express integrin β4. However the 1089 β6 cell line has been engineered to over express integrin β6. Integrin β6 is up-regulated in myoepithelial cells in high grade DCIS (Jones *et al.* 2009). Thus the original myoepithelial cell line (1089) is an *in vitro* model of *wildtype* cells and the 1089 β6 is more DCIS-like.

Myoepithelial cells and luminal epithelial cells both express several markers including E-cadherin, integrin β1, α2, α3, and α6, though α6 is expressed at lower levels in luminal epithelial cells compared to myoepithelial cells (Gordon *et al.* 2003). However there are some differences, which enable luminal cell populations to be distinguished from myoepithelial cells and vice versa. Integrin β4, Vimentin, CK14, tenascin, and α-smooth muscle actin are all expressed in myoepithelial cells and are not expressed in luminal epithelial cells (Gordon *et al.* 2003). Likewise in contrast to myoepithelial cells, luminal epithelial cells express CK18 (Gordon *et al.* 2003).

To characterise 1089 β4 and 1089 β6 cell lines several cell markers were selected and analysed via western blot (Figure 3.1; Methods 2.17; 2.20-2.23). However these cell lines were prone to phenotypic drift with changes in marker

profiles and cell morphology occurring soon after entering culture (Figure 3.1). Both 1089 β4 and 1089 β6 cell lines displayed altered morphology from a 'cobble stone' like appearance to a more elongated fibroblastic like appearance (Figure 3.1). In concurrent to these morphological changes the cell lines lost E-cadherin and CK14 expression and occasionally gained CK18 expression (Figure 3.1).

The dynamic phenotype of the myoepithelial cell lines were determined to be inappropriate for experiments designed to investigate the long term consequences of ADAMTS-15 expression. It would have been difficult to determine and distinguish the effects of ADAMTS-15 expression from those that were due to phenotypic drift. Therefore alternative *in vitro* models of breast cancer were utilised.



e

	1089 β4		1089 β6		MCF-7		MDA-MB-231	
	LJ	DE	LJ	DE	LJ	DE	LJ	DE
E-Cadherin	+	+?	+	+?	+	+	-	-
CK18	-	+	-	-	+	+	+	+
CK14	+	-	+	-	-	-	+/-	-

Figure 3.1: Myoepithelial cells display a dynamic phenotype in culture. Myoepithelial cell lines 1089 β4 and 1089 β6 undergo a phenotypic drift in culture. Images (**a, b**) show evidence that the cells display altered morphology and western blot analysis from cell lysates of the expression of cell markers (**c**) E-Cadherin (135kDa), bands at the bottom are GAPDH loading control. (**d**) Cytokeratin 18 (45kDa). (**f**) A summary table compares our results with those from Prof. Louise Jones lab. + = expressed, - = not expressed, +/- = weak expression, LJ = Louise Jones lab result and DE = Dylan Edwards lab result. Numbers in brackets represent the predicted molecular weights of the respective proteins. Cell lysates from MDA-MB-231, MCF-7 and G361 cells were used as controls.

3.2.2 Breast cancer cells

The luminal epithelial cells lie in close proximity to the surrounding layer of myoepithelial cells within the ductal structures of the breast (Figure 1.8; Gordon *et al.* 2003; Hsiao *et al.* 2011; Porter *et al.* 2004). Therefore it is likely that the luminal epithelial cells come into contact with ADAMTS-15, expressed by neighbouring myoepithelial cells (Figure 1.8; Gordon *et al.* 2003; Hsiao *et al.* 2011; Porter *et al.* 2004). And since the majority of breast cancers are thought to originate from an epithelial-like cell it is appropriate to investigate the functional consequences of ADAMTS-15 expression on luminal epithelial cell types. Several well established *in vitro* models of epithelial-like breast cancers exist (Lacroix and Leclercq, 2004). From amongst them, MCF-7 cells and MDA-MB-231 cells, both of which endogenously express ADAMTS-15 were chosen as appropriate models (Figure 3.3; Table 2.3; Table 3.1; Lacroix and Leclercq, 2004).

MCF-7 cells are a luminal epithelial-like, metastatic ductal carcinoma cell line. MCF-7 cells express an array of luminal epithelial markers (e.g. CK18, and progesterone receptor) and *in vivo* tumours derived from these cells display histological features that are representative of those found in patients (Figure 3.1; Table 3.1; Lacroix and Leclercq, 2004). The molecular profiles of MCF-7 cells (ER+/PgR+/ErbB2-) also correlate well with epithelial-like tumours found in patients, though there are some functional differences. MCF-7 derived tumours do not readily grow *in vivo* unless supplemented with oestrogen (Table 3.1; Lacroix and Leclercq, 2004). Though this has proved to be useful as a model for understanding oestrogen dependent tumour growth, and the development of tumour resistance to anti-oestrogen therapies e.g. Tamoxifen (Table 3.1;

Lacroix and Leclercq, 2004). Also whilst cancer cells from patients with a similar molecular profile to that of MCF-7 cells are able to metastasise, in animal models MCF-7 cells are poorly invasive and rarely metastasise (Table 3.1; Lacroix and Leclercq, 2004). However the relatively low invasive potential of MCF-7 cells is useful, particularly as a comparator model against more invasive breast cancer cell lines, such as MDA-MB-231 cells.

MDA-MB-231 cells are an invasive mesenchymal-like, metastatic ductal carcinoma cell line. The morphology and behaviour of MDA-MB-231 cells is overtly different to that of MCF-7 cells (Figure 3.2; Table 3.1). MDA-MB-231 cells appear more elongated and fibroblastic than the more epithelial-like MCF-7 cells (Figure 3.2). Also in contrast to MCF-7 cells, MDA-MB-231 cells are highly invasive and metastatic *in vivo* (Table 3.1; Lacroix and Leclercq, 2004). MDA-MB-231 cells may have originally derived from a luminal epithelial cell type, though it is difficult to determine since these cells have 'turned off' several epithelial markers (e.g. E-Cadherin) and have 'turned on' expression of a variety of mesenchymal markers including Vimentin (Figure 3.1; Table 3.1; Lacroix and Leclercq, 2004). This 'epithelial to mesenchymal transition' (EMT) is thought to be a tumorigenic process in which epithelial cells acquire the morphological and molecular phenotypes associated with invasiveness (Figure 3.2; Table 3.1; Lacroix and Leclercq, 2004). MDA-MB-231 cells are therefore likely to be a de-differentiated cell type and in concordance with their molecular profile (ER-/PgR-/ErbB2-, triple negative) are representative of the basal breast cancer subtype which frequently form poorly differentiated tumours (Grade III) (Table 1.1; Table 3.1; Lacroix and Leclercq, 2004). Indeed MDA-MB-231 cells were originally derived from a patient with a poorly differentiated tumour (Lacroix and

Leclercq, 2004). In order to provide additional characterisation data an expression profile via qRT-PCR was carried out to determine the baseline expression (mRNA) levels of several hyalectanases, syndecans and integrins (figure 3.3; Appendix A7).

	MDA-MB-231	MCF-7
Description	Invasive mesenchymal-like, metastatic ductal carcinoma cell line	Luminal epithelial-like, metastatic ductal carcinoma cell line
Original Tissue	metastasis; pleural effusion	metastasis; pleural effusion
Modal Chromosome number(s)	64, 69–70	88,86,79,65
ER status	-	+
PgR status	-	+
ErbB2 (Her2/neu) status	-	-/+ (low)
Tumourigenicity of cell line in mouse models	Aggressive, highly invasive and high incidence of metastatic spread particularly to the Lungs of nude and SCID mice.	Cells are viable but require estrogenic supplementation for tumourigenesis in nude mice. Cells are also poorly invasive and rarely metastatic in mice.

Table 3.1 Comparison of MDA-MB-231 and MCF-7 human mammary cancer cell lines. Adapted from Lacroix and Leclercq, 2004.

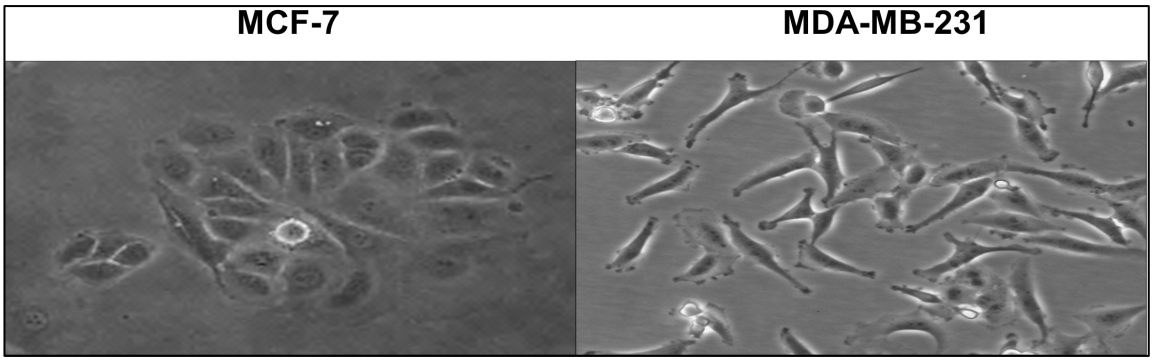


Figure 3.2: Images of MCF-7 cells and MDA-MB-231 cells.

	MDA-MB-231	MCF7
ADAMTS1	26	34
ADAMTS4	21	40
ADAMTS5	36	40
ADAMTS8	40	40
ADAMTS9	26	40
ADAMTS15	29	30
ADAMTS20	37	36
TIMP3	26	29
Syndecan 1	28	26
Syndecan 4	25	26
Integrin β1	25	27
Integrin α5	24	27

Ct 15-25	Very high expression
Ct 26-30	High expression
Ct 31-35	Moderate expression
Ct 36-39	Low expression
Ct <40	Very low / undetectable expression

Figure 3.3: Expression profile of MDA-MB-231 and MCF-7 cells. qRT-PCR analysis of gene expression in MDA-MB-231 and MCF-7 cells. Relative expression levels are shown as a Ct value heat-map. The displayed Ct values are averaged from three independently collected RNA samples. See appendix A7 to see an expanded version of this data set.

3.3 Generation of ADAMTS-15 expressing mammary cancer cell lines

3.3.1 Validation of ADAMTS-15 constructs

To investigate the functional consequences of ADAMTS-15 expression in *in vitro* models (MCF-7 and MDA-MB-231 cells) of breast cancer several ADAMTS-15 constructs were generated by Dr. Laura Wagstaff (Figure 2.1). In addition to full length ADAMTS-15, a metalloproteinase inactive form of ADAMTS-15 (E362A) and several truncated ADAMTS-15 constructs (ΔA, ΔB, and ΔC) which lack C-terminal domains were also generated. These constructs were PCR cloned into pcDNA 3.2 plasmid (Invitrogen #K2440-20). A FLAG tag was also cloned in-frame into the C-terminal sequence of all ADAMTS-15 constructs to enable purification and detection of ADAMTS-15 protein.

These constructs were validated through sequencing, which confirmed that these constructs had been cloned as expected, and via restriction digest. Empty vector (EV) pcDNA 3.2 contains a unique BamHI recognition sequence, which results in a linearized plasmid (Figure 3.4). During the construction of constructs TS15, E362A and ΔA, ΔB, ΔC the BamHI site was removed from the vector backbone. However ADAMTS-15 contains a unique BamHI site, therefore when digested with BamHI a single linearised product was formed as expected (Figure 3.4). ΔC did not linearise because the BamHI site is positioned immediately prior to the first TSR, a region which is lost in the ΔC truncated form of ADAMTS-15 (Figure 3.4; Figure 3.11a).

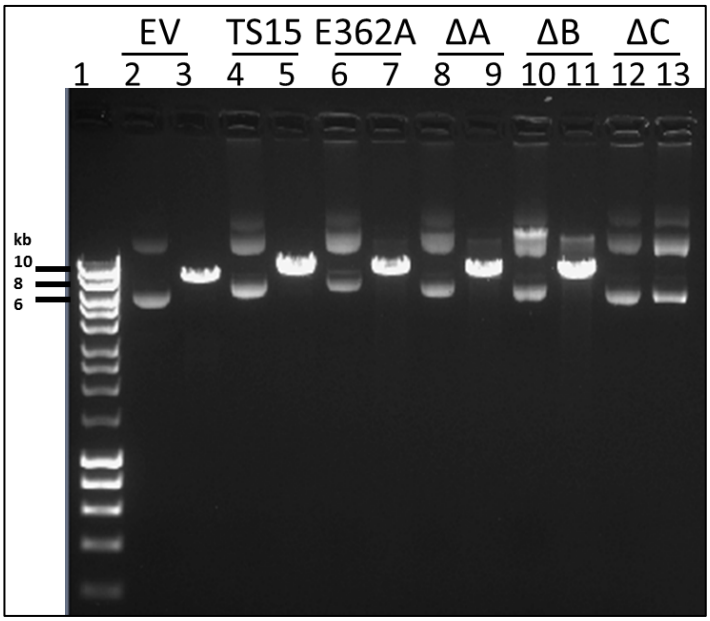


Figure 3.4: Validation of ADAMTS-15 pcDNA 3.2 constructs. 200ng of DNA loaded into each lane except for lane 1 which contained 10kb DNA ladder to approximate restriction digest product sizes. Even numbered lanes contained uncut circularised plasmid DNA and odd numbered lanes contained plasmid DNA that were linearised with *Bam*HI restriction enzyme. Predicted sizes pcDNA 3.2 (EV) 7.7kb, TS15 and E362A 10.6kb, ΔA 10.2kb, ΔB 9.7kb and ΔC 9.21kb.

3.3.2 Transient expression of ADAMTS-15 in human breast cancer cell lines

ADAMTS-15 constructs were validated through transient transfection into MCF-7 and MDA-MB-231 cell lines. Western blot analysis (Methods 2.17; 2.20-2.23) of cell lysate samples collected 72 hours post transfection confirmed expression of ADAMTS-15 and E362A constructs (Figure 3.5). Transient expression of ADAMTS-15 was tolerated by both cell types. However since ADAMTS-15 is potentially protective in breast cancer it is possible that these cell types might reject and/or mutate the ADAMTS-15 plasmid over time. Also on a technical note transient transfection of plasmids into MDA-MB-231 cells are generally inefficient. Therefore systems that facilitate the stable expression of ADAMTS-15 were deemed more appropriate for longer-term assays.

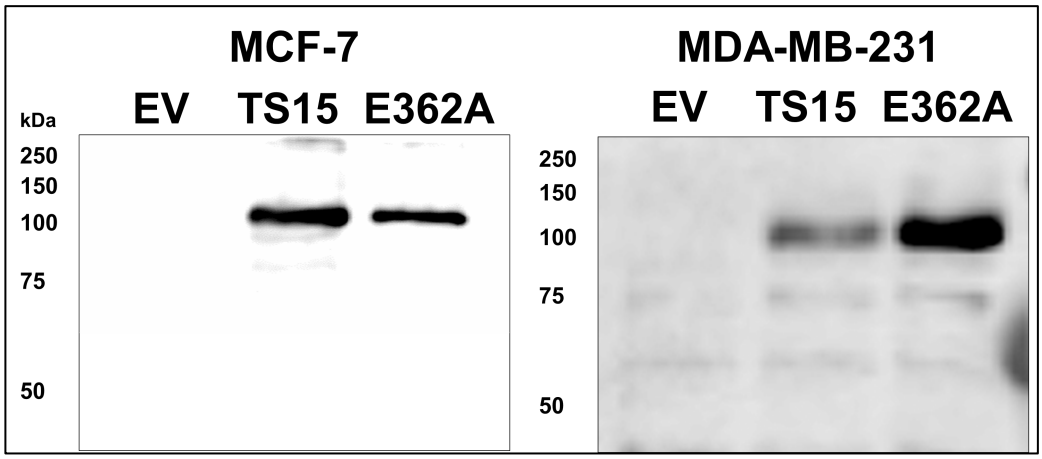


Figure 3.5: Characterisation of ADAMTS-15 transient expression in human breast cancer cell lines. Western blot analysis of human breast cancer cells transfected with pcDNA3.2 empty vector control (EV), *wildtype* ADAMTS-15 (TS15) or a metalloproteinase inactive form of ADAMTS-15 (E362A). Anti-Flag antibodies were utilised to detect ADAMTS-15 from cell lysate samples. These data are representative of several experiments.

3.3.3 Constitutive expression of ADAMTS-15 in human breast cancer cell lines

ADAMTS-15 constructs empty vector (EV) control, *wildtype* ADAMTS-15 (TS15) and a metalloproteinase inactive form of ADAMTS-15 (E362A) were stably transfected as described in materials and methods (Methods 2.4.1) into MDA-MB-231 and MCF-7 cells. Polyclonal pools were used in all experiments and antibiotic selection (G418) was utilised to select for ADAMTS-15 or E362A overexpressing cells respectively.

The expression of ADAMTS-15 was determined via western blot analysis (Methods 2.17; 2.20-2.23) of cell lysate samples (Figure 3.6) and qRT-PCR (Figure 3.8; Methods 2.14-2.16). Whilst several commercial ADAMTS-15 antibodies were tested, none of them could reliably detect ADAMTS-15 protein (Appendix 5). Therefore detection of ADAMTS-15 protein was via an anti-FLAG antibody. A limitation of this approach was that endogenous ADAMTS-15 protein could not be detected, however qRT-PCR primers and probes were designed to detect both endogenous and exogenous (over expression) ADAMTS-15 mRNA levels. Whilst mRNA levels may not always correlate with protein level, for several reasons (including protein stability, degradation *etc*.) the pattern of ADAMTS-15 expression and protein levels did match. In MDA-MB-231 cells overexpression of TS15 and E362A were approximately 8x higher (3 Ct values lower) than endogenous ADAMTS-15 expression in EV cells (Figure 3.8). TS15 and E362A protein levels were also equal (Figure 3.6). In contrast to this the levels of stable overexpression of TS15 (Ct 20) and E362A (Ct 25) in MCF-7 cells were markedly different. This pattern could also be observed at the protein level (Figure 3.6; 3.8). Although MCF-7 EV, TS15 and

E362A cells were utilised, the expression levels of TS15 and E362A were more comparable in the MDA-MB-231 cells and were therefore chosen as the focus for the majority of the project.

ADAMTS-15 overexpression was maintained throughout culture, suggesting that overexpression of ADAMTS-15 is not inherently toxic to the cells. Indeed both MDA-MB-231 and MCF-7 cells maintained their correct cell marker profile even when overexpressing ADAMTS-15 constructs (Figure 3.7). There were also few differences in the expression profiles of the hyalectanases and TIMPs examined via qRT-PCR in MDA-MB-231 EV, TS15 and E362A cells (Figure 3.8). These expression profiles also remained comparable when comparing empty vector control cells with the appropriate parental cell lines (Figure 3.3).

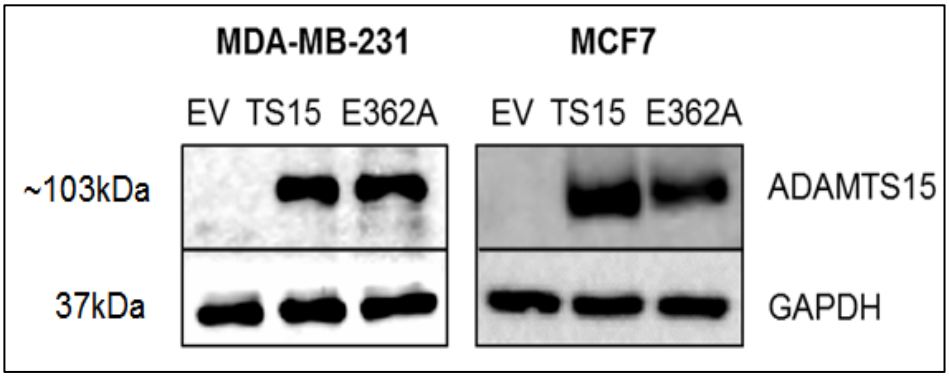


Figure 3.6: Characterisation of ADAMTS-15 expression in human breast cancer cell lines. Western blot analysis of human breast cancer cells engineered to constitutively express pcDNA3.2 empty vector control (EV), *wildtype* ADAMTS-15 (TS15) or a metalloproteinase inactive form of ADAMTS-15 (E362A). Anti-Flag antibodies were utilised to detect ADAMTS-15 from cell lysate samples. These data are representative of multiple experiments.

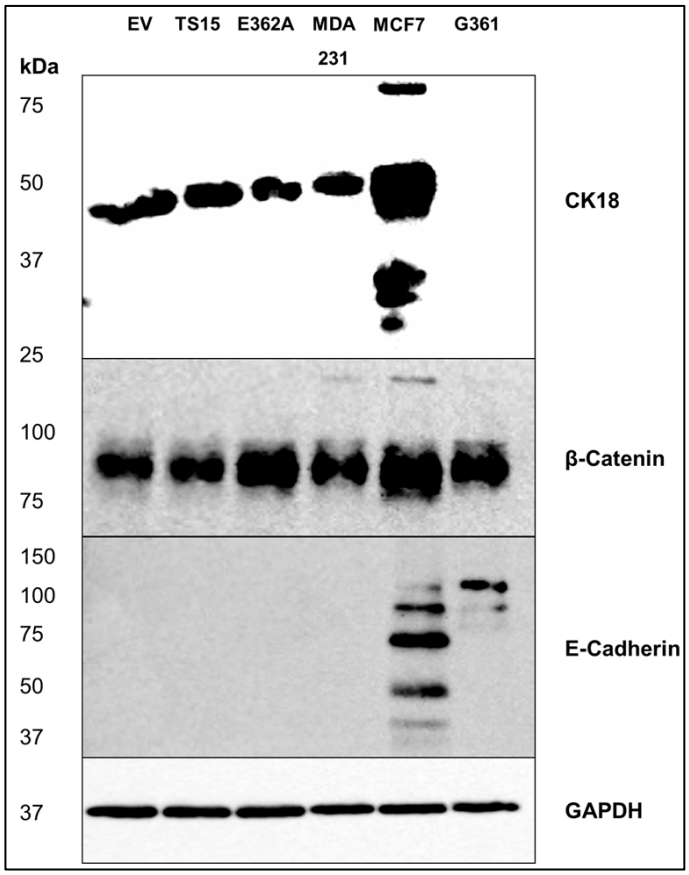


Figure 3.7: Western blot analysis of MDA-MB-231 EV, TS15 and E362A cells. Western blot analysis of cell lysates from MDA-MB-231 cells engineered to constitutively express pcDNA3.2 empty vector control (EV), *wildtype* ADAMTS-15 (TS15) or a metalloproteinase inactive form of ADAMTS-15 (E362A). Cell markers that are known to be involved with epithelial-mesenchymal transition (EMT) were chosen; Cytokeratin 18 (45kDa), β-Catenin (92kDa), E-Cadherin (135kDa) and GAPDH loading control (37kDa). Numbers in brackets represent the predicted molecular weights of the respective proteins. Cell lysates from MDA-MB-231, MCF-7 and G361 cells were used as controls.

	MDA-MB-231		
	EV	TS15	E362A
ADAMTS1	25	24	24
ADAMTS4	21	20	20
ADAMTS5	35	34	34
ADAMTS8	40	40	40
ADAMTS9	30	31	30
ADAMTS15	26	23	23
ADAMTS20	37	36	37
TIMP3	24	24	23

MCF-7		
EV	TS15	E362A
31	20	25

Ct 15-25	Very high expression
Ct 26-30	High expression
Ct 31-35	Moderate expression
Ct 36-39	Low expression
Ct <40	Very low / undetectable expression

Figure 3.8: Expression profile of MDA-MB-231 EV, TS15 and E362A cells. qRT-PCR analysis of gene expression in MDA-MB-231 EV, TS15 and E362A cells. Relative expression levels are shown as a Ct value heat-map. The displayed Ct values are averaged from three independently collected RNA samples. See appendix A7 to see an expanded version of this data set.

3.4 Analysis of cell viability and proliferation

MTT assays and flow cytometry were utilised to evaluate the effects of ADAMTS-15 on breast cancer cell viability, proliferation and apoptosis. Overexpression of ADAMTS-15 did not affect MCF-7 or MDA-MB-231 cell viability (Figure 3.9a-b). MDA-MB-231 EV, TS15 and E362A cells did not show any differences in proliferation in either normal tissue culture conditions (10% FCS) or stress conditions (0% FCS) as determined by cell counts over a period of 4 days (Figure 3.9c; Methods 2.5). In corroboration with these results a flow cytometric analysis (Methods 2.26) of the cell cycle profiles of ADAMTS-15 expressing cells did not identify any significant differences either (Figure 3.9d). An examination of the sub-G1 population also highlights that ADAMTS-15 does not appear to induce apoptosis (Figure 3.9d). From these data it can be concluded that ADAMTS-15 does not affect breast cancer cell viability or proliferation. A greater understanding of the context (localisation) in which ADAMTS-15 operates may shed some light into the tumour suppressive function(s) of this metalloproteinase.

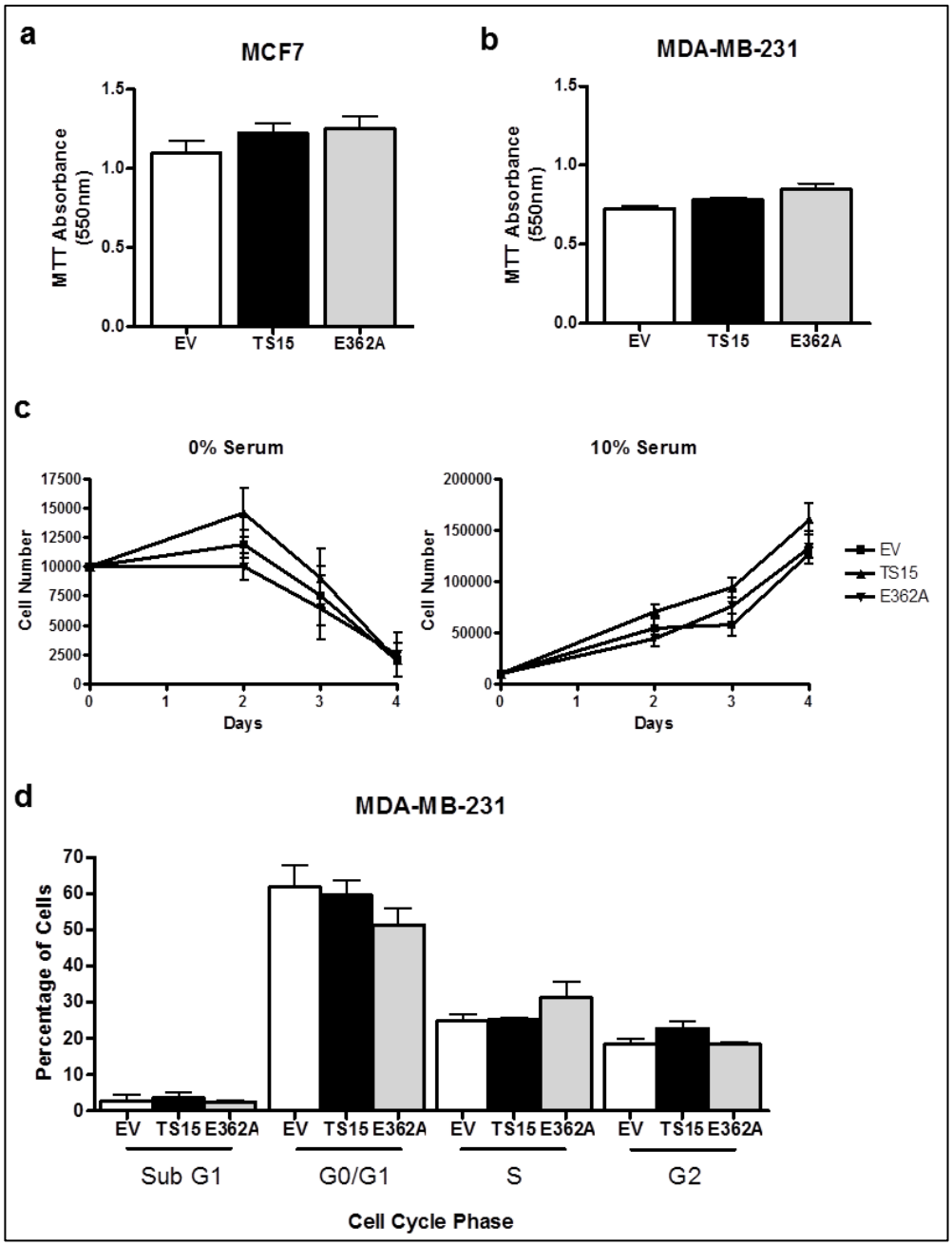


Figure 3.9: ADAMTS-15 does not affect breast cancer cell viability or proliferation. MTT cell viability assay of MCF7 (**a**) and MDA-MB-231 (**b**) EV, TS15 or E362A human breast cancer cells. These data are based upon three independent experiments, error bars represent s.e.m. statistical tests revealed no significant differences. (**c**) Cell growth assay of MDA-MB-231 EV, TS15 and E362A expressing cells. Cells were grown with media containing either 0% or 10% FCS. Total cell numbers were counted at the indicated time points post seeding, data were averaged across four wells per cell group, each day, error bars represent s.e.m. statistical tests revealed no significant differences. (**d**) Cell cycle analysis of MDA-MB-231 EV, TS15 and E362A cells was analysed via flow cytometry as described in materials and methods, data are represented by four independently collected cell samples, error bars represent s.e.m. statistical tests revealed no significant differences.

3.5 ADAMTS-15 localisation

Association with the extracellular matrix is one of the defining characteristics of the ADAMTS family. The association of ADAMTSs with the ECM is largely mediated by the ADAMTS ancillary domains, whose broader functions also include the regulation of ADAMTS proteolytic activity and substrate preferences (Kelwick and Edwards, 2014). Thus ECM association is functionally important and also plays a role in determining the context in which the ADAMTSs operate. Since the localisation of ADAMTS-15 within the context of *in vitro* models of breast cancer is unknown, yet may be functionally important several experiments were designed to examine this.

MDA-MB-231 EV, TS15 and E362A cells were seeded in serum containing media onto coverslips and allowed to adhere overnight. Immunofluorescence antibody staining (Methods 2.25) confirmed that ADAMTS-15 is an ECM associated metalloproteinase (Figure 3.10a). ADAMTS-15 E362A as expected also associates with the ECM (Figure 3.10a). Generally as can be seen in the images ADAMTS-15 sits in the pericellular space and may potentially interact with cell surface proteins (Figure 3.10a).

To provide additional insight into ADAMTS-15 localisation ADAMTS-15 constructs were transiently transfected. At 72 hours post transfection cell lysate, conditioned media and extracellular matrix samples were taken and the localisation of ADAMTS-15 was examined via western blot (Methods 2.17-2.23). Since the samples were proportionately loaded, absolute measurements of ADAMTS-15 localisation cannot be inferred however a qualitative view can be determined. In MCF-7 cells ADAMTS-15 was associated with the ECM,

however ADAMTS-15 was also detected in the conditioned media (Figure 3.10b). It should also be noted that the association of ADAMTS-15 with the ECM via western blot was generally difficult to detect in both MDA-MB-231 and MCF-7 cells (Figure 3.10b). Though of course only exogenous, FLAG tagged ADAMTS-15 could be detected and so it is possible that a portion of ECM binding sites were already taken up by endogenously expressed ADAMTS-15. Thus an overexpression of ADAMTS-15 may have been unable to bind to a saturated ECM. Interestingly HEK293T cells, readily expressed high levels of ADAMTS-15 constructs though the greatest proportion of ADAMTS-15 remained in the cell lysate (Figure 3.10b). ADAMTS-15 expressed in HEK293T cells did associate with the ECM (Figure 3.10b). However in comparison to the efficiency of the transfection, relatively little ADAMTS-15 associated with the ECM or the conditioned media (Figure 10b). It is possible therefore that ADAMTS-15 may localise differently in different cell types.

Viloria *et al.* 2009 reported that the two C-terminal TSR's in ADAMTS-15 are required for ECM binding, since transient transfection of truncated forms of ADAMTS-15 in HCT-116 (colorectal cancer cell lines) display reduced association with the ECM. In confirmation with reports in the literature these data also indicate that ADAMTS-15 associates with the ECM via its ancillary domain (Figure 3.11). Expression of truncated constructs, ΔA, ΔB or ΔC, which lack regions of the ancillary domain display a reduced level of association with the ECM in comparison to full-length ADAMTS-15 (Figure 3.11). Future studies may find it useful to further optimise and characterise these constructs, particularly within breast tumour cell lines to provide additional insight into ADAMTS-15 localisation.

It should also be noted that whilst ADAMTS-15 protein is shown to be ~103kDa (Figure 3.5, 3.6 and 3.10b) which is the predicted and reported molecular weight of ADAMTS-15 (Viloria *et al.* 2009). In several blots, different band sizes for ADAMTS-15 have been observed, which might correspond to pro and active (furin processed) forms of ADAMTS-15 (Figure 3.10c). However it is also possible that ADAMTS-15 may undergo post translational modification, though no conclusive evidence of this is provided in this thesis.

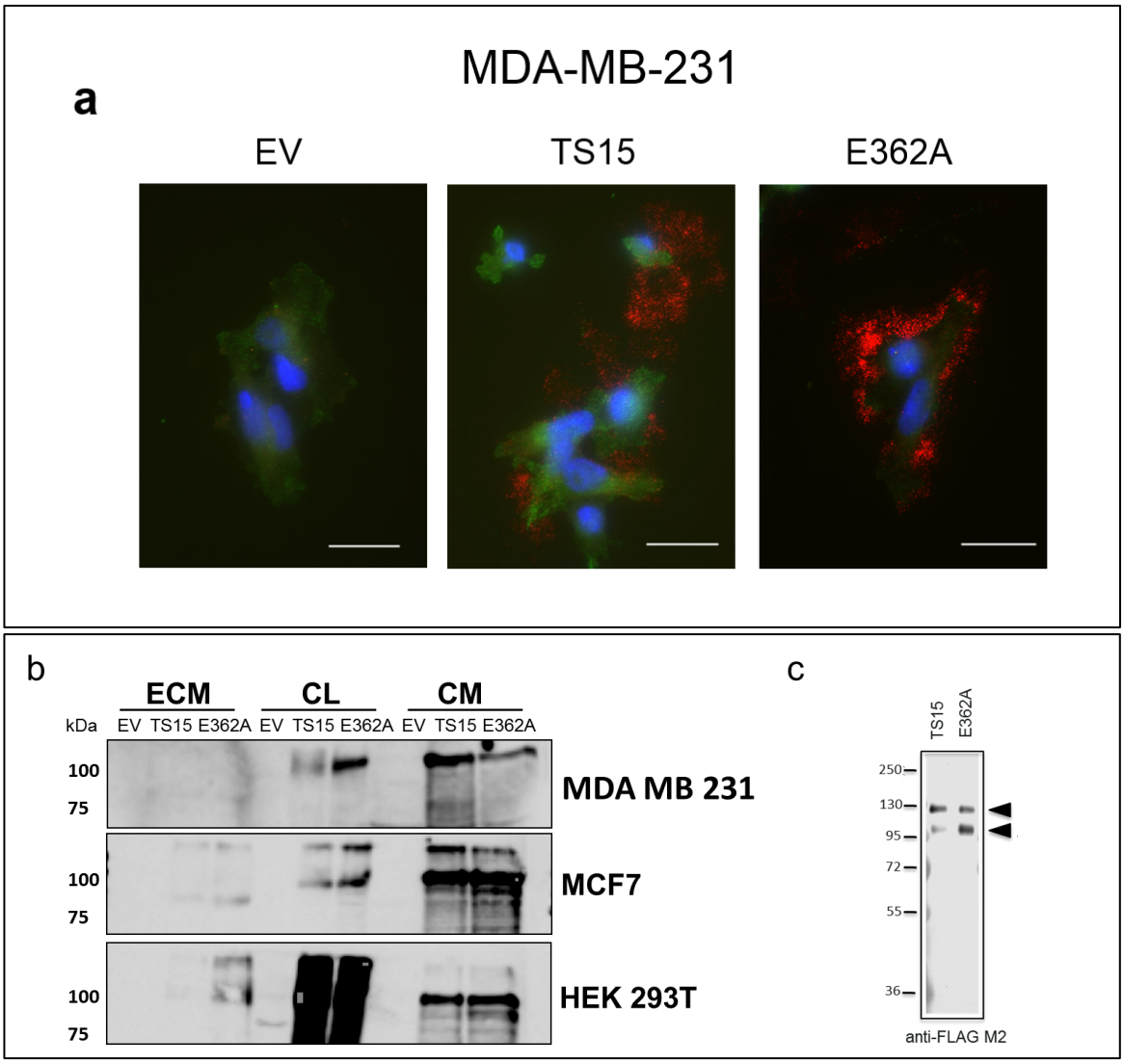


Figure 3.10: ADAMTS-15 associates with the ECM. (a) The expression and localisation of ADAMTS-15 (red) and active Integrin β1-12G10 (green) in MDA-MB-231 cells was analysed by immunofluorescence. Cell nuclei are DAPI stained (blue), white scale bars represent 20μm in length data in collaboration with Dr. Christian Roghi. (**b**) Western blot analysis of ADAMTS-15 localisation. EV, TS15 and E362A were transiently transfected into MCF7 and HEK 293T cells. At 48h post transfection extracellular matrix (ECM), cell lysate and conditioned media samples were taken and proportionally loaded (40% of the respective sample). These data were generated in collaboration with Dr. Laura Wagstaff. (**c**) ADAMTS-15 and E362A protein was purified via flag tag from MDA-MB-231 TS15 and E362A cells and then analysed by western blot. Data provided by Dr. Kazu as part of collaborations with Professor Hideaki Nagase's group.

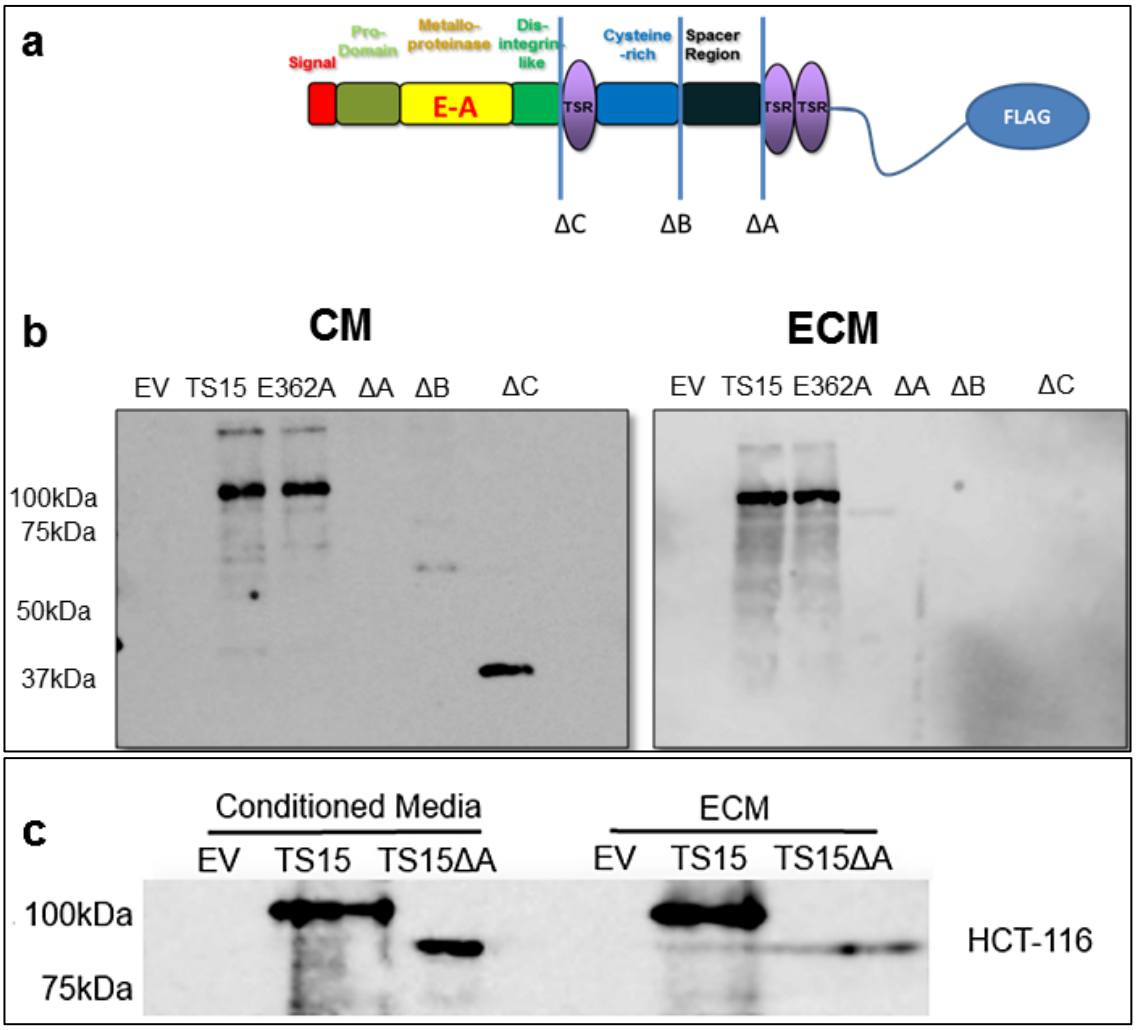


Figure 3.11: ADAMTS-15 associates with the ECM via it's ancillary domain. **(a)** Schematic of ADAMTS-15. Ancillary domains to the right of the blue lines are missing in the three truncation constructs ΔA, ΔB and ΔC respectively (**b and c**) HCT-116 colorectal cancer cells were transiently transfected with either pcDNA 3.2 empty vector control (EV), TS15 or ΔA, ΔB or ΔC truncated constructs. Extracellular matrix (ECM) and conditioned media (CM) samples were taken and proportionally loaded (40% of the respective sample).

3.6 Aggrecanase assay

ADAMTS-15 is reported as an aggrecanase in the literature, primarily due to phylogenetic similarities with the hyalectanases several of which have demonstrable aggrecanolytic activity. Whilst ADAMTS-15 is a proven versicanase, beyond a patent there is no evidence that clearly demonstrates ADAMTS-15 aggrecanolytic activity (Stupka *et al.* 2013; Yamaji *et al.* 2000). Therefore it seemed appropriate to examine ADAMTS-15 aggrecanolytic activity (Methods 2.7).

ADAMTS-15 and E362A were purified using anti-FLAG M2 antibody-conjugated agarose beads from MDA-MB-231 TS15 and E362A conditioned media. Purified pro-forms of ADAMTS-15 and E362A were then activated with furin to ensure that only active ADAMTS-15 was used in each aggrecanase activity assay. Aggrecanolytic activity was detected via western blot using antibodies that can detect an aggrecan neo-epitope (anti-AGEG). Incubation of bovine aggrecan with *wildtype* ADAMTS-15 (TS15) but not the metalloproteinase inactive form (E362A) was able to cleave aggrecan (Figure 3.12). In comparison with ADAMTS-5, ADAMTS-15 displays weak activity. Incubation with N-TIMP3 inhibited ADAMTS-15 aggrecanase activity, indicating that ADAMTS-15 is inhibited by TIMP3 (Figure 3.12). These data also indicate that ADAMTS-15 E362A is catalytically inactive. Therefore in all future assays metalloproteinase independent effects can be discerned.

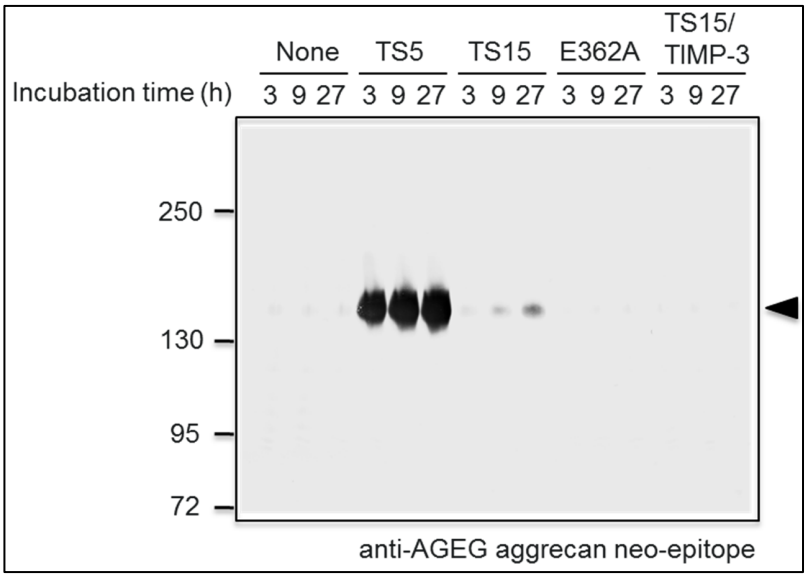


Figure 3.12: ADAMTS-15 aggrecanase activity is inhibited by N-TIMP-3. Aggrecanase activity of ADAMTS-15, as determined by western blot analysis of Aggrecan AGEG neo-epitope. Unconditioned media was used as a negative control and recombinant ADAMTS-5 (TS5) was used as a positive control. Data in collaboration with Dr. Kazuhiro Yamamoto from Professor Hideaki Nagase group.

3.7 Discussion

The overall aim of this thesis is to investigate the role of ADAMTS-15 in breast cancer, since previous studies have shown that ADAMTS-15 is potentially protective (Porter *et al.* 2004; 2006). Yet the functional consequences of ADAMTS-15 expression in breast cancer are unknown. *In vitro* models, particularly immortalised cell lines are amenable to genetic manipulation and are suitable for long term functional studies. An array of mammary cell lines exist that represent several healthy and breast cancer tumour subtypes (Lacroix and Leclercq, 2004). Thus *in vitro* models can be utilised to gain an initial insight into the functional consequences of ADAMTS-15. Whilst *in vivo* studies are arguably more physiologically relevant, they are better designed if informed by *in vitro* studies. To this end several human mammary cell culture models were proposed as an appropriate starting point from which to explore the functional consequences of ADAMTS-15 in breast cancer.

Within the ductal structures of the breast, the luminal epithelial cells and the myoepithelial cells are two physiologically and structurally important cell types. Of the two cell types the myoepithelial cells were determined to be the most appropriate for functional studies since it is the myoepithelial cells and not the luminal epithelial cells which express ADAMTS-15. Also a subset of aggressive breast cancer tumours express myoepithelial markers such as (α6β4 integrin) and therefore may be of myoepithelial cell-like origin (Gordon *et al.* 2003; Jones *et al.* 2004; Polyak and Hu, 2005; Tan and Ellis 2013). Even in the majority of breast cancers which are thought to originate from luminal epithelial cells, an absence of a myoepithelial cell population is observed during the progression to invasive disease (Gordon *et al.* 2003; Hsiao *et al.* 2011; Porter *et al.* 2004).

Thus the loss of *wildtype* myoepithelial cells and/or the loss of the protective factors expressed by these cells contribute to the transformation of *in situ* tumour cells into invasive disease (Gordon *et al.* 2003; Hsiao *et al.* 2011; Porter *et al.* 2004). Since ADAMTS-15 is potentially one of those myoepithelial protective factors, an investigation of the consequences of ADAMTS-15 expression in myoepithelial cell populations would be informative. We acquired two immortalised cell lines 1089 β4 and 1089 β6 respectively. These cell lines were kindly gifted to our lab from Professor Louise Jones, Queen Mary University of London. However in our hands these cell lines were found to be prone to phenotypic drift with changes in marker profiles and cell morphology occurring soon after entering culture. Thus the dynamic phenotype of the myoepithelial cell lines were determined to be inappropriate for long term functional studies.

Luminal epithelial cells were chosen as an appropriate alternative model for several reasons. Luminal epithelial cells are likely to come into contact with ADAMTS-15 expressed by neighbouring myoepithelial cells and the majority of breast cancers are thought to originate from a luminal epithelial-like cell. Several well established *in vitro* models of epithelial-like breast cancers exist, which have been extensively characterised in the literature. From amongst them MCF-7 cells and MDA-MB-231 cells were chosen as the most appropriate *in vitro* models since they retain the molecular characteristics of the tumours from which they were derived. However the majority of this thesis focused on MDA-MB-231 cells, since these cells are representative of poorly differentiated (grade III) tumours. This is biologically important since ADAMTS-15 expression inversely correlates with tumour grade in breast cancer, with expression

significantly reduced in grade III breast tumours compared to grade I or II tumours (Porter *et al.* 2004). ADAMTS-15 expression also inversely correlates with tumour grade in colorectal cancer (Viloria *et al.* 2009).

Several ADAMTS-15 constructs which were originally generated by Dr. Laura Wagstaff were validated as part of this thesis via sequencing and restriction digest. In addition to full length ADAMTS-15, a metalloproteinase inactive form of ADAMTS-15 (E362A) and several truncated ADAMTS-15 constructs (ΔA, ΔB, and ΔC) which lack C-terminal domains were validated for this project. Whilst these ADAMTS-15 constructs were successfully transiently transfected into several cell lines, since ADAMTS-15 is potentially tumour suppressive it is possible that these cell lines might reject and/or mutate the ADAMTS-15 plasmid over time. Therefore systems that facilitate the stable expression of ADAMTS-15 were deemed more appropriate for longer term assays.

MDA-MB-231 and MCF-7 cells, which stably constitutively express ADAMTS-15 constructs, were used in preference to transient transfection experiments, since long term expression could be maintained via antibiotic selection. The generation of these stable cell lines utilised a transfection strategy that involved the random integration of constructs into the genome of these cells. To avoid clonal effects as a result of random integration, polyclonal pools were used in all functional assays. One caveat of the use of polyclonal pools is that the pools represent the average expression of the monoclonal populations. Thus those monoclonal pools which excessively overexpress the gene of interest, dominate the polyclonal population. Indeed the level of overexpression of ADAMTS-15 constructs in MCF-7 cells was found to be excessive and lacked comparability. In contrast to this the overexpression of TS15 and E362A in the MDA-MB-231

stable polyclonal pools was comparable and only around 8x higher than endogenous ADAMTS-15 expression in EV control cells. Therefore the MDA-MB-231 stable cells were deemed to be more appropriate since the overexpression is arguably more physiologically relevant and fairer comparisons between TS15 and E362A cells can be made.

Despite earlier concerns about the potential for tumour cells to reject ADAMTS-15, stable constitutive expression of ADAMTS-15 in MCF-7 and MDA-MB-231 cells did not affect cell viability or proliferation. There were no observable toxicities associated with expression of ADAMTS-15 and expression was maintained throughout culture. Thus it can be concluded that ADAMTS-15 does not affect breast cancer cell growth or viability.

Since ADAMTS-15 does not affect cell proliferation any insight into the localisation and by inference the functional context in which ADAMTS-15 operates may be informative. To this end ADAMTS-15 localisation was examined in several cell lines, though particular attention was paid to localisation in human mammary cancer cell lines. As expected ADAMTS-15 was secreted and localised to the extracellular matrix in both MDA-MB-231 and MCF-7 breast cancer cell lines. Unfortunately due to time constraints the localisation of ADAMTS-15 truncation constructs expressed in breast cancer cell lines was not examined. However an examination of ADAMTS-15 localisation in other cell lines provided further insight including the importance of the ancillary domain for determining ADAMTS-15 localisation. The two C-terminal ADAMTS-15 TSR's are required for ECM binding, since transient transfection of truncated forms of ADAMTS-15, ΔA-C, or G849fs respectively in HCT-116 (colorectal cancer cells) display reduced ECM association. This is

functionally important as the loss of ECM association results in the mis-localisation of ADAMTS-15 away from the pericellular space thus preventing interactions with cell surface proteins. Viloria *et al.* 2009 confirmed that ECM localisation was functionally important as expression of ADAMTS-15 G849fs did not reduce HCT-116 cell invasion to the same extent as *wildtype* ADAMTS-15. Though no ADAMTS-15 substrates or protein interaction partners were identified that could explain this observation (Viloria *et al.* 2009). However based on the reported sheddase activity of ADAMTS-1 it is possible to identify Syndecan-4, heparan-binding epidermal growth factor (HB-EGF) and amphiregulin (AR) as potential interaction candidates since ADAMTS-1 shares a 66% metalloproteinase domain identity with ADAMTS-15 (Liu *et al.* 2006; Rodríguez-Manzaneque *et al.* 2009). Also whilst there are no reports of an ADAMTS directly interacting with an integrin, it is possible that an ADAMTS may be able to interact with an integrin via it's TSR domains. Whilst the identification of ADAMTS-15 interaction partners may be relevant, at the start of this project very little evidence about the hyalectanase activity of ADAMTS-15 was known.

It is only recently that reports have clearly shown that ADAMTS-15 is a proteolytically competent hyalectanase. Stupka *et al.* 2013 reports that ADAMTS-15 is an important versicanase within the context of muscle development. Whilst evidence of ADAMTS-15 versicanase activity is clear, with the exception of a Japanese patent there is no clear evidence of ADAMTS-15 aggrecanolytic activity (Yamaji *et al.* 2000). In this thesis, these data demonstrate evidence of ADAMTS-15 aggrecanolytic activity (Figure 3.12). Though it should be noted that since absolute protein concentrations were not

utilised, this assay is qualitative. Therefore, whilst ADAMTS-15 aggrecanase activity appears to be weaker than ADAMTS-5, no absolute comparisons can be made. Several Aggrecan neo-epitopes were analysed, though only AGEG could be detected from ADAMTS-15 aggrecanase activity. Again this suggests that ADAMTS-15 is a relatively weak aggrecanase and therefore Aggrecan may not be the major substrate for ADAMTS-15. Additionally these data indicate that TIMP-3, the broadest inhibitor of the ADAMTSs is able to inhibit ADAMTS-15 aggrecanolytic activity. However, this assay could be improved through the addition of a positive control, such as ADAMTS-5, which is known to be inhibited by TIMP-3. In contrast to *wildtype* ADAMTS-15, the catalytically inactive form of ADAMTS-15 (E362A), did not display aggrecanase activity (Figure 3.12). Therefore the metalloproteinase independent effects of ADAMTS-15 can be discerned in all future functional assays.

3.8 Chapter summary

In summary, whilst the myoepithelial cell lines displayed a dynamic phenotype in culture, several well-established *in vitro* models of breast cancer have been characterised. Transient or stable expression of ADAMTS-15 does not alter the fundamental characteristics of these models and therefore the functional consequences of ADAMTS-15 expression in these systems can be examined. Fundamental aspects about ADAMTS-15 localisation and activity have also been discerned:

- ADAMTS-15 does not affect breast cancer cell growth or viability.
- ADAMTS-15 associates with the ECM via it's ancillary domain.
- ADAMTS-15 has aggrecanase activity that is inhibited by N-TIMP-3.

The following chapter investigates whether ADAMTS-15 modulates the migratory behaviour of human breast cancer cell lines.

Chapter Four: ADAMTS-15 Modulates the Migratory Behaviour of Human Breast Cancer Cell Lines

4.1 Introduction

Functional *in vitro* studies have demonstrated that ADAMTS-15 reduces invasion in colorectal cancer cell lines (Viloria *et al.* 2009). However the functional consequences of ADAMTS-15 expression on breast cancer cell migration are unknown.

4.2 Analysis of migration in 2D assays

4.2.1 Scratch wound assay

The scratch wound assay is typically described as an *in vitro* wound healing model, since inflicted scratch wounds remove the underlying matrix along with a section of the cell layer (Ashby and Zijlstra, 2012; Hsu *et al.* 2012; Krampert *et al.* 2005). Whilst different parameters can be analysed, typically the closure ('healing') of the wound is tracked over time (Methods 2.13). The scratch wound assay is also an informative migration assay, as the directed movement of individual cells into the wound area can also be tracked. A potential limitation of this model is that wound healing can be the result of both cell migration and cell proliferation. Since ADAMTS-15 does not affect breast cancer cell proliferation (Figure 3.9) any observable effects are likely due to a modulation of cell migration.

MDA-MB-231 EV, TS15 and E362A cells were seeded at high density onto tissue culture plastic in medium containing 10% (vol/vol) serum and allowed to

adhere overnight. The next day 'scratch wounds' were introduced and wound closure was monitored over a period of 24 hours via time-lapse video microscopy. Data are expressed as the percentage of wound closure at 12 and 24 hours post wound. Expression of either *wildtype* (TS15) or catalytically inactive (E362A) ADAMTS-15 in MDA-MB-231 cells were associated with reduced scratch wound recovery in comparison to control (EV) (Figure 4.1). From these data it can be concluded that ADAMTS-15 inhibits breast cancer cell migration in a metalloproteinase independent manner.

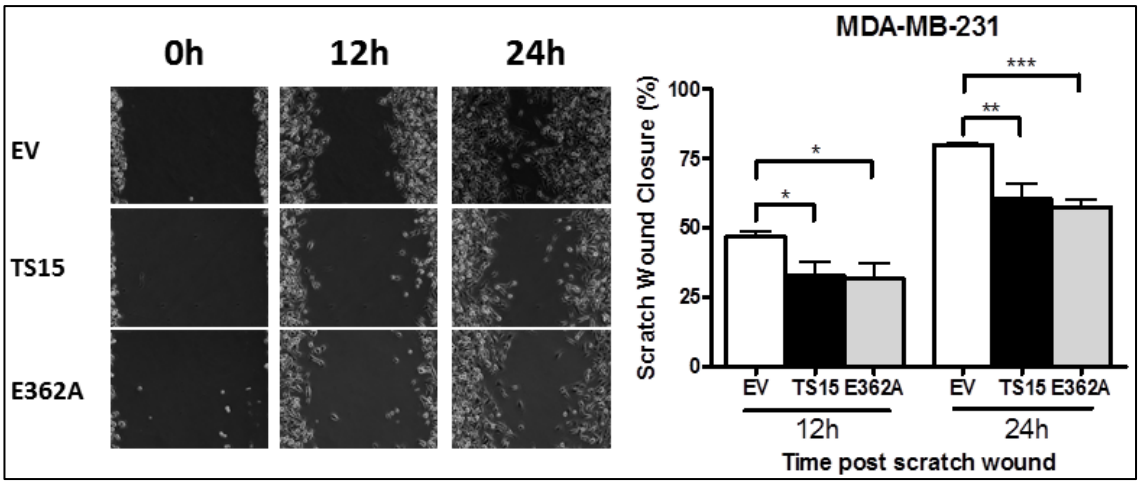


Figure 4.1: ADAMTS-15 reduces scratch wound recovery of human breast cancer cells in a metalloproteinase independent manner. Scratch wounds were introduced into monolayers of MDA-MB-231 EV, TS15 and E362A cells. Wound recovery was determined at the indicated time points, data represent 3-5 individual wells analysed per cell group, error bars represent s.e.m. *p<0.05, **p<0.01, ***p<0.001.

4.2.2 Analysis of random cell migration

To monitor the migration of individual cells a lower seeding density was utilised than that used during the scratch assays. Another notable difference between the scratch wound assay and a 2D cell migration assay is that random cell migration rather than directional migration is tracked (Methods 2.11; Ashby and Zijlstra, 2012).

Individual cells were tracked over a period of 24 hours and Image J plugins ('manual tracking' and 'chemotaxis') were used to quantify migration velocity (μm/min). Expression of either *wildtype* (TS15) or catalytically inactive (E362A) ADAMTS-15 inhibited cell migration in MDA-MB-231 and MCF-7 cells (Figure 4.3). To substantiate that these effects were attributable to ADAMTS-15 cells were transfected either with a smartpool of ADAMTS-15 or control non-targeting siRNAs (Methods 2.11.3). Migrating-siRNA transfected cells were tracked and analysed over a period of 24 hours. Knockdown of *wildtype* ADAMTS-15 (TS15) or E362A restored cell migration to control (EV) levels (Figure 4.3). Western blot analysis of ADAMTS-15 protein in siRNA transfected cells confirmed that ADAMTS-15 knockdown was successful. In concordance with the scratch wound assay these data confirm that ADAMTS-15 reduces breast cancer cell migration in a metalloproteinase independent manner.

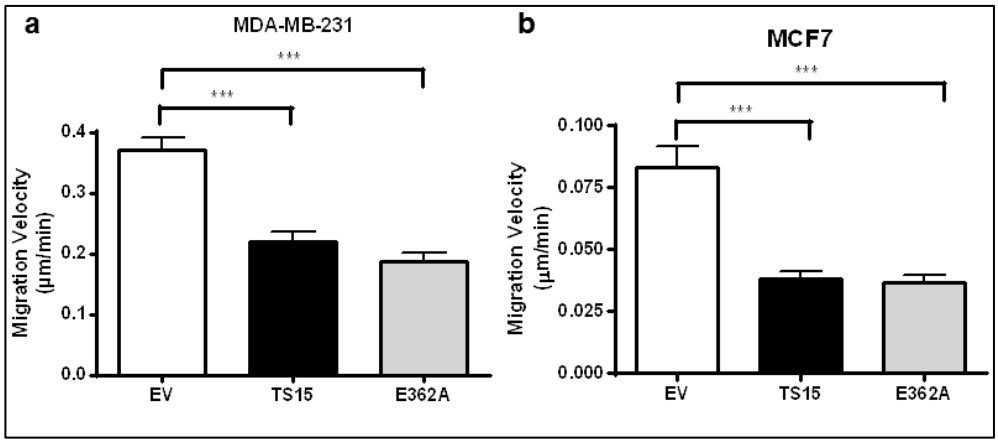


Figure 4.2: ADAMTS-15 reduces 2D migration of human breast cancer cells in a metalloproteinase independent manner. 2D migration velocity of MDA-MB-231 (**a**) or MCF7 (**b**) human breast cancer cells that constitutively express empty vector control (EV), *wildtype* ADAMTS-15 (TS15) or metalloproteinase inactive ADAMTS-15 (E362A) was determined with time-lapse microscopy over a period of 24 hours, data represent 60 individually tracked cells per group, error bars represent s.e.m. ***p<0.001.

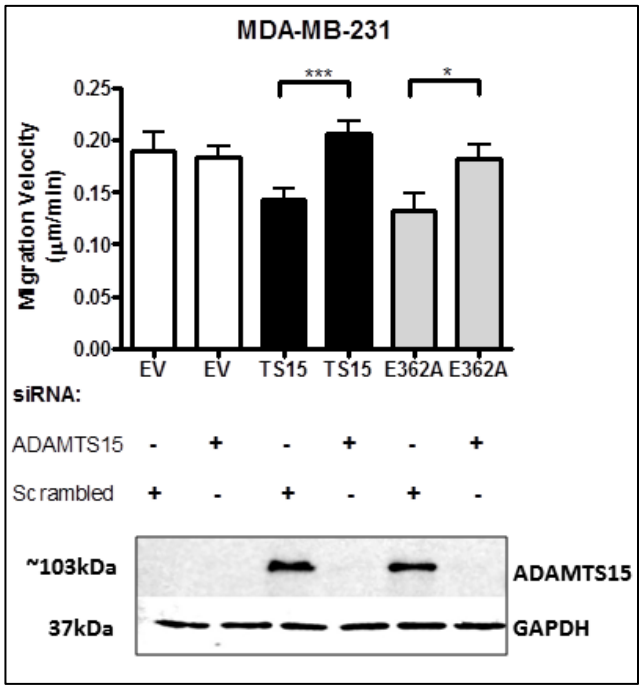


Figure 4.3: Knockdown of ADAMTS-15 abrogates the anti-migratory phenotype. MDA-MB-231 EV, TS15 or E362A cells were transfected with either non-targeting siRNA (scrambled) or ADAMTS-15 siRNA. Cell migration was tracked via time-lapse microscopy over a period of 24 hours, data represent 20-28 individually tracked cells per group, error bars represent s.e.m. *p<0.05, ***p<0.001. Accompanying western blot images confirm knockdown of ADAMTS-15. ADAMTS-15 and E362A were detected via anti-FLAG antibody using cell lysate samples of siRNA transfected cells.

4.3 Analysis of matrix specific effects on cell migration

4.3.1 Conditioned matrix migration assay

A conditioned matrix experiment was designed based upon several assays reported in the literature (Methods 2.11.2; Khalkhali-Ellis and Hendrix, 2007; Phillips *et al.* 2008). MDA-MB-231 EV, TS15 and E362A cells were seeded at high density and cultured for three days in serum conditions until 100% confluency. Cells were removed with EDTA/EGTA to preserve the underlying conditioned matrices. Visual conformation and methylene blue staining was utilised to confirm that EDTA/EGTA treatment successfully removed the cell monolayer (Figure 4.4b). Naïve MDA-MB-231 EV cells were then seeded at low density onto each of the conditioned matrices and 2D migration velocity was tracked.

MDA-MB-231 (EV) cell migration was inhibited when seeded onto ADAMTS-15 conditioned matrices. (Figure 4.4). E362A conditioned matrices also inhibited MDA-MB-231(EV) cell migration, however this was not statistically significant ($p=0.06$) (Figure 4.5). This may be due to differences in matrix deposition and/or the stability of ADAMTS-15 protein in comparison to E362A protein, however in these assays these parameters were not controlled for. Although matrices from MDA-MB-231 EV, TS15 and E362A were qualitatively analysed in a Coomassie blue-stained gel and no obvious differences were observed (data not shown). Based upon previous assays it is likely that these conditioned matrices inhibited cell migration due to ECM associated ADAMTS-15 deposited by the conditioning cells. To explore further how ADAMTS-15 might influence

cell-ECM interactions, MDA-MB-231 EV, TS15 and E362A cells were seeded onto physiologically relevant matrix components.

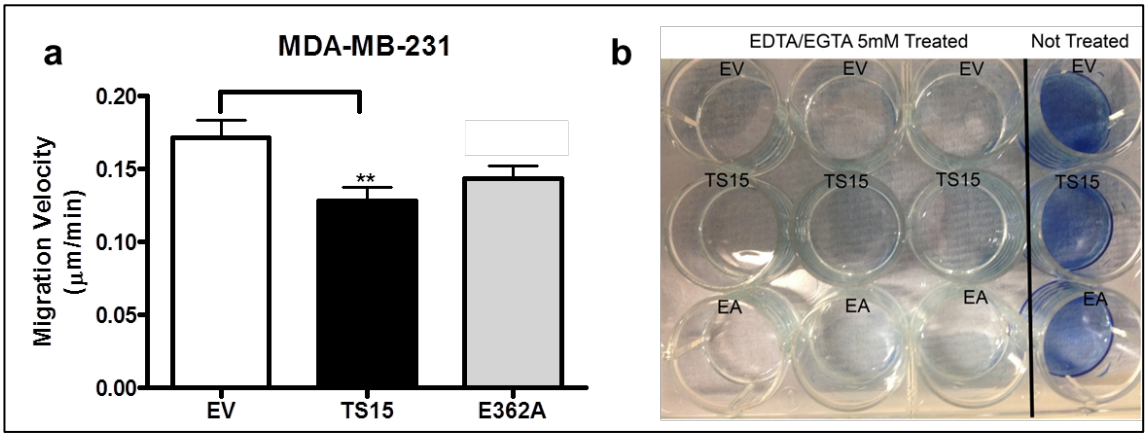


Figure 4.4: ADAMTS-15 conditioned matrix reduces the migration of human breast cancer cells. MDA-MB-231 human breast cancer cells that constitutively express EV, TS15 or E362A were cultured for 72h to allow the formation of matrices. Cells were then removed with EDTA/EGTA (5mM) to preserve the underlying matrix. Naive MDA-MB-231 EV cells were then seeded onto the conditioned matrices. (**a**) 2D migration velocity of MDA-MB-231 (EV) cells was determined with time-lapse microscopy over a period of 24 hours, data represent 60 individually tracked cells per group, error bars represent s.e.m. **p<0.01. E362A was not statistically in comparison to EV, p0.06. (**b**) Methylene blue staining was utilised to demonstrate that the matrix conditioning cells had been successfully removed prior to the seeding of naïve EV cells.

4.3.2 2D migration on fibronectin, collagen type I and laminin

To understand how ADAMTS-15 might influence the migratory behaviour of breast tumour cells within a mammary matrix context MDA-MB-231 EV, TS15 and E362A cells were seeded onto surfaces pre-coated with either collagen type I, fibronectin or laminin (Methods 2.11.1). These matrices were selected since they are important within the context of human mammary tissue architecture (Butcher, *et al.* 2009; Gusterson *et al.* 1982; Hancox *et al.* 2009; Mao and Schwarzbauer, 2005).

MDA-MB-231 EV, TS15 and E362A expressing cells adhered to and migrated on collagen type I at comparable levels (Figure 4.5). However matrix-specific effects were observed on fibronectin and laminin. Expression of TS15 or E362A inhibited the migration of MDA-MB-231 cells on fibronectin and laminin compared to control EV cells (Figure 4.5; Figure 4.6c). The anti-migratory effect is explained in part by the adhesive behaviour of cells which express ADAMTS-15. TS15 and E362A expressing cells displayed a relatively more spread out morphology and in adhesion assays more strongly adhered to laminin and fibronectin in comparison to control cells (Figure 4.5). Migration time lapse images revealed that TS5 and E362A expressing cells adhered strongly enough that cell detachment, particularly from laminin, required extensive cellular force (Figure 4.6a). In some cases adhesion was so strong that cells ruptured their cell membranes in order to de-adhere (Figure 4.6a).

Migration directionality, expressed as a ratio between the Euclidian distance (distance between cell start and finish points) and the accumulated distance

(total migrated distance in a given time period) were also quantified in these assays (Figure 4.5). Cells which persistently migrate in a straight line will have a directionality of one, since the distance between the cell start and finish points and the total migrated distance will be equal (Figure 4.6d). Cells with more random migration patterns have a lower level of directionality and could potentially be zero since a cell could migrate back to its original starting point (Figure 4.6d).

Within an *in vivo* context, cells which adhere and migrate along fibronectin fibrils are likely to exhibit more orderly or persistent migration patterns (Bass *et al.* 2007; Mao and Schwarzbauer, 2005; Petrie, Doyle and Yamada, 2009). Since aberrant regulation of cell adhesion processes is likely to result in random migration patterns directionality can provide a qualitative indicator of the ability of cells to modulate behaviours in response to the ECM (Bass *et al.* 2007; Petrie *et al.* 2012; Petrie, Doyle and Yamada, 2009; Rodríguez-Manzaneque *et al.* 2009; Spiering and Hodgson, 2011). Expression of ADAMTS-15 and E362A enhanced directionality on fibronectin and laminin matrices (Figure 4.5). Therefore there may be differences in the level or activity of cell adhesion receptors in cells, which express ADAMTS-15. To this end the integrin profiles of these cells were examined.

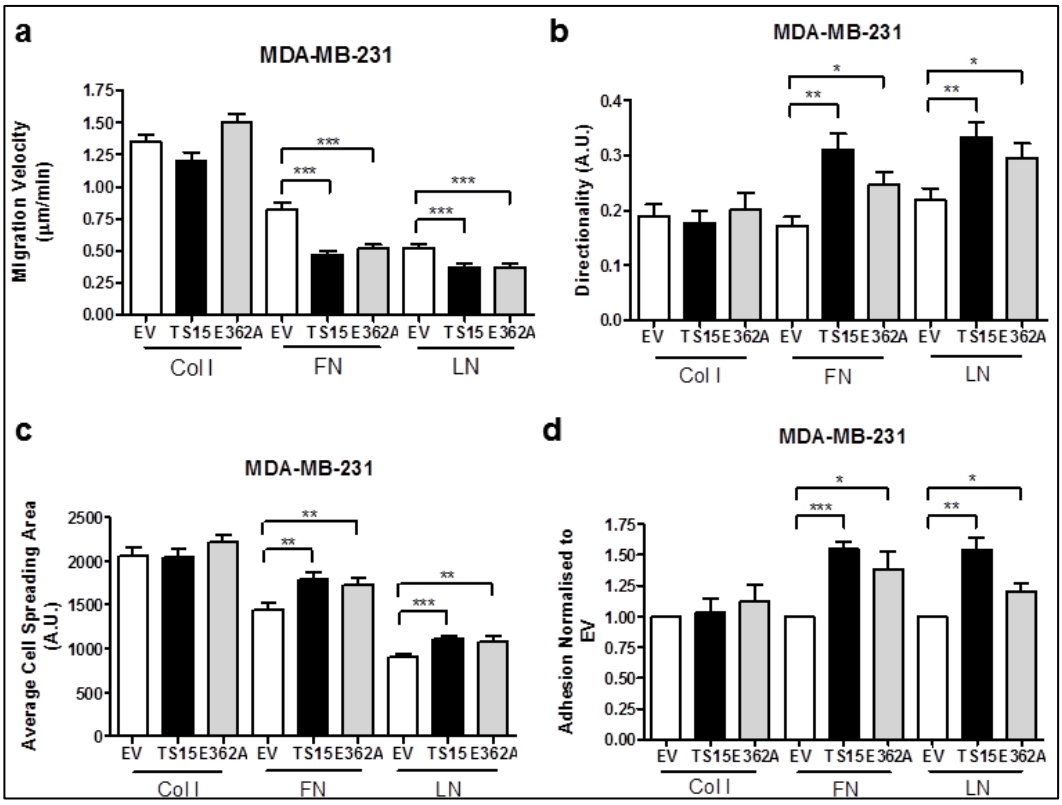


Figure 4.5: ADAMTS-15 reduces 2D migration on Fibronectin and Laminin. (**a**) MDA-MB-231 EV, TS15 or E362A cells were seeded onto 24 well plates that were pre-coated with either Collagen type I, Laminin or Fibronectin. Cell migration was tracked via time-lapse microscopy over a period of 24 hours, data represent 20-39 individually tracked cells per group, error bars represent s.e.m.; ***p<0.001. (**b**) The migration directionality (Euclidian distance / accumulated distance) of each individually tracked cell was also determined. Data represent 20-39 individually tracked cells per group, error bars represent s.e.m. *p<0.05, **p<0.01. (**c**) MDA-MB-231 EV, TS15 or E362A cells were seeded onto 24 well plates that were pre-coated with either Collagen type I, Laminin or Fibronectin. At 4hours post seeding the cell spread area (pixel area) of individual cells was determined using ImageJ, data represent 57 individually analysed cells per group, error bars represent s.e.m.; **p<0.01, ***p<0.001 (**d**) Adhesion assay. MDA-MB-231 EV, TS15 or E362A cells were seeded onto 96 well plates that were pre-coated with either Collagen type I, Laminin or Fibronectin. Adhered cells were fixed and stained with Methanol/Methylene Blue. Absorbance (630nm) was measured and TS15, E362A values were normalised to the average EV absorbance value, six wells per cell group were analysed, error bars represent s.e.m.; *p<0.05, **p<0.01, ***p<0.001.

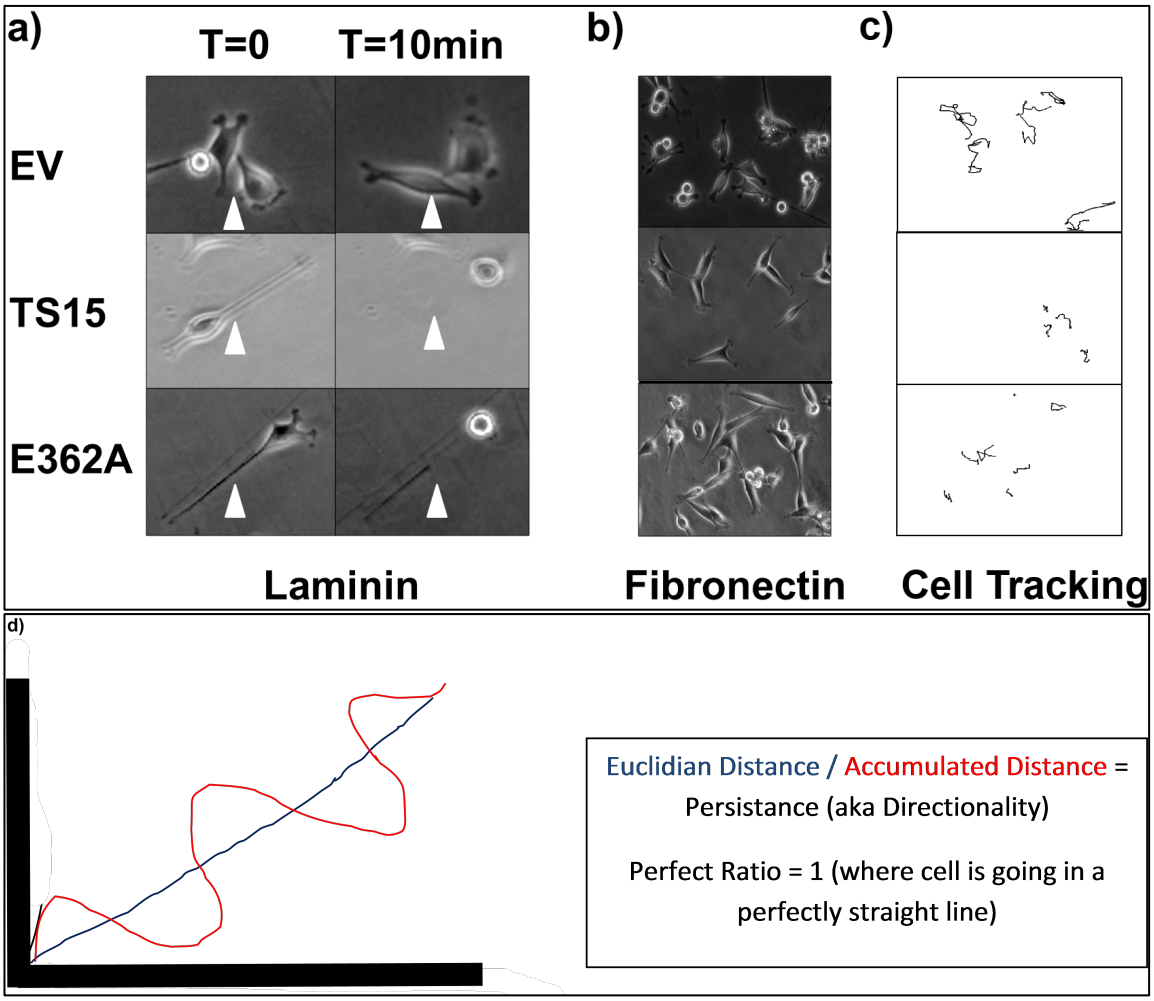


Figure 4.6: MDA-MB-231 EV, TS15 and E362A cells on laminin and fibronectin. (**a**) Images of MDA-MB-231 EV, TS15 and E362A cells that have adhered to a laminin-coated surface. The white arrows highlight the adhesive behaviour of representative cells. TS15 and E362A cells adhered strongly to laminin in comparison to EV cells, to the extent that detachment from the matrix often resulted in a tearing of the cell membrane. (**b**) Images of MDA-MB-231 EV, TS15 and E362A cells that have adhered to a fibronectin-coated surface. (**c**) Cell migration pathways of cells adhered to a fibronectin-coated surface as analysed by Image J plugins manual tracking and chemotaxis. All images are representative of the matrix migration assays in figure 4.5 (**d**) Schematic explanation of directionality.

4.3.3 ADAMTS-15 expression is associated with an altered integrin profile

Adhesion to fibronectin and laminin was enhanced in TS15 and E362A expressing cells. Therefore there may be differences in the level of expression or activity of cell adhesion receptors in cells which express ADAMTS-15. To this end the integrin profiles of these cells were examined.

Integrin α5 partners with integrin β1 to bind to fibronectin (Humphries *et al.* 2006). Expression of integrin α5, as determined my qRT-PCR, was approximately 2-fold higher in TS15 and E362A expressing cells, which potentially explains why these cells adhered more strongly to fibronectin (Figure 4.5; Figure 4.8). However due to time constraints integrin α5 was not examined at the protein level.

Laminin is the ligand for several integrin heterodimers including, α6β1 and α6β4 (Humphries *et al.* 2006). Expression of integrin β1, as determined my qRT-PCR, was approximately 2-fold higher in TS15 and E362A expressing cells, which potentially explains why these cells adhered more strongly to laminin (Figure 4.5; Figure 4.6; Figure 4.8). However at the protein-level there were no overt differences in either integrin β1 or α6 (Figure 4.7a). Flow cytometric analysis did not detect any differences in the surface levels of integrin β1 either (Figure 4.7b). To examine whether integrin β1 was differentially activated, cells were stained with β1-12G10 antibody, which preferentially binds to active integrin β1 (Methods 2.25; Humphries *et al.* 2005). However no differences were observed in the level of active integrin β1 in these cells (Figure 3.10a). However differences in the laminin binding integrin β4 were detected. MDA-MB-

231 TS15 and E362A expressing cells were associated with an increased level of total integrin β4 in comparison to control EV cells (Figure 4.7a). Whilst EV and indeed parental MDA-MB-231 cells do express integrin β4, the levels of total β4 were consistently higher in TS15 and E362A expressing cells in multiple independent western blots. As noted before the ligand for integrin α6β4 is laminin, thus increased levels of β4 may explain the adhesion and migration phenotype observed in TS15 and E362A cells. However, a caveat to this analysis is that based upon these flow Cytometry data there are no differences in the surface level of integrin β4 in either control (EV), TS15 or E362A expressing cells (Figure 4.7b; Methods 2.26).

It should also be noted that not all laminin and fibronectin binding integrin heterodimers were examined in this study. An in depth examination of integrin signalling, localisation and trafficking may be required in future studies to fully characterise the matrix specific effects observed in TS15 and E362A expressing cells.

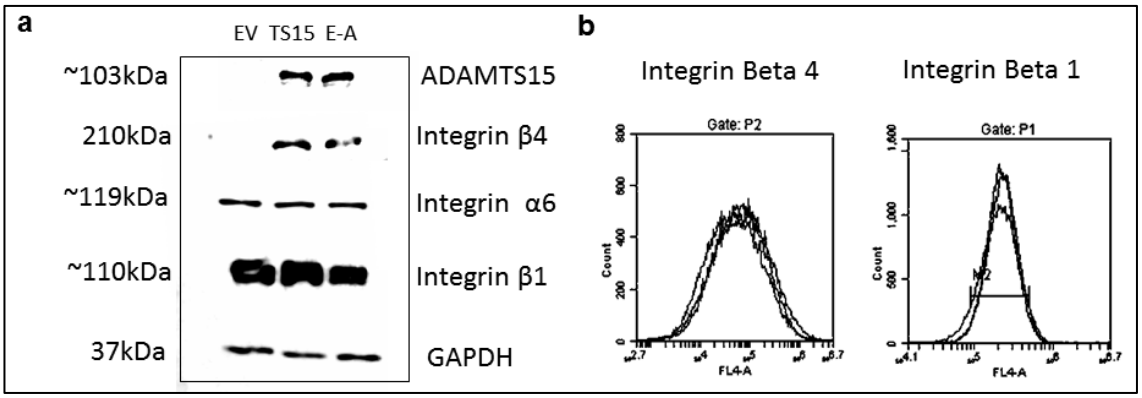


Figure 4.7: ADAMTS-15 expression is associated with an altered integrin profile. (**a**) Western blot analysis of the integrin profiles of MDA-MB-231 cells that constitutively express EV, ADAMTS-15 or E362A. Data are representative of three independently collected samples (**b**) Flow Cytometry analysis of the surface levels of Integrin β4 and Integrin β1 on MDA-MB-231 EV, TS15 and E362A cells. Data are representative of three independently collected cell samples.

	MDA-MB-231		
	EV	TS15	E362A
ADAMTS1	25	24	24
ADAMTS4	21	20	20
ADAMTS5	35	34	34
ADAMTS8	40	40	40
ADAMTS9	30	31	30
ADAMTS15	26	23	23
ADAMTS20	37	36	37
TIMP3	24	24	23
Syndecan 1	26	26	25
Syndecan 4	24	24	25
Integrin β1	27	26	26
Integrin α5	25	24	24

MCF-7		
EV	TS15	E362A
31	20	25

Ct 15-25	Very high expression
Ct 26-30	High expression
Ct 31-35	Moderate expression
Ct 36-39	Low expression
Ct <40	Very low / undetectable expression

Figure 4.8: Expression profile of MDA-MB-231 EV, TS15 and E362A cells. qRT-PCR analysis of gene expression in MDA-MB-231 EV, TS15 and E362A cells. Relative expression levels are shown as a Ct value heat-map. The displayed Ct values are averaged from three independently collected RNA samples. These data are an extended set from that shown in figure 3.8. See appendix A7 to see an expanded version of this data set.

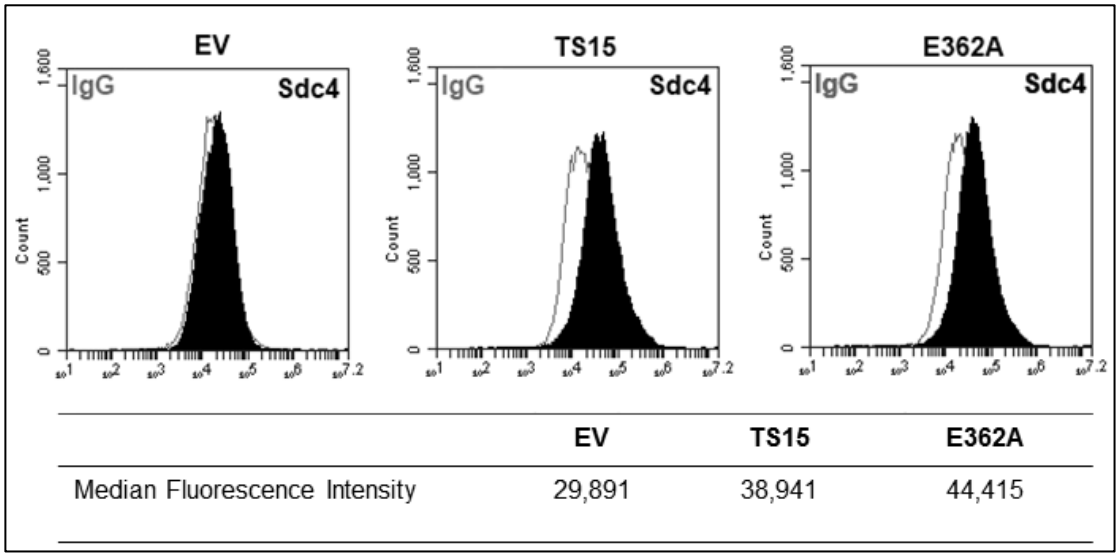
4.4 Investigation of potential involvement of syndecan-4 in ADAMTS-15 effect on cell migration

As discussed in the introduction chapter there seems to be an intimate connection between the syndecans and the ADAMTSs, which co-ordinate with other metalloproteinases to regulate cell-ECM interactions. This is particularly apparent since ADAMTS-1 shedding of syndecan-4 from the cell surface results in a functional loss of cell adhesion and promotes a pro-migratory phenotype with a concomitant loss of migration directionality (Bass *et al.* 2007; Choi *et al.* 2011; Rodríguez-Manzaneque *et al.* 2009).

In light of these studies and since both ADAMTS-15 and syndecan-4 are anti-migratory there may be a functional link. Interestingly, expression of TS15 and E362A in MDA-MB-231 cells were associated with increased surface levels of syndecan-4 in comparison to control EV cells (Figure 4.9). Yet expression of the syndecan-4 sheddases ADAMTS-1 and -4 were approximately 2-fold higher in TS15 and E362A expressing cells (Figure 4.8). Thus it could be inferred that ADAMTS-15 may be protecting syndecan-4 from sheddase activity.

In agreement with this hypothesis knockdown of syndecan-4 in MDA-MB-231 TS15 and E362A expressing cells abrogated the anti-migratory phenotype (Figure 4.10). Directionality was also lost upon knockdown of syndecan-4 in TS15 and E362A expressing cells (Figure 4.10), whilst syndecan-4 knockdown in control cells elicited no effect (Figure 4.10). Based on these data it is possible that syndecan-4 and ADAMTS-15 are functionally linked within the context of regulating breast tumour cell migration. ADAMTS-15 may protect syndecan-4 from sheddase action, thus promoting the anti-migratory effects of syndecan-4.

To explore this link further, the effects of modulation of PKCα signalling, which lies downstream of syndecan-4, were examined.



	EV	TS15	E362A
Median Fluorescence Intensity	29,891	38,941	44,415

Figure 4.9: ADAMTS-15 expression is associated with increased levels of syndecan-4 on the cell surface. Flow Cytometry analysis of the surface levels of Syndecan-4 (Black) on MDA-MB-231 EV, TS15 and E362A cells. Graphs are representative and median fluorescence intensity values were averaged from three independently collected cell samples. IgG isotype control (White) antibody was utilised as a negative control.

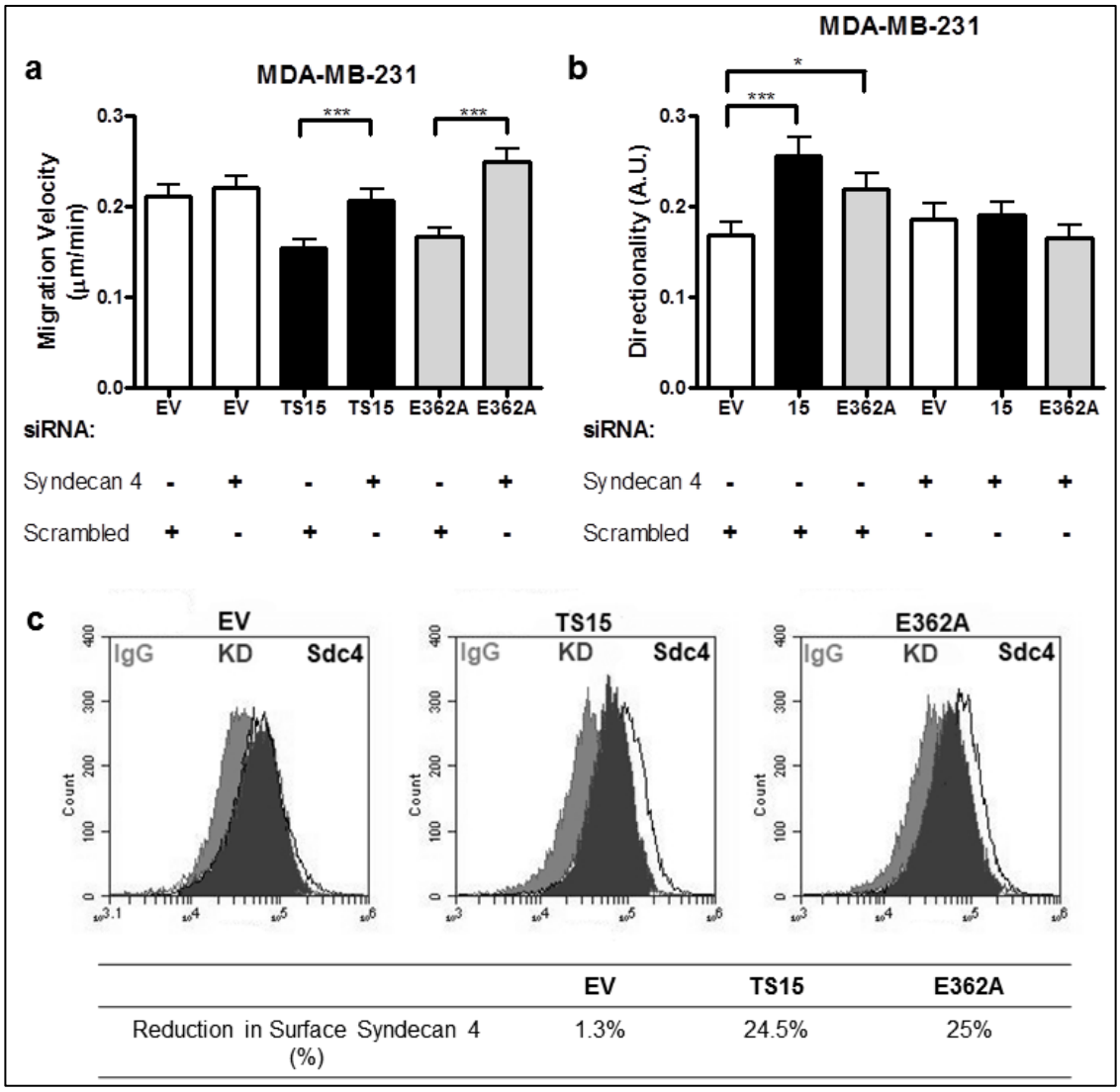


Figure 4.10: Knockdown of Syndecan-4 abrogates ADAMTS-15 anti-migratory phenotype. (**a**) MDA-MB-231 EV, TS15 or E362A cells were transfected with either non-targeting siRNA (control) or Syndecan-4 siRNA and cell migration tracked via time-lapse microscopy over a period of 24 hours, data represent 50 individually tracked cells per group, error bars represent s.e.m.; ***p<0.001. (**b**) The migration directionality (Euclidian distance / accumulated distance) of each individually tracked cell was also determined, data represent 50 individually tracked cells, error bars represent s.e.m.; *p=0.05, ***p<0.001. (**c**) Flow cytometry analysis of surface levels of Syndecan-4 on MDA-MB-231 EV, TS15 and E362A cells that were transfected with either non-targeting control siRNA (White) or syndecan-4 siRNA (Black). Isotype secondary antibody, IgG (Grey), was utilised as a staining control. Reduction in surface Syndecan-4 was determined as a percentage reduction of median fluorescence from cells transfected with syndecan-4 siRNA vs. control siRNA. Graphs are representative and median fluorescence intensity values were averaged from three independently collected cell samples.

4.5 Analysis of the involvement of signalling pathways that might be involved in mediating the ADAMTS-15 anti-migratory phenotype

4.5.1 PKC signalling

PKCα signalling is intimately connected to syndecan-4 since none of the other syndecan family members contain a PKCα binding motif within their cytoplasmic domains (Keum *et al.* 2004; Oh, Woods and Couchman, 1997). Upon engagement with fibronectin, the cytoplasmic domain of syndecan-4 recruits, stabilises and activates PKCα at the plasma membrane (Keum *et al.* 2004; Oh, Woods and Couchman, 1997). PKC α signalling feeds into cell adhesion and migration pathways through the activation of Rac1 and RhoA (Avalos *et al.* 2009; Carr *et al.* 2013). Since PKCα mediates syndecan-4 signalling, pharmacological activation or inhibition of PKCα may provide some insight into the role of syndecan-4 in the ADAMTS-15 anti-migratory phenotype.

MDA-MB-231 EV, TS15 and E362A cells were treated with 0-100nM of phorbol 12-myristate 13-acetate (PMA), a known activator of PKC signalling (Methods 2.11; Keum *et al.* 2004; Oh, Woods and Couchman, 1997). Treatment of MDA-MB-231 cells with either 50nM or 100nM PMA stimulated cell migration (Figure 4.11a). Thus PMA-mediated activation of PKCα abrogated the anti-migratory phenotype.

However, PMA is not a specific activator of PKCα, since it is known from previous studies that in addition to PKCα, PMA also stimulates PKCδ (Brodie *et al.* 2004; Murakami *et al.* 2004; Shanmugam *et al.* 1998). PKCδ serves as a

negative regulator of PKCα activity, since overexpression of PKCδ is associated with decreased PKCα activity, at least in endothelial cells (Murakami *et al.* 2004). Whilst PKC α establishes pro-adhesive and regulated migratory structures, PKCδ signalling on the other hand favours impaired cell migration (Brodie *et al.* 2004; Murakami *et al.* 2004). Therefore PMA stimulation of PKC signalling may have imposed an imbalance in these signalling pathways, towards the observed pro-migratory phenotype (Figure 4.11a).

PMA also activates ERK1/2 signalling which is known to enhance tumour cell migration (Brodie *et al.* 2004; von Thun *et al.* 2012). Whilst this may suggest that PMA treatment may have stimulated migration independently of syndecan-4 signalling, PKC δ has been shown to activate ERK1/2 (Brodie *et al.* 2004; Keshamouni, Mattingly and Reddy, 2002).

Another interpretation of these data is that PMA may have accelerated the shedding of syndecan-4 through an induction of the expression of several metalloproteinases, including the syndecan-4 sheddase ADAMTS-1 (Brule *et al.* 2006; Rodríguez-Manzaneque *et al.* 2009; Worley *et al.* 2009). Thus PMA stimulation may have accelerated the shedding of syndecan-4 such that any protective effects elicited by ADAMTS-15 were overcome (Rodríguez-Manzaneque *et al.* 2009).

Conversely treatment with the PKC inhibitor, bisindolylmaleimide I (BIM I) suppressed migration in control cells, yet had no effect on TS15 or E362A expressing cells (Figure 4.11a; Methods 2.11; Avalos *et al.* 2009; Bass *et al.* 2007; Keum *et al.* 2004). Arguably PKC signalling in TS15 and E362A cells is already reduced such that the addition of an inhibitor has no effect. It can be

speculated that these data highlight that PKC signalling may be tightly regulated in ADAMTS-15 expressing cells such that PKC signalling is localised to syndecan-4 focal adhesion complexes. However until syndecan-4/PKC activity and localisation is examined in greater detail these data are supportive, but not conclusive of a functional link between ADAMTS-15 and syndecan-4. To this end several other signalling pathways were also examined.

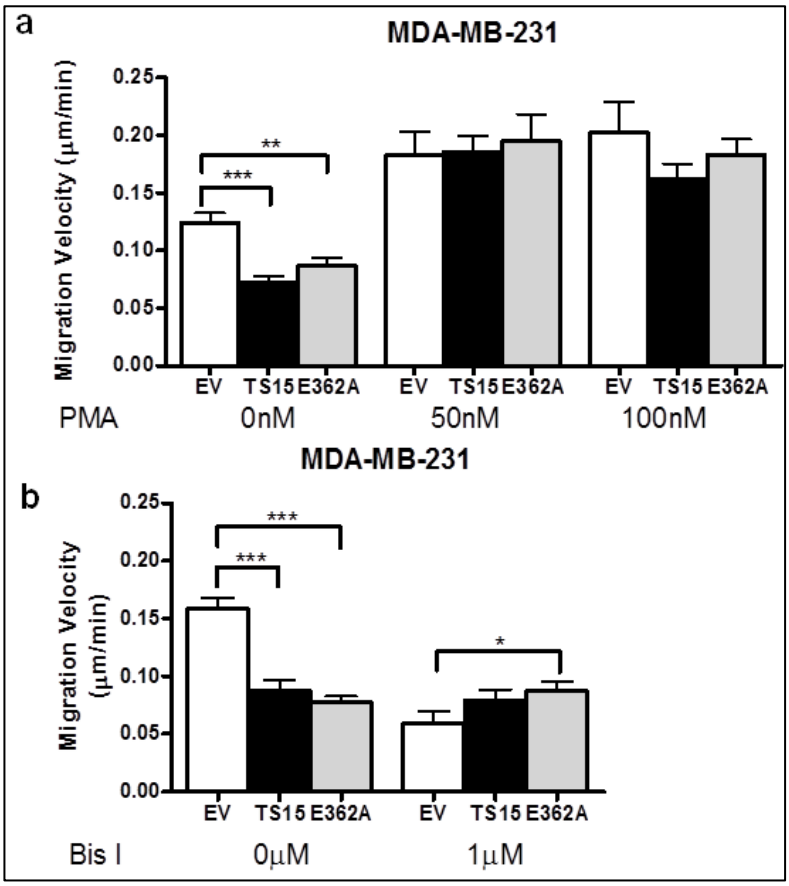


Figure 4.11: Modulation of signalling pathways down-stream of Syndecan-4 abrogates ADAMTS-15 anti-migratory phenotype. (**a**) MDA-MB-231 EV, TS15 or E362A cells were treated with 0-100nM phorbol 12-myristate 13-acetate (PMA) and cell migration tracked via time-lapse microscopy over a period of 24 hours. Data represent the mean of at least 15 individually tracked cells per group, error bars represent s.e.m.; **p<0.01, ***p<0.001. (**b**) MDA-MB-231 EV, TS15 or E362A cells were treated with 0 μM or 1μM BIM I and cell migration tracked via time-lapse microscopy over a period of 24 hours, Data represent 30 individually tracked cells per group, error bars represent s.e.m. *p<0.05, ***p<0.001.

4.5.2 EGF signalling

Following EGF stimulation MDA-MB-231 cells expressing either TS15 or E362A displayed reduced 2D migration at EGF concentrations up to 50ng/ml (Figure 4.12; Methods 2.11). However, at 100ng/ml EGF, the migration levels of cells expressing TS15 or E362A were restored to control (EV) levels. The effect of ADAMTS-15 expression on pERK activation was investigated in greater detail via a pERK activation time course assay. MDA-MB-231 EV, TS15 and E362A cells were treated with EGF (50ng/ml) for the indicated times and then analysed via western blot (Figure 4.12). Whilst these western blots are not definitive it appears that EV cells show an increase in activation of pERK at 30 minutes post EGF stimulation, whereas TS15 and E362A expressing cells display a delayed activation of pERK at 60 minutes post EGF stimulation. This pattern was generally consistent across the two independent experiments shown; however there was some inter-experimental variability.

Together these data indicate that independent of its catalytic activity, ADAMTS-15 reduces the sensitivity of cells to the migration stimulatory effects of EGF. This is consistent with reports that ADAMTS-15 expression inhibits ERK activation in colorectal cancer cells (Viloria *et al.* 2009).

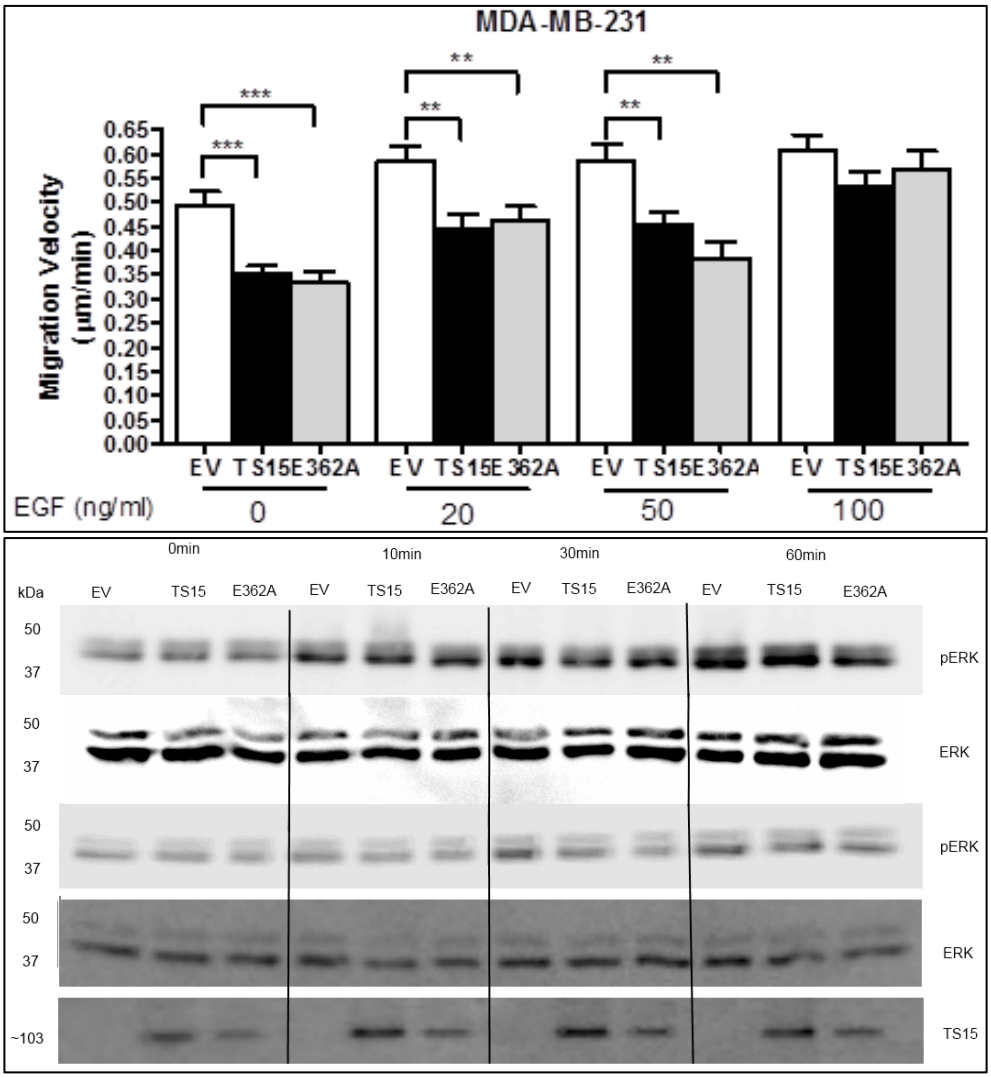


Figure 4.12: ADAMTS-15 reduces the sensitivity of breast cancer cells to the migration stimulatory effects of EGF. (Top) MDA-MB-231 EV, TS15 or E362A cells were treated with 0-100ng/ml EGF and cell migration tracked via time-lapse microscopy over a period of 24 hours, n=30-40, error bars represent s.e.m.; ** p≤0.01, *** p≤0.001. (**Bottom**) WB analysis of pERK activation post EGF stimulation (50ng/ml final concentration). Two independent experiments are shown. Protein sizes: ADAMTS-15 ~103kDa, ERK/pERK 42/44 kDa).

4.5.3 ROCK signalling

MDA-MB-231 EV, TS15 and E362A cells were treated with an established and specific inhibitor of the Rho-associated kinases (ROCK) (Brew *et al.* 2009; Narumiya, Ishizaki and Uehata, 2000). Treatment with Y-27632 inhibited the migration of MDA-MB-231 control cells (EV), yet had no statistical effect on the migration of ADAMTS-15 expressing cells (Figure 4.13). Whilst not statistically significant, Y-27632 may have slightly enhanced the migration of E362A-expressing cells (Figure 4.13). It is well established that inhibition of ROCK signalling has an anti-migratory effect, since ROCK-inhibited cells fail to form stable focal adhesion complexes from which cells can generate the forces needed to migrate (Brew *et al.* 2009; Narumiya, Ishizaki and Uehata, 2000). Therefore it is not surprising that Y-27632 inhibited the migration of MDA-MB-231 control cells (EV). It is however interesting that inhibition of ROCK signalling did not completely abolish the migratory capacity of ADAMTS-15 expressing cells. These data suggest that ROCK signalling is to some extent already inhibited in ADAMTS-15 expressing cells. Since RhoA (and Rac1) is frequently overexpressed in invasive breast tumours, lower levels of ROCK activation in ADAMTS-15 expressing cells may be a putative anti-migratory mechanism (Brew *et al.* 2009).

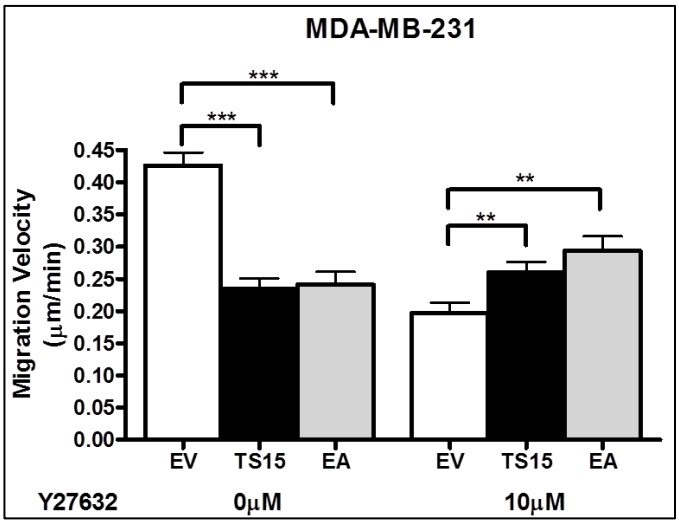


Figure 4.13: ROCK inhibitor Y27632 does not affect the ADAMTS-15 anti-migratory phenotype. MDA-MB-231 EV, TS15 or E362A cells were treated with 0-10μM Y27632 and cell migration tracked via time-lapse microscopy over a period of 24 hours, data represent 27-37 individually tracked cells per group, error bars represent s.e.m. **p<0.01, ***p<0.001.

4.5.4 Global effects on kinase phosphorylation

To examine global effects on cell signalling pathways that are associated with expression of ADAMTS-15 an R&D phosphokinase array was utilised (Methods 2.24). Cell lysates from MDA-MB-231 EV, TS15 and E362A cells were incubated with membranes that were pre-printed with anti-phospho-kinase antibodies for 43 different kinases. The membranes were imaged using HRP and phosphokinase levels determined through an analysis of the pixel density of antibody detection spots. Pixel density is dependent upon the level of phosphokinases in cell lysate samples that bind to the anti-phospho-kinase antibodies. Raw data were plotted and analysed according to the observed patterns (Figure 4.14; Appendix 6).

Kinases with increased (activity-independent activation) or decreased (activity-independent inhibition) phosphorylation levels in both TS15 and E362A cells compared to EV, may indicate functional consequences that are independent of ADAMTS-15 metalloproteinase proteolytic activity. The phosphorylation levels of several kinases including Fyn, Lyn, Lck and Yes were all reduced in TS15 and E362A cells compared to control. These kinases lie downstream of integrin β4 and are involved in regulating cell migration pathways (Figure 4.14b).

Kinases with increased (TS15-dependent activation) or decreased (TS15-dependent inhibition) phosphorylation levels specifically in TS15 cells may indicate functional consequences of ADAMTS-15 proteolytic activity. Interestingly ERK 1/2 phosphorylation levels were higher specifically in TS15 overexpressing cells (TS15 dependent-activation). Yet ADAMTS-15 has been reported to reduce basal levels of pERK in ADAMTS-15 transfected HCT-116

cells (Viloria *et al.* 2009). Perhaps these differences indicate differences in breast and colorectal cancer cell lines or that ADAMTS-15 functions differently depending upon context. Conversely ERK signalling is involved in regulating cell proliferation, yet over expression of ADAMTS-15 does not affect breast cancer cell proliferation (Figure 3.4). Several pERK antibodies are reported to be sensitive enough to detect as little as 50pg of phosphorylated MAP kinase protein, therefore it is possible that these signal differences may only represent small differences in detectable protein levels. In addition to this only one cell lysate sample was used for each cell line and therefore this result may be an outlier.

Several kinases showed no difference (c-Jun, MSK1/2 and JNK pan) or were ambiguous (p53-S392, Pyk2, Chk-2 and eNOS) in that the observed changes did not show a consistent pattern, and may simply be background 'noise'. Kinases with increased (E362A-dependent activation) or decreased (E362A-dependent inhibition) phosphorylation levels specifically in E362A cells were equally puzzling yet 21% of the kinases examined displayed an E362A-dependent pattern. It is unclear how the metalloproteinase inactive form of ADAMTS-15 (E362A) could have a 'gain-of-function', though it is possible that E362A could act as a dominant-negative against endogenous ADAMTS-15. However no E362A specific effects have been observed in any functional assay and therefore it is possible that these patterns are also random 'noise'.

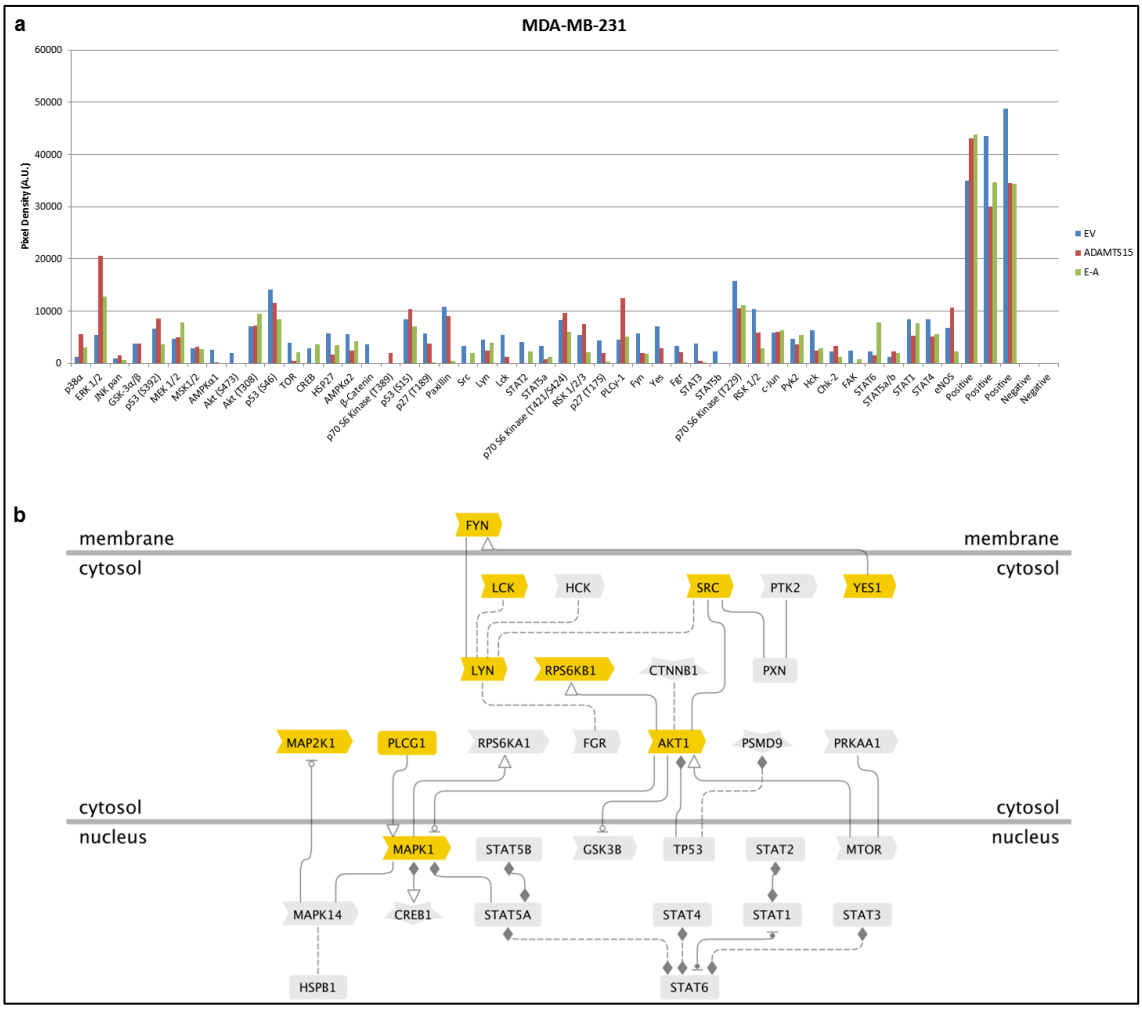


Figure 4.14: Global effects on kinase phosphorylation in ADAMTS-15 expressing cells. Cell lysates from MDA-MB-231 EV, TS15 and E362A were incubated with membranes that were pre-treated with anti-phosphokinase antibodies as part of the R&D Human phosphokinase array kit. (**a**) An analysis of HRP signal pixel density was utilised to determine the levels of kinase activation compared to control spots on the membrane. (**b**) Genomatix cloud based software was utilised to highlight kinases (yellow) that were less active in TS15 and E362A cells compared to control EV cells. Genes shown in grey are there to demonstrate several cell migration/integrin signalling pathways that the highlighted kinases are a part of. Data represent one cell lysate sample per cell group.

4.6 Analysis of migration in 3D matrices

The ADAMTS-15 anti-migratory phenotype has largely been characterised within a 2D context, yet cells exist within a 3D environment *in vivo*. Whilst 2D assays are informative cells may exhibit behaviours that are more physiologically relevant when seeded within 3D matrices. For instance cells exhibit different modes of migration when seeded into a 3D context in comparison to 2D tissue culture environments (Ashby *et al.* 2012; Blobel *et al.* 2010; Even-Ram and Yamada, 2005; Friedl *et al.* 2012; Petrie *et al.* 2012). To address this, ADAMTS-15 expressing breast tumour cells were seeded within either collagen type I gels or matrigel (Methods 2.12). Because cells could migrate up or down through these 3D gels, multiple planes on the Z-axis were captured. This was an appropriate precaution since cells did migrate into and out of the ideal focal plane. However cells could still be tracked and analysed in a similar manner to the method established for 2D assays.

Another notable difference between 2D and 3D assays is that cells may need to proteolytically degrade the 3D matrix in order to clear a migration pathway. Therefore the surface levels of the collagenase MT1-MMP were analysed since this membrane bound metalloproteinase has been shown to enhance the invasive potential of tumour cells, in part through the degradation and clearing of collagen (Eisenach *et al.* 2012; Ota *et al.* 2009; Sabeh *et al.* 2004). Surface levels of MT1-MMP were equivalent in MDA-MB-231 EV, TS15 and E362A expressing cells and in agreement with 2D migration data ADAMTS-15 expression does not affect cell migration either on or through collagen type I (Figure 4.5; 4.15b-c).

Interestingly expression of TS15 or E362A did reduce 3D migration of MDA-MB-231 cells, through matrigel in comparison to empty vector (EV control) cells (Figure 4.15). Since Matrigel is a mix of several basement membrane matrix components including laminin these data are in agreement with the matrix specific effects of ADAMTS-15 observed in 2D migration assays (Figure 4.5).

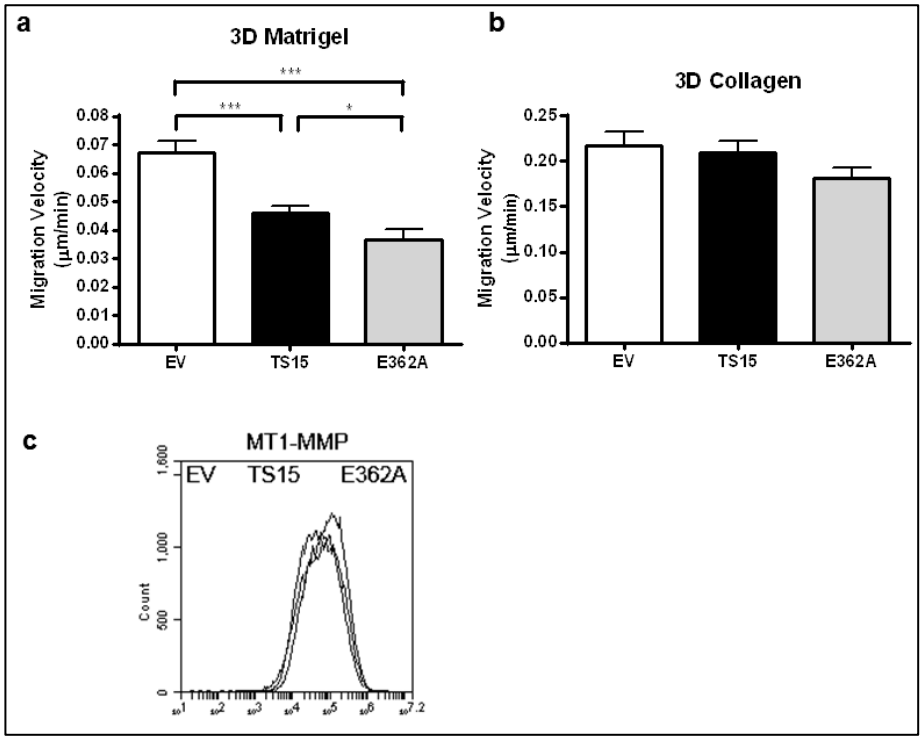


Figure 4.15: ADAMTS-15 reduces 3D migration through matrigel but not collagen type I in a metalloproteinase independent manner. MDA-MB-231 EV, TS15 and E362A cells were seeded into Matrigel (**a**) or Collagen type I gels (**b**). 3D cell migration tracked via time-lapse microscopy over a period of 24 hours, data represent 43 individually tracked cells per group, error bars represent s.e.m. *p<0.05, ***p<0.001 (**c**) Flow cytometry analysis of surface levels of MT1-MMP on MDA-MB-231 EV, TS15 and E362A cells. Data are representative of three independently collected cell samples per group.

4.7 Discussion

4.7.1 ADAMTS-15 inhibits cell migration in a metalloproteinase independent manner

ADAMTS-15 is emerging as an anti-migratory metalloproteinase, which so far has been shown to reduce the invasive potential of colorectal cancer cell lines *in vitro* and *in vivo* (Viloria *et al.* 2009). Thus an inhibition of the invasive potential of tumour cells may be the putative protective mechanism that underpins the increased survival prospects for breast and colorectal cancer patients that express ADAMTS-15 (Porter *et al.* 2006; Viloria *et al.* 2009). In light of these studies the aim of this chapter was to investigate the functional consequences of ADAMTS-15 expression on human breast cancer cell migration. To this end several migration assays were utilised, including the scratch wound assay.

The scratch wound assay is typically described as an *in vitro* wound healing model, since inflicted scratch wounds remove the underlying matrix along with a section of the cell layer (Ashby and Zijlstra, 2012; Hsu *et al.* 2012; Krampert *et al.* 2005). Wounding is typically a manual process, which adds a level of variability to these assays since scratches may not always be perfectly uniform and cells along the wound edges may be inadvertently damaged. To ensure consistency, cell density and wounding strategies were optimised prior to the experiments and non-uniform scratches were excluded from the analysis.

These data show for the first time, that expression of ADAMTS-15 reduces the scratch wound recovery of human breast cancer cells (Figure 4.1). Wound closure can also be a consequence of cell proliferation, though based upon data

presented earlier, ADAMTS-15 does not affect breast cancer cell proliferation (Figure 3.9), and thus it is likely that ADAMTS-15 inhibited scratch wound closure through an inhibition of cell migration (Figure 4.1). In addition to this the expression of the metalloproteinase-inactive form of ADAMTS-15 (E362A) also reduced scratch wound recovery (Figure 4.1). Therefore, ADAMTS-15 inhibition of cell migration is independent of its catalytic activity.

The scratch wound assay can be adapted to examine individual or collective migration. For collective migration assays, the cell monolayers at either side of the scratch have the potential to collectively migrate into the wound (Bindschadler and McGrath, 2007; Riahi *et al.* 2012). However, close examination of the time-lapse images reveals that in these assays, the cells tended to migrate individually into the scratch wound (Figure 4.1). In light of this, these data are not sufficient to examine any influence ADAMTS-15 might have on collective cell migration. Nevertheless, future studies should examine this, since collective migration during embryonic development and tumour cell invasion may be mediated by several metalloproteinases (Ismat, Cheshire and Andrew, 2013). It is therefore possible that ADAMTS-15 might have a role in the regulation of collective cell migration. To investigate this Matrigel spheroid assays could be utilised as previously described (Dang, Prechtl, and Pearson, 2011; Serres *et al.*, 2013).

Whilst collective cell migration could not be examined, cell density is still an important factor to consider since cell-cell interactions are known to influence cell behaviour. However, in this case, expression of ADAMTS-15 inhibited breast cancer cell migration even when cells were seeded at a low density (Figure 4.2). These 2D migration assays were also adapted such that siRNA

knockdown of TS15 or E362A restored migration to control, EV levels (Figure 4.3). In combination these data demonstrate a robust and reproducible association between ADAMTS-15 expression and a metalloproteinase independent inhibition of breast cancer cell migration.

4.7.2 ADAMTS-15 modulates cell-ECM interactions

Previously, ECM associated ADAMTS-15 has been shown to reduce the invasive potential of colorectal cancer cell lines (Viloria *et al.* 2009). Whilst the mechanism was not identified, these data suggest that ADAMTS-15 might modulate cell-ECM interactions and influence cell migration. To determine whether ECM associated ADAMTS-15 is functionally capable of modulating breast cancer cell migration, several matrix assays were established, including an adapted conditioned matrix assay (Khalkhali-Ellis and Hendrix, 2007; Phillips *et al.* 2008). To summarise these conditioned matrix data, ADAMTS-15 and to a lesser extent, E362A conditioned matrices inhibited the migration of naïve EV control cells (Figure 4.4). Therefore these data provide additional evidence that ECM-deposited ADAMTS-15 inhibits breast cancer cell migration and by extension ADAMTS-15 modulates cell-ECM interactions.

A potential limitation of the conditioned matrix assay is that the absolute amount of deposited matrix was not quantified. Instead conditioned matrix samples were visualised in Coomassie blue-stained gels (data not shown). Whilst there were no qualitative differences in matrix deposition, a quantitative analysis of matrix deposition may be required for future studies. Thus matrix variability could potentially explain why E362A conditioned matrices did not inhibit cell migration to the same extent as TS15 conditioned matrices. Differences in

protein stability between *wildtype* and E362A forms of ADAMTS-15 may have also contributed to these disparities. Nevertheless, as previously discussed, attempts were made to control for these factors. For instance conditioned cells were seeded at a density that was informed through the optimisation of the scratch wound assay and as discussed in chapter 3, the mRNA and protein levels of TS15 and E362A were comparable between the appropriate cell lines (Figure 3.6; Figure 3.8). In summary, reasonable controls were enacted to ensure that MDA-MB-231 EV, TS15 and E362A cells would lay down comparable levels of matrices.

In addition to the conditioned matrix assays, MDA-MB-231 EV, TS15 and E362A cells were seeded onto surfaces pre-coated with either collagen type I, fibronectin or laminin. These matrices were selected since they are ECM components that are found within human mammary tissue architecture (Butcher, *et al.* 2009; Gusterson *et al.* 1982; Hancox *et al.* 2009; Mao and Schwarzbauer, 2005). As expected ADAMTS-15 inhibited cell migration in a metalloproteinase-independent manner, however in these assays the effects were matrix specific. Expression of TS15 or E362A inhibited cell migration on fibronectin and laminin but not collagen type I (Figure 4.5). In all other assays (e.g. scratch wound) cells were seeded with serum-supplemented media. Since serum contains fibronectin, essentially ADAMTS-15 was inhibiting cell migration on a fibronectin-enriched matrix.

These matrix specific effects can be explained in part by the adhesive behaviour of ADAMTS-15 expressing cells. TS15 and E362A expressing cells display a relatively more spread out morphology and in adhesion assays more strongly adhered to laminin and fibronectin in comparison to EV control cells

(Figure 4.5). There were no differences in cell adhesion on collagen type I (Figure 4.5). Migration time-lapse images also revealed that TS15 and E362A expressing cells adhered to the extent that cell detachment, particularly from laminin, required extensive cellular force (Figure 4.6a). In some cases cell adhesion was so strong that cells ruptured their cell membranes in order to de-adhere (Figure 4.6a).

These matrix specific effects were also recapitulated within 3D migration models that are more representative of the 3D *in vivo* tissue environment (Ashby *et al.* 2012; Blobel *et al.* 2010; Even-Ram and Yamada, 2005; Friedl *et al.* 2012; Petrie *et al.* 2012). MDA-MB-231 EV, TS15 and E362A cells were seeded within either collagen type I gels or Matrigel, a basement membrane matrix solution, that includes laminins, collagen type IV and fibronectin. In agreement with the 2D migration assays, the inhibition of 3D cell migration was also matrix specific: ADAMTS-15 inhibited breast cancer cell migration in a metalloproteinase-independent manner through Matrigel but not collagen type I gels (Figure 4.15). Thus ADAMTS-15 modulates interactions between breast tumour cells and specific components of the surrounding extracellular matrix. As an extension of this, it can be argued that these matrix specific effects provide insight into the broader significance of ADAMTS-15 during breast tumourigenesis.

4.7.3 Importance of ADAMTS-15 during breast tumourigenesis

Within the ductal structures of the breast, it is the ADAMTS-15 expressing myoepithelial cells that maintain breast tissue architecture (Adriance *et al.* 2005; Butcher *et al.* 2009; Gordon *et al.* 2003). Since ADAMTS-15 enhances cell adhesion to basement membrane proteins, it is possible that expression of ADAMTS-15 is one of the mechanisms through which myoepithethial cells maintain breast tissue integrity (Figure 4.5; Figure 4.15; Adriance *et al.* 2005; Butcher *et al.* 2009; Gordon *et al.* 2003). In other words, during breast tumourigenesis ADAMTS-15 may be protective since it may function to restrain tumour cells within the basement membrane. Tumour cells might tolerate the presence of myoepithelial derived ADAMTS-15 during the earlier stages of tumourigenesis, due to ADAMTS-15 having no observable influence on breast tumour cell proliferation (Figure 3.9). Indeed there are no differences in ADAMTS-15 expression between cancer and normal tissues (Porter *et al.* 2004; 2006). During the transition to invasive disease however, breast architecture becomes increasingly aberrant (poorly differentiated), the myoepithelial-basement membrane interface is lost and tumour cells are more freely able to invade into the surrounding tissue (Adriance *et al.* 2005; Butcher *et al.* 2009; Gordon *et al.* 2003; Hsiao *et al.* 2011; Porter *et al.* 2004; 2006; Jones, 2006; Polyak and Hu, 2005). Within this context, ADAMTS-15 expression is lost and as a consequence the invasive potential of luminal epithelial cells is enhanced (Porter *et al.* 2004; Porter *et al.* 2006; Viloria *et al.* 2009).

As well as the loss of the basement membrane, additional changes in the ECM also occur during breast tumourigenesis. The extracellular matrix ‘stiffens’ due

to the increased deposition of collagen type I and other matrix components. These changes enhance the invasive and metastatic potential of tumour cells (Gusterson *et al.* 1982; Hojilla *et al.* 2008; Kakkad *et al.* 2012; Mueller and Fusenig, 2004; Spivey *et al.* 2012; Svetlana*et al.* 2007). As a side note, the MDA-MB-231 cells used in this study were also chosen since they are intrinsically capable of proliferating, adhering and migrating within stiff matrix environments (Tilghman *et al.* 2010). Therefore, the poorly differentiated MDA-MB-231 cells, within the 3D collagen type I gels are an appropriate model of the 'stiff-matrix' environment that occurs post-DCIS. Within this 'stiff-matrix' environment ADAMTS-15 expression did not alter the intrinsic capabilities of MDA-MB-231 cells to adhere and migrate on or through collagen type I (Figure 4.5; Figure 4.15). However, like other ADAMTSs, ADAMTS-15 may have additional tumour suppressor functions, beyond the ability to restrain tumour cells within the breast ductal structure, which will be covered in the subsequent chapter. Before moving onto these additional tumour suppressor functions , these matrix specific effects were examined further. To provide a greater mechanistic insight, the integrin profiles of MDA-MB-231 EV, TS15 and E362A cells were examined, since differences in the expression of fibronectin and laminin binding integrins could explain why ADAMTS-15 expressing cells adhered more strongly to these matrices.

Several integrins were profiled that are known to bind to either fibronectin and/or laminin. Integrin β1 was examined since it is capable of forming into 6 laminin and 3 fibronectin adhesion complexes, depending upon which α subunit forms the heterodimer pair (Table 1.5). Expression of integrin β1, as determined by qRT-PCR, was approximately 2x higher in MDA-MB-231 TS15 and E362A

cells in comparison to control EV cells (Figure 4.8). These data are suggestive that ADAMTS-15 expressing cells may express higher levels of integrin β1 containing adhesion complexes. Yet at the protein level there were no differences in either total or active integrin β1 (Figure 3.10a; Figure 4.7). The surface levels of integrin β1 were also unaffected by expression of ADAMTS-15 (Figure 4.7). Expression of integrin α5, as determined by qRT-PCR, was also approximately 2x higher in TS15 and E362A expressing cells (Figure 4.8). Since α5β1 binds fibronectin these data may provide mechanistic insight into the ADAMTS-15 associated matrix specific effects (Table 1.5).

Integrin α6 can partner with either integrin β1 or β4, to form a laminin binding adhesion complex (Bass, Morgan and Humphries, 2007; Wang *et al.* 2010). Whilst there were no differences in α6 or β1 total protein, interestingly total integrin β4 protein is increased in MDA-MB-231 TS15 and E362A cells (Figure 4.7). Earlier discussions have presented the context through which ADAMTS-15 may function to maintain breast tissue integrity. This has clear synergies with what is already known about integrin β4 and its role in regulating cell adhesion and migration, however at this point a functional link between ADAMTS-15 and β4 is largely circumstantial. Nevertheless this will be of interest to future studies and therefore warrants some discussion.

An increase in total integrin β4 may have a biological significance in breast tissues, since integrin α6β4 signalling regulates epithelial cell polarity (Litjens, de Pereda and Sonnenberg, 2006; Gilcrease *et al.* 2007; Natali *et al.* 1992). Integrin α6β4 is an essential component of the hemidesmosome adhesion complex, which functions to anchor cells, such as epithelial cells to laminin-5 (332) in the basement membrane (Litjens, de Pereda and Sonnenberg, 2006;

van der Neut *et al.* 1996; Wang *et al.* 2010). In combination these integrin α6β4-mediated processes act to establish and maintain the integrity of breast tissue architecture. The regulation of hemidesmosomes, particularly in a cancer context, is complex and involves extensive signalling crosstalk between α6β4, EGFR, syndecans and other cell surface receptors (Mariotti *et al.*, 2001; Wang *et al.* 2010). Interactions between α6β4 and plectin are known to initiate the formation of type II hemidesosomes (Litjens, de Pereda and Sonnenberg, 2006). The tetraspanin CD151 may also join during the early stages of hemidesomosme formation, followed by BP180 and BP230 (Litjens, de Pereda and Sonnenberg, 2006). Typically, integrin α3β1, syndecans and EGFR are recruited once the hemidesomosme reaches a mature state (Litjens, de Pereda and Sonnenberg, 2006).

During the transition to invasive breast cancer, hemidesmosomes are disassembled and integrin α6β4 is re-distributed along the leading edge of the migrating cell (Mariotti *et al.* 2001; van der Neut *et al.* 1996; Wang *et al.* 2010). A re-distribution of α 201 to the lamellipodia is associated with increased invasive potential and a poor clinical outcome for breast cancer patients (Gabarra *et al.* 2010; Gordon *et al.* 2003; Natali *et al.* 1992). Hemidesmosome disassembly is mediated either by activated EGFRs which can phosphorylate several tyrosine residues (Tyr- 1257, 1440, 1494 and 1526) and/or PKC-mediated phosphorylation of several serine residues (Ser-1356, 1360, and 1364) within the β4 signalling domain (Mariotti *et al.*, 2001; Rabinovitz, Tsomo, and Mercurio, 2004; Wang *et al.* 2010). These signalling events alter the conformation of the β4 cytoplasmic domain, perturb β4-plectin interactions and

ultimately lead to the physical disassembly of the hemidesmosome complex (Mariotti *et al.*, 2001; Wang *et al.* 2010).

Interestingly, ADAMTS-15 and E362A expressing breast tumour cells are relatively insensitive to both EGF and PMA (PKC activator) (Figure 4.11, 4.12). Thus it is possible that ADAMTS-15 may inhibit signalling pathways which lead to the disassembly of hemidesmosomes. This hypothesis is also consistent with the phosphokinase array data. Several kinases, which lie downstream of integrin β4, such as Fyn, show reduced activation in TS15 and E362A expressing cells (Figure 4.14). It is already known that an inhibition of Fyn increases the stability of hemidesmosomes and that stable hemidesmosome complexes suppress cancer cell migration and invasion (Kashyap *et al.* 2011; Mariotti *et al.*, 2001; Rabinovitz, Tsomo, and Mercurio, 2004). Therefore it is possible that ADAMTS-15 and α6β4 might act synergistically to anchor epithelial cells to the laminin-enriched basement membrane. In doing so, ADAMTS-15 may restrain epithelial cell migration and act to maintain breast tissue integrity. There is a distinct possibility that ADAMTS-15 might directly interact with hemidesmosomal integrin β4 or β1 via its disintegrin domain and thus act to stabilise hemidesmosomes directly. Although as of yet, there is no evidence of direct ADAMTS:integrin interactions. As an additional factor to consider, ADAMTS-15 expression did not alter the surface levels of integrin β4, as analysed by flow cytometry (Figure 4.7). Therefore the functional distribution and recycling of β4, rather than simply its total protein level may be more informative in terms of understanding how ADAMTS-15 inhibits cell migration on laminin. Future studies could utilise more sophisticated co-culture assays, such as the organoid models utilised by Holliday *et al.* 2009. These systems are

better equipped to examine any co-incidental link between hemidesmosome and basement membrane disassembly, the redistribution of α6β4 into lamellipodia and the loss of ADAMTS-15, which may all temporally coincide during the transition to invasive breast cancer.

4.7.3 ADAMTS-15 and syndecan-4

ADAMTS-15 may influence other cell adhesion receptors, such as syndecans and this thesis contains data in support of this. As previously discussed the syndecans are a family of four heparan sulfate proteoglycans that sit within the cell membrane as type I transmembrane receptors (Choi *et al.* 2011). Syndecans are functionally diverse though they may primarily function to recruit and activate integrins or growth factor receptors at matrix adhesion sites (Couchman and Woods, 1999; Choi *et al.* 2011; Morgan *et al.*, 2007; Wang *et al.*, 2010). Thus, the syndecans participate in the regulation of cell adhesion and migration signalling (Choi *et al.* 2011; Morgan *et al.*, 2007; Wang *et al.*, 2010). More specifically syndecan-1, which is broadly expressed in epithelial cells (also see Figure 4.8) has been shown to directly activate αvβ3 and αvβ5 integrins during breast tumour cell invasion (Beauvais, Burbach and Rapraeger, 2004; Wang *et al.*, 2010). Syndecan-4, on the other hand acts as a co-receptor for fibronectin in conjunction with integrin α5β1 (Bass, Morgan and Humphries, 2007; Wang *et al.* 2010). In some contexts syndecan-4 competes with syndecan-1 for binding to the laminin receptor α6β4 (Bass, Morgan and Humphries, 2007; Wang *et al.* 2010). As a generalisation, when syndecan-1 is bound to α6β4 they become a part of a complex involving Src family kinases and ErbB2 (Wang *et al.* 2010). Syndecan-1 is not required for the formation of the complex, however the binding of syndecan-1 to the distal region of the α6β4

cytoplasmic domain, enables Fyn to more efficiently phosphorylate and thus activate β4 (Wang *et al.* 2010). In concert the activated ErbB2, $\alpha 6\beta 4$ and Fyn complexes promote a pro-migratory phenotype (Wang *et al.* 2010). In contrast to this, whilst syndecan-4 may also mediate a similar process, there is growing evidence that syndecan-4, is anti-migratory (Rodríguez-Manzaneque *et al.*, 2009). Some reports describe syndecan-4 as being able to sequester and recycle integrin α6β4 within intracellular vesicles (Wang *et al.* 2010). Thus syndecan-1 and -4 may compete with one another to regulate the localisation and activity of integrins towards sites of active matrix engagement (Wang *et al.* 2010). As a result the levels of syndecans and integrins are a complex and highly regulated balance, with a net effect that is either pro or anti-migratory.

Interestingly, ADAMTS-15 and E362A expression are associated with increased surface levels of syndecan-4 (Figure 4.9). Thus it is possible, that in this study, the MDA-MB-231 TS15 and E362A cells are compensating for increased syndecan-4 mediated recycling of integrin $\beta 4$, through an up regulation of total $\beta 4$ protein (Figure 4.7). Thus a balance would be established where surface β4 would be largely maintained (Figure 4.7). Yet the up regulation of β4 protein in ADAMTS-15 expressing cells is not offsetting the pro-adhesive and anti-migratory activity of syndecan-4 (Rodríguez-Manzaneque *et al.*, 2009). So it is unclear why the tumour cells would compensate in this way and not through a more robust up regulation of the syndecan-4 sheddases ADAMTS-1 and -4 (Figure 4.8). A profiling of all laminin and fibronectin integrins in future studies will be revealing. It could be that as suggested here, the increase in total β4 is a side effect of the main mechanism(s) through which ADAMTS-15 mediates the

anti-migratory phenotype, namely that ADAMTS-15 protects syndecan-4 from being shed from the cell surface (Figure 4.9; Figure 4.16).

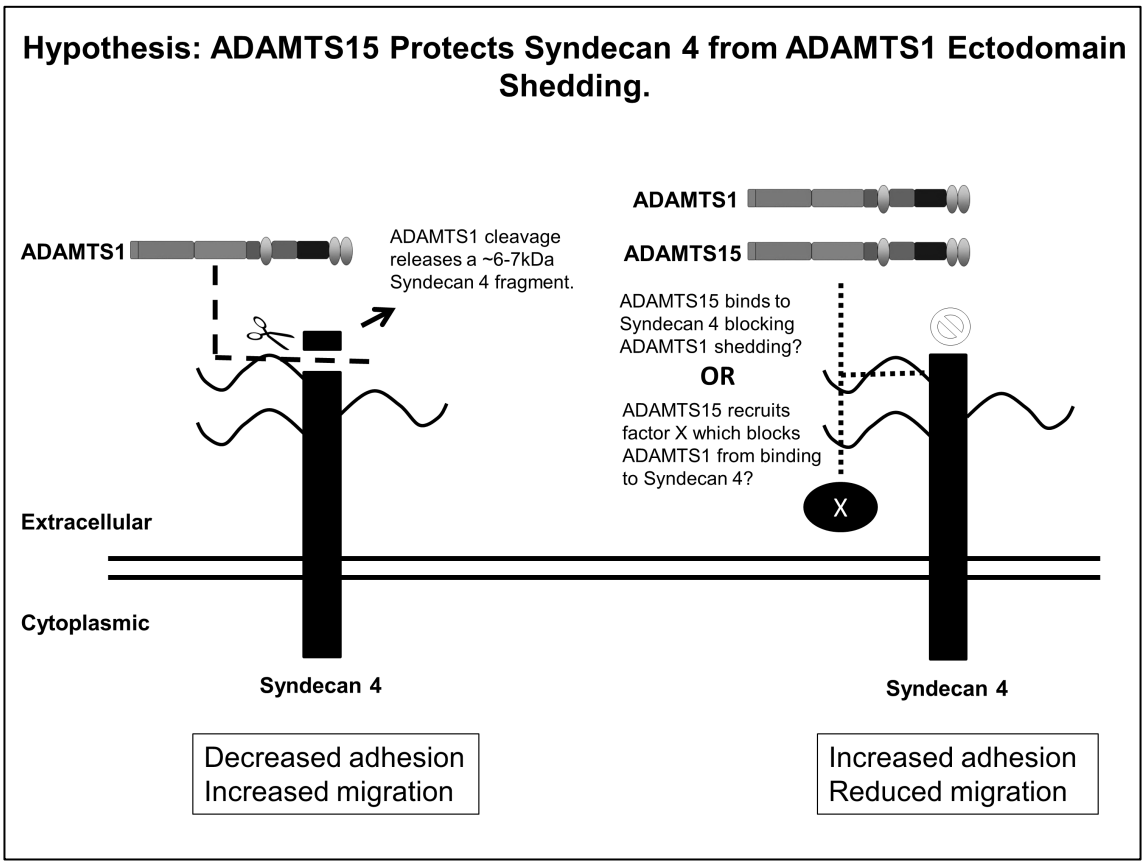


Figure 4.16: ADAMTS-15 may protect syndecan-4 from being shed from the cell surface. (**left**) Known ADAMTS-1 shedding activity is depicted (**right**) ADAMTS-15 directly or in-directly prevents ADAMTS-1 shedding of syndecan-4.

Expression of TS15 and E362A in MDA-MB-231 cells were associated with increased surface levels of syndecan-4 in comparison to control EV cells (Figure 4.9). Yet expression of the syndecan-4 sheddases ADAMTS-1 and -4 were approximately 2x higher in TS15 and E362A expressing cells (Figure 4.8). Thus it could be inferred that ADAMTS-15 may be protecting syndecan-4 from sheddase activity. In agreement with this hypothesis knockdown of syndecan-4 in MDA-MB-231 TS15 and E362A expressing cells abrogated the anti-migratory phenotype (Figure 4.10). Directionality was also lost upon knockdown of

syndecan-4 in TS15 and E362A expressing cells, whilst syndecan-4 knockdown in control cells elicited no effect (Figure 4.10).

Based on these data it is possible that syndecan-4 and ADAMTS-15 are functionally linked within the context of regulating breast tumour cell migration, such that ADAMTS-15 may protect syndecan-4 from sheddases, thus promoting the syndecan-4-associated anti-migratory phenotype. Whilst this thesis does not present any evidence of a direct association between ADAMTS-15 and syndecan-4, experimental modulation of syndecan-4 or downstream PKC signalling indicates that there is a functional link.

Stimulation with the PKC activator phorbol-12-myristate-13-acetate (PMA), increased the migration rate of EV cells, and it also overcame the inhibitory effects of TS15 and E362A, resulting in the cells displaying the same level of migration as control EV cells (Figure 4.11). The interpretation of these data is potentially complex (thesis section 4.5.2), however based upon what is known in the literature, it is possible that PMA stimulated an increase in the expression of the syndecan-4 sheddases. Thus PMA stimulation may have accelerated the shedding of syndecan-4 such that any protective effects elicited by ADAMTS-15 were overcome (Rodríguez-Manzaneque *et al.* 2009). Alternatively, ADAMTS-15 may influence the ability of syndecan-4 to activate downstream PKC signalling. In this case, PMA stimulation bypassed the ADAMTS-15 mediated inhibition of PKC activation and ultimately restored cell migration to that of control levels. In agreement with this perspective, treatment with the PKC inhibitor, bisindolylmaleimide (bis I) reduced the migration of EV control cells to the same level as that of TS15 and E362A expressing cells (Figure 4.11). Therefore as previously suggested it could be argued that PKC signalling in

TS15 and E362A cells is already reduced such that the addition of an inhibitor has no effect. However, until syndecan-4/PKC activity and localisation is examined in greater detail these data are supportive, but not conclusive of a functional link between ADAMTS-15 and syndecan-4.

4.8 Chapter summary

- ADAMTS-15 reduces 2D and 3D migration of human breast cancer cell lines in a metalloproteinase independent manner.
    - Expression of ADAMTS-15 or E362A inhibits 2D migration of human breast cancer cell lines on fibronectin and laminin but not collagen type I.
    - Expression of ADAMTS-15 or E362A inhibits 3D migration of human breast cancer cell lines through matrigel but not collagen type I.
    - Expression of ADAMTS-15 or E362A is associated with an altered integrin profile, including an increase in total integrin β4 protein.
- ADAMTS-15 anti-migratory function is likely due to syndecan-4
    - ADAMTS-15 and E362A expression is associated with increased levels of syndecan-4 on the cell surface.
    - Knockdown of syndecan-4 or modulation of syndecan-4 signalling abrogates the ADAMTS-15 mediated anti-migratory phenotype.

Chapter Five: The Effects of ADAMTS-15 on Angiogenesis and Metastasis

5.1 Introduction

The aim of this chapter is to investigate the anti-angiogenic potential of ADAMTS-15 and to discern whether ADAMTS-15 influences the ability of human breast cancer cells to metastasise.

5.2 Angiogenesis assays

Whilst several hyalectanases including ADAMTS-1, -4, -5, -8 and -9 have been reported to have anti-angiogenic effects, the role of ADAMTS-15 in angiogenesis is unknown (Hsu *et al.* 2012; Koo *et al.* 2010; Kumar *et al.* 2012; Luque *et al.* 2003; Vázquez *et al.* 1999). The hyalectanases are not the only ADAMTSs shown to modulate angiogenesis, since ADAMTS-2 and ADAMTS-12 have demonstrable anti-angiogenic effects (Dubail *et al.* 2010; El Hour *et al.* 2010; Llamazares *et al.* 2007). Indeed the central TSR found in all ADAMTSs may confer an implicit anti-angiogenic potential since peptides derived from the central TSR domains of ADAMTS-4, -16, and -18, termed adamtsostatins, are being developed as anti-angiogenic therapeutic agents (Karagiannis and Popel, 2007).

ADAMTS-1, -4 and -5 require their central TSR to bind VEGF, resulting in diminished downstream pro-angiogenic signalling (Freitas *et al.* 2013; Hsu *et al.* 2012; Kumar *et al.* 2012; Luque *et al.* 2003). However the anti-angiogenic activities of several ADAMTSs require proteolytic activity. ADAMTS-1 cleavage of thrombospondin (TSP)-1 and -2 releases anti-angiogenic fragments (Lee *et*

al. 2006). ADAMTS-1 is also capable of autolytic cleavage, which releases C- and N- terminal ADAMTS-1 fragments that are anti-angiogenic (Liu *et al.* 2006). And in the case of ADAMTS-9, only the active metalloproteinase was able to reduce HUVEC tube formation in an *in vitro* model of angiogenesis (Koo *et al.* 2010).

5.2.1 HUVEC tube formation

Human umbilical vein endothelial cells (HUVECs) form tube-like structures when cultured in 3D matrix environments (Cooley *et al.* 2010). Tube formation is enhanced when HUVECs are stimulated with pro-angiogenic growth factors and conversely anti-angiogenic factors reduce tube formation (Cooley *et al.* 2010). Thus HUVEC tube formation assay is an established and informative model of angiogenesis. In the context of the ADAMTS family, tube formation assays have previously been utilised to determine the anti-angiogenic properties of ADAMTS-2, -9 and -12 (Dubail *et al.* 2010; Koo *et al.* 2010; Llamazares *et al.* 2007).

In this assay HUVECs were suspended into Collagen type I gels and treated with conditioned media from MDA-MB-231 EV, TS15 or E362A cells (Methods 2.27). At 24 hours post-seeding, cells were fixed with 4% PFA, stained (Phalloidin and DAPI) and tube formation was quantified as described in materials and methods. ADAMTS-15 but not E362A (metalloproteinase inactive ADAMTS-15) reduced HUVEC tube formation in comparison to control (EV) (Figure 5.1). This data demonstrates for the first time that ADAMTS-15 is anti-angiogenic and that it's proteolytic activity is required. To further confirm these results an aortic ring assay was utilised.

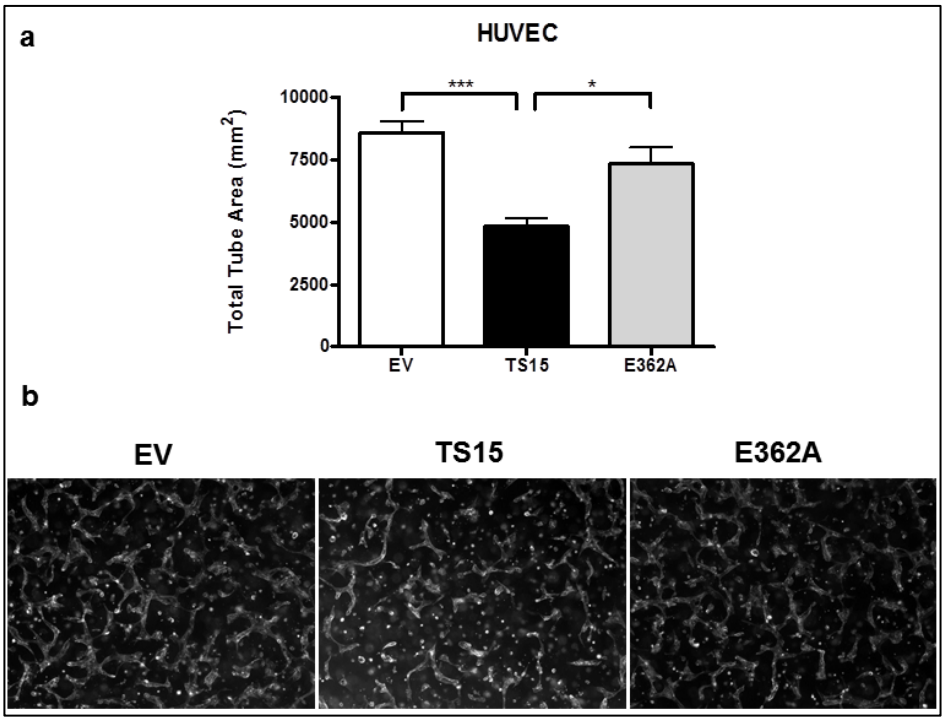


Figure 5.1: ADAMTS-15 reduces HUVEC tube formation in a metalloproteinase dependent manner. HUVECs seeded into Collagen type I gels were treated with conditioned media from MDA-MB-231 EV, TS15 or E362A cells and cultured for 24 hours to allow tube-like structures to form. Cells were stained, images taken and analysed as described in materials and methods, data represent 4 wells per group, error bars represent s.e.m.; *p<0.05, ***p<0.001. Data in collaboration with Dr. Lin Cooley.

5.2.2 Aortic ring assay

The murine aortic ring assay is an *ex vivo* model of angiogenesis. Since this model utilises multiple cell types, including endothelial cells, pericytes and fibroblasts the assay is physiologically analogous to *in vivo* angiogenesis (Baker *et al.* 2012). The assay has precedent within the context of the metalloproteinase field. The aortic ring assay was utilised by El Hour *et al.* 2010 to demonstrate that addition of exogenous ADAMTS-12 reduced sprouting back to *wildtype* levels in aortic rings excised from Adamts12 -/- mice.

In these experiments aortic rings were excised from mice and embedded in collagen type I (Methods 2.27; Baker *et al.* 2012). Aortic rings were treated with MDA-MB-231 EV, TS15 or E362A conditioned media and VEGF (30 ng/ml). Six days later, rings were fixed and the number of sprouting micro vessels was quantified under phase contrast. ADAMTS-15 but not E362A (metalloproteinase inactive ADAMTS-15) reduced sprouting in comparison to control (EV) (Figure 5.2). The data complements the HUVEC tube formation assay in confirming that *wildtype* ADAMTS-15 is anti-angiogenic. Interestingly *wildtype* ADAMTS-15 was able to reduce sprout formation even though aortic rings were also treated with pro-angiogenic VEGF (Figure 5.2). Thus the anti-angiogenic effect of ADAMTS-15 is robust. Whilst like other ADAMTSs, ADAMTS-15 may be able to bind VEGF, E362A had no effect on sprouting. Therefore determining ADAMTS-15 proteolytic substrates will be more productive in determining the anti-angiogenic mechanism.

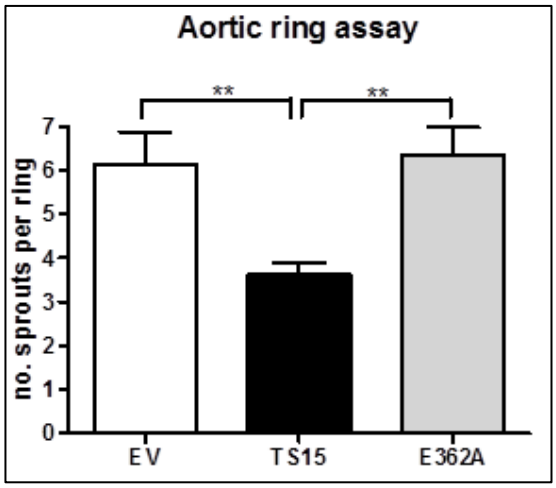


Figure 5.2: ADAMTS-15 reduces aortic ring sprouting in a metalloproteinase dependent manner. Thoracic aortae were isolated from 6- to 8-week-old mice and prepared for embedding in collagen matrix as described in Baker *et al.* 2012. Aortic rings were treated with EV, TS15 or E362A conditioned media as described (Thesis chapter 2.27.1) and VEGF (30 ng/ml). Six days later, rings were fixed and the number of micro-vessels were quantified under phase contrast. Data represent 24 wells per cell group, error bars represent s.e.m. **p<0.01. Data in collaboration with Dr. Stephen Robinson.

5.3 Experimental metastasis (tail vein assay)

MDA-MB-231 EV, TS15 and E362A cells were injected intravenously into the tail veins of immune-compromised CD1 nu/nu mice. Prior to injection cells were tagged by retroviral transduction with a β-galactosidase marker to enable the detection of disseminated tumour cells. At 35 days post injection mice were culled and several organs were removed for staining and analysis of micro-metastasis as previously described (Methods 2.28; Elkin and Vlodavsky, 2001; Krüger *et al.* 1994).

The overall number of micro-metastasis observed in the Liver was much lower than that of the lungs (**Figure 5.3; 5.5**). With only 25-50% of mice positive for micro-metastasis in the Liver, yet 80-100% of mice were positive micro-metastasis in the Lungs (**Figure 5.3; 5.5**). In patients basal-like tumours preferentially metastasise to the lungs, brain and bone, yet comparatively speaking exhibit a lower rate of liver metastasis (Kennecke *et al.* 2010) These global patterns may therefore be indicative of the general metastatic potential of MDA-MB-231 cells, which are a basal-like human breast cancer cell line (Table 3.1).

ADAMTS-15 reduced metastatic spread to Liver in a metalloproteinase independent manner (Figure 5.3), yet ADAMTS-15 enhanced metastatic spread to the lungs in a metalloproteinase dependent manner (Figure 5.5). ADAMTS-15 did not affect metastatic spread to the bone marrow (Figure 5.4).

These data reveal that ADAMTS-15 has organ specific effects on the ability of mammary cancer cells to colonize and grow that can be either dependent or independent of its catalytic activity.

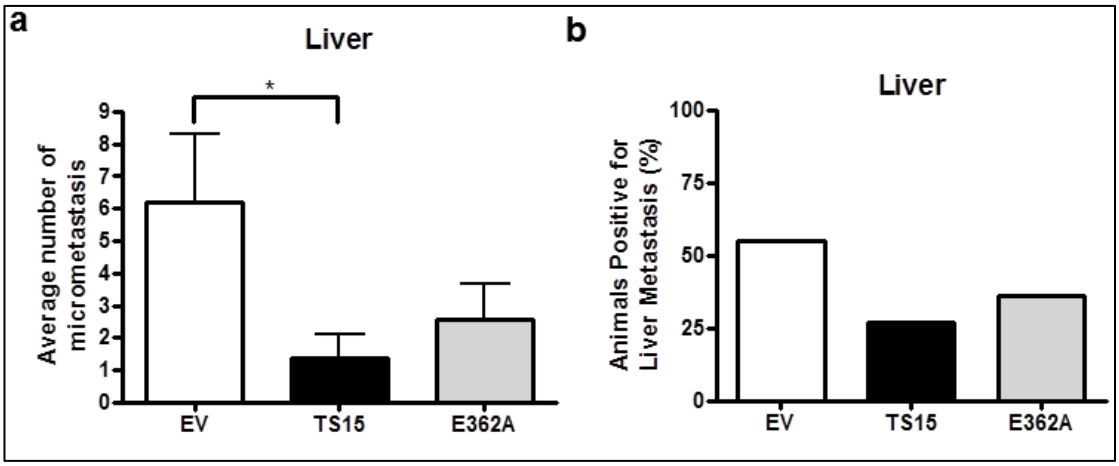


Figure 5.3: ADAMTS-15 reduces metastatic spread to Liver in a metalloproteinase independent manner. ADAMTS-15 reduces metastatic spread to Liver *in vivo*. LacZ tagged MDA-MB-231 EV, TS15, and E362A cells were intravenously injected into the tail veins of CD1 nu/nu mice. At 35 days post injection mice were culled. (**a**) Livers were excised and stained to enable visualisation of micro-metastatic foci. (**b**) The percentage of mice with visible metastatic foci present in the Liver. Data represent 10-11 mice per group, error bars represent s.e.m. *p<0.05. *In vivo* experiments were designed according to three R principles.

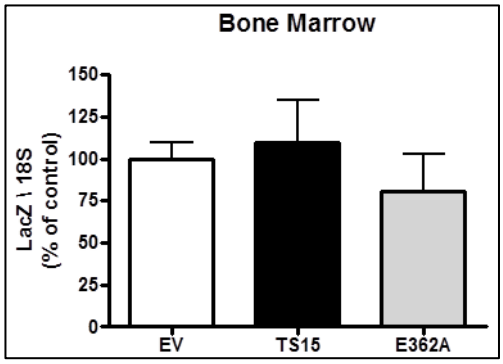


Figure 5.4: ADAMTS-15 does not affect metastatic spread to the bone marrow. LacZ tagged MDA-MB-231 EV, TS15, and E362A cells were intravenously injected into the tail veins of CD1 nu/nu mice. At 35 days post injection mice were culled. qRT-PCR analysis of LacZ was used to determine if MDA-MB-231 EV, TS15 or E362A cells had metastasised to bone marrow. Data represent 3-6 tissue samples per group, error bars represent s.e.m, *p<0.05.

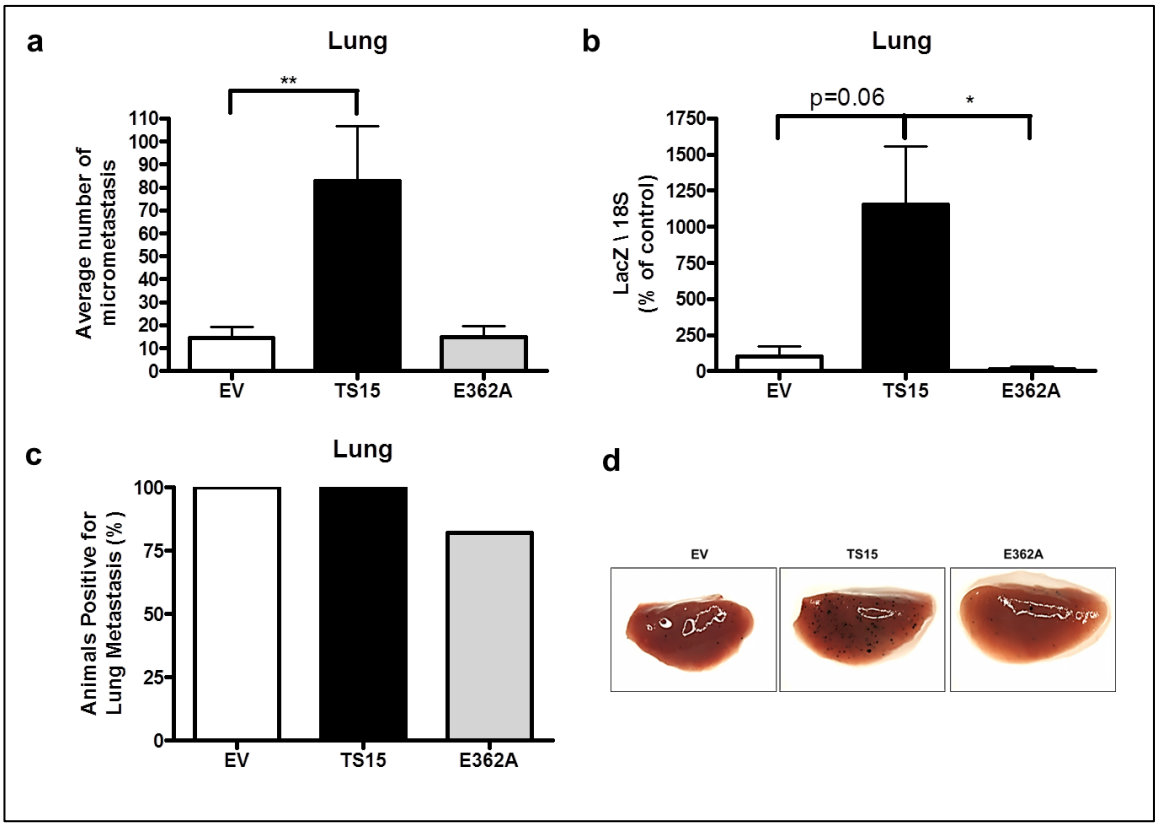


Figure 5.5: ADAMTS-15 enhances metastatic spread to the Lungs in a metalloproteinase dependent manner. LacZ tagged MDA-MB-231 EV, TS15, and E362A cells were intravenously injected into the tail veins of CD1 nu/nu mice. At 35 days post injection mice were culled. (**a**) Lungs were excised and stained to enable visualisation of micro-metastatic foci. Data represent 10-11 mice per group, error bars represent s.e.m. **p<0.01(**b**) qRT-PCR analysis of LacZ was used to determine if MDA-MB-231 EV, TS15 or E362A cells had metastasised to the lungs, Data represent 3-6 tissue samples per group, error bars represent s.e.m, *p<0.05. (**c**) The percentage of mice with visible metastatic foci present in the Lung. Data represent 10-11 mice per group (**d**) Images show representative lungs. *In vivo* experiments were designed according to three R principles.

5.4 Discussion

5.4.1 Angiogenesis

The initial interest in understanding the angiogenic potential of the ADAMTSs began in 1999 when ADAMTS-1 and -8 were found to have a high level of anti-angiogenic activity in *in vivo* angiogenesis models (Vázquez *et al.* 1999). Subsequent studies have identified several ADAMTSs with anti-angiogenic potential including ADAMTS-1, -2, -4, -5, -8, -9 and -12 (Dubail *et al.* 2010; Hsu *et al.* 2012; Koo *et al.* 2010; Kumar *et al.* 2012; Llamazares *et al.* 2007; Luque *et al.* 2003; Vázquez *et al.* 1999). The hyalectans feature predominantly in the list of known anti-angiogenic ADAMTSs, although it should be noted that the ADAMTSs may have an intrinsic anti-angiogenic potential that is mediated by the central TSR, a structural feature present in all family members. ADAMTS-1, -4 and -5 are all capable of binding the pro-angiogenic signalling molecule VEGF via their central TSR, thus diminishing downstream pro-angiogenic signalling (Freitas *et al.* 2013; Hsu *et al.* 2012; Kumar *et al.* 2012). This intrinsic potential is being commercially exploited. Peptides derived from the central TSR domains of ADAMTS-4, -16, and -18, termed adamtsostatins, are being developed as anti-angiogenic therapeutic agents (Karagiannis and Popel, 2007).

However not all ADAMTSs, including ADAMTS-9 are able to bind VEGF (Koo *et al.* 2010). Instead ADAMTS-9 requires its proteolytic activity to mediate its anti-angiogenic effect (Koo *et al.* 2010). ADAMTS-1 is also capable of modulating angiogenesis through several mechanisms that require its proteolytic activity and is perhaps the most insightful comparator to assess the angiogenic

potential of ADAMTS-15 since ADAMTS-1 is the most well studied family member.

ADAMTS-1 is involved in array of mechanisms that can have both pro and anti-tumorigenic consequences in cancer (Esselens *et* al. 2010; Liu *et al.* 2006; Vázquez *et al.* 1999). The catalytic activities of ADAMTS-1 seem to lie at the heart of these seemingly conflicting reports. Full length ADAMTS-1 is able to cleave and/or act as a sheddase for an array of substrates that ultimately have pro-tumorigenic and pro-angiogenic consequences. Liu *et al.* 2006 reported that full length ADAMTS-1 was involved in the shedding of the transmembrane precursors of heparan-binding epidermal growth factor (HB-EGF) and amphiregulin (AR). ADAMTS-1 was also found to be associated with the activation of ErbB-2 and EGF receptors (Liu *et al.* 2006) In combination these proteolytically mediated activities contribute to an inhibition of apoptosis and enhanced tumour angiogenesis (Liu *et al.* 2006).

Conversely the autolytic cleavage of ADAMTS-1 leads to the generation of two fragments, an N-terminal fragment and a C-terminal fragment both of which are capable of inhibiting metastasis and tumour angiogenesis (Liu *et al.* 2006). ADAMTS-1 can also cleave thrombospondin (TSP)-1 and -2 the result of which is the release of anti-angiogenic fragments (Lee *et al.* 2006). Thus ADAMTS-1 requires its proteolytic activity to be both pro and anti-angiogenic, depending on whether itself or another substrate is targeted for proteolytic activity.

To explore the angiogenic potential of ADAMTS-15 an *in vitro* model of angiogenesis was adapted from the one described by Cooley *et al.* 2010. HUVECs embedded in collagen type I gels were treated with conditioned media

from MDA-MB-231 EV, TS15 and E362A cells. Primary HUVECs were utilised which can be highly sensitive and difficult to culture in comparison to immortalised cell lines. So to ensure optimal assay conditions, conditioned media was generated with HUVEC media and 2% fetal calf serum, rather than the DMEM media that MDA-MB-231 cells were routinely cultured in. This precaution was taken since the HUVECs did not tolerate being cultured in DMEM conditioned media, whereas MDA-MB-231 cells were fine in HUVEC media throughout the 48h media-conditioning period.

The anti-angiogenic activity of ADAMTS-15 was assayed again in the murine aortic ring assay. The aortic ring assay is an *ex vivo* model of angiogenesis and since this model utilises multiple cell types, including endothelial cells, pericytes and fibroblasts the assay is physiologically analogous to *in vivo* angiogenesis (Baker *et al.* 2012). As in the case of the tubulogenesis assays aortic rings were treated with MDA-MB-231 EV, TS15 or E362A conditioned media. Based on experiences of generating conditioned media for the tubulogenesis assays HUVEC media was also used to generate the conditioned media. However, assay conditions could be further optimised, since sprouting was three fold higher in the no treatment control, in which neat media was used (data not shown). In future assays recombinant ADAMTS-15 could be utilised to overcome any general MDA-MB-231 conditioned media effects. Another advantage to using recombinant proteins over conditioned media is that the absolute levels of ADAMTS-15 or E362A protein were not known. The levels of pro- and anti-angiogenic factors in the conditioned media, such as VEGF were also not examined.

However in defence of these assays the MDA-MB-231 EV, TS15 and E362A cells used to generate conditioned media were extensively characterised to ensure that the levels of expression of ADAMTS-15 and E362A were comparable. The anti-angiogenic activities of the other hyalectanases can also be excluded since all the stables cell lines expressed comparable levels of ADAMTS-1, -4, -5, -8, -9 and -20.

Both *wildtype* ADAMTS-15 and E362A contain the same central TSR and spacer domains both of which are required for VEGF binding (Freitas *et al.* 2013; Hsu *et al.* 2012; Kumar *et al.* 2012). Since E362A did not have any observable anti-angiogenic effect it is unlikely that ADAMTS-15 sequestered VEGF signalling in these assays. Therefore from these data it can be concluded that the anti-angiogenic activity of ADAMTS-15 requires its proteolytic activity.

Also unlike ADAMTS-1, -2, -4, and -5, no evidence has been found to suggest that ADAMTS-15 undergoes autolytic cleavage at least not in the cell lines examined in this project (Colige *et al.* 2005; Flannery *et al.* 2002; Kashiwagi *et al.* 2004; Liu *et al.* 2006; Zeng *et al.* 2006). Therefore ADAMTS-15 does not seem to be producing autolytic fragments with anti-angiogenic potential.

It could be speculated that like ADAMTS-1, ADAMTS-15 might release anti-angiogenic fragments through the cleavage of thrombospondin (TSP)-1 or -2 (Lee *et al.* 2006). Though this may not be a reliable assumption since ADAMTS-9 mediates its anti-angiogenic effects via proteolytic activity, yet ADAMTS-9 does not cleave either TSP-1 or TSP-2 (Koo *et al.* 2010). Indeed the relevant anti-angiogenic substrates for both ADAMTS-9 and -15 are currently unknown and will be of interest for future studies.

5.4.2 Metastasis

In vivo data from FVB-PyMT mice, a transgenic model of highly metastatic mammary carcinoma has provided some insight into the protective nature of ADAMTS-15 in breast cancer. In a time course experiment ADAMTS-15 expression levels were reduced in FVB-PyMT mice compared to age-matched *wildtype* FVB mice (Porter *et al.* 2006). In particular ADAMTS-15 expression was significantly reduced in FVB-PyMT mice beyond week 7. Essentially ADAMTS-15 expression was lost as the tumours in these mice became poorly differentiated and more metastatic (Porter *et al.* 2006). The loss of ADAMTS-15 expression *in vivo* matches histological data from breast cancer patients in which ADAMTS-15 expression inversely correlate with tumour grade (Porter *et al.* 2004; Porter *et al.* 2006).

A similar pattern has also been observed in colorectal cancer, knockdown of ADAMTS-15 in SW-620 colorectal cancer cells resulted in an invasive phenotype *in vitro* and the invasive phenotype acquired by these cells enhanced their tumourigenic potential *in vivo* (Viloria *et al.* 2009). Subcutaneous injection of SW-620 cells into severe combined immunodeficient mice (SCID) resulted in the formation of tumours (Viloria *et al.* 2009). Knockdown of ADAMTS-15 in these cells enhanced the number and size (volume) of the tumours which formed (Viloria *et al.* 2009). In agreement with histological analysis of human samples, SW-620 tumours with low ADAMTS-15 expression were poorly differentiated (Porter 2006; Viloria *et al.* 2009).

These *in vivo* studies have demonstrated concordance between the protective mechanisms of ADAMTS-15 *in vitro* and histological analysis of patient

samples. However the role of ADAMTS-15 catalytic activity in determining metastatic potential is unknown. To this end an experimental metastasis assay was conducted in which MDA-MB-231 EV, TS15 and E362A cells were injected into the tail veins of CD1nu/nu mice (Elkin and Vlodavsky; 2001; Krüger *et al.* 1994). Since injected cells were able to freely circulate to the lungs, liver and other organs, differences in the metastatic potential of these human mammary cancer cell lines could be observed.

ADAMTS-15 reduced the metastatic spread of MDA-MB-231 cells to the Liver in a metalloproteinase independent manner. In combination with the anti-migratory effects of ADAMTS-15 these data suggest that ADAMTS-15 might elicit its protective effects through an inhibition of the metastatic potential of breast tumour cells. However, the protective effects of ADAMTS-15 *in vivo* may be complex since these data indicate that expression of ADAMTS-15 influences the metastatic potential of human breast cancer cells differently depending upon the tissue context (Figures 5.3-5.5) and interestingly, some of these effects require its proteolytic activity. For instance, in contrast to the Liver, ADAMTS-15 enhanced the metastatic spread of MDA-MB-231 cells to the lungs in a metalloproteinase dependent manner. Additionally, the anti-angiogenic activity of ADAMTS-15 also requires its proteolytic activity.

Therefore these data indicate that ADAMTS-15 modulates the metastatic ability of breast cancer cells via metalloproteinase dependent and independent processes. Through these processes ADAMTS-15 influences the ability of breast tumour cells to metastasise and colonise different tissues.

5.5 Chapter summary

- The anti-angiogenic activity of ADAMTS-15 requires its proteolytic activity.
    - ADAMTS-15 reduced HUVEC tube formation in a metalloproteinase dependent manner.
    - ADAMTS-15 reduced aortic ring sprouting in a metalloproteinase dependent manner.
- ADAMTS-15 has organ specific effects on the ability of mammary cancer cells to metastasise that can be either dependent or independent of its catalytic activity.
    - ADAMTS-15 reduces metastatic spread to Liver in a metalloproteinase independent manner.
    - ADAMTS-15 enhances metastatic spread to the Lungs in a metalloproteinase dependent manner.

Chapter Six: Final discussion and Future Perspectives

The human ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are a family of 19 secreted, matrix-associated zinc metallo-endopeptidases that have diverse roles in the regulation of tissue morphogenesis, cell migration, inflammation, and angiogenesis (Esselens *et al.* 2010; Kelwick and Edwards, 2014; Porter *et al.* 2005; Rodríguez-Manzaneque *et al.* 2009; Wagstaff *et al.* 2011). Whilst the loss of individual ADAMTSs may in some cases be phenotypically mild, broad changes in ADAMTS expression, function or localisation are associated with an array of arthritis and cancer patho-physiological processes (Kelwick and Edwards, 2014; Kevorkian *et al.* 2004; Lin and Liu, 2010; Porter *et al.* 2004; Wagstaff *et al.* 2011). In the case of ADAMTS-15, expression of this metalloproteinase inversely correlates with tumour grade in both breast and colorectal cancer, such that ADAMTS-15 expression is significantly reduced in grade III tumours compared to grade I or II (Porter *et al.* 2004; Viloria *et al.*, 2009). In colorectal cancer, ADAMTS-15 is genetically inactivated through the emergence of somatic mutations, which impair the ability of the protein to suppress the invasive potential of colorectal cancer cell lines (Viloria *et al.* 2009). ADAMTS-15 is also protective in breast cancer, where ADAMTS-15 expression is associated with increased overall survival (Porter *et al.* 2006). However, in breast cancer the functional basis of ADAMTS-15 tumour suppression is unknown and therefore merits further investigation. To this end, this thesis set out to explore the effects of ADAMTS-15 on human mammary cell behaviour.

MDA-MB-231 and MCF-7 cells were engineered for stable, constitutive expression of either *wildtype* (TS15) or metalloproteinase inactive (E362A) ADAMTS-15. These expression systems were initially characterised to explore ADAMTS-15 localisation and proteolytic activity before moving onto functional assays. To summarise these data, ADAMTS-15 associated with the ECM via its ancillary domain, however the proportion of ECM bound ADAMTS-15 varied between different cell lines. In terms of proteolytic activity, ADAMTS-15 is a reported versicanase (Stupka *et al.* 2013) and in this study, these data confirm ADAMTS-15 as an active, though weak, aggrecanase. However, more detailed kinetic analyses are required in order to quantifiably compare ADAMTS-15 aggrecanase activity against other aggrecanase, such as ADAMTS-5. Addition of N-TIMP-3 was sufficient to inhibit aggrecanolytic activity and as expected E362A was incapable of aggrecan cleavage. Thus ADAMTS-15 is now sufficiently characterised amongst the hyalectanases.

In functional assays, expression of ADAMTS-15 did not affect breast cancer cell viability or growth, as determined via MTT, growth curves and a cell cycle analysis. Instead the major functional consequences of ADAMTS-15 expression centre upon breast tumour cell migration, angiogenesis and metastasis (Figure 6.1). Breast tumour cells expressing either *wildtype* (TS15) or metalloproteinase inactive (E362A) ADAMTS-15 displayed reduced cell migration and upon knockdown of ADAMTS-15, cell migration was restored to the level of control cells. Therefore it can be concluded that ADAMTS-15 inhibits breast cancer cell migration in a metalloproteinase independent manner. More specifically, in 2D migration assays, ADAMTS-15 inhibited cell migration on fibronectin and laminin but not collagen type I. Interestingly, these matrix specific effects were

also consistent across 3D migration assays, where ADAMTS-15 inhibited cell migration through matrigel but not collagen type I gels. The mechanistic basis for this inhibition of cell migration can be explained, in part, through the observation that ADAMTS-15 expressing cells adhere more strongly to fibronectin and laminin. Thus on these matrices, cell adhesion was strong enough to inhibit cell migration. Additionally, the integrin profiles of ADAMTS-15 expressing cells differ in comparison to control cells. Integrin α5 and β1 subunits, which form a major fibronectin-binding integrin, were found to be up-regulated two fold in ADAMTS-15 expressing cells. However ADAMTS-15 expression did not alter the activity or surface level of integrin β1. From amongst the laminin binding integrins, α6 and β4 were examined. Unexpectedly, ADAMTS-15 expression was associated with an increase in total β4 protein. However this association is complex, since ADAMTS-15 expression did not alter the level of integrin β4 on the cell surface. Also, there were no differences in total α6 protein. Nevertheless, an examination of integrin β4 will be of interest in future studies, particularly in terms of the role integrin β4 plays in regulating hemidesmosome adhesion complexes, which function to anchor epithelial cells to laminin-5 (332) in the basement membrane (Litjens, de Pereda and Sonnenberg, 2006; van der Neut *et al.* 1996; Wang *et al.* 2010). A potential function of myoepithelial derived ADAMTS-15 may be to induce integrin β4 expression, which in turn will act to restrain epithelial cells within the ductal structures of the breast. In this case, the functional distribution of β4, within hemidesmosomal structures rather than simply its total protein level will be more informative. Therefore in future studies, more sophisticated co-culture assays could be utilised, such as the organoid model described in Holliday *et*

al., (2009). These systems are better equipped to examine any co-incidental link between hemidesmosome and basement membrane disassembly, the redistribution of pro-migratory α6β4 into lamellipodia and the loss of ADAMTS-15, which may all temporally coincide during the transition to invasive breast cancer.

In order to reconcile the ADAMTS-15 anti-migratory phenotype, syndecans-1 and -4 were examined. These proteoglycans are broadly expressed in epithelial cells, are known to regulate the activity of several fibronectin and laminin-binding integrins and have functional consequences on cell adhesion and migration (Bass, Morgan and Humphries, 2007; Beauvais, Burbach and Rapraeger, 2004; Couchman and Woods, 1999; Rodríguez-Manzaneque *et al.* 2009; Wang *et al.* 2010). Within the context of breast cancer, ADAMTS-1 or -4 shedding of syndecan-4 results in a functional loss of cell adhesion and promotes a pro-migratory phenotype with a concomitant loss of directionality (Bass *et al.* 2007; Choi *et al.* 2011; Rodríguez-Manzaneque *et al.* 2009). In this study these data reveal that expression of *wildtype* or inactive E362A mutant ADAMTS-15 by breast cancer cells is associated with an increase in the surface level of syndecan-4. It is possible therefore that ADAMTS-15 may be protecting syndecan-4 from sheddases, thus promoting its accumulation on the cell surface. Since *wildtype* ADAMTS-15 elicits the same protective capacity as the inactive E362A form, it is conceivable that ADAMTS-15 may be an inefficient syndecan-4 sheddase (in contrast to ADAMTS-1 and -4) and therefore protection could be a result of ADAMTS-15 blocking access of the more efficient hyalectanases to their substrate. An examination of syndecan-4 shedding in the presence or absence of ADAMTS-15 and other hyalectanses will provide the

experimental evidence required to ratify this hypothesis. An inducible ADAMTS-15 expression system where ADAMTS-15 expression is induced upon the removal of doxorubicin (TET-OFF) has been established and could be utilised to test this hypothesis.

Whilst this thesis does not present any evidence of a direct association between ADAMTS-15 and syndecan-4, experimental modulation of syndecan-4 or downstream signalling indicates that there is a functional link. In particular, knockdown of syndecan-4 restored the migration of ADAMTS-15 expressing cells to control levels. PMA stimulation of PKC signalling also abrogated the anti-migratory phenotype; this likely involves changes in actin dynamics but also may lead to increased expression of potent syndecan-4 sheddases such as ADAMTS-1, and -4 (Rodríguez-Manzaneque *et al.* 2009). Conversely treatment with the PKC inhibitor, bisindolylmaleimide I suppressed migration in control cells, yet had no effect on ADAMTS-15 expressing cells. In combination these data suggest that ADAMTS-15 influences cell migration in a metalloproteinase-independent manner by altering cell surface syndecan-4 function or turnover via a mechanism that is upstream of Protein Kinase-C (PKC)-regulated events. Nevertheless in future studies it will still be worthwhile to fully characterize the ADAMTS-15 anti-migratory phenotype, including those signaling events that lie downstream of PKC. The activity of the core migration signaling machinery (RhoA/Rac1/Cdc42) could be examined through several commercially available activity assays, which primarily utilize antibodies to recognize their active forms. Additionally actin dynamics, which are altered in syndecan-4 shed cells, could be examined in real time through live cell imaging, in which ADAMTS-15

expressing cells could be transfected with a GFP tagged actin expression system.

Several hyalectanases including ADAMTS-1, -4, -5, -8 and -9 have been reported to have anti-angiogenic effects, yet the anti-angiogenic potential of ADAMTS-15 is unknown (Hsu *et al.* 2012; Koo *et al.* 2010; Kumar *et al.* 2012; Luque *et al.* 2003; Vázquez *et al.* 1999). To assess the angiogenic potential of ADAMTS-15, growth media were conditioned by MDA-MB-231 ADAMTS-15 expressing cells and then incorporated into HUVEC tube formation assays. *Wildtype* but not the metalloproteinase-inactive (E362A) ADAMTS-15 conditioned media reduced HUVEC tubulogenesis compared to control. These conditioned media were also utilised in an *ex vivo* angiogenesis model, the murine aortic ring assay. In these assays *wildtype* ADAMTS-15, but not E362A conditioned media inhibited angiogenic sprouting. Taken together these data demonstrate that ADAMTS-15, like several other ADAMTSs, displays anti-angiogenic activity, but in contrast to its effects on cell migration, its effects on endothelial sprouting and tube formation require its metalloproteinase activity. Based upon what is known from other ADAMTSs, likely anti-angiogenic substrates include thrombospondin (TSP)-1 and -2, which upon cleavage release anti-angiogenic fragments (Lee *et al.* 2006). However this may not be a reliable assumption since ADAMTS-9 mediates its anti-angiogenic effects via proteolytic activity, yet ADAMTS-9 does not cleave either TSP-1 or TSP-2 (Koo *et al.* 2010). Instead the relevant anti-angiogenic substrates for both ADAMTS-9 and -15 are currently unknown and will be of interest for future studies. Additionally, proteomics based approaches are identifying novel ADAMTS substrates that lie beyond the current ADAMTS sub-group descriptors, and by

inference allude to novel biological functions (Esselens *et al.* 2010; Lopez-Otin and Overall, 2002; Rodríguez-Manzaneque *et al.* 2009).

The identification of novel ADAMTS-15 substrates will ultimately help to unravel the complex influence this metalloproteinase has on the metastatic potential of breast tumour cells. In experimental metastasis assays, ADAMTS-15 expression in MDA-MB-231 cells inhibited metastatic spread to the liver in a metalloproteinase-independent manner. Yet in contrast to this, *wildtype* but not metalloproteinase inactive (E362A) ADAMTS-15 enhanced metastatic spread to the lungs. The availability of hyalectanase substrates within the tissue microenvironment will likely influence the metastatic propensity of ADAMTS-15 expressing cells. Additionally, since ADAMTS-15 has matrix specific effects on cell adhesion and migration it is possible that some tissue environments may be more favourable to the establishment and development of a metastatic niche (Freitas *et al.* 2013; Kaplan *et al.* 2005; Kopitz *et al.* 2007; Hanahan and Weinberg, 2011; Liu *et al.* 2006)

These seemingly contradictory outcomes are not uncommon in the ADAMTS family. For example, expression of catalytically active ADAMTS-1 enhances the metastatic propensity of TA3 mammary carcinoma and Lewis Lung Carcinoma cells *in vivo* (Liu *et al.* 2006). In other contexts, including breast cancer, ADAMTS-1 shedding of syndecan 4 or semaphorin 3C enhances the migratory behaviour of cancer cell lines (Esselens *et al.* 2010; Rodríguez-Manzaneque *et al.* 2009). Conversely ADAMTS-1 proteolytic activity can also be anti-metastatic. ADAMTS-1 proteolytic cleavage of thrombospondins -1 and -2 releases anti-angiogenic fragments (Lee *et al.* 2006). ADAMTS-1 also displays auto-catalytic

behaviour, at a cleavage site within its own spacer region (Liu *et al.* 2006). This autocatalytic event releases N- terminal and C-terminal anti-metastatic fragments that act both as anti-angiogenic and metastasis-inhibitory factors (Liu *et al.* 2006). ADAMTS-1 can also elicit anti-tumorigenic effects in a metalloproteinase independent manner, through the binding of VEGF. The binding of VEGF sequesters both pro-angiogenic and pro-migratory signalling (Freitas *et al.* 2013; Luque *et al.* 2003). An *Adamts15*-knockout mouse has been established (UC Davis knock out mouse project https://www.komp.org/geneinfo.php?project=VG10097), which may provide additional insight. One approach would be to intercross such animals with the mouse mammary cancer model MMTV-PyMT mice, to determine the effect on disease progression. Using these models an examination of spontaneous mammary tumour formation, progression and tumour angiogenesis could be carried out that may shed additional light into the protective role of ADAMTS-15 in breast cancer.

In conclusion this work has contributed to our understanding of the fundamental characteristics of ADAMTS-15; namely its association with the ECM and aggrecanase activity. This work has also provided mechanistic insight into the anti-tumourigenic roles of ADAMTS-15 in modulating breast tumour cell migration, metastasis and angiogenesis. However in continuation of this study, more research is needed to unravel the complexities of the metalloproteinase dependent and independent functions of ADAMTS-15 in breast cancer.

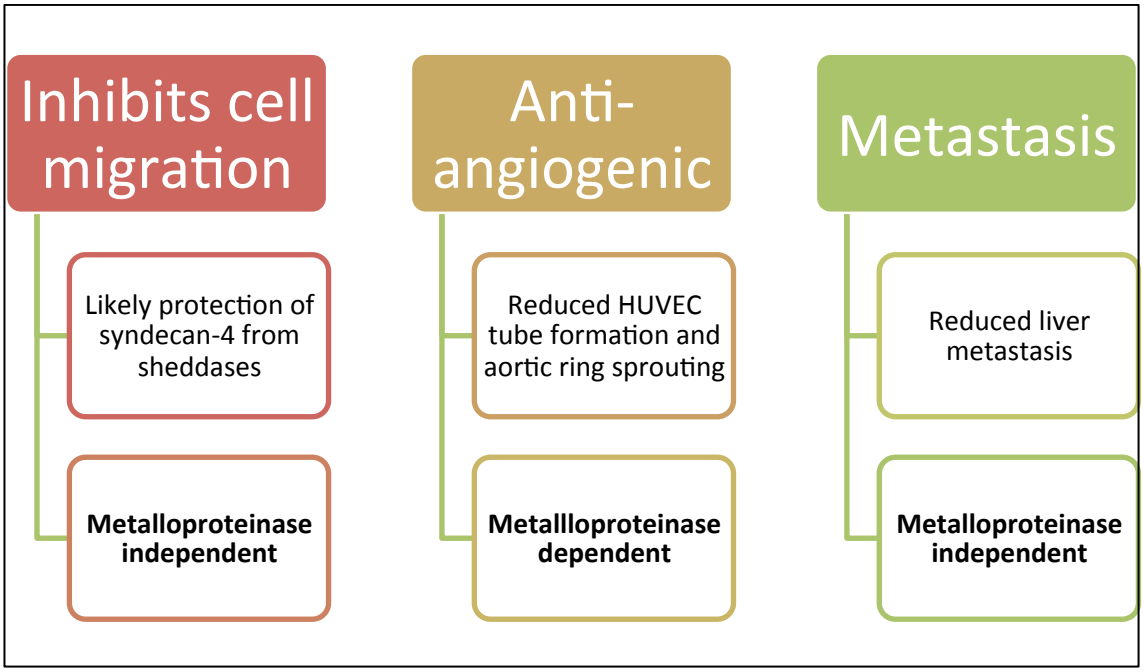


Figure 6.1: Summary of ADAMTS-15 protective effects in breast cancer that have been experimentally verified in this thesis.

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Appendix

Appendix 1: ADAMTS evolutionary history

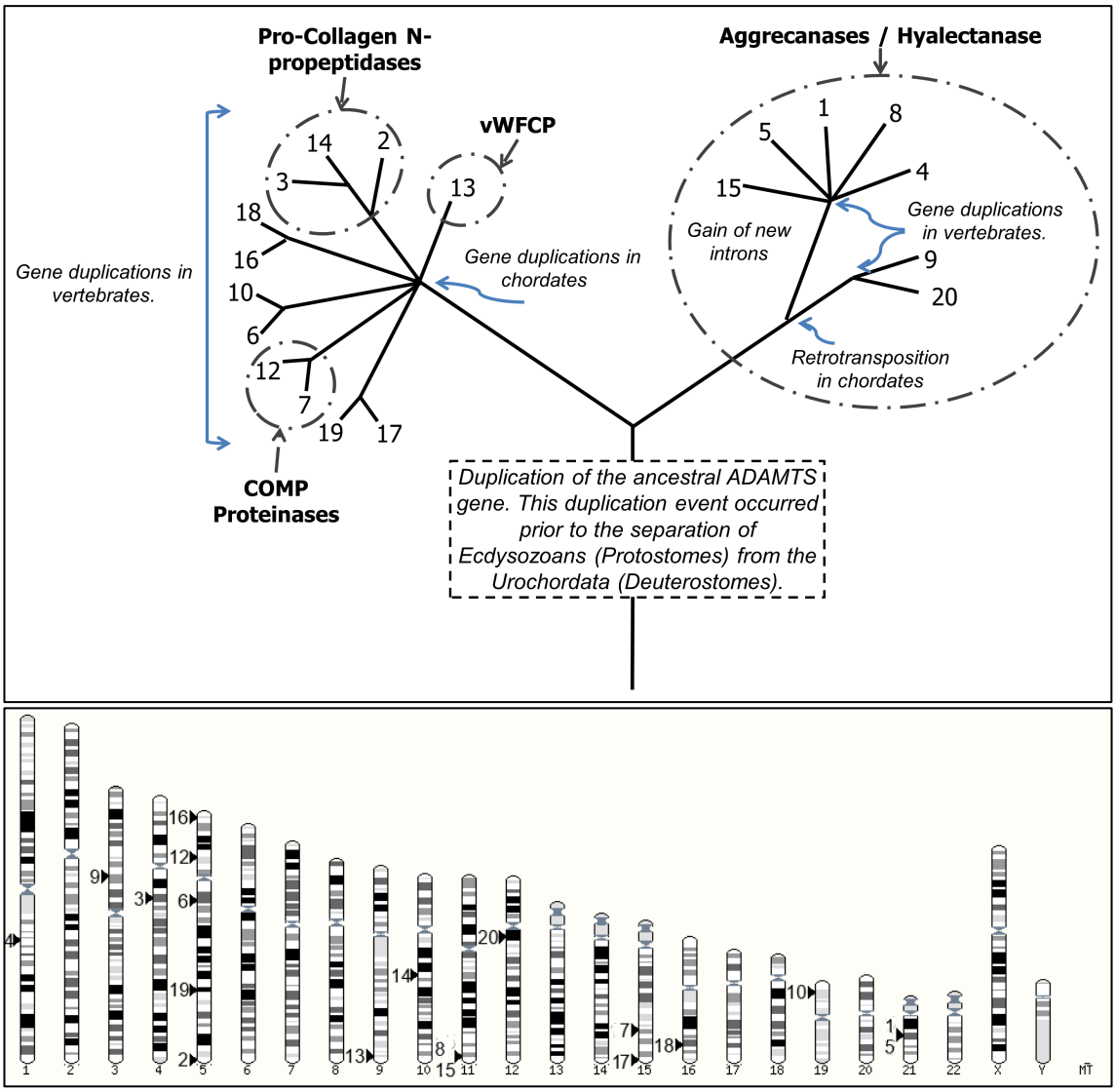

Figure A1: ADAMTS evolutionary history and genomic locations. (Top) The figure shows a representation of the likely gene duplications and a retrotransposition event that gave rise to the expansion of the ADAMTS family. The figure is not drawn to scale, in terms of evolutionary distance. Adapted from Nicholson *et al.* 2005; Huxley-Jones *et al.* 2005; Angerer *et al.* 2006; Brocker *et al.* 2009; Kelwick and Edwards, 2014. **(Bottom)** The genomic locations of the 19 human ADAMTS genes.

Organism	Number of ADAMTS Genes
Mus musculus **(Mouse)**	**19** ADAMTS-1,-2,-3,-4,-5,-6,-7,-8,-9,-10,-12,-13,-14,-15,-16,-17,-18,-19 and -20
Rattus norvegicus **(Rat)**	**19** ADAMTS-1,-2,-3,-4,-5,-6,-7,-8,-9,-10,-12,-13,-14,-15,-16,-17,-18,-19 and -20
Danio rerio **(Zebrafish)**	**15** ADAMTS-1,-2,-3, -6,-8a-d,-9,-10,-12,-13,-14,-15a-c,-16,-17,-18 and -20
Takifugu rubripes **(Puffer Fish)**	**16** ADAMTS-1,-2,-3,-4,-5,-6,-7,-8,-9,-10,-12,-14,-15,-16,-18, and -20
Ciona intestinalis **(Sea Squirt)**	**6** ADAMTS**-a** [2,3,14], **-b** [16,18], **-c** [7,12], **-d** [9,20], **-e** [6,10] and **–f** [1,4,5] [human orthologues]
Drosophila melanogaster **(Fruit Fly)**	**3** **CG4096** [7,12], **CG6107** [9,20], **CG14869** [none] [human orthologues]

Table A1: ADAMTS orthologues. Data adapted from Ensemble genome browser (last accessed 1/1/13); Huxley-Jones *et al.* 2005; Huxley-Jones *et al.* 2007; Nicholson *et al.* 2005

	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS10	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20
TS1	**100**																		
TS2	28	**100**																	
TS3	28	**84**	**100**																
TS4	**61**	28	28	**100**															
TS5	**57**	29	31	46	**100**														
TS6	37	35	35	40	40	**100**													
TS7	41	35	34	42	42	**52**	**100**												
TS8	**60**	31	32	**54**	**52**	33	37	**100**											
TS9	**52**	35	36	49	46	49	48	**50**	**100**										
TS10	39	34	33	39	38	**77**	**52**	32	46	**100**									
TS12	38	29	28	39	38	**52**	**73**	35	48	**51**	**100**								
TS13	27	29	29	28	26	31	34	25	27	29	28	**100**							
TS14	29	**77**	**81**	29	32	37	35	33	38	37	30	32	**100**						
TS15	**66**	33	31	**58**	**56**	40	46	**56**	**53**	40	42	27	33	**100**					
TS16	33	33	34	35	33	45	**50**	32	45	45	46	30	32	39	**100**				
TS17	32	31	31	35	32	38	40	33	39	40	38	29	33	33	41	**100**			
TS18	33	37	36	38	34	48	49	34	44	47	49	27	35	38	**75**	44	**100**		
TS19	31	26	26	33	33	40	38	29	34	40	39	28	30	32	43	**61**	44	**100**	
TS20	46	33	34	44	43	41	46	45	**62**	39	45	30	37	45	37	36	39	32	**100**

Functional Groups
Aggrecanases / Hylectanases ADAMTS-1, 4, 5, 8, 9, 15, 20
Procollagen N propeptidases ADAMTS-2, 3, 14
vWFCP ADAMTS-13
COMP Proteinases ADAMTS-7, 12
Orphan/Unknown ADAMTS-6, 10, 16, 17, 18, 19

High Level of Homology
Low Level of Homology

Figure A1.2: ADAMTS metalloproteinase sequence identity. ClustalW analysis of the sequence identities between human ADAMTS metalloproteinase domains. Domains that show the greatest similarities (≥50% identity) are shaded in grey. Adapted from Kelwick and Edwards, 2014

Appendix 2: miRNA regulation of ADAMTSs

Gene	Predicted miRNA
ADAMTS-1	miR-142-5p miR-628-3p miR-340 miR-15a miR-98 miR-10b miR-34a miR-155 miR-380 miR-338-3p
ADAMTS-2	miR-27a miR-95 miR-7 miR-10a miR-10b miR-217 miR-223 miR-200b miR-27b miR-141
ADAMTS-3	miR-199a-3p miR-501-3p miR-16 miR-95 miR-101 miR-144 miR-9 miR-30e miR-429 miR-202
ADAMTS-4	miR-125b miR-182 miR-342-3p miR-339-5p miR-345 miR-384 miR-329 miR-485-3p miR-202 miR-495
ADAMTS-5	miR-16 miR-105 miR-181a miR-203 miR-211 miR-224 miR-140-5p miR-145 miR-134 miR-382
ADAMTS-6	miR-29b miR-221 miR-203 miR-140-5p miR-9 miR-369-3p miR-515-5p miR-587 miR-605 miR-330-5p
ADAMTS-7	miR-29b miR-767-5p miR-541 miR-370 miR-596 miR-920 miR-921 miR-29a miR-129-5p miR-29c
ADAMTS-8	let-7f miR-203 miR-153 miR-346 miR-448 miR-519a miR-450b-5p let-7a let-7b let-7c
ADAMTS-9	miR-205 miR-338-5p miR-548a-5p miR-216b miR-29a miR-30a miR-32 miR-29b miR-103 miR-182
ADAMTS-10	miR-27a miR-29a miR-101 miR-149 miR-296-5p miR-331-3p miR-512-3p miR-637 miR-767-5p miR-361-3p
ADAMTS-12	let-7a let-7b let-7c let-7d let-7e let-7f miR-98 miR-198 miR-214 let-7g
ADAMTS-13	miR-525-5p miR-520a-5p miR-509-3p miR-596 miR-769-5p miR-766 miR-197 miR-34a miR-149 miR-302a
ADAMTS-14	miR-218 miR-636 miR-615-5p let-7d miR-24 miR-30b miR-328 miR-202 miR-516b miR-766
ADAMTS-15	miR-29a miR-29b miR-10a miR-217 miR-223 miR-137 miR-153 miR-150 miR-29c miR-362-5p
ADAMTS-16	miR-142-5p miR-545 miR-124 miR-524-5p miR-567 miR-587 miR-595 miR-612 miR-340 miR-892a
ADAMTS-17	miR-758 miR-19a miR-101 miR-29b miR-144 miR-134 miR-362-5p miR-324-3p miR-346 miR-486-5p

ADAMTS-18	miR-181a miR-302a miR-16 miR-19a miR-29b miR-148a miR-205 miR-130a miR-302c miR-382
ADAMTS-19	miR-369-3p miR-380 miR-26b miR-148a miR-214 miR-219-5p miR-133a miR-154 miR-301a miR-374a
ADAMTS-20	miR-19b miR-27a miR-29b miR-200b miR-27b miR-141 miR-186 miR-200a miR-379 miR-429

Table A2: miRNA regulators of the ADAMTSs. Data in table adapted from: http://www.mirnabodymap.org/index.php and Kelwick and Edwards, 2014.

Appendix 3: ADAMTS domain sizes

	Signal/Prodomain	Metallo	Disintegrin	TSR1	Cysteine Rich	Spacer	TSR2	TSR3	TSR4	TSR5	TSR6	TSR7	TSR8	TSR9	TSR10	TSR11	TSR12	TSR13	TSR14	TSR15	Mucin	Procollagen	CUB1	CUB2	GON1	PLAC
TS1	248	230	64	53	112	133	47	56																		
TS2	255	227	65	53	108	135	57	59	50													183				31
TS3	245	227	65	53	108	136	56	58	46													228				32
TS4	208	231	64	53	112	152																				
TS5	257	230	63	53	111	147	52																			
TS6	240	240	64	53	105	127	56	57	55	51																35
TS7	228	236	60	53	106	127	55	60	49	49	56	47	56								420					57
TS8	209	233	67	53	110	146	54																			
TS9	283	228	63	53	111	131	52	58	53	53	55	54	55	52	54	54	54	54	60	53					197	
TS10	229	240	64	53	105	123	56	57	55	51																35
TS12	236	232	59	53	33	114	55	59	49	49	57	46	57								320					62
TS13	70	231	66	53	118	130	57	59	52	55	58	52	55										146	109		
TS14	248	227	65	53	109	136	56	53	45													207				32
TS15	201	237	63	53	113	160	52	54																		
TS16	275	233	63	53	107	127	49	56	53	55	51															32
TS17	219	247	63	54	104	103	53	52	49	48																34
TS18	280	231	63	53	108	127	54	52	49	55	51															33
TS19	312	247	63	49	104	129	58	57	49	74																34
TS20	249	230	63	53	111	132	52	58	53	53	55	53	53	52	54	54	54	54	61	52					196	

Figure A3: ADAMTS domain sizes. The size (numbers of amino acid residues) of every domain in the human ADAMTS family is shown. The grey bars are a visual representation of the data. Data adapted from Kelwick and Edwards, 2014

Appendix 4: ADAMTS-15 sequences

>ADAMTS-15_protein

MLLLGILTLAFAGRTAGGSEPEREVVVPIRLDPDINGRRYYWRGPEDSGDQGLIFQITAFQEDFYLHLTP
DAQFLAPAFSTEHLGVPLQGLTGGSSDLRRCFYSGDVNAEPDSFAAVSLCGGLRGAFGYRGAEYVISPLP
NASAPAAQRNSQGAHLLQRRGVPGGPSGDPTSRCGVASGWNPAILRALDPYKPRRAGFGESRSRRRSG
*RAKR*FVSIPRYVETLVVADESMVKFHGADLEHYLLTLLATAARLYRHPSILNPINIVVVKVLLLRDRDSG
PKVTGNAALTLRNFCAWQKKLNKVSDKHPEYWDTAILFTRQDLCGATTCDTLGMADVGTMCDPKRSCSVI
EDDGLPSAFTTAH**E**LGHVFNMPHDNVKVCEEVFGKLRANHMMSPTLIQIDRANPWSACSAAIITDFLDSG
HGDCLLDQPSKPISLPEDLPGASYTLSQQCELAFGVGSKPCPYMQYCTKLWCTGKAKGQMVCQTRHFPWA
DGTSCGEGKLCLKGACVERHNLNKHRVDGSWAKWDPYGPCSRTCGGGVQLARRQCTNPTPANGGKYCEGV
RVKYRSCNLEPCPSSASGKSFREEQCEAFNGYNHSTNRLTLAVAWVPKYSGVSPRDKCKLICRANGTGYF
YVLAPKVVDGTLCSPDSTSVCVQGKCIKAGCDGNLGSKKRFDKCGVCGGDNKSCKKVTGLFTKPMHGYNF
VVAIPAGASSIDIRQRGYKGLIGDDNYLALKNSQGKYLLNGHFVVSAVERDLVVKGSLLRYSGTGTAVES
LQASRPILEPLTVEVLSVGKMTPPRVRYSFYLPKEPREDKSSHPKDPRGPSVLHNSVLSLSNQVEQPDDR
PPARWVAGSWGPCSASCGSGLQKRAVDCRGSAGQRTVPACDAAHRPVETQACGEPCPTWELSAWSPCSKS
CGRGFQRRSLKCVGHGGRLLARDQCNLHRKPQELDFCVLRPC

Legend: Signal/Pro-domain **Furin cleavage site RAKR** Metalloproteinase domain **E362A mutation position** Disintegrin domain TSR1 Cysteine rich domain Spacer domain TSR2 TSR3

Figure A4: ADAMTS-15 Sequence. The figure highlights the key ADAMTS-15 protein domains and the location of the E362A mutation in the metalloproteinase inactive construct used in this study.

Appendix 5: Validation of ADAMTS-15 antibodies

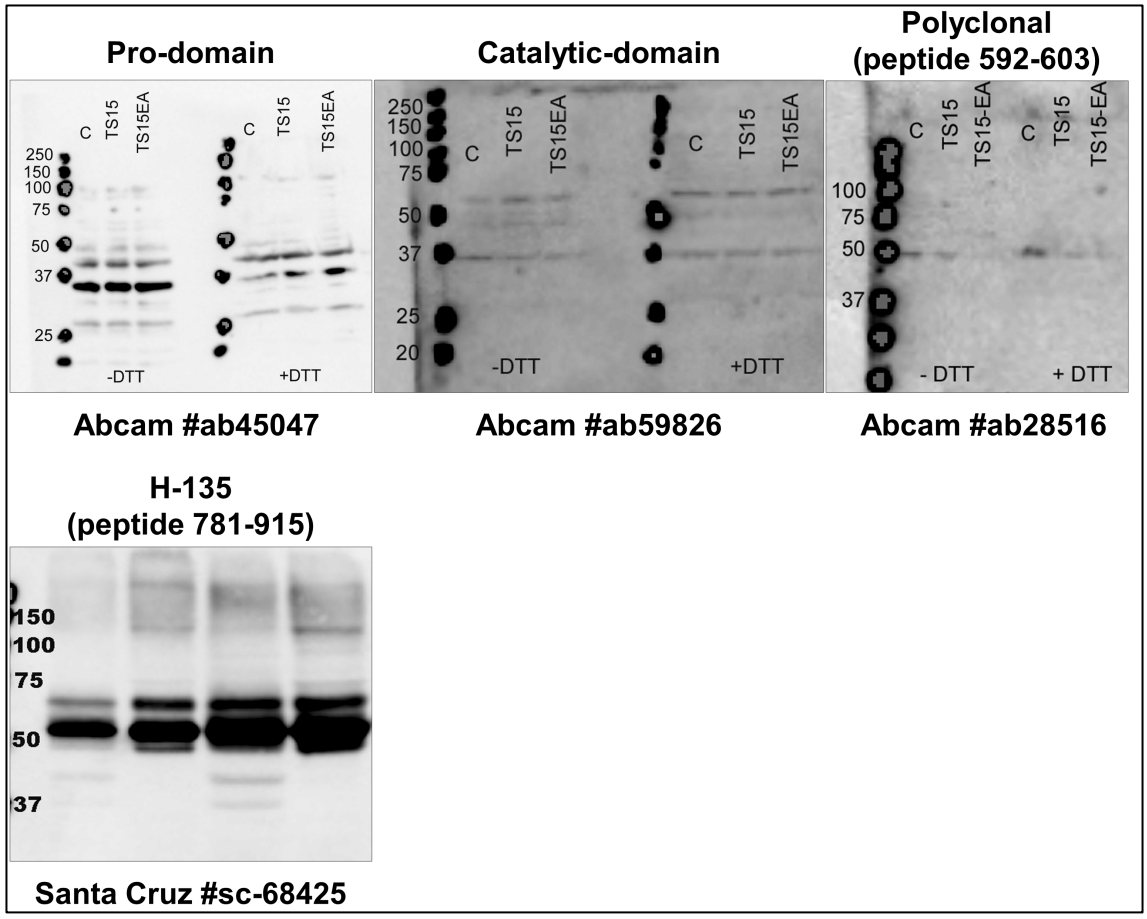

Figure A5: Commercial ADAMTS-15 antibodies do not reliably detect endogenous ADAMTS-15. Several commercially available ADAMTS-15 antibodies were tested and none were able to reliably detect ADAMTS-15 (predicted size ~103kDa) from cell lysate samples of human breast cancer cells. Santa Cruz antibody H-135 is reported to detect ADAMTS-15 (Viloria *et al.* 2009) however in our hands ADAMTS-15 could not be detected in either MCF-7 (lanes 1-2) or MDA-MB-231 ADAMTS-15 expressing cells (lanes 3-4). For Abcam antibodies, cell lysates from MDA-MB-231 EV (C, control), TS15 and E362A constitutive cells were used. Some antibodies were generated using ADAMTS-15 peptides that correspond to the indicated residues.

Appendix 6: Phosphokinase array data

	EV	TS15	E-A	Pattern
p38α	1185.5	5532	2983.5	
ERK 1/2	5381	20524.5	12870	
p70 S6 Kinase (T389)	0	2044	0	
p53 (S15)	8431	10382.5	7131	
PLCγ-1	4545.5	12460	5165.5	
TOR	3974	479.5	2170.5	
CREB	2874.5	0	3666	
HSP27	5813	1660	3555	
AMPKα2	5541.5	2513.5	4235	
Src	3302.5	0	1994	
STAT2	4106	0	2336	
STAT1	8453.5	5274.5	7674.5	
MEK 1/2	4663	4969	7825.5	
Akt (T308)	7125.5	7273.5	9537	
STAT6	2316.5	1478.5	7822.5	
GSK-3α/β	3723	3870	0	
p27 (T189)	5814	3739	254.5	
Paxillin	10795	9110.5	541	
Fgr	3368.5	2110.5	176.5	
p70 S6 Kinase (T421/S424)	8351	9599.5	6041	
RSK 1/2/3	5420.5	7507	2139	
p27 (T175)	4368.5	2066	354.5	
STAT5a/b	1189	2235.5	1994	
AMPKα1	2654	54.5	0	
Akt (S473)	2009	0	0	
p53 (S46)	14121.5	11647	8447.5	
β-Catenin	3651	0	0	
Lyn	4520.5	2462	3896	
Lck	5429.5	1196.5	0	
STAT5a	3413	839	1293.5	
Fyn	5687	2059	1778	
Yes	7057.5	2953.5	0	
STAT3	3818	451.5	146	
STAT5b	2307.5	0	0	
p70 S6 Kinase (T229)	15865.5	10568	11207.5	
RSK 1/2	10328	5881.5	2951.5	
STAT4	8388.5	5179.5	5626.5	
Hck	6368.5	2415	2965.5	
FAK	2399.5	0	863	
p53 (S392)	6602	8638	3667.5	
Pyk2	4661	3618	5511.5	
Chk-2	2287	3303.5	1228.5	
eNOS	6763.5	10692	2249	
c-Jun	5956	6122	6413.5	
MSK1/2	2950.5	3150	2769	
JNK pan	966.5	1570	654	
Positive	34951.5	43093	43891.5	
Positive	43525	29869	34727.5	
Positive	48821	34532	34335	
Negative	0	0	0	
Negative	0	0	0	

Activity Dependant Activiation	Peak at TS15
Activity Dependant Inhibition	Reduction at TS15
E-A Dependant Activation	Peak at E-A
E-A Dependant Inhibition	Reduction at E-A
Activity Independent Activation	Peak for TS15 and E-A compared to EV
Activity Independent Inhibition	Reduction for TS15 and E-A compared to EV
No Differnce	No difference between samples
Controls	Control Samples
Ambiguous	TS15 and E-A cause different effects

Figure A6: Human phosphokinase array data. Cell lysates from MDA-MB-231 EV, TS15 and E362A were incubated with membranes that were pre-treated with anti-phosphokinase antibodies as part of the R&D Human phosphokinase array kit. An analysis of HRP signal pixel density was utilised to determine the levels of kinase activation compared to control spots on the membrane. Colour coding is assigned according to pixel density values, where red denotes high values and green denotes low values. The diagrams in the right hand column in combination with the coloured categories (see figure legend) are intended to highlight phosphokinase patterns associated with ADAMTS and/or E362A expression compared to control EV cells.

Appendix 7: qRT-PCR data

	MDA-MB-231	MCF7
ADAMTS1	26 ±0.40	34 ±0.40
ADAMTS4	21 ±0	40 ±0
ADAMTS5	36 ±0	40 ±0
ADAMTS8	40 ±0	40 ±0
ADAMTS9	26 ±0.04	40 ±0
ADAMTS15	29 ±0.03	30 ±0.41
ADAMTS20	37 ±0.20	36 ±0.47
TIMP3	26 ±0.14	29 ±0.27
Syndecan 1	28 ±0.06	26 ±0.04
Syndecan 4	25 ±0.18	26 ±0.04
Integrin β1	25 ±0.19	27 ±0.01
Integrin α5	24 ±0.07	27 ±0.07

Ct 15-25	Very high expression
Ct 26-30	High expression
Ct 31-35	Moderate expression
Ct 36-39	Low expression
Ct <40	Very low / undetectable expression

Figure A7: Expression profile of MDA-MB-231 and MCF-7 cells. qRT-PCR analysis of gene expression in MDA-MB-231 and MCF-7 cells. Relative expression levels are shown as a Ct value heat-map. The displayed Ct values, ±standard deviation, are averaged from three independently collected RNA samples. These data are the same as that shown in figure 3.3, except in this figure the standard deviation of three independently collected RNA samples are also shown.

	MDA-MB-231			MCF-7		
	EV	TS15	E362A	EV	TS15	E362A
ADAMTS1	25 ±0.16	24 ±0.15	24 ±0.23			
ADAMTS4	21 ±0.36	20 ±1.26	20 ±0.01			
ADAMTS5	35 ±1.20	34 ±0.56	34 ±0.82			
ADAMTS8	40 ±0.00	40 ±0.00	40 ±0.00			
ADAMTS9	30 ±1.16	31 ±0.41	30 ±0.75			
ADAMTS15	26 ±0.81	23 ±0.42	23 ±0.53	31 ±1.48	20 ±0.21	25 ±0.13
ADAMTS20	37 ±0.74	36 ±0.65	37 ±0.71			
TIMP3	24 ±1.33	24 ±0.57	23 ±0.47			
Syndecan 1	26 ±0.89	26 ±0.65	25 ±0.00			
Syndecan 4	24 ±0.01	24 ±0.12	25 ±0.38			
Integrin β1	27 ±1.37	26 ±1.12	26 ±0.09			
Integrin α5	25 ±0.93	24 ±0.81	24 ±0.56			

Ct 15-25	Very high expression
Ct 26-30	High expression
Ct 31-35	Moderate expression
Ct 36-39	Low expression
Ct <40	Very low / undetectable expression

Figure A8: Expression profile of MDA-MB-231 EV, TS15 and E362A cells. qRT-PCR analysis of gene expression in MDA-MB-231 EV, TS15 and E362A cells. Relative expression levels are shown as a Ct value heat-map. The displayed Ct values, ±standard deviation, are averaged from three independently collected RNA samples. These data are the same as that shown in figures 3.8 and 4.8, except in this figure the standard deviation of three independently collected RNA samples are also shown.