ADAMTS-1 and syndecan 4 intersect in the regulation of cell migration and angiogenesis

Jordi Lambert

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University of East Anglia

School of Biological Sciences

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Abstract

The extracellular proteoglycanase ADAMTS-1 has critical roles in organogenesis and angiogenesis. We demonstrate here the functional convergence of ADAMTS-1 and the transmembrane heparan sulfate proteoglycan syndecan 4 in influencing adhesion, migration, and angiogenesis *in vitro*. Knockdown of ADAMTS-1 resulted in a parallel reduction in cell surface syndecan 4 that was not due to altered syndecan-4 expression or internalisation, but was attributable to increased expression and activity of matrix metalloproteinase 9 (MMP-9), a known syndecan 4 sheddase. Knockdown of either syndecan 4 or ADAMTS-1 led to enhanced endothelial cell responses to exogenous vascular endothelial growth factor (VEGF), and increased microvessel sprouting in ex vivo aortic ring assays, correlating with reduced ability of the cells to sequester VEGF. On fibronectin, but not type 1 collagen matrices, knockdown of either ADAMTS-1 or syndecan 4 elicited increased migration and altered focal adhesion morphologies, with a higher proportion of larger focal adhesions and formation of long fibrillar integrin α5-containing focal adhesions. Integrin α5-null endothelial cells also displayed enhanced migration in response to ADAMTS-1/syndecan 4 knockdown, indicating that integrin α5 was not the mediator of the altered migratory behaviour. Plating of naïve endothelial cells on cell-conditioned matrix from ADAMTS-1/syndecan 4 knockdown cells demonstrated that the altered behaviour was matrix dependent. Fibulin-1, a known ECM co-factor of ADAMTS-1, was expressed at reduced levels in ADAMTS-1/syndecan 4 knockdown cells. These findings support the notion that ADAMTS-1 and syndecan 4 are functionally interconnected in regulating cell migration and angiogenesis, via the involvement of MMP-9 and fibulin-1 as collaborators.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin repeats
C-terminal	Carboxyl-terminus
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
DMEM	Dulbecco's Modified Eagle Medium
DS	Dermatan sulfate
EC	Endothelial cell
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FA	Focal adhesion
FAK	Focal adhesion kinase
FBS	Foetal Bovine Serum
FGF2	Fibroblast growth factor 2
FN	Fibronectin
GAG	Glycosaminoglycan
GAP	GTPase-activating proteins
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
HRP	Horseradish peroxidase
HS	Heparan sulfate
HSC70	Heat shock 70
HSPG	Heparan sulfate proteoglycan
HUVEC	Human umbilical vein endothelial cell
MMP	Matrix metalloprotease
N-terminal	Amino-terminus,
PBS	Phosphate buffered saline
PDGF	Platelet derived growth
PKC	Protein kinase C
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SDC4	Syndecan 4
SEM	Standard error of the mean
siRNA	Small interfering RNA

TIMP	Tissue inhibitor of metalloproteinase
TSP	Thrombospondin
TSRs	Thrombospondin type 1 repeats
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth muscle cell
Y	Tyrosine

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1 Introduction

1.1 Preface

The extracellular matrix (ECM) is a complex and dynamic structure, which has direct control upon numerous essential cell behaviours such as proliferation, identity, and survival, which it regulates through both its physical and biochemical properties.

Cells and the ECM have a reciprocal relationship. Cells respond to their environment by transduction of ECM signals through cell adhesion receptors. Cells are also responsible for the production and secretion of ECM, and its remodelling by cellular proteases.

Angiogenesis, an essential physiological process, is regulated in a complex mechanism which requires convergence of the ECM, cell adhesion receptors and proteases in order for crucial stages such as degradation of the basement membrane and migration of endothelial cells (ECs) to occur in a co-ordinated and stepwise manner.

This thesis will explore the roles of the extracellular protease ADAMTS-1 and the cell surface adhesion receptor syndecan 4 in the ECM, and how they collaborate to regulate angiogenesis and cell migration.

1.2 The ECM environment

The ECM is the non-cellular component of organs, which provides structural support for cells and tissues. Alongside this structural role the ECM has important roles in adhesion, signalling and cell fate decisions, functioning both physically and biochemically (Frantz, Stewart and Weaver, 2010).

Physically, the ECM functions as a barrier, an anchorage site, or as a movement track for cell migration. The physical properties of the ECM such as rigidity, density, porosity, insolubility and topography provide physical clues to cells, controlling their behaviour (Yue, 2014).

The biochemical properties of the ECM can induce signal transduction pathways in cells, mediated by adhesion receptors. The ECM is by no means inert and can also function as a reservoir of growth factors, mediating their bioavailability and thereby regulating signalling.

There are two major ECM structures within tissues; the interstitial matrix, which consists of threadlike fibrils that form a porous and fibrous network surrounding cells, and the basement membrane, a sheet like structure which serves as a platform for cells and a boundary between tissue components (Akalu and Brooks, 2006).

Vertebrate genomes encode hundreds of ECM proteins. While diverse and varied, ECM proteins share some universal properties, such as large size and complex, multi-domain structures (Hynes, 2009). ECM proteins are largely comprised of three major classes of bio-molecule; structural proteins (collagen, fibrillin, elastin), specialised proteins (fibronectin, laminin) and proteoglycans (aggrecan, versican, perlecan) (Figure 1.1) (Slack, 2007).

Extracellular matrix proteolysis and turnover is an essential part of healthy tissue maintenance and embryogenesis, and is achieved via a balance between proteases and their inhibitors (Rozario and DeSimone, 2010).

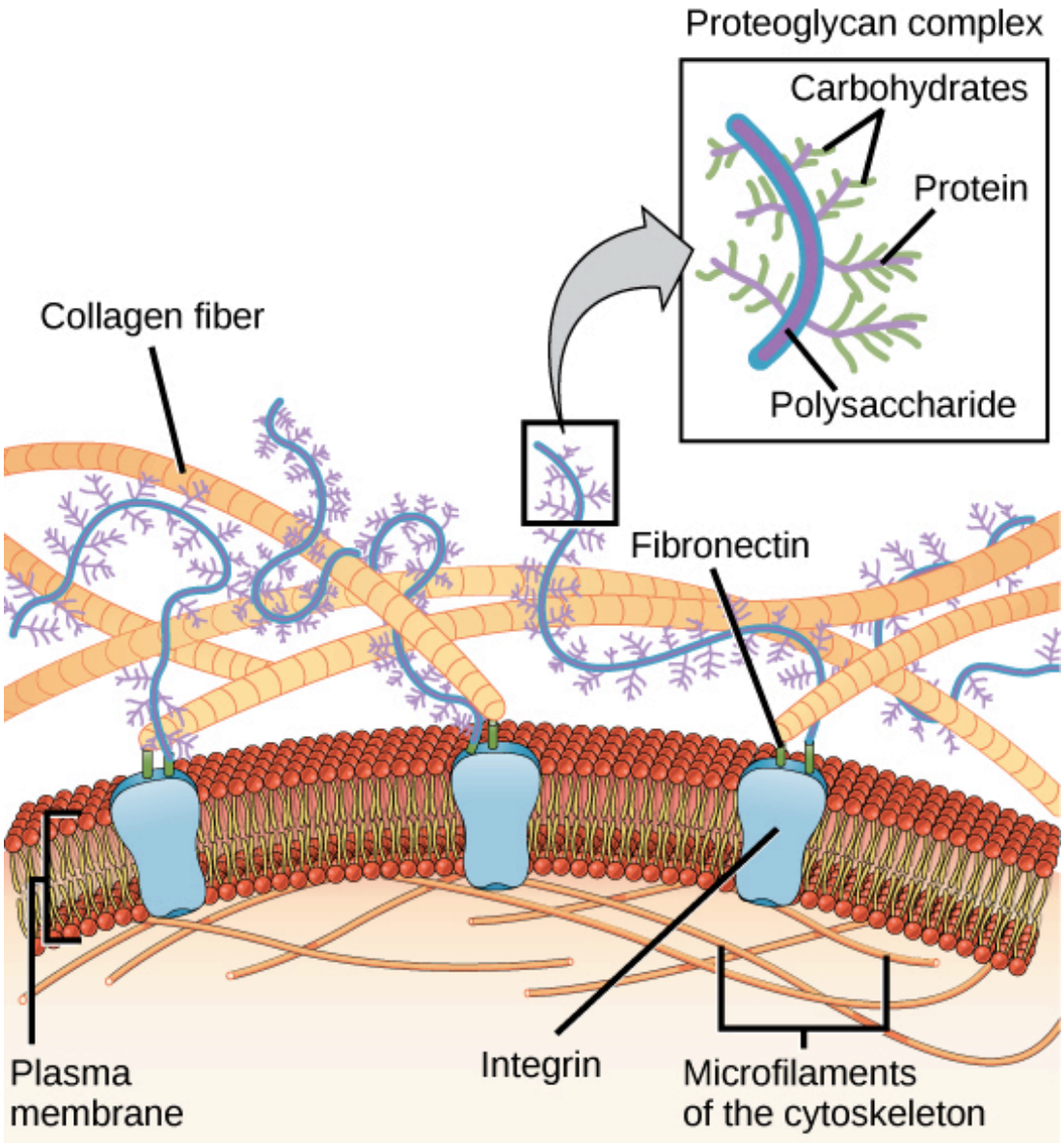


Figure 1.1 Schematic representation of the cell-ECM environment. The ECM is primarily composed of collagen, fibronectin and proteoglycans. Cells interact with the ECM via cell adhesion receptors such as integrins, which serve to connect the ECM to the cytoskeleton. Image reproduced from OpenStax biology (Rye *et al.*, 2018).

1.2.1 Fibronectin

Fibronectin (FN) is a high molecular weight extracellular matrix glycoprotein, consisting of roughly 4-9% carbohydrate (Pankov and Yamada, 2002). It is widely expressed by multiple cell types and responsible for mediating a variety of interactions within the ECM, and plays vital roles in adhesion and migration. Fibronectin is critically important in embryogenesis, exemplified by the early embryonic lethality of mice with *FN* inactivation (George *et al.*, 1993).

Fibronectin is secreted as a dimer, its structure consists of two nearly identical 250 kDa subunits, covalently linked near the C-termini by a pair of disulfide bonds. Each monomer is assembled from three repeating units, termed FN repeats; 12 type I, two type II, and 15-17 type III (Rocco *et al.*, 1987). Sites for glycosylation are primarily found within the type III repeats (Figure 1.2).

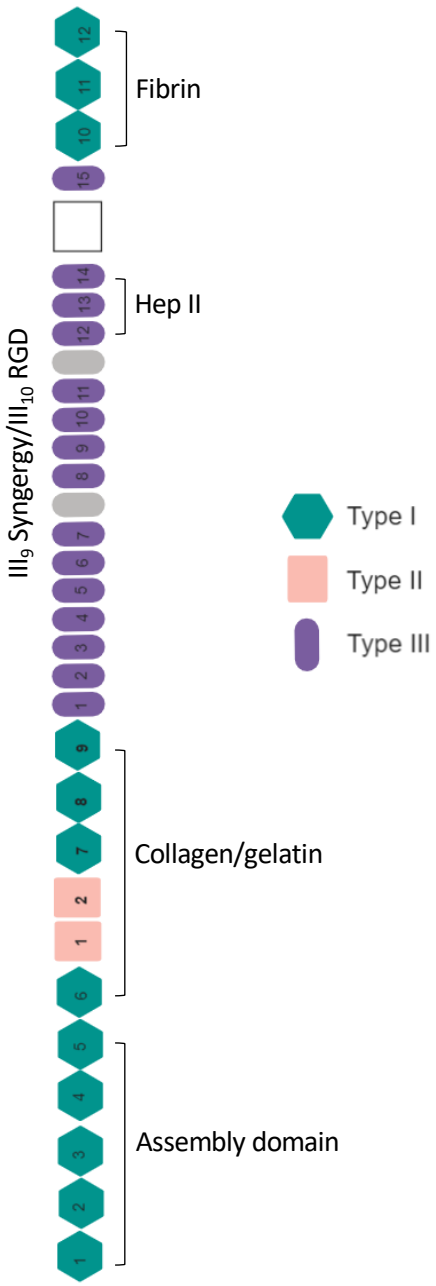


Figure 1.2 Structure of a fibronectin monomer. Fibronectin monomers consist of three types of repeating unit, type I, type II and type III. Binding sites for integrins and other ECM proteins are highlighted.

Fibronectin is a ligand for members of the integrin receptor family, with α5β1 regarded as the canonical FN receptor. Integrin binding to FN occurs at the tripeptide sequence RGD, located in FN repeat III_{10}, this binding is complex and dependent on flanking residues, 3D presentation and individual features of the integrin binding pocket. For example, in the case of α5β1, a second site in FN repeat III_9 (the synergy site – PHSRN, in the central binding domain of FN) promotes specific binding (Feng and Mrksich, 2004).

Fibronectin also has binding sites for heparin, collagen, gelatin and fibrin. The two major heparin binding domains are heparin II in the C-terminal, and the weaker heparin I in the N-terminal, the heparin II domain is also capable of binding GAG and CS. The collagen binding domains are found in repeats I_{6-9} and $II_{1,2.}$ Fibronectin-fibrin interaction is important for cell adherence to and migration into fibrin clots (Makogonenko *et al.*, 2002). The two major fibrin binding sites are found in the N-terminal and formed by type I repeats 4 and 5 (Singh, Carraher and Schwarzbauer, 2010).

Fibronectin molecules are the product of a single gene, which in humans can be alternatively spliced to generate as many as 20 variants (Ffrench-Constant, 1995). Fibronectin is split into two categories, soluble plasma FN which is synthesised primarily by hepatocytes in the liver, and shows a relatively simple splicing pattern, and insoluble cellular FN, which is a much larger and more heterogenous group of proteins.

Fibronectin is a vital part of the insoluble ECM, and most of its effects are attributed to this fraction. Fibronectin matrix assembly, termed fibrillogenesis, is a cell-mediated process in which soluble dimeric FN is converted into a fibrillar network (Mao and Schwarzbauer, 2005). It proceeds in a series of regulated steps which drive FN to self-associate, and interact with other ECM proteins

Fibronectin is secreted as compact dimers that will not polymerize into fibres without being activated in a cell-dependent mechanism (Singh, Carraher and Schwarzbauer, 2010). Initiation of fibronectin matrix assembly begins at focal contacts, these are

sites that are rich in paxillin, vinculin and β1 and β3 integrins. Fibronectin activation is induced by interaction with cell surface receptors in these focal contacts, usually integrin α5β1.

Integrins form an initial contact with FNI1-5, along with the essential steps of integrin α5β1 binding to the RGD loop on FNIII10, and the neighbouring PHSRN sequence. Integrin binding to the ligand fibronectin induces integrin clustering, bringing together the bound fibronectin, increasing its local concentration (Wierzbicka-Patynowski and Schwarzbauer, 2003).

The next stage in assembling fibrils is unfolding of the compact FN structure into an extended one, followed by elongation. As a ligand FN induces integrin clustering, this brings together bound FN, increasing its local concentration. As FN fibrils form on the outside of the cell, the cytoplasmic domains of integrins organise cytoplasmic proteins, linking FN to the actin cytoskeleton and stimulating signalling pathways essential for propagation of FN fibril formation. Integrin linkage of FN to the actin cytoskeleton generates tension via actin-myosin contractility, inducing a conformational change in surface bound FN (Zhang, Magnusson and Mosher, 1997). Fibronectin unfolding is dependent on β1 integrin-FN translocation from focal contacts into tensin-rich fibrillar adhesion complexes. This unfolding reveals hidden binding sites which allow for FN self-interaction, and interaction with other ECM components.

Extended FN dimers overlap and stagger to form initial thin 5 nm fibrils, which then become laterally associated into thicker 6-22 nm diameter fibrils (Winklbauer and Stoltz, 1995). Continuous FN fibril interactions allow for the formation of high molecular weight, complex, branched fibrillar FN matrices. These matrices are not static, and FN fibrils are constantly polymerised and remodelled within the matrix.

The major fate of FN lost from the matrix is endocytosis and lysosomal degradation. The large size of fibronectin means in cannot be effectively internalised by cells until it has been cleaved. Fibronectin is a substrate of many proteases, including matrix

metalloproteases (MMPs), which regulate fibronectin remodelling by promoting fibronectin cleavage (Shi and Sottile, 2011).

1.2.2 Collagen

Collagen is the most abundant protein in mammals, constituting ~30% of total protein mass, and forms the major structural element of the ECM. It is generally secreted into the ECM by stromal fibroblasts, where it forms supramolecular assemblies (De Wever *et al.*, 2008). There are 28 collagen types, which can be categorised into four groups: fibril-forming collagens (types I, II, III), network-forming collagens (basement membrane collagen type IV), and fibril-associated collagens (types IX, XII), and others (type VI) (Ricard-Blum, 2011).

Cells interact with collagen in multiple ways. Fibril-forming collagens can be cleaved by MMP family members -1, -8, -13 and -14, and collagen is a ligand for β1 subunit-containing integrin heterodimers (Heino, 2000).

1.2.3 Laminin

Laminins are high molecular weight glycoproteins which form a major constituent of the basement membrane. They are composed of three disulfide-linked polypeptide chains, α,β, and γ. In humans, there are 11 laminin genes coding for five α, three β, and three γ laminin subunits. Chains show tissue- and cell-specific distribution, but every basement membrane contains at least one member of the laminin family (Aumailley, 2013)

Laminin has numerous biological activities, it is a cell adhesion molecule, and can influence cell growth, morphology and migration (Kleinman *et al.*, 1985). Laminins physically bridge the intracellular and extracellular compartments, relaying signals critical for behaviour. In general, this is mediated through interactions between the C-terminal ends of laminins and receptors anchored in the plasma membranes of cells adjacent to basement membranes. The N-terminal end of laminin is mainly involved in interaction with other ECM molecules (Sasaki, Fässler and Hohenester, 2004).

Almost all laminin receptors bind the globular G domain at the C-terminus of the laminin α chains; the C-terminus of these α chains is 865-900 residues longer than the β, and γ chains, and is folded into 5 sub-domains, LG1 to LG5 which make up the large laminin globular domain. Depending on the laminin isoform, laminin-cell interactions are mediated by one of four different laminin binding integrins: α3β1, α6β1, α6β4, and α7β1 (Aumailley, 2013).

1.2.4 Proteoglycans

Proteoglycans are a key part of the ECM, and a vast and heterogenous group. All proteoglycans consist of a protein core with glycosaminoglycan (GAG) chains covalently linked to serine residues of the core protein. In general, they possess a single type of GAG chain such as heparan sulfate (HS), chondroitin sulfate (CS) or dermatan sulfate (DS), and can be categorised based on these side chains (Mizumoto and Sugahara, 2013).

The chondroitin sulfate proteoglycans (CSPG) are generally secreted and serve as ECM molecules in the developing and mature CNS. Examples of the secreted CSPG include the lectican family (aggrecan, versican, brevican, neruocan), and tenascins (Stanton *et al.*, 2011). Few are expressed on the cell surface, examples include the transmembrane CD44, and GPI-anchored brevican.

The heparan sulfate proteoglycans (HSPG) can be both cell surface and extracellular, and fall into three groups depending on location: Membrane (syndecans, glypicans), secreted (agrin, perlecan, type XVIII collagen) and the secretory vesicle proteoglycan serglycin (Sarrazin, Lamanna and Esko, 2011). Many have a modular domain structure and can form bridges between cells and the ECM, and they are widely thought to be co-receptors for a variety of ligands (Park *et al.*, 2017).

The protein cores of proteoglycans are synthesised in the rough endoplasmic reticulum. In the golgi, core proteins are then posttranslationally modified with glycosyltransferases in a complex biosynthetic pathway (Pedersen *et al.*, 2000). GAG

chain formation is initiated by the synthesis of a tetrasaccharide linkage region, shared by both HSPG and CSPG. The linkage region is covalently linked to serine residues in the core protein, and is assembled by the stepwise addition of, by corresponding specific glycosyltransferases: a single xylose, two successive galactose residues, and a single glucuronic acid. Addition of N-acetylgalactosamine to this linker triggers formation of chondroitin backbone, characterised by repeating disaccharide of glucuronic acid and N-acetylgalactosamine. HS consists of the repeating disaccharide of N-acetylglucosamine and glucuronic acid, therefore addition of N-acetylglucosamine (GlcNAc) would trigger HS backbone formation (Mikami and Kitagawa, 2013). In HS, some of the N-acetylglucosamine is deacetylated and N-sulfated (NS). These sulfated domains then undergo epimerization to uronic acid, some of which is then 2-*O*-sulfated (Couchman and Pataki, 2012).

Dynamic interactions between growth factors and the ECM are essential, and in here lies a key role of the proteoglycans. Binding and release of growth factors by the ECM can serve to sequester growth factors and control bioavailability, or may enhance growth factor activity. Heparan sulfate GAG side chains are highly anionic due to the presence of acidic sugar residues and readily bind many growth factors such as fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs). It is a generally held view that HSPGs act as a sink for growth factors which can be released by degradation of the ECM, or the glycosaminoglycan component of HSPG (Forsten-Williams *et al.*, 2008).

1.3 Roles of extracellular proteases in ECM turnover and function

Cells are constantly rebuilding and remodelling the ECM through synthesis and degradation. These processes are essential for morphogenesis, and their dysregulation can contribute to diseases such as osteoarthritis and fibrosis, therefore the ECM is tightly regulated to maintain tissue homeostasis (Bonnans, Chou and Werb, 2014).

Cleavage of ECM components is the main process during ECM remodelling and is important for regulating ECM abundance, composition, and structure, as well as releasing biologically active molecules such as growth factors (Bonnans, Chou and Werb, 2014).

The degradation of the extracellular matrix is essential in angiogenesis; not only must the basement membrane be digested to allow a new sprout to develop, but digestion of ECM proteins can also release ECM-bound growth factors, or expose cryptic regions which may have pro-, or anti- angiogenic activity (Yue, 2014).

1.3.1 The metzincin superfamily of proteases

The major players in ECM remodelling belong to the metzincin gene superfamily of zinc dependent proteases, which encompasses astacins (BMP1, meprin), serralysins, matrixins/matrix metalloproteinases (MMPs), and adamalysins/reprolysins (Escalona *et al.*, 2018).

The metzincins are multidomain proteins, synthesised as zymogens. They contain a pro-domain, followed by a globular catalytic domain containing a characteristic 'Met-turn' (a conserved methionine downstream of the catalytic Zn^{2+} binding motif, which causes a structural turn in the molecule giving the active site cleft its structure) and the consensus zinc binding motif **HE**XX**H**XX**G**XX**H** (Bode *et al.*, 1996) (Figure 1.3).

1.3.2 The Matrixins/MMPs

The MMPs are the prototypical matrix proteinases, and can be secreted or membrane bound. MMPs contribute to the physiological process of bone remodelling, immunity and wound healing. Their activity is tightly controlled at the transcriptional level, and by pro-peptide activation and tissue inhibitors of metalloproteases (TIMPs) (Chakraborti *et al.*, 2003).

The MMP pro-domain directs proper folding and maintains latency via a molecular complex between a single cysteine residue and the zinc atom in the catalytic domain, blocking the active site. Protein activation can be achieved via numerous mechanisms, including activating factors such as the plasminogen-plasmin cascade, other MMPs, furin-like pro-protein convertases (PPCs), non-proteolytic compounds, and denaturants such as urea, which all cause the dissociation of the cysteine residue and removal of propeptide region, leading to activation via the so-called 'cysteine switch mechanism' (Van Wart and Birkedal-Hansen, 1990).

There are 24 *MMP* genes in humans, including duplicated *MMP23* genes, therefore there are 23 MMP proteins. MMPs are multifunctional, but can be broadly categorised into the collagenases (MMP-1, MMP-8, MMP-13), the gelatinases (MMP-2, MMP-9), the stromelysins (MMP-3, MMP-10, MMP-11) the matrilysins (MMP-7,

MMP-26), the membrane type MMPs (MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP), GPI anchored MMPs (MT4-MMP, MT6-MMP) and others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28) (Nagase, Visse and Murphy, 2006).

Remodelling the ECM is a key role of the MMPs. Collagen proteolysis, predominantly by collagenases, occurs at a site three quarters from the N-terminus, cleavage fragments denature and form gelatin, which can in turn be degraded by gelatinases MMP-2 and -9 (Löffek, Schilling and Franzke, 2011). Basement membrane collagen IV can also be cleaved by gelatinases, as well as the stromelysins and matrilysins. Laminin, fibronectin, elastin, and aggrecan are substrates for most, if not all MMPs (Löffek, Schilling and Franzke, 2011).

Cell surface and membrane spanning proteins are also substrates for certain MMPs, for example cell-cell adhesion receptors: MMP-7 and MMP-3 cleave E-cadherin, and MT1-MMP cleaves CD44 (Rodríguez, Morrison and Overall, 2010). The cleavage and release of membrane proteins, or 'ectodomain shedding', can function to regulate signalling in multiple ways, such as releasing bioactive fragments or ligands, as well as cleaving, and thereby preventing activation of receptors (Löffek, Schilling and Franzke, 2011)**.**

The MMPs have multiple roles in angiogenesis, ECM degradation is an important stage in vascular cell migration, alongside this MMPs can liberate proangiogenic growth factors that are sequestered in the matrix, and can conversely generate anti-angiogenic molecules by cleaving plasma proteins, matrix molecules, or proteases themselves, making them highly important in the co-ordination of new vessel growth (Nabeshima *et al.*, 2002).

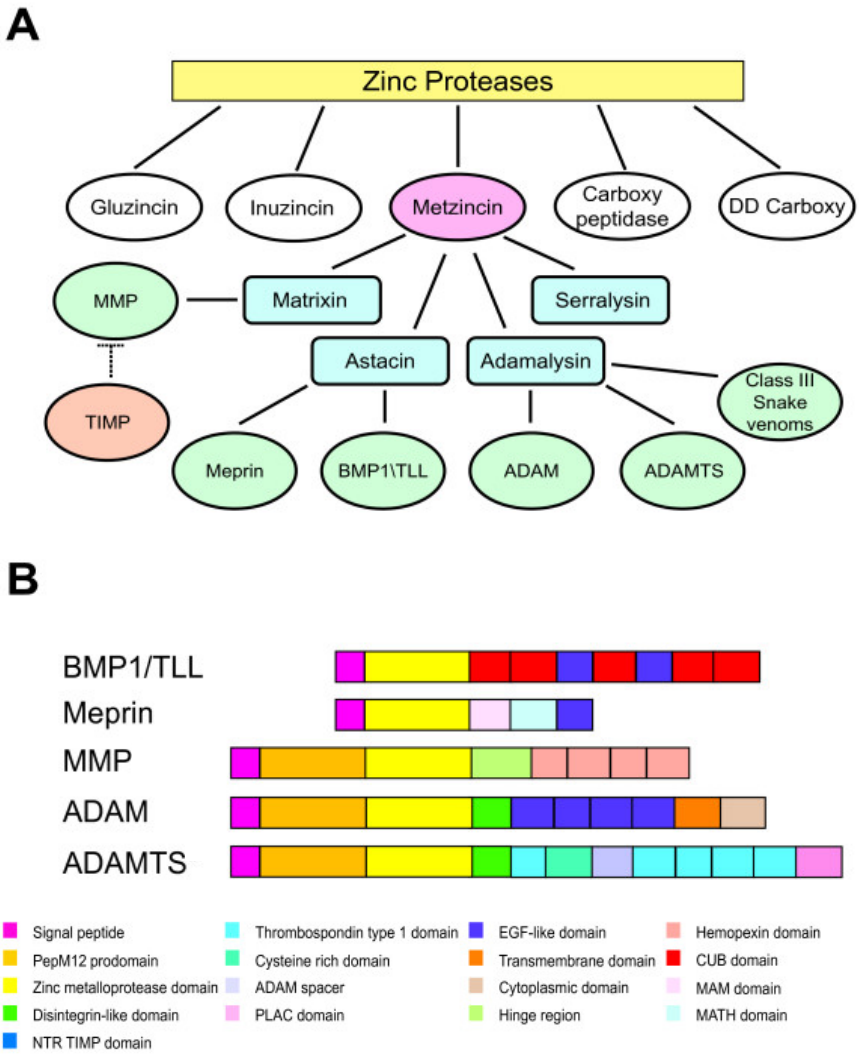


Figure 1.3 The metzincin superfamily of proteases. A) Family hierarchy. The metzincin family consists of the subfamilies matrixins, astacins, adamalysins and serralysins. B) Domain structure of family members. All metzincin proteases contain a zinc metalloprotease domain in the N-terminal, C-terminal domains are specific to subfamilies. Figure adapted from Bode *et al.* (1996).

1.3.3 TIMPs

The tissue inhibitors of metalloproteases (TIMPs) are the endogenous inhibitors of MMPs, as well as ADAMs and ADAMTS. Expression of TIMPs allows for precise regulation of proteases and prevents uncontrolled ECM turnover. There are 4 TIMP genes, TIMP -1, -2, -3, and -4. Each protein consist of two distinct domains, an 125 amino acid reside N-terminal, and a 65 residue C-terminal, the conformation of each domain is stabilised by three disulfide bonds (Williamson *et al.*, 1990; Baker, 2002). TIMPs are secreted and free, with the exception of TIMP-3, which is found tightly bound to glycosaminoglycans such as heparan sulfate in the ECM (Yu *et al.*, 2000).

TIMP-1 was the first member of the family to be discovered in the early 1970s, and was shown to inhibit gelatinases and collagenases. In general, the TIMPs are broad spectrum MMP inhibitors. TIMP-1 is slightly more restricted than the others and has low affinity for membrane-type MMPs. TIMP-3 has the broadest inhibition spectrum, and inhibits several members of the ADAM and ADAMTS family, in particular ADAMTS-1 and -4 (Nagase, Visse and Murphy, 2006). Interestingly, the affinity of TIMP-3 to ADAMTS-1 and -4 is much stronger than its affinity for MMPs, suggesting inhibition of aggrecanases may be its primary physiological function (Kashiwagi *et al.*, 2001).

The understood mechanism of inhibition is based on the structures of TIMP-MMP complexes. TIMPs have a wedge shaped ridge in the N-terminal domain which slots into the active site of the target MMP, preventing its catalytic activity (Murphy, 2011).

1.3.4 Adamalysins/reprolysins

The adamalysin/reprolysin family consists of the reprolysins, originally isolated from snake venom, the closely-related ADAMs (a disintegrin and metalloprotease) and the more recently discovered ADAMTS (a disintegrin and metalloprotease with thrombospondin repeats).

1.3.4.1 A disintegrin and metalloprotease (ADAM)

The ADAMs are implicated in numerous biological mechanisms including tumour metastasis, angiogenesis, and growth factor shedding. C-terminal to the metalloprotease domain they possess a disintegrin domain, a cysteine-rich region, an EGF-like domain, a transmembrane domain, and a cytoplasmic domain.

There are 22 known *ADAM* genes in human, of which at least 12 are proteolytically active. In contrast to the MMPs, proteolytically active ADAMs are membrane-bound, and thought to primarily play a role in adhesion, acting as molecular switches via ectodomain shedding of type I and type II transmembrane proteins (Mullooly *et al.*, 2016).

1.3.4.2 A disintegrin and metalloprotease with thrombospondin repeats (ADAMTS)

The *Adamts* family was identified in 1997, when a screen of genes expressed in murine colon adenocarcinoma identified a novel cDNA encoding for ADAMTS-1 (Kuno *et al.*, 1997). Like the ADAMs, the ADAMTS have an N-terminal signal peptide followed by a pro-domain, metalloprotease, and disintegrin domain, however the ADAMTS are secreted proteases, and therefore do not have transmembrane or cytoplasmic domains. Instead, downstream of the disintegrin domain, they have multiple thrombospondin type 1 repeats (TSRs) followed by an ancillary region composed of a cysteine-rich region, a spacer region, and occasionally additional specialist domains. The ancillary region determines substrate specificity, as in ADAMTSs the protease domain alone unable to process native substrates (Kelwick, Desanlis, *et al.*, 2015).

With the exception of ADAMTS-6 and -10, all ADAMTS family members are predicted to be catalytically active, and can be broadly categorised into clades based on substrate: aggrecanases/proteoglycanases, procollagen N-propeptidases, cartilage oligomeric matrix protein-cleaving enzymes, von Willebrand factor proteinases, and a group of orphan enzymes (Figure 1.4). There is no evidence for the cysteine switch mechanism of activation well described for MMPs, however excision of the pro-

peptide is typically a pre-requisite of catalytic activity, and all ADAMTSs contain at least one site for furin-like PPCs.

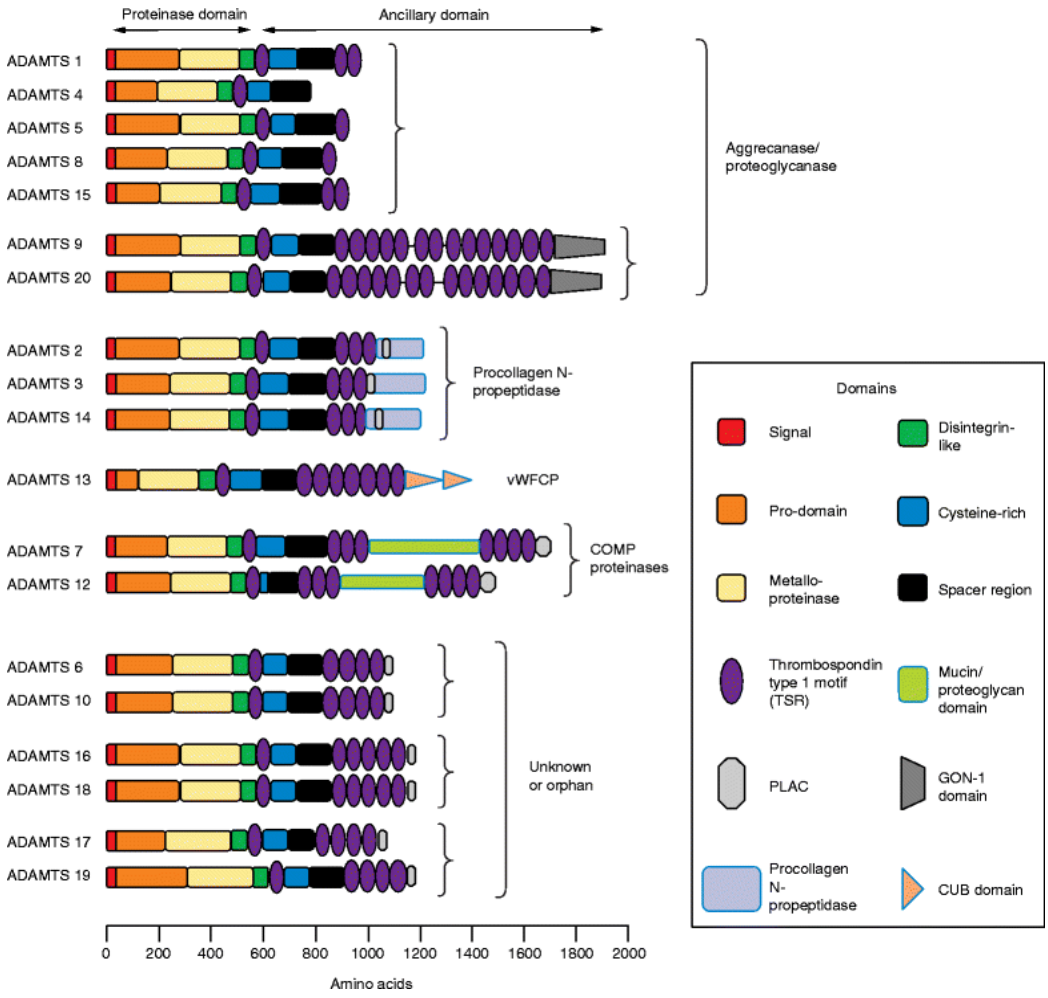


Figure 1.4 The ADAMTS proteases. The ADAMTS family can be sub-grouped into 5 clades based on substrate; aggrecanases, procollagen N-propeptidases, vWFCP, COMP proteinases and a group of orphan enzymes. All members share structural features of a signal peptide, a pro-domain followed by a metalloproteinase domain, a disintegrin-like domain, a thrombospondin type 1 motif and a spacer region, the C-terminal is specific to each protease. Figure adapted from Kelwick, Desanlis, *et al.* (2015).

1.3.4.3 The pro-collagen N-propeptidases

The pro-collagen N-propeptidases (ADAMTS-2, -3 and -14) are essential for the maturation of triple helical collagen fibrils. Triple-helical procollagens are secreted with pro-peptides at both extremities, and removal of both the N- and C-terminal pro-domain of type I and II procollagen is required to generate soluble collagen monomers which can assemble into elongated and cylindrical collagen fibres.

ADAMTS-2,-3, and -14 fulfil this role in the case of the amino terminal pro-peptide (Bekhouche and Colige, 2015). The importance of the pro collagen N-propeptidases is highlighted in human disease; mutations in ADAMTS-2 can give rise to Ehlers-Danlos type VIIC disease, as a result of abnormal polymerisation of collagen fibres containing the N but not C propeptide (Colige *et al.*, 2002).

1.3.4.4 Cartilage oligomeric matrix protein cleaving enzymes

The cartilage oligomeric matrix protein (COMP) proteases (ADAMTS-7 and -12), digest COMP, a prominent non collagenous component of cartilage. In the case of ADAMTS-12 the interaction is mediated via the EGF domain of COMP and the 4 C-terminal TSP repeats of the enzyme (C. Liu *et al.*, 2006).

Due to the importance of COMP in cartilage, the roles of ADAMTS-7 and -12 in arthritis, a disease characterised by cartilage breakdown, have been closely investigated. Degraded fragments of COMP have been observed in arthritic patients, as has an upregulation of ADAMTS-7 and -12, and indeed Liu *et al*, showed that ADAMTS-7 and -12 degradation of COMP plays an important role in the initiation and progression of the arthritis (C. Liu *et al.*, 2006; C.-J. Liu *et al.*, 2006).

1.3.4.5 von Willebrand factor proteinase

ADAMTS-13's major function is cleavage of von Willebrand factor (VWF) anchored on the endothelial surface, in circulation, and at sites of vascular injury. Cleavage of VWF is essential for proper haemostasis, and mutations in ADAMTS-13 can result in thrombocytopenic purpura (TTP), a disorder of blood coagulation where extensive

clots form in small blood vessels, characterised by the presence in the plasma of large VWF strings (Zheng, 2015).

1.3.4.6 The Orphan Enzymes

Several 'orphan' enzymes exist, whose physiological substrates are yet to be identified, these include ADAMTS-6, -10, -16, -17, -18 and -19. The importance of these ADAMTSs is highlighted by their role in human disease, but may be distinct from catalytic functions. For example, ADAMTS-10 is resistant to furin cleavage due to lack of conservation of the furin cleavage site in its pro-domain, and does not appear to be catalytically active, however it has an essential role in fibrillin microfibril function (Kutz *et al.*, 2011). Mutations in ADAMTS-10 or fibrillin-1 result in Weill Marchesani syndrome (WMS), characterised by short stature, eye defects, hypermuscularity and thickened skin (Mularczyk *et al.*, 2018). ADAMTS-17 is also capable of binding, but not cleaving, fibrilin-1, and ADAMTS-17 defects have been shown to lead to similar dysfunctions. Autosomal recessive ADAMTS-17 mutations cause WMS-like syndrome, affected individuals have short stature and eye abnormalities but lack joint stiffness and brachydactyly associated with WMS (Hubmacher *et al.*, 2017).

1.3.4.7 The aggrecanase/proteoglycanase clade

The aggrecanase clade consists of 7 proteases (ADAMTS-1, -4, -5, -8, -9, -15 and -20), and is often referred to as the 'angioinhibitory clade'. The primary substrates for this group are chondroitin sulfate proteoglycans of the hyalectan family, of which there are four members, aggrecan, brevican, neurocan and versican (Stanton *et al.*, 2011). Whilst expression of neurocan and brevican is largely restricted to neural tissue, aggrecan is a major component of cartilage ECM. Studies have identified ADAMTS-4 and -5 as the major physiologically relevant aggrecanases, and their proteolytic activity is important in osteoarthritis (Verma and Dalal, 2011). Subsequent studies have identified limited aggrecanase activity amongst ADAMTS-1, -8, -9 and -15 however it is unlikely that this is the major role of these enzymes.

Versican forms highly hydrated complexes with hyaluronan, and is widely distributed in the provisional matrix of mammalian embryos. In cardiac development, immature versican-rich ECM is replaced by a collagen, proteoglycan and elastin containing matrix. Myocardial compaction, endocardial cushion remodelling, myogenesis and interdigital web regression are all developmental processes where ADAMTS-mediated cleavage of versican is key (Nandadasa, Foulcer and Apte, 2014). ADAMTS-1, -4, -5, -9, -15 and -20 can act as versicanases, and their importance is this context is highlighted by knockout models, *Adamts-1, 5* and *9* -/- mice all display phenotypes resulting from defective cardiac development, alongside cleft palate and syndactyly (Kelwick, Desanlis, *et al.*, 2015).

ADAMTS-1 was the first member of the ADAMTS family to be identified (Kuno *et al.*, 1997; Kuno, Terashima and Matsushima, 1999). It is widely expressed in developing mammalian embryos; *Adamts-1 -/-* mice show growth retardation, morphological defects in the kidney, adrenal gland and adipose tissue, and infertility in females due to ineffective cleavage of versican during ovarian maturation, demonstrating an essential role for this enzyme in development (Shindo *et al.*, 2000). *Adamts-1* -/- mice also present with a delayed wound healing response, which, alongside the upregulation of ADAMTS-1 seen in wounded skin, suggests a role for the protease in cell migration and angiogenesis (Krampert *et al.*, 2005).

Once secreted, ADAMTS-1 binds to the ECM (Hashimoto, Shimoda and Okada, 2004; Kelwick, Desanlis, *et al.*, 2015). This ECM anchorage is mediated by its spacer region and three TSRs. When cultured with heparin, this interaction is disrupted and ADAMTS-1 can be found in cell culture media, indicating that the ADAMTS-1 ECM interaction is mediated by HSPG (Kuno and Matsushima, 1998). By binding ECM components such as GAGs, ADAMTS-1 can modulate growth factor availability and cell adhesion.

1.4 Cellular adhesion molecules and the ECM

Cells rely upon adhesion molecules, cell surface glycoproteins, to interact with and respond to the ECM. The principal players in cell matrix interaction are the integrins, however other molecules such as selectins and syndecans can bind the ECM.

Cell adhesion receptors also serve to form a link between the ECM and the cell cytoskeleton. Sites of ECM-cytoskeleton connection occur at focal adhesions (FAs), large and dynamic macromolecular structures. FAs are composed of adhesion receptors such as integrins and syndecans, which recruit adaptor and scaffold proteins to stabilise the adhesion (Lo, 2006). FAs provide both mechanical linkage, as well as allowing for the transmission of extracellular signals. The formation and turnover of adhesions in response to the environment is also essential for mesenchymal migration.

1.4.1 Integrins

The integrins are a family of heterodimeric transmembrane glycoproteins, which act as cell adhesion receptors. They form a major component of focal adhesions, and modulate dynamic interactions between the ECM and actin cytoskeleton during cell motility. Integrin molecules contain 2 subunits, an α and a β, both of which are type I transmembrane proteins. In mammals, there are 18 α and 8 β subunits, which form at least 24 distinct heterodimers (Humphries, Byron and Humphries, 2006). Integrin heterodimers can be roughly classified into four major classes based on ligand specify; RGD receptors, collagen receptors, leukocyte specific receptors and laminin receptors (Figure 1.5).

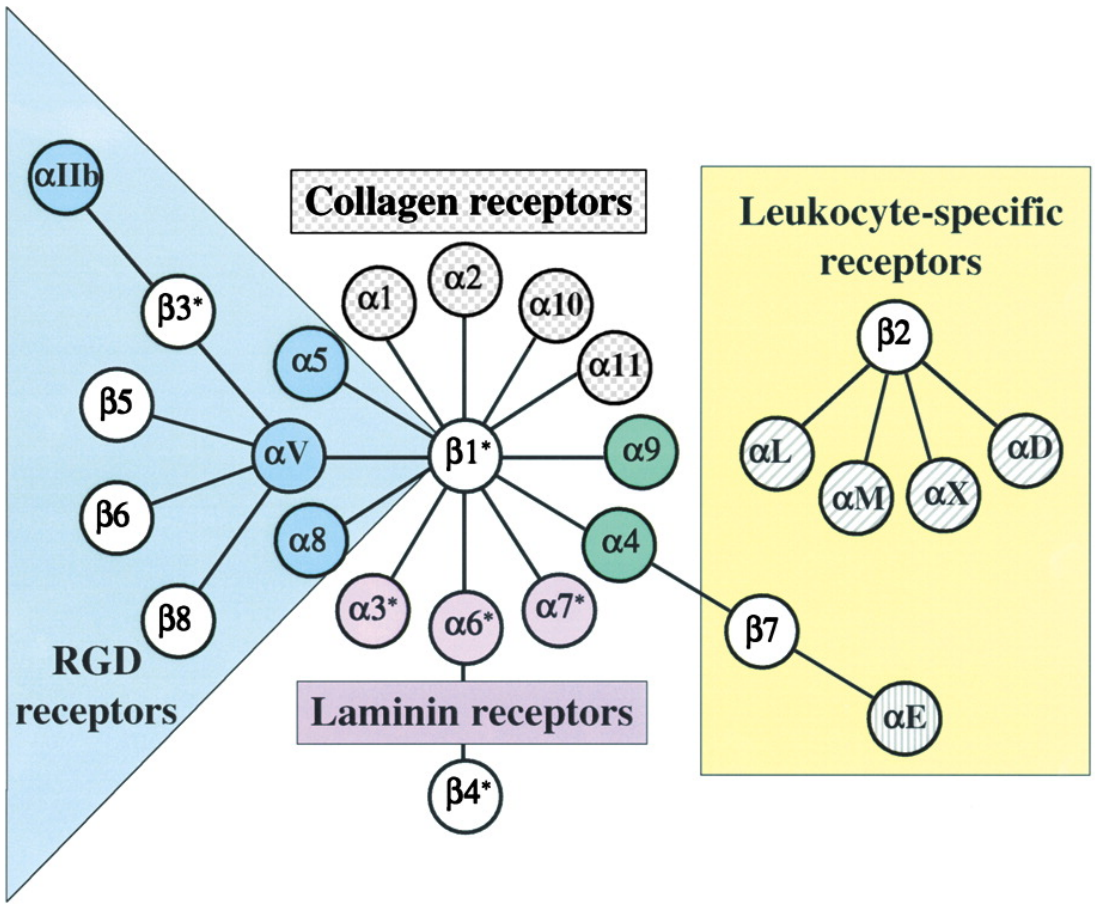


Figure 1.5 Integrins form 24 distinct heterodimers. The figure shows the mammalian subunits and their possible α/β combinations. Grey hatching represents inserted A/I domains (the principal ligand-binding domain), asterisks denote alternatively spliced cytoplasmic domain. Figure reproduced from Hynes (2002).

As well as functioning mechanically, linking the cell cytoskeleton to the ECM, integrins function biochemically, and signal bi-directionally in pathways called outside-in and inside-out signalling. In inside-out signalling, integrins respond to intracellular stimuli, which initiates conformational changes influencing how the extracellular 'head' interacts with extracellular ligands. During outside-in signalling, integrins respond to ligand binding and mediate the transmission of extracellular signals across the plasma membrane, activating intracellular signalling machinery and mediating cellular responses such as migration or proliferation (Harburger and Calderwood, 2008).

1.4.1.1 Integrin Inside-out signalling

Inside-out signalling, or integrin activation, referrers to the ability of integrins to regulate their affinity for extracellular ligands. Integrins on the cell surface are normally in a low affinity state, and are 'activated' in response to intracellular signalling acting upon the integrin cytoplasmic tails.

In a resting state α and β subunits are in close proximity, in order for activation to occur the non-covalent complex between the cytoplasmic tails of α and β subunits must be unclasped, this in turn unclasps the association of transmembrane helices, and allows transmission of a conformational change into the extracellular domain, exposing the ligand binding site (Plow, Meller and Byzova, 2014).

The short cytoplasmic tail of the β subunit is responsible for interaction with proteins which regulate the inside out signalling process. One such key regulator is talin, a protein consisting of a N- terminal globular head, followed by an extended rod of helical bundles, containing an actin-binding motif (Moser *et al.*, 2009). A FERM sub-domain in the head region of talin binds the β subunit cytoplasmic tail, displacing it from the α subunit. Several proteins can inhibit integrin activation by competing with talin for integrin binding, for example Dok1 and ICAP1 (Critchley and Gingras, 2008; Harburger and Calderwood, 2008).

1.4.1.2 Integrin outside-in signalling

The binding of integrins to ligands initiates outside-in signalling. Integrins form an initial talin-mediated connection between the cytoskeleton and ECM; additional proteins are then recruited and integrins cluster, stabilising and reinforcing this link. This results in the formation of a focal adhesion complex at the integrin cytoplasmic tail, and activation of downstream signal pathways which regulate cell shape, migration, growth and survival (Hu and Luo, 2013).

Proteins recruited by integrins can be broadly grouped into 3 categories, integrin-binding proteins, adaptors/scaffolding proteins and enzymes/effectors. Integrin cytoplasmic tails themselves have no intrinsic catalytic activity, and therefore signalling requires the recruitment of effector proteins, in particular the non-receptor tyrosine kinases focal adhesion kinase (FAK) and Src are essential in integrin signalling cascades. Serine threonine kinases such as PKC are also regulated by integrin clustering, as are lipid kinases of the PI3K family. Other important signalling molecules are small G proteins of the Rho GTPase family (Bustelo, Sauzeau and Berenjeno, 2007).

1.4.1.3 RHO GTPases in integrin outside-in signalling

The Rho family is a subgroup of the Ras superfamily of GTP hydrolases. One of the main functions of the family is to assemble or activate proteins at the cytoplasmic surface of membranes, and these signalling proteins are required to mediate assembly, disassembly, and organization of actin filament structures. Members of the Rho family are classified into six subgroups: Rho, Rac, Cdc42, Rnd, RhoBTB and RhoT/Miro (Bustelo, Sauzeau and Berenjeno, 2007).

Most Rho GTPases act as molecular switches, active when bound to GTP, and inactivated when this is hydrolysed to GDP. This switch is regulated by guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs). The GEFs promote the exchange of GDP for GTP, encouraging activation the G protein, leading to stimulation of signalling cascades and cellular responses. Conversely, GAPs

promote hydrolysis of bound GTP, catalysing the return to the inactive state (Bustelo, Sauzeau and Berenjeno, 2007).

Different Rho GTPases hold different cellular functions; Rac is required for lamellipodial protrusion, Cdc42 is required to maintain cell polarity, including localizing lamellipodial activity to the leading edge of migrating cells, Rho is required to maintain cell adhesion during movement, and Ras regulates focal adhesion and stress fibre turnover (Figure 1.6) (Nobes and Hall, 1999).

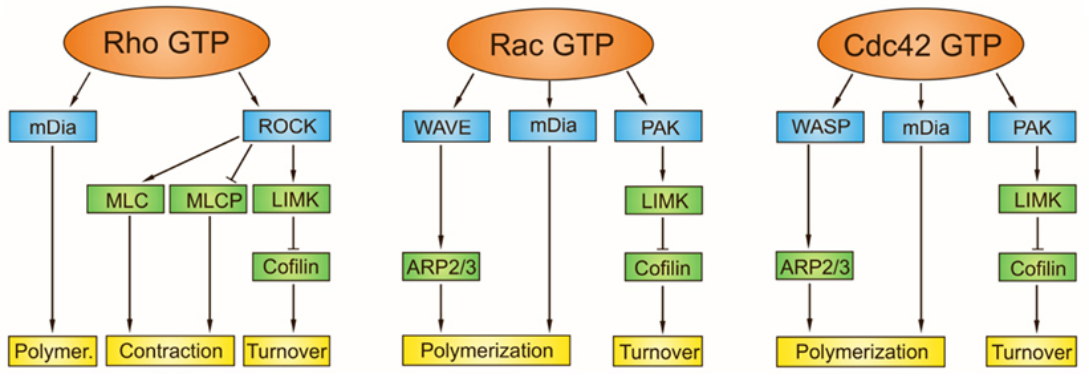


Figure 1.6 Rho family GTPases have different cellular functions. Charts show members of the family, their downstream effectors, and outcomes for the actin cytoskeleton including polymerisation, turnover and contraction. Figure adapted from Dráber, Sulimenko and Dráberová (2012).

Integrin inside-out signalling through small GTPases is vital during integrin-dependent cell spreading and retraction. Rac activity, necessary for lamellipodia formation, is stimulated by integrin signalling. Integrin ligation induced by outside in signalling also dynamically regulates Rho, necessary for regulation of cell retraction.

One proposed mechanism for the link between integrin ligation and downstream small GTPase activation is through Src family kinase activation, in particular c-Src, which has been shown to mediate phosphorylation of various GEFs for Rac (Figure 1.7).

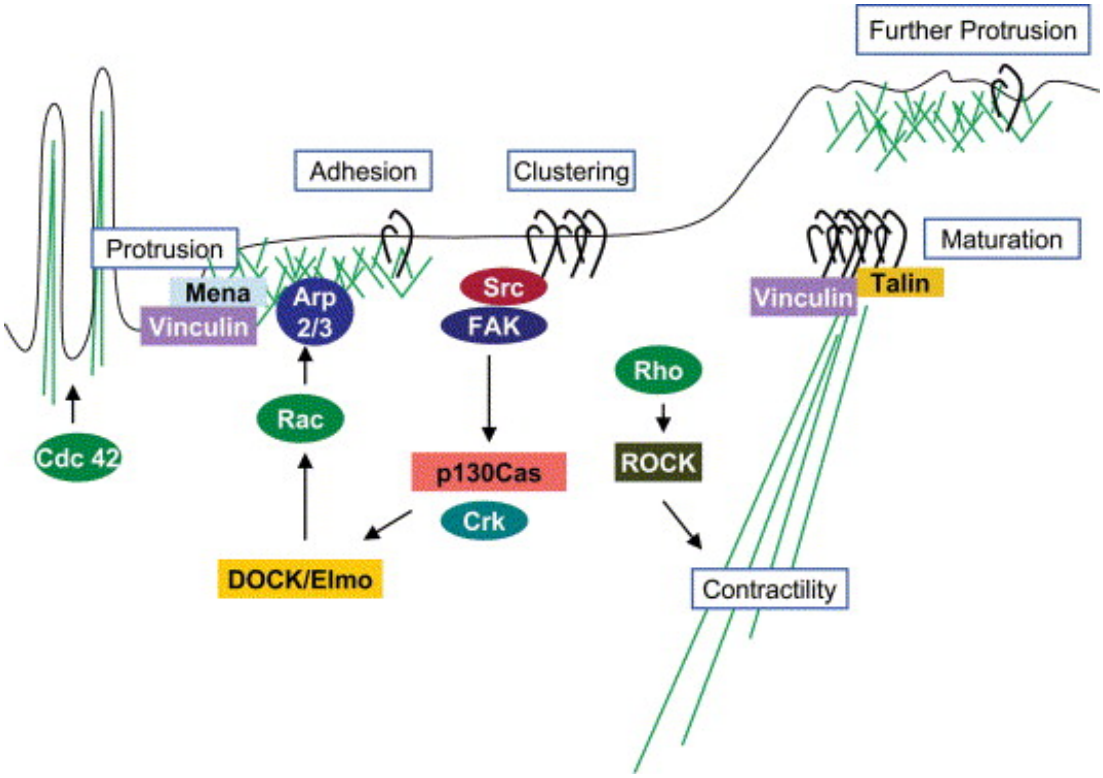


Figure 1.7 Small G proteins are essential for co-ordinated cell migration. CDC42 and Rac drive forward protrusions of the cell membrane, these are stabilized by initial adhesions. Integrin clustering strengthens adhesions and leads to signalling via Src and FAK, activating adaptor molecules Cas/CRK. Rho-mediated contractility through ROCK assembles stress fibres and leads to maturation of adhesions. Figure reproduced from Shen, Delaney and Du (2012).

1.4.1.4 Integrin trafficking

Integrin functions are dependent on the relative surface availability of heterodimers, which is regulated by membrane trafficking. Nine different integrins can bind fibronectin, therefore cells adherent to fibronectin can have adhesions containing many different integrins that can differentially affect adhesion dynamics. An antagonistic relationship between the recycling of α5β1 and αvβ3 has been demonstrated to regulate migration. On fibronectin, α5β1 adhesions are more dynamic, whereas αvβ3 mediated adhesions are associated with more persistent migration (Huttenlocher and Horwitz, 2011).

Recycling of αvβ3, but not α5β1, is controlled by the Rab4 short loop recycling pathway. Recycling through this route promotes formation of αvβ3 integrin containing cell-matrix adhesions at the leading edge of migrating cells, which supports Rac and Arp2/3 mediated lamellipodial extension and maintains directionally persistent migration in fibroblasts. Disruption of this recycling causes lamellipodial collapse and promotes rapid, random migration due to an increase in Rab11-RCP dependent recycling of α5β1 promoting activation of the RhoA-ROCK-Cofilin pathway, which drives ruffling protrusions and rapid migration (López-Otín and Matrisian, 2007; Paul, Jacquemet and Caswell, 2015).

1.4.2 The syndecans

A second family highly important in cell adhesion is the syndecans. The syndecans are a family of type I transmembrane proteoglycans, with roles in development, angiogenesis, wound healing, tumour growth and neurogenesis (Leonova and Galzitskaya, 2013). There are four family members, each consisting of a protein core (20-40kDa) with extracellular CS or HS GAG chains attached, which allow them to engage the ECM and growth factors (Morgan, Humphries and Bass, 2007). They possess a single transmembrane domain and short cytoplasmic tail which contains a pair of conserved regions and a unique variable region. The cytoplasmic domain of all syndecans contains a binding site for PDZ-binding proteins such as syntenin (Figure 1.8).

Syndecans 1 and 3 possess both types of GAG chains, whereas syndecan 4 and 2 only have HS chains. Heparan sulfate chains consist of 50-150 repetitions of disaccharide units (mainly glucuronic acid and *N*-acetylglucosamine) with alternating high and low sulfate domains attached to conserved Ser-Gly dipeptides in the extracellular core protein.

Expression of syndecans varies throughout cell types. Syndecan 1 is highly expressed in epithelia, syndecan 2 in endothelia and fibroblasts, whereas high expression of syndecan 3 can mostly be found in neuronal tissues. Syndecan 4 is the only member of the family to be ubiquitously expressed and is the major syndecan in endothelial cells (Leonova and Galzitskaya, 2013; Vuong *et al.*, 2015)

The syndecans interact with a large array of ligands through their GAG chains, and are considered to be ligand gatherers, working in co-operation with signalling receptors; alongside this syndecans have the capacity to signal independently (Couchman, 2003).

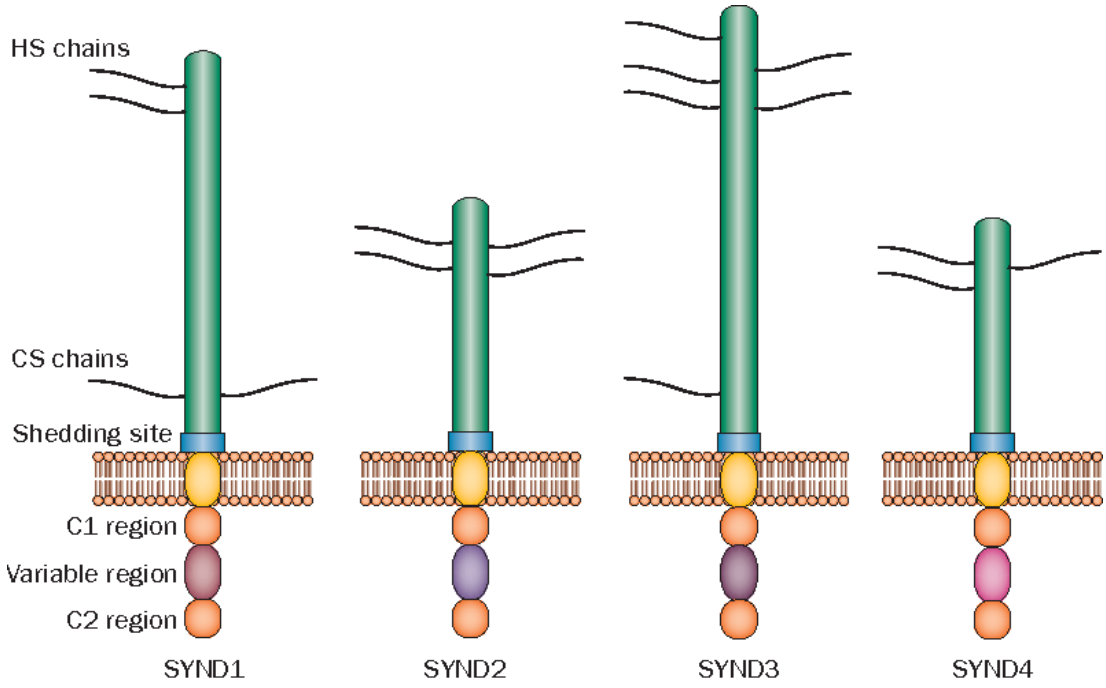


Figure 1.8 The syndecan family of transmembrane proteoglycans. The syndecan family has four members, each has an intracellular tail with two conserved regions and a variable domain, a transmembrane region, and an extracellular domain to which HS or CS chains are covalently attached. Syndecans have an extracellular shedding site close to the membrane. Figure reproduced from Pap and Bertrand (2013).

1.4.2.1 Syndecan 4

Syndecan-4 is of particular interest as it is the only member of the syndecan family reported to be enriched in focal adhesions (Woods and Couchman, 1994). The HS GAG chains of syndecan 4 allow it to interact with heparin-binding growth factors such as FGFs, VEGFs and PDGFs. In this way syndecan 4 can generate variable spatial distributions of growth factors and ECM components, although the physiological significance of this remains unclear.

Syndecan 4 can act as a co-receptor, stabilising the interaction between growth factors and other cell membrane receptors. This is best defined in the case of FGFs. Although FGFs bind FGFRs with high affinity, this interaction and subsequent signalling events are amplified and can be prolonged by the presence of heparan sulfate chains (Sperinde and Nugent, 2000). Syndecan 4 can also function as a receptor in its own right; upon ligand binding syndecan 4 clusters and translocates to lipid rafts, specialised membrane regions rich in cholesterol and sphingolipid (Tkachenko and Simons, 2002). In the case of FGF2 binding, clustering of syndecan 4 in lipid rafts leads to non-clathrin mediated, Rac-1 and CDC42-dependent endocytosis of FGF2-syndecan 4 complexes; cellular uptake of FGFs is believed to be necessary for them to exhibit their full mitogenic affect (Tkachenko *et al.*, 2004).

Syndecan 4 also has a role in lymphangiogenesis; the formation of lymphatic vessels from pre-existing lymphatic vessels, believed to be similar to angiogenesis. Syndecan 4 has been shown to interact with VEGF receptor 3 on the lymphatic endothelial cell surface, and potentiate its activity. Supporting this, pathological lymphangiogenesis is impaired in *Sdc4*-/- mice (Johns *et al.*, 2016). Conversely, Wang *et al.* found that syndecan 4 deletion lead to excessive expansion of lymphatic vasculature during embryonic development (Wang *et al.*, 2016). It has therefore been proposed that syndecan 4 may act in a context-dependent mechanism.

1.4.2.2 Syndecan ectodomain shedding

Shedding refers to the proteolytic cleavage and release of syndecan ectodomains, believed to have a number of physiological and pathological roles. Proteases of the

metzincin family are largely responsible for syndecan cleavage. Mapping of MMP cleavage sites on syndecan 1 and 2 showed the preferred region for cleavage is two membrane proximal sites, 2 and 15 residues from the transmembrane domain. Other cleavage sites were found 35-40 residues C-terminal from HS chain substitution sites (Manon-Jensen, Multhaupt and Couchman, 2013).

Known shedders of syndecans include MMP-7, MT1-MMP, MT3-MMP (syndecan 1) (Li *et al.*, 2002; Endo *et al.*, 2003), and MMP-2 and MMP-9 (syndecan 1, 2 and 4) (Brule *et al.*, 2006; Fears, Gladson and Woods, 2006). It is not yet clear how extracellular stimuli influence these sheddases to mediate syndecan cleavage, but different agonists appear to activate distinct intracellular signalling pathways to activate shedding.

Syndecan 4 shedding by proteases can serve to downregulate signal transduction at the cell membrane, however released soluble ectodomains retain their GAG chains and ability to bind growth factors and serve as important signalling molecules; they can acts as paracrine or autocrine effectors, as antagonists or competitive inhibitors of growth factors and can compete with intact syndecan for extracellular ligands (Steinfeld, Van Den Berghe and David, 1996; Manon-Jensen, Itoh and Couchman, 2010).

1.5 Other molecular players in the ECM

1.5.1 Fibulin 1

Fibulin I is a secreted glycoprotein found in association with ECM structures such as fibronectin fibrils and basement membranes. It is capable of binding a number of matrix components, including the hepII domain of fibronectin, nidogen-1, and certain proteoglycans (Balbona *et al.*, 1992).

Fibulin 1 is a ligand for the c type lectin domain of the proteoglycans aggrecan and versican (Aspberg *et al.*, 1999). The physiological relevance of the association is not yet fully elucidated, however tissue co-localisation of fibulin-1 with versican or aggrecan implies a functional association; fibulin 1 and versican are both found in the subintimal layer of the tunica media of rat aorta, and the expression of both is upregulated in damaged skin (Fässler *et al.*, 1996; Aspberg *et al.*, 1999). As in the case of skin, versican expression is greatly upregulated in response to damage in blood vessels, but it is unclear if fibulin 1 responds in the same way (Bode-Lesniewska *et al.*, 1996).

As aggrecan and versican are known substrates of ADAMTS-1, it seems logical to find a connection between ADAMTS-1 and fibulin I, and indeed a yeast two-hybrid screen using the c-terminal region of ADAMTS-1 as bait identified fibulin 1 as an interacting molecule (Lee *et al.*, 2005). Immunoprecipitation substantiated the ability of the proteins to interact, and similar expression patterns were seen *in vivo*. Functional investigation revealed that fibulin 1 was not a substrate for ADAMTS-1, but instead its presence enhanced the capacity of ADAMTS-1 to cleave aggrecan (Lee *et al.*, 2005).

Fibulin 1 has been found to have profound effects on cell adhesion and migration; the incorporation of fibulin 1 into fibronectin matrices inhibited cell attachment, spreading and migration, and cells transfected to overexpress fibulin 1 displayed reduced velocity. The motility suppressing effects of fibulin 1 appeared to be FN specific, but fibulin 1 did not perturb interactions between α5β1 or heparan sulfate proteoglycans with fibronectin. Fibulin 1 was found to inhibit ERK, and it was posited

that the effects were due to modulation of actomyosin contractility (Twal *et al.*, 2001).

A more recent study comparing the migration inhibitory effects of fibulin 1 and tenascin C (another matrix glycoprotein) saw similar inhibition of cell adhesion, and demonstrated that this was correlated with inhibition of FAK and ERK activation. It was found that these inhibitory effects could be bypassed by activation of RhoA, and that the fibroblast response to fibulin 1 was dependent on expression of syndecan 4 (Williams and Schwarzbauer, 2009).

Studies of the extracellular protein calumenin have highlighted the importance of MMP proteolysis in the regulation of fibulin 1. MMP-13 cleaves fibulin 1; calumenin binding to fibulin 1 prevents this degradation, leading to inactivation of ERK1/2 and inhibition of migration. This inhibition of ERK was dependent on calumenin and fibulin 1 interaction with fibronectin in an α5β1 and syndecan-4 dependent mechanism. These data highlight that although fibulin 1 may not perturb α5β1 or heparan sulfate proteoglycan interaction with fibronectin, their presence is essential for it to exert its anti-migratory effects.

1.6 Cell migration

Cell migration, essential for growth, development, wound healing, and angiogenesis, is greatly influenced by interaction with the ECM. The best characterised mode of cell migration is mesenchymal migration, a crawling style of cell migration which requires polarisation of cells into a leading and trailing edge. To achieve this polarisation, the actin cytoskeleton, as well as adhesion and chemosensory receptors, must be redistributed. Actin polymerisation drives membrane protrusion, new adhesions can then form at this leading edge, allowing cells to generate traction required for actin polymerisation driven translocation of the cell body. Adhesions and the trailing edge then disassemble and are recycled to allow for persistent and continuous migration (Huttenlocher and Horwitz, 2011).

In 3D tissues cell migration requires regulated extracellular proteolysis and degradation of the ECM in order to overcome physical barriers in tissue environments, achieved through engagement of matrix metalloproteinases at the cell surface (Friedl and Wolf, 2003).

1.6.1 Formation and turnover of focal adhesions

An initial nascent adhesion forms where a small extended membrane protrusion makes contact with the ECM. This early adhesive focus is formed of an integrin-ECM linkage, ligand binding to integrin induces clustering of integrins, and this interaction is then stabilised by the binding of the cytoskeletal scaffold protein talin, which mediates crosslinking with F-actin. Talin also facilitates the linkage of adaptor proteins such as paxillin and actin-binding proteins such as vinculin and α-actinin, as well as the recruitment of early effector proteins such as the non-receptor tyrosine kinase FAK (Critchley and Gingras, 2008).

This initial ‘focal contact’ is immature and often short lived, with the potential to develop into a larger mature focal adhesion, or be recycled, depending on the environment. Maturation into a focal adhesion is controlled by tension force and local actin polymerisation: actomyosin contraction induces conformational changes in talin, which exposes further vinculin-binding sites, and promotes the formation of

more stable focal adhesions (De Pascalis and Etienne-Manneville, 2017). The final stage of development into a mature fibrillar adhesion is regulated by Rho activity, and characterized by the recruitment of tensin and zyxin (Nobes and Hall, 1999; Wozniak *et al.*, 2004).

Focal adhesions grasp the ECM to generate forces necessary to pull the cell body forward, and therefore continued cell movement requires release and disassembly of trailing edge adhesions (Wolfenson, Lavelin and Geiger, 2013). The generation of retractive force is dependent on actin-myosin interaction, stimulated by phosphorylation of the myosin light chain, which occurs when Rho activates its downstream effector Rho kinase (ROCK) inducing phosphorylation and inhibition of myosin light chain phosphatase (Shen, Delaney and Du, 2012). Extension of microtubules into FAs triggers their disassembly and internalisation of integrins at the cell surface, in a mechanism mediated by the GTPase dynamin, recruited by FAK (Figure 1.9) (Wang *et al.*, 2011).

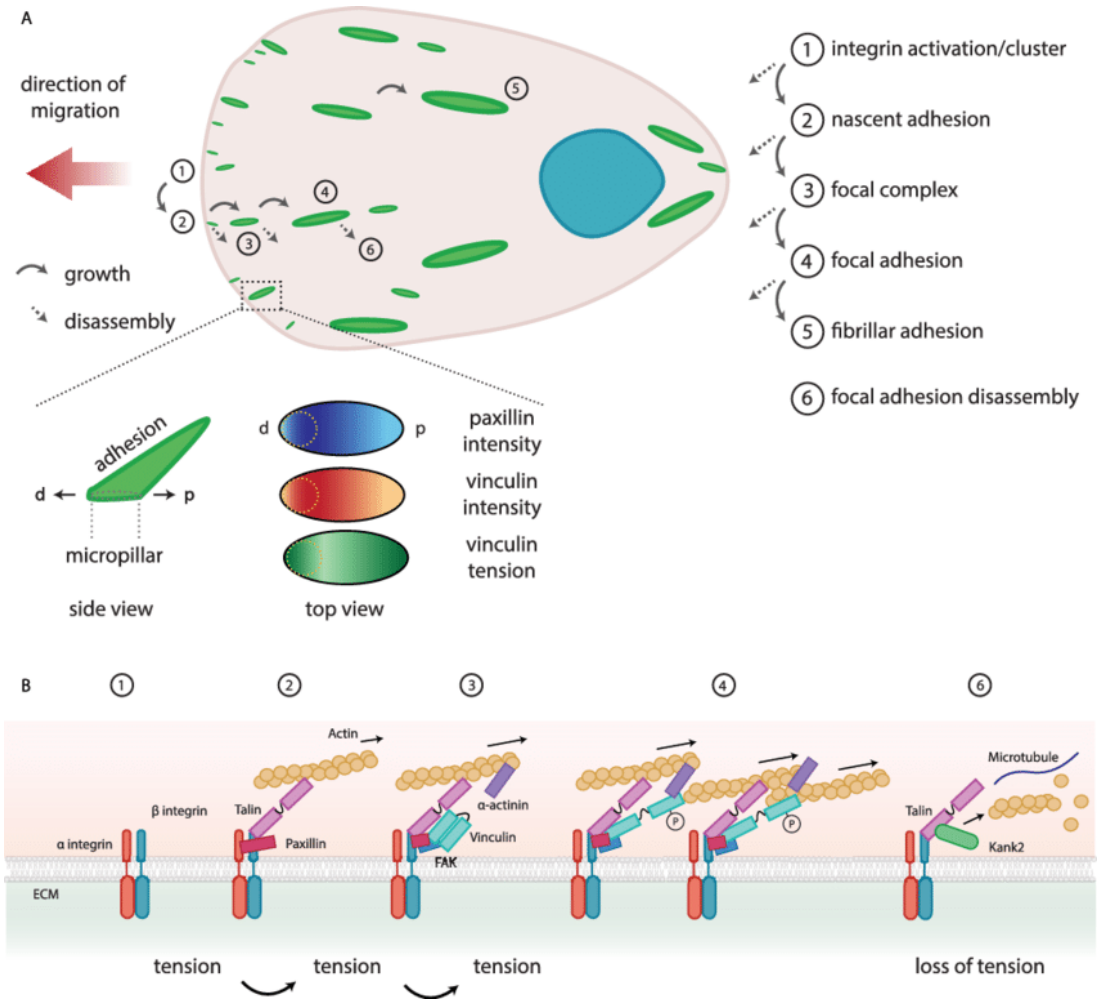


Figure 1.9 Formation, maturation and turnover of focal adhesions. An initial integrin ECM linkage forms a nascent adhesion, this matures via the recruitment of adaptor proteins such as talin and vinculin. In order for sustained migration adhesions at the trailing end must disassemble, triggered by the expansion of microtubules into the adhesion. Figure adapted from De Pascalis and Etienne-Manneville (2017).

1.6.2 Syndecan 4 and cell migration

The role of syndecan 4 in focal adhesions is well appreciated; syndecan 4 serves as a direct link between the ECM and extracellular signalling proteins, since it can simultaneously bind fibronectin and the intracellular actin-associated protein α-actinin, and can recruit other proteins to sites of focal adhesions, a key example being the signalling molecule protein kinase C α (PKCα) (Greene *et al.*, 2003). PKCα is essential for adhesion-mediated signal transduction; the Syndecan 4 cytoplasmic domain, when bound to phosphatidylinositol 4,5-bisphosphate, recruits PKCα to sites of focal adhesions through interaction with its catalytic domain and mediates its activation (Lim *et al.*, 2003). This interaction also mediates the location and stability of PKCα (Oh, Woods and Couchman, 1997b).

Another important role of syndecan 4 in focal adhesions is attributed to the activation of FAK, leading to the potentiation of many downstream signalling pathways. Fibroblasts spreading onto a fibronectin fragment lacking the HepII binding domain attach but do not fully spread or form focal adhesions, furthermore, FAK activation by autophosphorylation of Tyr_{397} does not occur in cells spreading on this HepII lacking fibronectin. Syndecan 4 binds to the HepII-binding domain of fibronectin, suggesting its importance in this context, and indeed syndecan 4 null fibroblasts spreading on fibronectin show reduced levels of FAK Tyr_{397}. Further supporting a role for syndecan 4, focal adhesion and stress fibre formation can be rescued by activation of Rho or PKCα, or by clustering of syndecan 4 with antibodies against the syndecan 4 extracellular domain, indicating that syndecan 4 activates FAK in a Rho-dependent manner (Saoncella *et al.*, 1999; Woods *et al.*, 2000; Wilcox-Adelman, Denhez and Goetinck, 2002).

Despite the predicted importance of syndecan 4 in focal adhesion formation, fibroblasts isolated from *Sdc4*-/- mice retain the ability to form focal adhesions, likely as a result of the plasticity of adhesion mechanisms; adhesion receptors are numerous and varied, and it is likely cells isolated from *Sdc4*-/- mice adapt, and an alternate receptor may fulfil the roles of syndecan 4 (Echtermeyer *et al.*, 2001). Many

important functions of syndecan 4 in focal adhesion formation occur in concert with the integrins.

1.6.3 Co-operation of syndecans and integrins in adhesion and migration

Nearly all ECM proteins have a binding site for both syndecans and integrins, and substantial evidence suggests that full adhesion response requires dual engagement of HSPG and integrins; one well defined example is the dual engagement of integrin and syndecan 4 in adhesion to fibronectin, where the two adhesion receptors work in conjunction to promote focal adhesion and actin stress fibre formation.

Initial sites of attachment between α5β1 integrin and extracellular fibronectin have the potential to mature into focal adhesions, however this is dependent of the ability of syndecan 4 GAG chains to bind the heparin-binding fragment of fibronectin, and to active PKCa (Oh, Woods and Couchman, 1997a; Woods *et al.*, 2000; Greene *et al.*, 2003).

Syndecan 4 and integrin α5β1 dynamically regulate Rac1 and RhoA to regulate membrane protrusion. Rac1 functions to drive cell motility by formation of lamellopodia, whereas Rho signals cause membrane retraction. The balance of Rac and Rho activation therefore co-ordinates membrane protrusion and retraction; sites with high Rac 1 will supress RhoA and produce lamellopodia (Chauhan *et al.*, 2011).

Engagement of α5β1 alone is not sufficient to drive Rac1 activation; full activation requires the presence of syndecan 4. Syndecan 4 drives localised Rac1 activation at sites of ECM engagement in a PKCα-dependent manner, localising Rac1 activity to the leading edge of cells. Syndecan 4 regulation of Rac1 is mediated by RhoG, a small RhoGTPase (Bass *et al.*, 2008).

At the same time as the wave of Rac1 activation α5β1 and syndecan 4 inhibit RhoA. Integrin α5β1 and syndecan 4 make individual contributions to the suppression of RhoA during matrix engagement, in pathways that converge upon the GTPase activating protein p190 (p190RhoGAP). P190RhoGAP matrix induced tyrosine phosphorylation is stimulated independently by α5β1 integrin. Parallel engagement

of syndecan 4 causes redistribution of the phosphorylated pool of p190RhoGAP between membrane and cytosolic fractions in a mechanism that requires direct activation of PKC by syndecan 4. Activation of both pathways is essential for efficient regulation of Rho A, and focal adhesion formation (Bass *et al.*, 2008).

The regulation of integrin trafficking is also in part controlled by syndecan 4. Changes in surface expression of integrin α5β1 and αvβ3 results in differential adhesion stability and migration. The phosphorylation of syndecan 4 by Src drives syntenin binding to syndecan 4 via its PDZ domain, supressing the activity of the Ras superfamily GTPase ARF6, resulting in preferential endocytosis and degradation of α5β1 and upregulation of αvβ3 integrin at the cell surface, stabilising focal adhesion. The reverse occurs upon abrogation of syndecan 4 phosphorylation, destabilising adhesion complexes and disrupting migration (Morgan *et al.*, 2013).

1.7 Angiogenesis

Angiogenesis is the development of new blood vessel sprouts from the existing vasculature, a process essential for growth and development. In mature mammals, angiogenesis is generally only required under specific circumstances, for example in the placenta during pregnancy and during wound healing. Dysregulated angiogenesis can contribute to pathological process such as cancer and vascular retinopathies, and therefore both spatial and temporal regulation is essential (Cleaver and Melton, 2003).

Angiogenesis is tightly controlled by cell-cell and cell-ECM interactions, as well as by release of growth factors and morphogens. In particular, angiogenesis requires the proliferation, growth, differentiation and migration of endothelial cells, processes which require the ECM.

1.7.1 The Vascular system

The cardiovascular system is the network of vessels that transports blood throughout the body, delivering blood and nutrients and removing waste. Blood vessels are laid down embryonically in a process termed vasculogenesis, when mesenchymally-derived precursor angioblasts differentiate into ECs forming *de novo* networks. Angiogenesis is then required for the remodelling and expansion of this primitive network, and is triggered throughout life in hypoxic areas (Patan, 2004).

The system is comprised of five vessel types: arteries; which carry oxygenated blood away from the heart, arterioles, capillaries; small vessels where gas exchange between blood and tissues occurs, venules, and veins; which carry deoxygenated back to the heart.

The large vessels; arteries and veins, consist of three layers (Figure 1.10). The innermost layer, the tunica intima, consists of a single layer of endothelium supported by a basement membrane; this layer is anchored to the middle layer, the tunica media, with a layer of connective tissue, elastin and collagen. The tunica media is comprised of vascular mural cells such as vascular smooth muscle cells (VSMCs)

and pericytes, elastin and collagen. The outermost layer, the tunica externa, is primarily composed of connective tissue (Cleaver and Melton, 2003). Capillaries are the smallest type of blood vessel and unlike the larger vessels they have only one layer, a wall a single endothelial cell thick (Figure 1.10).

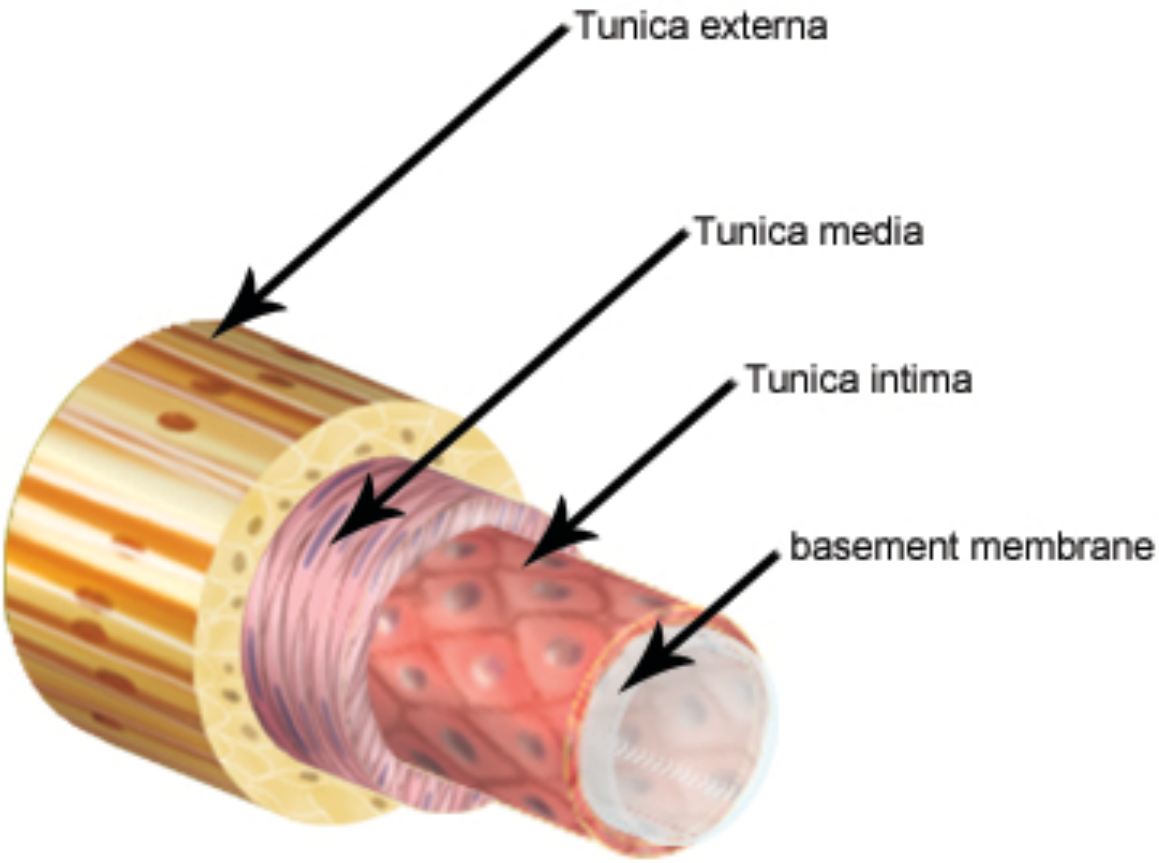


Figure 1.10 Structure of large blood vessels. The large vessels of the vascular system (veins and arteries) consist of three layers, the tunica intima, a single cell layer of endothelium anchored to a basement membrane, the tunica media containing vascular smooth muscle cells, elastin and collagen, and the tunica externa, the outermost layer which is comprised primarily of collagen.

1.7.2 The stages of angiogenesis

Sprouting angiogenesis, new blood vessels 'sprouting' from existing vasculature, is initiated in response to hypoxia. The process involves proteolytic degradation of the ECM, followed by chemotactic migration and proliferation of ECs. The process of sprouting angiogenesis occurs via a series of highly regulated steps, which all require interaction with and remodelling of the ECM (Yue *et al.*, 2007):

1. Reception of angiogenic signals (Figure 1.11)
2. Retraction of pericytes from abluminal surface of the vessel and proteolytic degradation of the ECM (Figure 1.12)
3. Chemotactic migration of ECs under the influence of angiogenic stimuli (Figure 1.13)
4. Proliferation of ECs and formation of lumen (Figure 1.14)
5. Recruitment of pericytes, deposition of new basement membrane, and initiation of blood flow

1.7.2.1 Reception of angiogenic signals

Angiogenesis is initiated under specific conditions upon reception of angiogenic signals. Hypoxia-inducible factors (HIFs) are transcription factors that respond to decreased oxygen availability, and are responsible for triggering angiogenesis in response to hypoxia. The HIFs exist as heterodimeric complexes, consisting of an oxygen destructible α-subunit, and a β subunit. In hypoxic conditions HIFs in parenchymal cells are no longer hydroxylated and targeted for degradation, and instead initiate expression of pro-angiogenic genes, including VEGF-A (Krock, Skuli and Simon, 2011).

Angiogenesis is also triggered after disruption of epidermal and vascular basement membranes, where damage releases ECM bound pro-angiogenic growth factors such as FGFs, which induce angiogenesis (Arbiser, 1996).

Numerous extracellular factors influence angiogenesis, including angiopoietins, FGFs, PDGFs, TNFα, interleukins, and the canonical regulators of angiogenesis, the VEGFs (Feucht, Christ and Wilting, 1997).

The mammalian VEGF family consists of five genes; alongside *VEGF-A* there is also *VEGF-B*, *VEGF-C*, *VEGF-D* and placenta derived growth factor (*P1GF*). The role of VEGF-B in vivo is not fully understood and knockout mice are largely healthy, however its expression can be detected in human tumours and it appears to have a role in pathological angiogenesis (Aase *et al.*, 2001; Zhang *et al.*, 2009). The structurally similar VEGF-C and -D are primarily involved in lymphangiogenesis, but also have roles in pathological angiogenesis, for example in carcinoma (Kukk *et al.*, 1996; Achen *et al.*, 2002; Jiang *et al.*, 2010). Placenta derived growth factor is a ligand for VEGFR-1, and plays an important role in trophoblast growth and differentiation during embryogenesis (Luttun, Tjwa and Carmeliet, 2002). The most potent stimulator of angiogenesis however is VEGF-A, its functions are so essential that even heterozygous deletion of the gene in mice is embryonic lethal due to extensive vascular structural defects (Carmeliet *et al.*, 1996).

The *VEGF-A* gene is found on the short arm of chromosome 6. It has 6 exons which can be alternatively spliced to generate 4 major isoforms, $VEGF_{121}$, $_{165}$, $_{189}$ and $_{206}$, and two minor isoforms, $VEGF_{145}$ and $_{183}$ (Chung and Ferrara, 2011). These variants have differential ability to bind HSPG in the ECM; $VEGF_{121}$ is freely diffusible, whereas $VEGF_{189}$ and $VEGF_{206}$ are almost completely bound to the ECM. The most abundant isoform, $VEGF_{165}$, is secreted, however a substantial fraction remains bound to the ECM.

Extracellular matrix-bound VEGF remains bioactive and can be converted into soluble forms. Addition of heparin or treatment with heparinase results in the release of VEGF from the ECM, as does processing by physiologically relevant proteases such as plasmin (Vempati, Mac Gabhann and Popel, 2010). Differential synthesis of VEGF isoforms alongside binding and release from the ECM are therefore key processes in

the regulation of angiogenesis, which allow for the generation of angiogenic gradients (Ferrara, 2010).

Vascular endothelial growth factors promote angiogenesis through the binding and activation of the VEGF receptor family of receptor tyrosine kinases (VEGFRs). Three VEGF receptors exist, VEGFR1, primarily a negative regulator of angiogenesis, VEGFR3, expressed on lymphatic endothelial cells and for which VEGF-A is not a ligand, and VEGFR2, the best studied of the VEGF receptors (Shibuya, 2011).

VEGFR1 can form homodimers, or heterodimers with VEGFR2. Binding of VEGF-A to VEGFR1-VEGFR2 heterodimers only induces a very weak phosphorylation, indicating that VEGFR1 acts as a decoy, controlling the amount of VEGF-A able to bind VEGFR2. VEGF-A has a much higher affinity for VEGFR1, however VEGFR2 is expressed 10-fold more on ECs, whereas VEGFR1 is mainly found expressed on monocytes, macrophages and vascular smooth muscle cells (Cao, 2009).

VEGF receptor 2 (VEGFR2) is the major receptor expressed on endothelial cells, and the major pro-angiogenic receptor. In the canonical signalling pathway, VEGF-A isoforms bind VEGFR2 inducing autophosphorylation of tyrosine residues in the cytoplasmic domain of VEGFR2. Phosphorylated VEGFR2 then initiates downstream signalling pathways, including the RAS/MAPK cascade, that produce cellular responses in ECs such as proliferation, migration and survival (Clegg and Mac Gabhann, 2015).

VEGFR3 is expressed on lymphatic vessels and binds VEGFC and D, initiating lymphogenesis (Simons, Gordon and Claesson-Welsh, 2016).

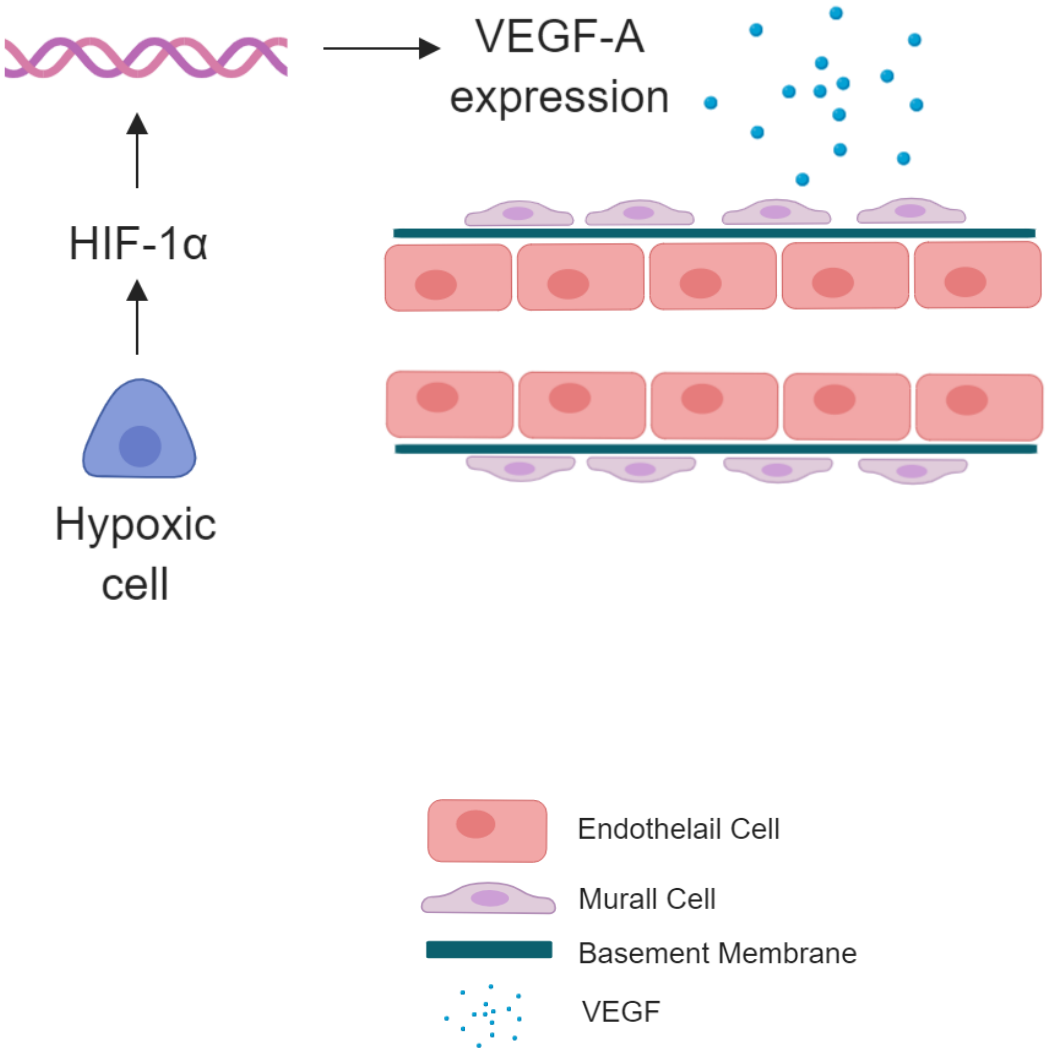


Figure 1.11 Activation of quiescent endothelium in response to angiogenic signals. In hypoxic environments, HIF-1α initiates transcription of pro-angiogenic cytokines including VEGF, these signal to the endothelium, initiating the process of angiogenesis.

1.7.2.2 Retraction of pericytes and degradation of the ECM

The basement membrane forms a sleeve around endothelial tubules, preventing ECs from leaving their positions. Generation of a new sprout requires both degradation of the endothelial basement membrane and ECM remodelling. Degradation of the membrane serves not only to liberate endothelial cells, but also to release sequestered growth factors and detach pericytes.

Mural cells (vascular smooth muscle cells (VSMCs) and pericytes) retract from the abluminal surface of existing vessels in response to angiopoietin 2 release from ECs (Huang *et al.*, 2010). TIE-1 and TIE-2 are receptor tyrosine kinases for the angiopoietins. TIE-2 deficient mice die embryonically due to failure of the vasculature to remodel and mature; this is correlated with a deficiency in supporting pericytes and smooth muscle cells, demonstrating its importance in the maintenance of mature vasculature (Augustin *et al.*, 2009). While angiopoietin 1 and 4 activate the TIE receptors promoting vessel maturation and stability, angiopoeitin-2 acts as a competitive antagonist, therefore its release by endothelial cells inhibits TIE-2, leading to detachment of mural cells (Scholz, Plate and Reiss, 2015).

The degradation of the vascular basement membrane and remodelling of the ECM is fulfilled by matrix proteases, in particular those belonging to the metzincin superfamily. Filopodia on tip cells express proteases such as the cell anchored MT1-MMP, which degrade the ECM creating a pathway for the newly forming sprout to develop into. Endothelial cells isolated from MT1-MMP deficient mice invade through Matrigel less efficiently than their WT counterparts, and the importance of MT1-MMP in this role is demonstrated by knockout mice, which show severe defects in angiogenesis (Zhou *et al.*, 2000; Oblander *et al.*, 2005).

The processing of the ECM by MMPs can release non-covalently bound growth factors and cytokines. One key example is the role of MMP-9 in regulation of VEGF bioavailability. Vascular endothelial growth factor binds to HSPG in the ECM and is released upon HSPG proteolysis by MMP-9, initiating angiogenesis (Hawinkels *et al.*, 2008). Another example of proteases releasing growth factors is found in TGF-β; TGF-

β is maintained in a latent state by latency associated protein (LAP), which is covalently bound to the fibrillin protein latent TGF binding protein (LTBP). Proteases can cleave these proteins to regulate TGF-β activity; LAP is a substrate of MMP-2, MMP-9, MMP-13 and MMP-14, and LTBP can be cleaved by MMP-7 (Robertson and Rifkin, 2016).

Activity of MMPs can also uncover bioactive fragments and cryptic epitopes of ECM proteins, for example cleavage of collagen IV by MMP-9 unveils a cryptic site HUIV26, necessary to initiate angiogenesis (Hangai *et al.*, 2002). Surprisingly, MMPs can also act in an angioinhibitory mechanism in some contexts, for example, MMP-12 is responsible for the release of angiostatin, an endogenous angiogenesis inhibitor, from plasminogen (Cornelius *et al.*, 1998). Endostatin can also be formed by cleavage of collagen XVIII (Heljasvaara *et al.*, 2005).

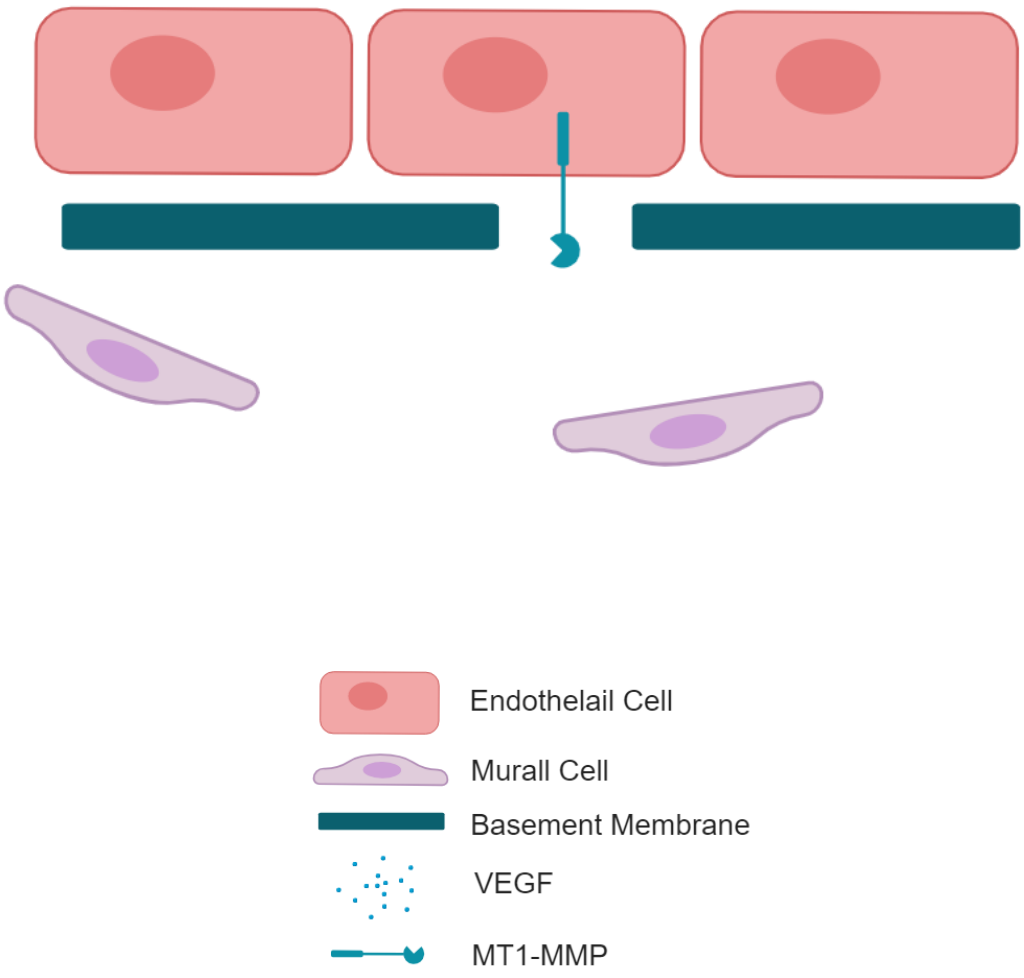


Figure 1.12 Mural cells retract and proteases degrade the basement membrane to allow a sprout to develop. TIE-2 – angiopoietin signalling stimulates retraction of mural cells, then MT1-MMP degrades the basement membrane.

1.7.2.3 Chemotactic migration and proliferation of ECs

Endothelial cells respond to VEGF-A gradients in specialised mechanisms, forming two distinct phenotypes – tip and stalk cells. Endothelial cells which lead the developing vasculature are termed 'tip cells'; these express high levels of VEGFR2 on long and dynamic filopodia, and migrate in response to angiogenic directional cues, but proliferate minimally. Endothelial 'stalk cells' proliferate as they follow behind the tip cell, allowing the capillary stalk to elongate. They produce few filopodia, but establish adherens and tight junctions, ensuring the stability for the new sprout (Gerhardt *et al.*, 2003).

This phenotypic specialisation of ECs is transient and reversible, and depends upon a balance of pro and anti-angiogenic signals. Of particular importance in determining branching pattern is Notch signalling; the Notch receptors are large transmembrane receptors, with ligands including JAG-1, DLL-1 and DLL-4, and their signalling inhibits angiogenesis. Tip cells express high levels of VEGFR2, and in response to VEGF-A signalling they produce DLL4, which activates Notch signalling in neighbouring cells in a lateral inhibition process (Kume, 2009).

Cellular proteolysis is essential for Notch signalling. Ligand-mediated activation of Notch leads to two proteolytic cleavage events, the first by ADAM10 and the second by γ-secretase. Cleavage results in the release of the Notch intracellular domain (NICD) which translocates to the nucleus and can stimulate transcription of target genes.

The tip cell is responsible for guiding the sprout properly, which relies on both attractive and repulsive cues. Endothelial cells express guidance receptors including ROBO4, UNC5B, PLEXIN-D1, NRPs and EPH family members (Garcia-Mata, Boulter and Burridge, 2011).

The basement membrane is also important in regulating the proliferation and migration of endothelial cells. Assembled basement membrane has a complex structure and is highly cross-linked, and signals to inhibit proliferation and promote an environment that facilitates cell-cell adhesion (Form *et al.*, 1986). As the basement

membrane is degraded cryptic domains of partially digested collagens are exposed, and these provide important pro-angiogenic cues. Detached endothelial cells are also in direct contact with the interstitial provisional matrix, which contains fibronectin, vitronectin and type I collagen, and this provisional matrix provides further proliferative cues (Kalluri, 2003).

Matrix metalloproteases support EC migration., and in particular, MT1-MMP is required for efficient endothelial migration on diverse ECM components, such as gelatin, collagen type I, fibronectin, or vitronectin (Gálvez *et al.*, 2001).

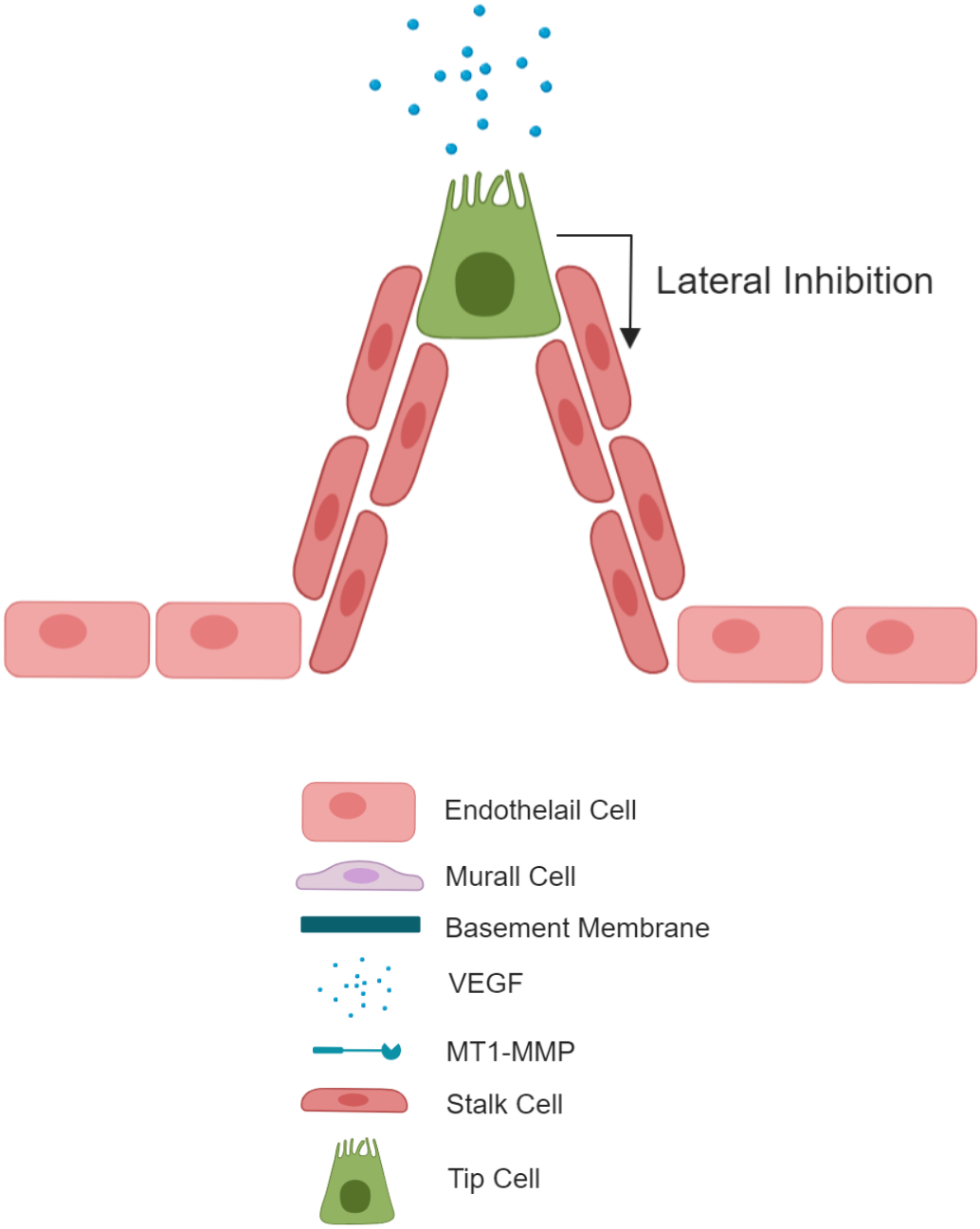


Figure 1.13 Tip cells guide a developing sprout, with stalk cells proliferating to develop the new vessel. Tip cells express VEGFR2 in high levels, and secrete Dll4 in response to VEGF-A, activating Notch and inhibiting the tip cell fate in nearby cells in a lateral inhibition process.

1.7.2.4 Lumen formation and vessel Maturation

A lumen forms when intracellular vacuoles develop and coalesce within a series of stalk cells. Maturation and stabilisation of capillary sprouts requires recruitment of pericytes and deposition of ECM. Major players in vessel maturation are TGF-β, PDGF-β and angiopoietin-1. TGF-β stimulates production of ECM, as well as mural cell induction, proliferation and migration (Pardali, Goumans and ten Dijke, 2010). Endothelial expressed PDGF-β activates the PDGF-β receptor on mural cells, stimulating their migration and proliferation (Gaengel *et al.*, 2009). Angiopoietin-1 activates TIE-2, stabilising vessels and promoting pericyte adhesion (Augustin *et al.*, 2009).

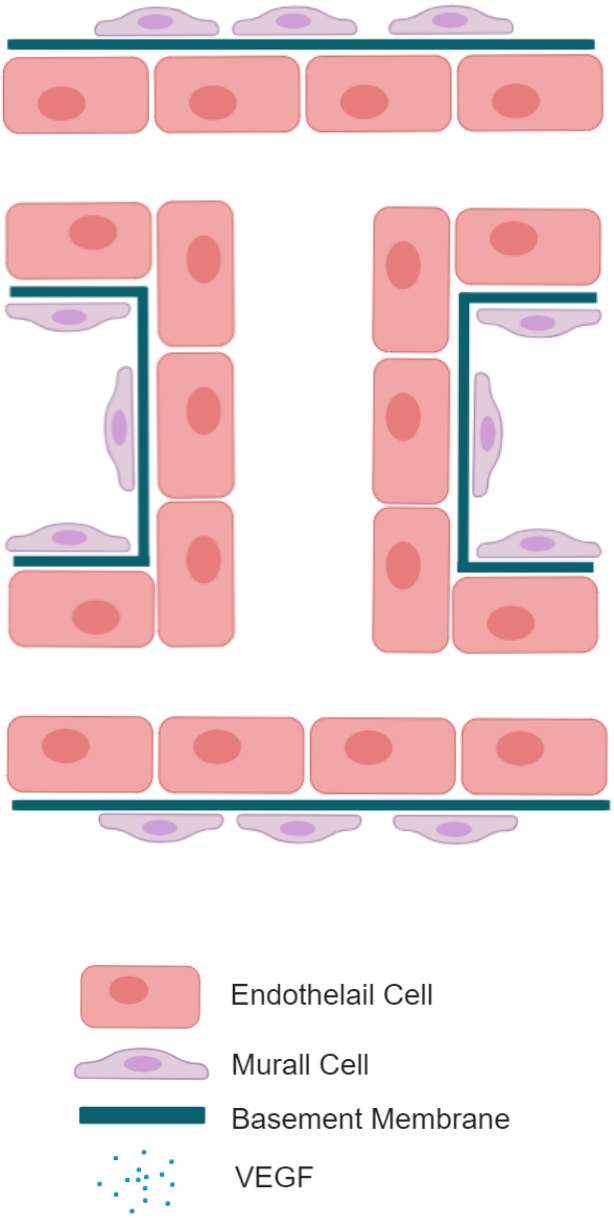


Figure 1.14 Vessel maturation requires basement membrane deposition, mural cell recruitment, and lumen formation. PDGF-β signalling is responsible for recruitment of mural cells, and TGF-β stimulates production of basement membrane

1.7.3 VEGFR2 signalling

In order to efficiently regulate angiogenesis, VEGFs must bind and activate receptors. VEGFR2 is the primary VEGF receptor expressed on endothelial cells, it is essential for angiogenesis as it is the main transducer of VEGF-A mediated cellular affects. *Vegfr2-/-* mice die *in utero* as a result of drastically impaired vasculogenesis, highlighting the necessity of this receptor (Shalaby *et al.*, 1995).

VEGFR2 is a type III transmembrane kinase receptor consisting of a 7 immunoglobulin (Ig)-like domain extracellular region, a short transmembrane domain, and a cytoplasmic region which contains the tyrosine kinase domain, split by a 70 amino acid insert (Figure 1.15) (Holmes *et al.*, 2007).

VEGF-A binding to the second and third extracellular Ig-like domains induces receptor dimerization, this is stabilised by low affinity interactions between the membrane proximal Ig domains, which allows for trans-autophosphorylation of intracellular tyrosine residues. Major phosphorylation sites are Y951 in the kinase insert domain, Y1054 and Y1059 within kinase domain 2, and Y1175 and Y1214 in the C-terminal tail, with Y1054 and Y1059 being critical for kinase activity (Matsumoto *et al.*, 2005).

VEGFR dimerization induces a switch in configuration of the transmembrane domains, and this rotation of dimers is essential for full kinase activity. VEGF-A is not the only ligand for VEGFR2; other VEGFs, including processed forms of VEGF-C and D can also bind giving different cellular effects. Different ligands induce difference degrees of rotation, thereby giving varying degrees of receptor activation, providing one mechanism by which differential effects are transduced (Sarabipour, Ballmer-Hofer and Hristova, 2016).

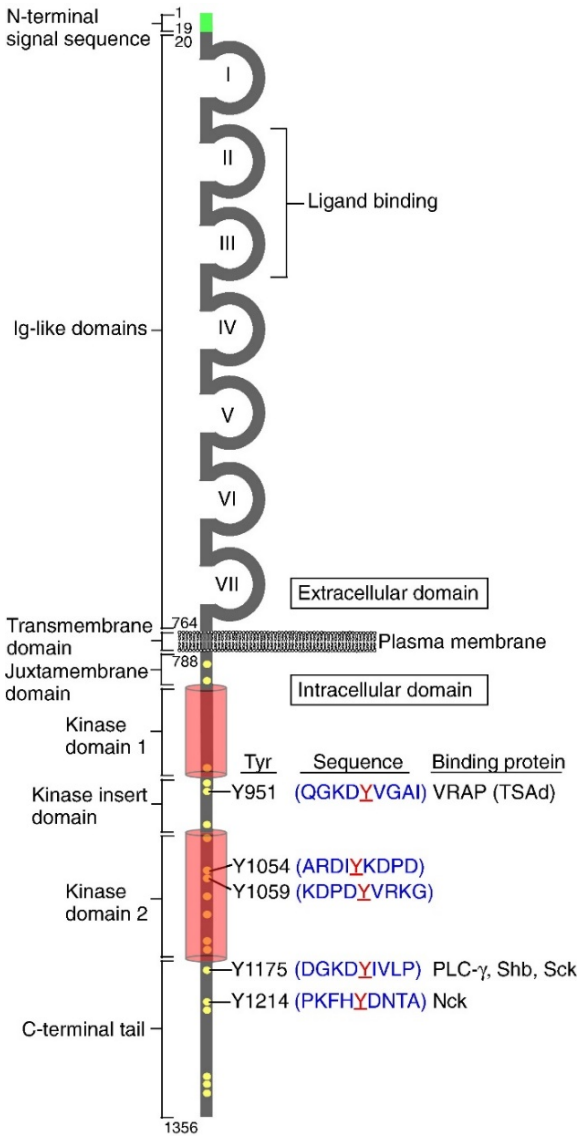


Figure 1.15 The structure of VEGFR2. VEGFR2 is a transmembrane protein with a cytoplasmic region containing two kinase domains separated by an insert, a single transmembrane pass, and an extracellular region composed of 7 Ig like domains, of which the second and third loop are responsible for ligand binding. Figure adapted from Holmes *et al.* (2007).

VEGFR2 signalling activates numerous pathways, controlling cell proliferation, migration, and survival. VEGFR2 induced activation of the classical extracellular signal regulated RAS/RAF/ERK/MAPK pathway is key for stimulating proliferation of endothelial cells in response to VEGF-A. Usually, docking proteins containing SH2 domains, such as GRB2, bind the phosphorylated tails of RTKs; GRB2 then binds the GEF SOS, allowing Ras to bind GTP and become active, however VEGFR2 does not appear to activate Ras via this traditional pathway. Instead, VEGF pY1175 dependent phosphorylation of PLCγ, its hydrolysis of PIP_2 and resulting accumulation of IP_3 causing calcium influx, and DAG activation of PKC, is responsible for activation of ERK (Takahashi, Ueno and Shibuya, 1999).

A potential mechanism for VEGFR2 activation of Ras has also proposed, where VEGFR2 stimulation of sphingosine kinase downstream of PKC results in the sequential activation of Ras, Raf and ERK (Shu *et al.*, 2002).

VEGFR2 promotes cell survival via activation of PI3K. Phosphorylation of PIP_2 by PI3K generates membrane bound PIP_3, resulting in membrane targeting and phosphorylation of Akt/PKB. Downstream, Akt then phosphorylates the apoptotic proteins Bcl-2 associated death protein (BAD) and caspase 9, inhibiting their activity to promote cell survival (Brunet *et al.*, 1999).

As well as mitogenic affects, VEGFR2 signalling is also responsible for driving the migration of endothelial cells. The adaptor protein Shb binds both VEGFR2 pY1175 and focal adhesion kinase (FAK). Upon VEGF stimulation, Shb is phosphorylated in a Src dependent manner, leading to PI3K phosphorylation and activation of focal adhesion kinase (FAK), promoting the formation and turnover of focal adhesions (Holmqvist *et al.*, 2004). VEGFR2 also regulates actin cytoskeleton reorganisation via activation of p38 MAPK and subsequently Hsp27, leading to actin polymerization and reorganisation into stress fibres (Lamalice *et al.*, 2004).

1.7.4 ADAMTSs and angiogenesis

The ADAMTS family proteases influence angiogenesis via both proteolytic and non-proteolytic mechanisms. ADAMTS-1 was identified based on a search for thrombospondin (TSP) homologous modules (Kuno *et al.*, 1997; Kuno, Terashima and Matsushima, 1999). Thrombospondins are secreted glycoproteins with antiangiogenetic properties; as all ADAMTS contain at least one type one thrombospondin like repeat (TSR) it was predicted they would all have anti-angiogenic capabilities, and research reveals a number of ADAMTS do regulate angiogenesis in TSR-dependent mechanisms (Iruela-Arispe *et al.*, 1999).

The structurally similar aggrecanases ADAMTS-1 and -8 have been demonstrated to inhibit FGF-induced vascularisation in the corneal pocket assay and VEGF-induced angiogenesis in the CAM model (Vázquez *et al.*, 1999). In the case of ADAMTS-1 this is partially attributed to its ability to sequester $VEGF_{165}$ via its C-terminal spacer region and 3 TSRs, blocking VEGFR2 phosphorylation and endothelial cell proliferation. This interaction is reversible, and dependent upon the heparin-binding domain of $VEGF_{165}$ – a splice variant lacking this domain, $VEGF_{121}$, was unable to bind ADAMTS-1 (Luque, Carpizo and Iruela-Arispe, 2003).

Another aggrecanase, ADAMTS-5, has also been shown to inhibit angiogenesis in a TSR-dependent mechanism, independent of proteolytic activity. The central TSR of ADAMTS-5, TSR1, inhibited endothelial tube formation on Matrigel, and reduced EC proliferation and promoted apoptosis (Sharghi-Namini *et al.*, 2008; Kumar *et al.*, 2012).

Although it is the prototypical anti-angiogenic ADAMTS clade, angioinhibitory functions are not restricted to the aggrecanase subgroup; the pro-collagen N-propeptidase ADAMTS-2 has been seen to inhibit angiogenesis in cellular and tumour models in a catalytically-independent mechanism, and the COMP protease ADAMTS-12 can inhibit angiogenesis in a mechanism reliant on its TSR (Llamazares *et al.*, 2007; Dubail *et al.*, 2010).

In contrast, ADAMTS-13 has been seen to both promote and inhibit angiogenesis. Full length ADAMTS-13 inhibited $VEGF_{165}$-induced angiogenesis, but a truncated version lacking TSR domains did not (Lee *et al.*, 2012). However, treatment of endothelial cells with full length ADAMTS-13 can also promote angiogenesis in a tube formation assay in a dose-dependent manner; surprisingly the mechanism also requires the TSR1 domain, and appears to result in upregulation of VEGFR2 (Kelwick, Desanlis, *et al.*, 2015; Lee *et al.*, 2015). ADAMTS-13 highlights the complex nature of angiogenic regulation, and is just one example of a protein with dual roles.

The ADAMTS proteases also have catalytic functions in the regulation of angiogenesis. Wild-type ADAMTS-15, but not a catalytically inactive mutant, is able to inhibit angiogenesis in aortic ring and VEGF-induced sprouting models (Kelwick, Wagstaff, *et al.*, 2015). Similarly, overexpression of wild-type ADAMTS-9, but not a catalytically dead form, reduced formation of tube-like structures on Matrigel in a mechanism that appears to involve downregulation of pro-angiogenic factors MMP-9 and VEGF-A (Koo *et al.*, 2010; Lo *et al.*, 2010). ADAMTS-1 can cleave thrombospondin 1 and 2, resulting in a pool of polypeptides with strong angioinhibitory affects. In *Adamts1-/-* mice, TSP1 proteolysis is absent, this is correlated with delayed wound repair and increased angiogenic response (Lee *et al.*, 2006). In a cancer context, the presence of ADAMTS-1 is also associated with increased proteolysis of the basement membrane glycoproteins nidogen 1 and 2, and reduced vessel density in tumour models (Martino-Echarri *et al.*, 2013).

1.7.5 Integrins in angiogenesis

Integrin signalling can influence cell migration, survival and proliferation, behaviours essential for the propagation of angiogenesis, implicating the integrins as key regulators of angiogenesis. Integrin heterodimers known to involved in angiogenesis include α1β1, α2β1, α4β1, α5β1, α6β1, α6β4, α9β1, αvβ3 and αvβ5 (Plow, Meller and Byzova, 2014).

Fibronectin is the major ECM protein deposited by endothelial cells during angiogenesis, suggesting fibronectin-binding integrins would be of particular

importance, and indeed the αv family of RGD-binding integrins are central to angiogenesis. The first integrin shown to regulate angiogenesis was αvβ3. It is widely expressed on tumour vasculature, but not on the quiescent blood vessels of normal tissue. The upregulation of αvβ3 is therefore a marker of angiogenesis, and is seen in skin wounding, arthritis and diabetic retinopathy, as well as in cancer. The expression of αvβ3 on endothelial cells is driven by bFGF, TNFα and IL-8. Ligation of αvβ3 can activate MAP kinase, FAK and Src, promoting proliferation, differentiation and migration (Friedlander *et al.*, 1995).

Integrin αv also dimerises with β1, 3, 5, 6 and 8. Different αv dimers function in distinct angiogenic pathways, demonstrated by the development of function blocking antibodies. Where αvβ3 antibodies block bFGF-driven angiogenesis, αvβ5 antibodies block VEGF dependent angiogenesis (Friedlander *et al.*, 1995). Activation of either pathway is protective from apoptosis, however the αvβ3 bFGF-driven pathway leads to activation of p21 activated kinase, Raf activation and MEK1-dependent protection from *extrinsic* mediated apoptosis, whereas the αvβ5 integrin pathway downstream of VEGF causes activation of FAK and Src, leading to activation of Raf, and Raf 1 mitochondrial translocation, resulting in MEK1-independent endothelial cell protection from *intrinsic* pathway apoptosis (Alavi *et al.*, 2003).

Much like αvβ3, α5β1 is poorly expressed on quiescent endothelium, but its expression is upregulated during tumour and corneal angiogenesis in response to a number of angiogenic stimuli, including bFGF and IL8. Adhesion mediated by α5β1 promotes migration and survival by suppressing the activity of PKA; α5β1 antagonists activate PKA, leading to activation of caspase 8 and inhibition of angiogenesis (Kim, Harris and Varner, 2000; Alavi *et al.*, 2003).

Integrins are important in VEGFR2 signalling, in particular β1 and β3. The full activation of VEGFR2 by VEGF-A requires VEGFR2-integrin β3 association. VEGF-A can also promote VEGFR2-integrin β1 complex formation, shifting VEGFR2 to focal adhesions and prolonging its activation (Somanath, Malinin and Byzova, 2009).

Integrin activation of Rho family GTPases is also involved in angiogenesis. Induction of Ras GTPases signals to the PI3K AKT and Ras MAP kinase pathways, which leads to the activation of transcription factors such as NF-kB and HOXD3, important regulators of the cell cycle and angiogenesis (Castellano and Downward, 2011). Activation of Rap1 is also partially dependent on αvβ3 activation, and promotes VEGF-A signalling in endothelial cells (Altemeier *et al.*, 2012).

Integrin signalling can also cross talk with other pathways, for example the Notch signalling pathway, important in vascular patterning as well as the recruitment of VSMC to maturing vessels. Vascular smooth muscle cell interaction with the Notch ligand Jagged 1 on endothelial cells leads to an upregulation of αvβ3, and allows the VSMC to adhere to the endothelial basement membrane (Scheppke *et al.*, 2012). Integrins also appear to play an important role in the activation of latent TGF-β, a key molecule in regulating apoptosis, proliferation and migration (Nishimura, 2009)

1.7.6 Syndecans in angiogenesis

The endothelial glycocalyx is a layer of membrane-bound macromolecules including HSPG, GAGs, glycoproteins and molecules adsorbed from plasma on the luminal surface of the vascular endothelium (Pries, Secomb and Gaehtgens, 2000). The glycocalyx is required for normal vascular development, and has roles in mechanotransduction, haemostasis, signalling, and blood cell–vessel wall interactions. Endothelial cell HSPGs (syndecans 1, 2, 4 and membrane-bound glypican-1) are thought to be a key component that anchors the glycocalyx to the cell, and syndecan 1 in particular is a major constituent of the endothelial glycocalyx (Savery *et al.*, 2013).

As with cell migration, syndecans can regulate angiogenesis in concert with the integrins. Syndecan 1 can associate with αvβ3 and αvβ5 integrins, and these integrins are dependent on syndecan 1 for their activation (Beauvais, Burbach and Rapraeger, 2004). Syndecan 1 silencing prevents HUVECs from spreading on the αvβ3 ligand vitronectin, and administration of a syndecan 1 inhibitor synstatin can interrupt syndecan 1-integrin interaction, blocking angiogenesis (Beauvais *et al.*, 2009). A

suggested mechanism for this involves insulin-like growth factor 1 receptor (IGF1R); the association between syndecan 1 ectodomain and integrin provides a docking site for IGF1R and this interaction is blocked by synstatin (Rapraeger *et al.*, 2013).

Syndecan 2 has been implicated in angiogenesis in contrasting mechanisms. The lack of syndecan 2 dramatically impairs developmental angiogenesis in zebrafish, in a mechanism dependent upon the syndecan 2 cytoplasmic domain, as re-expression of a truncated mutant could not rescue the phenotype (Chen, Hermanson and Ekker, 2004). Alternative data demonstrate an inhibitory role for the shed syndecan 2 extracellular core protein, inhibiting angiogenesis by preventing cell migration (Rossi *et al.*, 2014).

Little research has focused on syndecan 3 in a vascular context, as it is the major syndecan expressed in the nervous system. Syndecan 3 knockout mice are viable and exhibit normal development, however a bacterially-expressed syndecan 3 ectodomain (therefore lacking GAG chains) has been demonstrated to significantly inhibit angiogenesis in an aortic ring assay (De Rossi and Whiteford, 2013).

Syndecan 4 knockout mice are viable and healthy, however display significantly delayed healing of skin wounds, implying roles in cell migration and angiogenesis (Echtermeyer *et al.*, 2001). As is the case for syndecan 2, syndecan 4 shed by proteases has been shown to impair angiogenesis (Li *et al.*, 2016). Syndecan 4 has also been demonstrated to be essential in mediating the pro-angiogenic effect of TSP1 (Nunes *et al.*, 2008). Thrombospondin-1 is a highly anti-angiogenic protein, however under certain conditions it can also promote angiogenesis, and these pro-angiogenic activities have been attributed to the N-terminal heparin-binding domain of the glycoprotein (Chandrasekaran *et al.*, 2000). Nunes *et al*, showed that a syndecan 4 monoclonal antibody could block tubulogenesis induced by a recombinant protein encompassing the N-terminal residues of TSP-1 (Nunes *et al.*, 2008).

1.8 Functional associations of ADAMTS proteases and cell adhesion receptors

Several ADAMTS proteases have been demonstrated to rely on syndecan 4 in order to exert their functions. Notably, ADAMTS and syndecan 4 have been seen co-operate in the regulation of adhesion and migration. ADAMTS-10 and -6 have opposing roles in focal adhesion formation; ADAMTS-6 inhibits focal adhesion formation, whereas ADAMTS-10 is necessary. The C-termini of both proteases can bind syndecan 4, however in cells with ADAMTS-10 depleted, expression of syndecan 4 is able to rescue focal adhesions, whereas in cells with ADAMTS-6 depleted a glycocalyx forms (Cain *et al.*, 2016).

Syndecan 4 is essential for ADAMTS-15's regulation of migration; ADAMTS-15 inhibits migration on fibronectin in a catalytically dependent mechanism, and depletion of syndecan 4 is sufficient to attenuate this inhibition (Kelwick *et al.*, 2015).

Another instance of syndecan 4 dependent regulation of cell adhesion and migration is in the case of ADAMTS-1, which has been shown to cleave syndecan 4, however unlike in typical proteoglycan shedding this cleavage occurs near to the site of first GAG chain attachment. ADAMTS-1 cleavage of syndecan 4 results in decreased adhesion and increased cell migration; a similar phenotype was also seen in *Sdc4 -/-* endothelial cells (Rodríguez-Manzaneque *et al.*, 2009).

A possible interaction between ADAMTS and integrins is less clear cut. The ADAMs are the metzincins best known for their interactions with integrins; the disintegrin-like domains of many ADAMs are capable of acting as integrin ligands. Integrins known to interact with ADAM disintegrin-like domains include α4β1, α4β7, α5β1, α9β1, αvβ3 and αvβ5 (Bridges and Bowditch, 2005). ADAMTSs also possess a disintegrin domain, however the crystal structure of ADAMTS-1 revealed there was no structural homology to the disintegrin domains of other proteases, such as ADAM10, and suggests this may be a misnomer (Gerhardt *et al.*, 2007).

Despite this, a direct ADAMTS-integrin connection cannot be ruled out, as the ADAMs also interact with integrins via the cysteine rich domain, a domain which ADAMTSs also possess (Bridges and Bowditch, 2005).

1.9 Conclusions, research aims and objectives

The ECM is a complex and dynamic environment that can influence many essential cell behaviours. Cells interact with the ECM through adhesion receptors, and secrete proteases which allow them to dynamically remodel their environment.

Signals from the ECM, proteases, and adhesion receptors must converge in order to co-ordinate the multi-level regulation required for cell migration and angiogenesis. Members of the ADAMTS, syndecan and integrin families all contribute to this process.

Of particular interest are ADAMTS-1 and syndecan 4. ADAMTS-1 is anchored in the ECM and has potent angioinhibitory activity. One mechanism to which this is attributed is the sequestration of $VEGF_{165}$, an interaction that is predicted to require the contribution of HSPG such as a syndecan.

Syndecan 4 is of specific focus due to its expression in endothelial cells and localisation to focal adhesions, as well as its role in ADAMTS-15's regulation of cell migration (Kelwick *et al.*, 2015).

The possible involvement of integrins in mediating ADAMTS-1 and syndecan 4's effects also had to be considered, as these receptors are essential in migration and angiogenesis and they function in close relationship with syndecan 4. Integrins may link to ADAMTS-1 either directly, or via a syndecan 4 connection.

Given the links between ADAMTS-1 and syndecan 4, and the roles of these two proteins, this thesis therefore sets out to investigate any functional interaction between the two proteins and the consequences of any such interplay. As ADAMTS-1 is highly anti-angiogenic, a primary target of investigation will be endothelial cell behaviour. Roles of these proteins in cell migration will also be examined due to the

clear function of syndecan 4 in focal adhesion formation, and the importance of cell ECM interaction in driving endothelial cell migration, which is essential for angiogenesis.

This thesis explores the hypothesis that a functional interplay between syndecan 4 and ADAMTS-1 regulates cell migration and angiogenesis, via integrin family receptors.

Specific aims are as follows:

1. Investigate an ADAMTS-1 - syndecan 4 connection in fibroblasts and endothelial cells
2. Assess the contribution of syndecan 4 to angiogenesis using endothelial cells and *ex vivo* aortic ring assays
3. Evaluate the roles of ADAMTS-1 and syndecan 4 in cell migration using siRNA knockdown strategies and random migration assays
4. Examine the consequences of ADAMTS-1 or syndecan 4 depletion on integrin surface expression and trafficking.

2 Materials and Methods

2.1 Reagents and Antibodies

All chemicals were supplied by Sigma Aldrich (Poole, UK) unless otherwise indicated. Primary and secondary antibodies used are presented in **Error! Reference source not found.** and **Error! Reference source not found.**. qPCR probes are presented in **Error! Reference source not found.**. Details regarding siRNAs and shRNA are presented in **Error! Reference source not found.** and **Error! Reference source not found.** respectively.

Error! Reference source not found. List of primary antibodies used experimentally

Anti-	Clone/ cat #	Conjugate	Host	Source	Application
α5 integrin	14-0493-81	PE	Hamster	eBioscience	FC
α5 integrin	4705S		Rabbit	Cell Signalling Technology	WB
α5 integrin	ab150361		Rabbit	Abcam	ICC
αV integrin	12-0512-82	PE	Hamster	eBioscience	FC
β1 integrin	17-0291-82	APC	Hamster	eBioscience	FC
β3 integrin	11-0619-42	FITC	Hamster	eBioscience	FC
Biotin	3D6.6		Mouse	Jackson Immunoresearch	IP
BrdU	ab1893		Sheep	Abcam	ICC
BS1-lectin	L2895	FITC	Mouse	Sigma	ARA
ERK1/2 (p44/42 MAPK)	137F5 / #4695		Rabbit	Cell Signalling Technology	WB
Phospho ERK1/2	D13.14.4E #4370		Rabbit	Cell Signalling Technology	WB
Endomucin	sc-53941		Rat	Santa-Cruz Biotechnology	EC pos sort
FAK	#3285		Rabbit	Cell Signalling Technology	WB

Phospho FAK (Y407)	#OPA1-03887		Rabbit	Cell Signalling Technology	WB
Fibronectin	ab2413		Rabbit	Abcam	
Fibulin 1	ab175204		Rabbit	Abcam	WB, IP
GAPDH	ab8245		Mouse	Abcam	WB
HA tag	2-2.2.14		Mouse	Thermo Fisher	ICC
HA tag	66006-1-Ig		Mouse	Proteintech	ELISA
IgG control (IgG2a)	14-4724-82		Mouse	Invitrogen	FC, IP
Paxillin	ab32084		Rabbit	Abcam	ICC
Paxillin	# 2542		Rabbit	Cell Signalling Technology	WB
Phospho Paxillin (Y118)	#2541		Rabbit	Cell Signalling Technology	WB
Rac1	23A8		Mouse	Millipore	WB
Syndecan 4	KY/8.2		Rat	BD Pharmingen	FC
Syndecan 4	ab104568		Rabbit	Abcam	WB
VEGF-A	ab1316		Mouse	Abcam	IP
VEGFR2	55B11 / #2479		Rabbit	Cell Signalling Technology	WB
Phospho VEGFR2 (Y1175)	19A10 / #2478		Rabbit	Cell Signalling Technology	WB

Abbreviations: Aortic ring assay (ARA), Flow cytometry (FC), Immunocytochemistry (ICC), Immunoprecipitation (IP), Western blotting (WB), endothelial cell positive sorting (EC pos sort), and isotype control (IgG).

Error! Reference source not found. List of secondary antibodies used experimentally

Anti-	**Clone/ cat #**	**Conjugate**	**Host**	**Source**	**Application**
Mouse	P0447	HRP	Goat	Agilent Dako	WB
Mouse	a11002	alexa flour 532	Goat	invitrogen	ICC
Rabbit	P0448	HRP	Goat	Agilent Dako	WB
Rabbit	a21206	Alexa flour 488	Donkey	invitrogen	ICC
Rat	12-4817-82	PE	Mouse	eBioscience	FC
Sheep	ab150179	Alexa flour 647	Donkey	Abcam	ICC

Abbreviations: Aortic ring assay (ARA), Flow cytometry (FC), Immunocytochemistry (ICC), Immunoprecipitation (IP) and Western blotting (WB).

Error! Reference source not found. TaqMan qPCR assay probes

Gene	**Assay ID**	**Reporter/quencher**
18S	Mm03928990	FAM-MGB-NFQ
Itga5	Mm00439797	FAM-MGB-NFQ
Itgav	Mm00434486	FAM-MGB-NFQ
Itgb1	Mm01253230	FAM-MGB-NFQ
Itgb3	Mm00443980	FAM-MGB-NFQ
Adamts1	Mm01344169	FAM-MGB-NFQ
Adamts4	Mm00556068	FAM-MGB-NFQ
Mmp2	Mm00439498	FAM-MGB-NFQ
Mmp9	Mm00442991	FAM-MGB-NFQ
Mmp14	Mm00485054	FAM-MGB-NFQ
Sdc4	Mm00488527	FAM-MGB-NFQ
Vegfa	Mm00437306	FAM-MGB-NFQ
Kdr	Mm01222421	FAM-MGB-NFQ

Error! Reference source not found. List of siRNAs used to induce target depletion in ECs and 3T3s

Target (murine)	**Catalogue number**	**Targeted region**
Adamts1	J-04916-09	3'UTR
Mmp9	J-065579-09	ORF
Non-targeting control pool	D-001810-10	N/A
Sdc4	J-044221-05	ORF

Error! Reference source not found. List of shRNAs used to induce target depletion in HUVEC.

Target	**Sequence**	**TRC number**
Human *SDC4*	5'-CCGGCCTGATCCTACTGCTCATGTACTCGAGTACATGAGCAGTAGGATCAGGTTTTTG -3'.	TRCN0000123123
Human *ADAMTS1*	5'-CCGGTGAATTAGGCCACGTGTTTAACTCGAGTTAAACACGTGGCCTAATTCATTTTTG -3'.	TRCN0000294254

2.2 Cell culture, isolation and immortalisation

All cells were cultured at 37°C in a humidified chamber with 5% CO_2. For experimental analyses, plates and flasks were coated with either: 10 µg human plasma fibronectin (FN) (Millipore) or 10 µg collagen I (Col I) (Fisher Scientific) overnight at 4°C. VEGF-A_{164}, the mouse equivalent of human $VEGF_{165}$, was made in-house according to the method published by Krilleke *et al.* (2007). Mouse lung microvascular endothelial cell (MLEC) culture

Primary mouse lung endothelial cells were cultured in MLEC media, a 1:1 mix of Ham's F-12: low glucose DMEM (Invitrogen, California, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/mL penicillin/streptomycin (Invitrogen), 2 mM glutamax (Invitrogen), 50 µg/mL heparin, and 25 mg/L of endothelial mitogen (Bio-Rad, Kidlington, UK). Tissue culture flasks for primary endothelial cells were coated with a mixture of 0.1% gelatin, 10 µg/mL human plasma fibronectin (FN) (Millipore, Massachusetts, UK) and 10 µg/mL collagen I (Col I) (Thermo Fisher, Massachusetts, USA).

Immortalised mouse lung endothelial cells (ECs) were cultured in immortalised mouse lung endothelial cells (IMMLEC) media, a 1:1 mix of Ham's F-12: DMEM medium (low glucose) supplemented with 10% FBS, 100 units/mL penicillin/streptomycin (pen/strep), 2 mM glutamax, 50 µg/mL heparin. Flasks for routine sub-culture were pre-coated with 0.1% gelatin from porcine skin. Immortalised ECs were used between passages 5-22.

2.2.1 Other cell culture

Human umbilical vein endothelial cells (HUVECs) from pooled donors (Lonza, Slough, UK) were cultured in EBM-2 media supplemented with the SingleQuots™ kit (Lonza), and used between passages 1-6. HEK293 and 3T3 fibroblasts (ATCC, Virgina, USA) were cultured in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone, Invitrogen), 100 units/mL penicillin/streptomycin (Invitrogen), and 2 mM glutamax (Invitrogen).

2.2.2 EC isolation and immortalisation

The primary mouse lung endothelial cells used in this project were taken from in house stocks. Originally they were isolated from adult C57BL/6 mice as previously described in detail by Reynolds & Hodivala-Dilke (Reynolds and Hodivala-Dilke, 2006). Briefly, lungs were harvested from 6-8 week-old mice, followed by homogenisation and digestion in 0.1% collagenase I (Invitrogen). The cellular digests were passed once through a 16-gauge needle, and twice through a 19-gauge needle, followed by filtering through a 70 µm cell strainer (Thermo Fisher). Cells were collected by centrifugation at 300 x g, resuspended in MLEC and seeded onto a fibronectin, gelatin and collagen coated 10 cm dish. The following day, phosphate buffered saline (PBS) washes were performed to remove red blood cells. Once endothelial cell colonies were established, positive sorts were performed to remove contaminants (macrophages and fibroblasts) from the culture. Magnetic activated cell sorting (MACS) was used for the positive sort, cells were incubated with endomucin (1:1000 in PBS) for 30 minutes at 4°C, washed with PBS then incubated with sheep-anti-rat IgG coated magnetic beads for another 30 minutes. Cells were detached using trypsin and resuspended in MLEC media, and then placed on a magnet. The supernatant was removed, and after washing in media cells attached to the beads were resuspended in MLEC media and plated in a coated 6 cm dish. Once cells reached near confluency, a second positive sort was performed to ensure purity.

To immortalise, ECs were treated with polyoma-middle-T-antigen (PyMT) retroviral transfection as described by Robinson *et al.* (2009). PyMT conditioned media isolated from packaging GgP+E cells was added to the primary ECs with 8 µg/mL polybrene infection reagent for 6 hours at 37°C, this media was then replaced with MLEC. The PyMT-conditioned medium treatment was repeated the following day, after removal of the second PyMT media treatment, IMMLEC was added, and cells were henceforth cultured in IMMLEC.

2.3 RNAi and plasmid expression

Cells were manipulated with the use of siRNA and shRNA to induce target depletion, and plasmid overexpression of tagged ADAMTS-1 and syndecan 4.

2.3.1 siRNA transfection

ON-TARGET plus siRNAs (Dharmacon, Colarado, USA), were used for all siRNA transfections. Full details are outlined in **Error! Reference source not found.**.

For EC siRNA transfections, the electroporation method was used. Endothelial cells were transfected with 50 nM siRNA using the Amaxa Nucleofector System (Lonza) according to the manufacturer's instructions. Endothelial cells were trypsinised and counted, $1x10^6$ cells were resuspended in 100 µl transfection buffer (200 mM HEPES, 137 mM NaCl, 5 mM KCl, 6 mM D-glucose, 7 mM Na_2HPO_4), siRNA was added to a final concentration of 50 nM, and the T-005 nucleofector setting was used.

For 3T3 cells, lipid-based transfection was used. Cells were plated at a density of 250,000 cells / well into 6 well plates, and allowed to adhere overnight. Two transfection mixtures were then prepared, the first containing 6 µl Dharmafect 1 (Dharmacon), and 194 µl OptiMEM® medium (Invitrogen), the second containing 20 µl siRNA (20 nM final concentration) and 180 µl OptiMEM®. Mixtures were incubated at room temperature for 5 minutes, then combined and incubated for a further 20 minutes. The 400 µl transfection mix in 1600 µl OptiMEM® was then added to relevant wells, and incubated overnight. The following day, the transfection mixture was removed, and replaced with fresh DMEM.

For RNA analysis, cells were collected 24 hours post transfection while for protein analysis, 48 hours post transfection was allowed. Media used for siRNA transfections omitted the addition of pen/strep.

2.3.2 Transformation with and isolation of plasmid DNA

Several plasmid constructs were used throughout this work, the following stages outline how plasmids were amplified and purified for use in transfection of mammalian cells

2.3.3 Bacterial transformation

Plasmid DNA was introduced into One Shot™ Stbl3™ chemically competent *E. coli* by heat shocking. Bacterial vials had 1 µg of plasmid DNA added to them, followed by incubation at 4°C for 20 minutes, 42°C for 30 seconds, then returned to 4°C for a final 5 minutes. Bacterial cultures were added to 250 µl Super Optimal broth with catabolite repression (S.O.C) media (Thermo Fisher), and incubated for 1 hour at 37°C with shaking at 225 rpm. The bacterial transformation mix was then spread onto 1.5% agar plates with antibiotic selection (100 µg/mL ampicillin). Plates were incubated overnight at 37°C. The following day single colonies were picked using a sterile pipette tip, and inoculated into sterile lysogeny broth (LB) containing 100 µg/mL ampicillin. Colonies were grown overnight at 37°C with shaking at 225 rpm prior to plasmid isolation.

2.3.4 Plasmid preparation

Plasmids were isolated from bacterial cultures using Qiagen® spin kits (Qiagen, Hilden, Germany). For a mini prep, 2 ml of bacterial sample was used while for a maxi prep, it was 50 ml. Kits were used according to manufactures instructions. Plasmids were eluted in molecular biology grade water (Invitrogen), and concentrations were determined using a NanoDrop™ 1000 spectrophotometer

2.3.5 Transfection with ADAMTS-1 overexpression plasmids

For studies involving overexpression of ADAMTS-1 we utilised constructs generated by Rodriguez-Manzeneque *et al.* (Rodriguez-Manzaneque *et al.*, 2000; Rodríguez-Manzaneque *et al.*, 2002). Constructs consisted of a pcDNA3.1 backbone containing ampicillin and G418 resistance allowing for bacterial and mammalian selection respectively. The two constructs used were *ADAMTS1*-myc, which contained the full

length human *ADAMTS1* sequence and a carboxyl Myc tag, and ADAMTS-1-Z11, which in addition to the Myc tag had a zinc-binding site mutation, caused by a single base substitution A^{1154}>C.

To transfect cells with plasmid, $3x10^5$ 3T3 cells were seeded onto a 6 well plate with 4.5 ml DMEM and allowed to adhere overnight. The following day two transfection mixes were made, the first containing 0.2 ml OptiMEM® and 1 µg plasmid, the second with 0.2 ml OptiMEM® and 20 µl lipofectamine 2000 (Thermo Fisher), mixes were incubated at room temperate for 5 minutes, then combined and incubated for a further 30 minutes. The transfection mixture was then added to cells followed by incubation under normal tissue culture conditions for 15 hours. The media was then removed and replaced with fresh DMEM. To gain polyclonal pools of overexpressing cells, media was replaced with DMEM containing 500 µg/mL G418 48 hours post transfection. After incubation in G418 containing media for 1 week, presence of the Myc tag was determined by western blot. Transfected cultures were maintained in media containing G418 (500 µg/mL)

2.3.6 Lentivirus production in HEK293 cells and viral transfection

To generate stable knockdowns of syndecan 4/ADAMTS-1 in HUVECs, shRNA was used, shRNAs were Mission shRNA (Sigma Aldrich). Full details and sequences of shRNAs are outlined in **Error! Reference source not found.**.

For syndecan 4 immunocytochemistry and ELISA, ECs and 3T3s were transfected with HA-tagged *Sdc4* construct (a generous gift from James Whiteford). The construct contained the murine syndecan 4 cDNA mutated to contain the HA epitope tag between I32 and D33 upstream of an IRES element, followed by a sequence coding eGFP.

Constructs were packaged in HEK293 cells. Two mixes were prepared; in mix one 750 ng packaging plasmid (psPAX2) (Addgene, Massachusetts, USA) and 250 ng envelope plasmid (pMD2.G) (Addgene) were mixed with 1 µg shRNA plasmid or *Sdc4-HA* plasmid in 200 µl OptiMEM®. Mixture two contained 20 µl lipofectamine in 200 µl free OptiMEM®. Mixtures were incubated for 5 minutes at room temperature, then

combined and incubated for a further 30 minutes. The final mixture was transferred onto 80% confluent 293T cells in 10cm dishes for 15 h. The medium was replaced with high glucose DMEM containing 10% FBS and collected after 48 h. Media was centrifuged at 300 g to pellet cell debris, and supernatant containing virus was then filtered through a 0.45 µm filter. Virus containing media was aliquoted and stored at -80°C until use.

To induce shRNA depletion of targets, HUVEC were seeded at a density of $2.5x10^5$, media was removed and replaced with virus containing media and 8 µg/mL Polybrene. After 48 hours incubation, transfected cells were selected by incubating in media containing 2.2 µg/mL puromycin. For expression of HA-SDC4, 3T3s or ECs were seeded at a density of $2.5x10^5$, media was removed and replaced with virus containing media and 8 µg/mL Polybrene. After 48 hours incubation, transfected cells were selected using fluorescent activated cell sorting (FACS) for GFP.

2.4 Flow cytometry

For flow cytometric analysis, cells were removed from culture plates using citric saline buffer (1.35 M KCl, 0.15 M $Na_3C_6H_5O_7$). Cells were collected by centrifugation, resuspended in FACS buffer (5% FBS in PBS) then labelled with the appropriate primary antibodies for 1 hour at 4°C on a rocker (full antibody details in **Error! Reference source not found.**). Cells were washed three times in PBS, and resuspended in FACS buffer. If primary antibodies were not directly conjugated, cells were then incubated with a fluorophore-conjugated secondary antibody (eBioscience, California, USA) for 30 minutes at 4°C. Data was collected using a Beckman CytoFLEX (Beckman Coulter, California, USA) and analysed using FlowJo.

For treatment with MMP inhibitors, one of the following: 5 µM BB-94 (Abcam, Cambridge, UK), 10 µM CT1746 (CellTech, Slough, UK), 10 µM GM-6001 (Merck), 1 µM SB-3CT (Abcam) in 10 µL DMSO, or DMSO vehicle control, was added to cells 18 hours prior to flow cytometric analysis.

2.5 RNA extraction, reverse transcription–PCR and real-time quantitative RT–PCR analyses

2.5.1 RNA extraction

To collect RNA, media was removed from cultured cells, followed by two washes in PBS. The PBS wash was removed and cells were scraped into 200 µl RNA-Bee (Amsbio, Abingdon, UK) per $2.5x10^5$ cells. Phase separation was induced by addition of 100 µl chloroform, 15 seconds of agitation, then incubation at 4°C followed by centrifugation at 13,000 g for 15 minutes. The colourless top fraction was kept, and total cell RNA was extracted using the SV Total RNA Isolation Kit (Promega, Wisconsin, USA) according to the manufacturer's instructions. RNA concentration and quality were assessed using a Nanodrop (NR-1000, Labtech, Sussex, UK); 260 nm/ 280 nm absorbance ratios were used to identify any DNA contamination, and 260 nm/ 230 nm absorbance ratios to identify protein or phenol contamination. RNA samples were stored at -80°C.

2.5.2 Reverse transcription

RNA samples were reverse transcribed using GoScript™ Reverse Transcriptase system (Promega) according to manufacturer's instructions. Typically 1 µg of RNA was used per reaction. RNasin® inhibitor (Promega) was added to the reaction mix (40 U/µl). cDNA samples were stored at -20°C.

2.5.3 Taqman qPCR

To quantify relative levels of gene expression in samples Quantitative real-time TaqMan PCR was carried out as described previously (López-Otín and Matrisian, 2007). Briefly, 1 ng cDNA for housekeeping control (18s ribosomal RNA) or 5 ng cDNA for gene of interest (GOI) was included in a reaction mixture containing 1 µl of specific TaqMan mix (containing probe, and forward and reverse primers) (full details in **Error! Reference source not found.**) 8.33 µl qPCRBIO Probe Mix Lo-ROX (PCR Biosystems (London, UK), which was made up to a final reaction volume of 25 µl with nuclease free water (Promega). Reaction cycles were as follows: 2 minutes at 50°C, 10 minutes at 95°C then 40 cycles of 95°C for 15 seconds followed by 60°C for 1

minute. PCR was performed and data collected using 7500 Real-Time PCR System (Applied Biosystems, California, USA). The Comparative CT method was used to display relative gene expression (Schmittgen and Livak, 2008).

2.6 Immunocytochemistry

For visualisation of filamentous-(F) actin, siRNA transfected cells were seeded at a density of $2x10^5$ cells/well in 24 well plates on acid-washed, oven-sterilised glass coverslips pre-coated with FN, then allowed to adhere overnight. The following day, cells were serum starved for 3 hours in OptiMEM®, followed by stimulation with 30 ng/mL VEGF-A_{164} for 30 minutes at 37°C.

For analysis of paxillin containing focal adhesions, siRNA transfected cells were seeded at a density of $2x10^5$ cells/well on acid-washed, oven-sterilised glass coverslips pre-coated with FN or Col I for 90 and 180 minutes in IMMLEC media.

For imaging of syndecan 4, HA-SDC4-eGFP cells were siRNA transfected and seeded at a density of $2x10^5$ cells/well on acid-washed, oven-sterilised glass coverslips pre-coated with FN and allowed to adhere overnight.

Cells were fixed at indicated time points in 4% paraformaldehyde for 10 minutes, followed by 3 times washing in PBS, coverslips were then blocked and permeabilised with 0.3% Triton X-100, 10% serum (corresponding to species of secondary antibody) (Thermo Fisher) for 1 hour. To stain, coverslips were incubated with primary antibody diluted 1:100 in PBS for 1 h. Primary antibodies used from Table 2.1 were: anti-HA tag, anti-paxillin, anti-α5 integrin. Coverslips were washed with PBS, then incubated with the relevant Alexa-Fluor®-conjugated secondary antibody (Invitrogen) diluted 1:500 in PBS for 45 minutes in the dark. To stain for F-actin, Alexa-Fluor®-568–phalloidin (Invitrogen) was used 1:300 in PBS at the secondary-antibody incubation stage. Coverslips were washed in PBS again before mounting on slides with Prolong® Gold containing DAPI (Invitrogen). All stages were conducted at room temperature.

Focal adhesion area calculations were carried out in FIJI™. Images were adjusted by subtracting background with a rolling ball radius of 45, enhancing local contrasts

using the FIJI™ plugin CLAHE, which implements contrast-limited adaptive histogram equalisation using the following settings: blocksize 19, histogram bins 256, maximum slope 6, no mask, fast. Images were then auto-thresholded and the analyse particles command was used to calculate size and number of focal adhesions, with parameters set to size 0.2 – infinity.

Radial orientation of actin filaments was quantified using SOAX, a software for quantification of 2D and 3D biopolymer networks (available for download at: http://athena.physics.lehigh.edu/soax/), followed by graphing of density in R (Xu *et al.*, 2015).

2.7 Internalisation and recycling assays

Cell surface biotintylation based assays were used to quantify membrane protein internalisation and recycling.

2.7.1 Internalisation

For the internalisation assay, $1x10^6$ siRNA transfected cells were seeded into 10 cm dishes. After adhering overnight, cells were serum starved for 3 hours in OptiMEM®, then chilled on ice, followed by two ice cold PBS washes. Cells were surface labelled at 4°C with 0.3 mg/ml NHS-SS-biotin (Thermo Fisher) in Sorensen's buffer (SBS) (14.7mM KH2PO4, 2mm Na2HPO4, 120mM) for 30 min. Labelled cells were then washed in ice cold PBS, the wash was replaced with pre-warmed IMMLEC and cells were incubated at 37°C to allow internalization. At indicated time points, medium was removed, dishes were transferred to ice and washed twice with ice-cold PBS.

To allow for quantification of internalised protein biotin was removed from proteins remaining on the cell surface by incubation with the membrane impermeable reducing agent sodium methanethiolate (MesNa) (20 mM MesNa in 50 mM Tris-HCL (pH 8.6)) for 1 hour at 4°C. MesNa was quenched by the addition of 20 mM iodoacetamide (IAA) in SBS for 10 mins. Cells were lysed in 200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na_3VO_4, 7.5 mM EDTA, 7.5 mM EGTA, 1.5% Triton X-100, supplemented with protease inhibitor (1:200) (Merck, New Jersey, USA). Lysates

were cleared by centrifugation at 10,000 × g for 10 min. Levels of syndecan 4 internalisation were determined by capture ELISA. For α5 integrin internalisation analysis, the biotinylated protein was isolated by immunoprecipitation using anti-biotin (Jackson Immunoresearch, Ely, UK), and levels of α5 integrin assessed by SDS-PAGE.

2.7.2 Recycling

After surface labelling with biotin, cells were incubated in IMMLEC at 37°C for 20 minutes to allow internalization. Following removal of biotin from surface proteins using MesNA, the internalized fraction was then allowed to recycle to the membrane by returning cells to 37°C in IMMLEC. At the indicated times, cells were returned to ice and biotin was removed from recycled proteins by a second reduction with MesNa. Biotinylated SDC4 was then determined by capture-ELISA.

2.7.3 Capture-ELISA

96-well microplates (R&D Systems, Minnesota, USA) were coated overnight with 5 µg/mL HA-tag antibody (Proteintech) in PBS at room temperature. The plates were blocked in PBS containing 0.05% Tween-20 (0.05% PBS-T) with 1% BSA for 1 hr at room temperature. HA-SDC4 was captured by 2 hour incubation of 100 µl cell lysate (1 µg/µL) at room temperature. Unbound material was removed by extensive washing with 300 µl 0.05% PBS-T per well, for three washes. Wells were incubated with streptavidin-conjugated horseradish peroxidase (R&D Systems) in 0.05% PBS-T containing 1% BSA for 1 hr at room temperature. Following a further three washes, biotinylated HA-SDC4 was detected with Tetramethylbenzidine-based colour change reaction (R&D systems). Absorbance was read at 450 nm with wavelength correction set to 540 nm.

2.7.4 Immunoprecipitation and western blot

For each immunoprecipitation (IP) 400 µg of protein was used, and sample volumes were equalised to 1 ml. The immunoprecipitation protocol was then followed as in section 2.10, without the addition of NuPAGE® sample reducing agent (Life Technologies), followed by western blotting as described in Section 2.9.

2.8 Zymography

Zymography was performed using SDS-PAGE (7.5%) gels co-polymerized with 1 mg/ml gelatin. Media samples were collected from $1x10^6$ ECs cultured on a 10 cm dish, equal volumes were added to 5 µl 5X non-reducing sample buffer (Thermo Scientific). Gels were run for 1 hour 50 minutes at 100 V. After electrophoresis, gels were washed twice for 30 minutes in wash buffer (2.5% Triton X-100, 50 mM Tris HCl pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) at room temperature to remove SDS and allow refolding of proteases. Gels were then incubated overnight in incubation buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) at 37°C. Gels were stained in Coomassie Blue R 250 (Thermo Fisher) in a mixture of methanol:acetic acid:water (4:1:5) for 1 hour and destained in the same solution without dye. Gelatinase activity was visualized as distinct bands of digestion.

2.9 Western blot analysis

Samples for western blot analysis were collected using a cell scraper to scrape adherent cells into RIPA buffer (25mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, supplemented with protease inhibitor (1:200) (Merck), roughly 100 µl of RIPA was used per every 250,000 cells. Samples were centrifuged at 13,000 g for 10 minutes at 4°C to pellet nuclear debris. The supernatant was transferred to a new tube and protein levels quantified using the DC BioRad assay according to manufacturer's instructions, to ensure equal sample loading.

NuPAGE® sample reducing agent (500 mM dithiothreitol) and NuPAGE® LDS sample buffer (lithium dodecyl sulfate pH 8.4, Coomassie G250 and Phenol Red) (Life Technologies) were added to protein samples prior to boiling at 95°C for 5 minutes on a heating block. In general for a western blot, 20 µg protein per sample was loaded into 8% polyacrylamide gels. Following SDS-PAGE separation at 100 V for 1 hour 50 minutes, proteins were transferred onto a nitrocellulose membrane (Thermo) using a Bio-Rad Trans-Blot® wet transfer system, according to manufacturer's instructions. Proteins were transferred for 2 hours at 80 V. The nitrocellulose membrane was

blocked by incubation in 5% milk powder in PBS plus 0.1% Tween-20 (PBS-T) for 1 hr at room temperature (RT), followed by an overnight incubation in primary antibody diluted 1:1000 in 5% bovine serum albumin (BSA)/ PBS-T) at 4°C. The blots were then washed 3x with 0.1% PBS-T and incubated with the relevant horseradish peroxidase (HRP)-conjugated secondary antibody (Agilent, California, USA) diluted 1:2000 in 5% milk/PBS-T, for 2 hrs at RT. Chemiluminescence was detected following addition of Pierce ECL (Thermo) on a Bio-Rad Gel Doc XR + (Bio-Rad).

For VEGF time course assays, siRNA transfected ECs were seeded at $2.5x10^5$ cells per well into 6 well plates coated with 10 µg/ml FN. After 48 hours of recovery, cells were starved for 3 hours in serum free medium (OptiMEM®). VEGF-A_{164} was then added to a final concentration of 30 ng/ml. Cells were lysed at the indicated times in RIPA buffer. After protein quantification as above, 30 µg of protein from each sample was loaded onto 8% polyacrylamide gels. For paxillin analysis, samples were loaded onto a 4-12% gradient gel (Bio-Rad) for better resolution.

Primary antibodies (Table 2.1) used were as follows: anti-phospho (Y1175) VEGFR2); anti-VEGFR-2; anti-phospho (Thr202/Tyr204) p44/42 MAPK Erk1/2, anti-total p44/42 MAPK Erk1/2, anti-HSC70, anti-human SDC4, anti-phospho (Tyr118) paxillin, anti-phospho (Tyr925) FAK, anti-FAK, anti-GAPDH. All primary antibodies were used at a 1:1000 dilution.

FIJI™ was used for quantification of band densities. Band densities were normalised to loading controls.

2.10 Immunoprecipitation assays

HUVECs were grown to 80-90% confluency in 10 cm dishes coated with 10 µg/ml FN in PBS. Media was removed and cells washed in PBS. Cells were lysed in RIPA buffer containing protease inhibitor (1:200) (Merck). Samples were centrifuged at 13,000 g for 10 minutes at 4°C, the supernatant was kept, and protein concentration quantified.

Primary antibodies were diluted in 0.02% Tween in PBS and coupled to protein-G Dynabeads® (Invitrogen) by rotating incubation for 10 minutes at room temperature. The antibody solution was removed, and the coupled beads were resuspended in PBS (20 µl per sample). Coupled beads were added to samples, typically 400 µg of total protein from each sample was immunoprecipitated by incubation with coupled beads a on a rotator overnight at 4°C. Immunoprecipitated complexes were washed three times with 0.2 ml of RIPA buffer, beads were then resuspended in 100 µl PBS and transferred to a new tube, followed by one further PBS wash before being added to, and boiled in NuPAGE® sample reducing agent and sample buffer (Life Technologies) for western blotting as described in Section 2.9.

Primary antibodies used for immunoprecipitation were mouse-anti-human VEGF-A antibody and mouse anti-biotin as listed in Table 2.1

2.11 Active Rac1 pulldowns

To quantify active Rac1, the 'Rac1 Activation magnetic beads pulldown assay kit' (Merk, 7-10393) was used according to manufacturer's instructions. ECs were seeded at a density of $1x10^6$ cells/10 cm plate and allowed to adhere for 90 minutes, the plate was washed and adherent cells were lysed in a magnesium lysis buffer (MLB) (125 mM, HEPES pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50mM MgCl2, 5 mM EDTA and 10% glycerol, supplemented with protease inhibitor (1:200, Merk)). Lysates were incubated with Pak-1 p21 binding domain (PBD) magnetic beads for 45 minutes at 4°C with gentle agitation. Beads were washed in MLB, resuspended in NuPAGE® sample reducing agent and sample buffer (Life Technologies), boiled at 95°C for 5 minutes and loaded directly into a gel for western blotting as in 2.9. Membranes were probed using an anti Rac1 antibody at 1:1000 dilution.

2.12 VEGF ELISA

For quantification of free VEGF-A_{164} present in media, ECs were plated at a density of $2.5x10^5$ cells per well on a FN coated 6 well plate. Following adherence overnight, cells were chilled to 4°C, media was removed and cells were washed twice in ice cold

PBS. Media was replaced with ice cold OptiMEM® containing VEGF-A_{164} at a final concentration of 30 ng/ml, followed by incubation at 4°C for 30 minutes.

For the ELISA, media was removed from samples and diluted 1:10 in 1% BSA in PBS. VEGF-A_{164} concentration was quantified using a mouse VEGF duo-set ELISA, according to manufacturer's instructions (DY493, R&D Systems). Generation of standard curve and determination of concentrations was performed in R.

Cell lysates were collected to quantify VEGF signalling via western blotting as described in section 2.9.

2.13 BrdU Proliferation Assay

siRNA-transfected ECs were seeded onto 10 µg/ml FN-coated glass coverslips ($1.5x10^4$ cells/well of a 24 well plate). After 4 hours, the media was replaced with IMMLEC containing 10 nM BrdU (Abcam). Cells were incubated with BrdU for 12 hours, followed by fixation in 4% PFA. To stain, cells were incubated in 1 M HCl for 30 mins at room temperature, then permeabilized with PBS 0.25% Triton X-100 for 10 minutes and blocked by a 20 minute incubation in DAKO Block (Agilent). Incorporated BrdU was detected by incubation with anti-BrdU (ICR1, Abcam) diluted 1:100 in PBS for 1 hour at room temperature. Coverslips were washed in PBS, then incubated with anti-sheep Alexa-Fluor® 647 (Invitrogen) for 1 hour at RT. After further PBS washes, coverslips were mounted in Pro-long gold® containing DAPI (Invitrogen). The number of BrdU positive nuclei were counted, and expressed as a percentage of total DAPI positive nuclei.

2.14 Random-migration assay

siRNA-transfected ECs were trypsinised and seeded at a density of $1.5x10^4$ cells/well in 24-well plates coated with 10 µg/ml FN or 10 µg/ml Col I in PBS, and allowed to adhere overnight. The media was then replaced with fresh IMMLEC. One phase contrast image/well was taken live every 16 min in a fixed field of view using an inverted Axiovert (Zeiss, Oberkochen, Germany) microscope for 16 h at 37°C and 5%

CO_2. Individual cells were then manually tracked using the FIJI™ cell tracking plugin, MTrackJ and the speed of random migration was calculated in nM moved/second.

2.15 *Ex Vivo* Aortic Ring Assay

Thoracic aortae were isolated from 6 to 8 week-old adult C57BL6 mice and prepared for culture as described extensively by Baker *et al.* (2012). Briefly, aortas were removed and defatted, then sliced into rings roughly 1 mm thick, rings were distributed evenly into individual wells of a 24 well plate, one well per condition, with roughly 25 rings per well, and incubated in 800 µl OptiMEM®. Syndecan 4 or ADAMTS-1 depletion was induced using 1 µM siRNA. Transfection master mixes were created, mix 1 contained 1 µl siRNA (from 100 µm stock) and 184 µl OptiMEM®, mix 2 contained 3 µl oligofectamine and 12 µl OptiMEM®, mixes were combined and incubated for 20 minutes at room temperature, followed by addition to the appropriate wells of aortic rings. Rings were incubated overnight in siRNA, followed by embedding in a collagen matrix. Each ring was embedded into an individual well of a 96 well plate containing 1 mg/mL type collagen I from rat tail (Thermo Fisher) and 0.5 ml 10 x DMEM which was polymerized by incubation at 37°C for 1 hour. Rings were fed with OptiMEM® containing 2% FBS, which was replenished every 3 days, and incubated at 37°C. Where indicated, VEGF-A_{164} was added at 30 ng/mL. After 10 days, rings were fixed with 4 % PFA, permeabilized with 0.2% Triton, and stained with FITC BS1-Lectin. A Zeiss inverted microscope was used to visualise and count sprouting micro vessels.

To confirm siRNA knockdown of target genes, ~10 rings per condition were homogenized in 250 µl RNAbee, followed by RNA extraction and qPCR as in 2.5.3.

2.16 Conditioned matrix generation

To generate conditioned matrix (CM) for immunocytochemistry and western blotting, siRNA treated cells were seeded at ~70% confluence onto uncoated plates (glass coverslips in 24 well plates for ICC, 6 cm dishes for western blotting) and allowed to produce matrix for 48 hours. Under sterile conditions, media was removed and plates were washed with PBS. Adherent cells were removed while

preserving the ECM by incubation in a 20 mM ammonium hydroxide solution l (3 mL per 100 mm area). Plates were incubated for 5 minutes with gentle agitation, after which complete removal of cells was checked for microscopically. Ammonium hydroxide was removed, and cells were washed 5 times in sterile de-ionised H_2O with rocking. Untreated, or 'naïve' cells could now be plated onto this CM. For immunocytochemistry experiments $1.5x10^4$ naïve cells were seeded per well for 180 minutes, followed by fixation and staining as in section 2.6. For western blot analysis of signalling pathways $2.5x10^5$ cells were seeded per well, and collected at multiple time points as in section 2.9.

2.17 Statistics

Statistical analysis was conducted in R. Graphing was conducted in R or GraphPad. Where data were normally distributed a Student's T test or ANOVA (if comparing multiple groups) were used to determine statistically significant differences between conditions. Normal distributions were tested for using a Shapiro-Wilk test. Where data did not fit the normal distribution (focal adhesion sizes) a Kruskal-Wallis non-parametric test was used to determine statistical significance. Bar charts represent the mean and the standard error of the mean (SEM), unless otherwise stated. Asterisks represent P values as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

3 ADAMTS-1 regulates Syndecan 4 cell surface expression in an MMP-9 dependent mechanism

This chapter sought to investigate a potential connection between ADAMTS-1 and syndecan 4. The ADAMTS family of proteases, in particular those of the proteoglycanase clade, have been shown to function in collaboration with syndecan family members in several incidences.

One key example is in osteoarthritis; ADAMTS-4 and -5 are important in this context, and their roles require syndecans. The activation of ADAMTS-4 involves binding of the CS and HS chains of syndecan 1, and the expression of both ADAMTS-4 and -5 is dependent on syndecan 2 (Gao *et al.*, 2004; Yan *et al.*, 2018). Syndecan 4 is also involved in ADAMTS-5 activation, and its knockout in mice is cartilage protective; through direct engagement of its HS and the protease, syndecan 4 regulates MAPK dependent synthesis of the ADAMTS-5 activator MMP-3 (Echtermeyer *et al.*, 2009).

ADAMTS-syndecan functional links are also found in the regulation of cell adhesion and migration. In the case of ADAMTS-15, its regulation of adhesion and migration is dependent on syndecan 4; Kelwick *et al.* found that expression of ADAMTS-15 reduced migration of breast cancer cells, and that knockdown of syndecan 4 attenuated these effects (Kelwick, Wagstaff, *et al.*, 2015).

ADAMTS-1 is a particularly interesting member of the family. It belongs to the proteoglycanase clade, and has potent anti-angiogenic activity via a number of mechanisms, both requiring and independent of catalytic activity (Kuno *et al.*, 1997; Luque, Carpizo and Iruela-Arispe, 2003; Lee *et al.*, 2006). ADAMTS-1 has been linked to syndecan-4 under several circumstances.

The functions of ADAMTS-1 and syndecan 4 centre around the ECM. ADAMTS-1 is secreted and anchors to the matrix via its TSRs, in an interaction that is mediated by GAGs such as HS (Kuno and Matsushima, 1998). Syndecan 4 consists of a transmembrane core protein with extracellular covalently attached HS GAG chains,

which allow it to interact with growth factors and ECM proteins such as fibronectin, which contains syndecan 4 binding sites (Elfenbein and Simons, 2013).

In the context of angiogenesis, the interaction of ADAMTS-1 and VEGF in the ECM requires the contribution of a heparin-like molecule, and it has been hypothesised that syndecan 4 may fulfil this role, assisting in ADAMTS-1's sequestration of $VEGF_{165}$ by acting as a bridge between the two proteins (Luque, Carpizo and Iruela-Arispe, 2003). Circumstantially, these observations indicate that ADAMTS-1 and syndecan 4 co-exist in the same extracellular space, and may physically interact with each other.

Much like with ADAMTS-15 and syndecan 4, an ADAMTS-1 syndecan 4 interplay has been implicated in the regulation of cell adhesion and migration. N-terminal clipping of syndecan 4 by ADAMTS-1 results in defects in adhesion and promotion of migration. Complementing this, the overexpression of syndecan 4 was correlated with inhibition of migration, suggesting that the balance of this protein is essential for co-ordinated cell movement (Rodríguez-Manzaneque *et al.*, 2009).

ADAMTS-1 and syndecan 4 are clearly involved in cell migration and angiogenesis, and therefore research was focused on these ECM dependent functions. It was hypothesised that ADAMTS-1 and syndecan 4 may regulate cell migration and angiogenesis through downstream modification of the integrins, cell adhesion receptors essential for both processes (Barczyk, Carracedo and Gullberg, 2010).

Functional links between syndecan 4 and integrins have been widely reported. Syndecan 4 functions co-operatively with integrins in the formation of focal adhesions on fibronectin, and its phosphorylation by Src regulates integrin membrane traffic with consequences for cell migration (Saoncella *et al.*, 1999; Morgan *et al.*, 2013). The integrins are a large family of heterodimers; integrins α5β1 and αvβ3 were initially considered to be the most interesting, as these are the fibronectin binding integrins, their trafficking is regulated by syndecan 4, and their endothelial expression is upregulated during angiogenesis (Kim *et al.*, 2002; Bass *et al.*, 2008; Morgan *et al.*, 2013).

ADAMTS-1 has not been reported to interact with integrins, or to influence integrin behaviour, however its roles in cell migration and angiogenesis hint at a possible relationship. The expression of ADAMTS-15 was shown to alter the integrin profile in in MDA-MB-231 breast cancer cells, proposing a potential mechanism for its effects on cell motility. Due to the closely related structure and roles of ADAMTS-1 and -15 it is therefore possible that ADAMTS-1 regulates migration in a similar manner (Kelwick, Desanlis, *et al.*, 2015).

Predicted associations between ADAMTS-1, syndecan 4 (SDC4), fibronectin and selected integrins are demonstrated in a network map generated in STRING (Figure 3.1). Colours represent different lines of evidence used in predicting associations.

Based on these hypothesised interactions, this work sought to investigate the functional association of ADAMTS-1 and syndecan 4, and to explore mechanisms through which they may co-operate in the regulation of integrins, migration and angiogenesis. This chapter outlines the characterisation of the cell models utilised and demonstrates an interdependency between the expression of ADAMTS-1 and syndecan 4 in the context of both endothelial cells and fibroblasts.

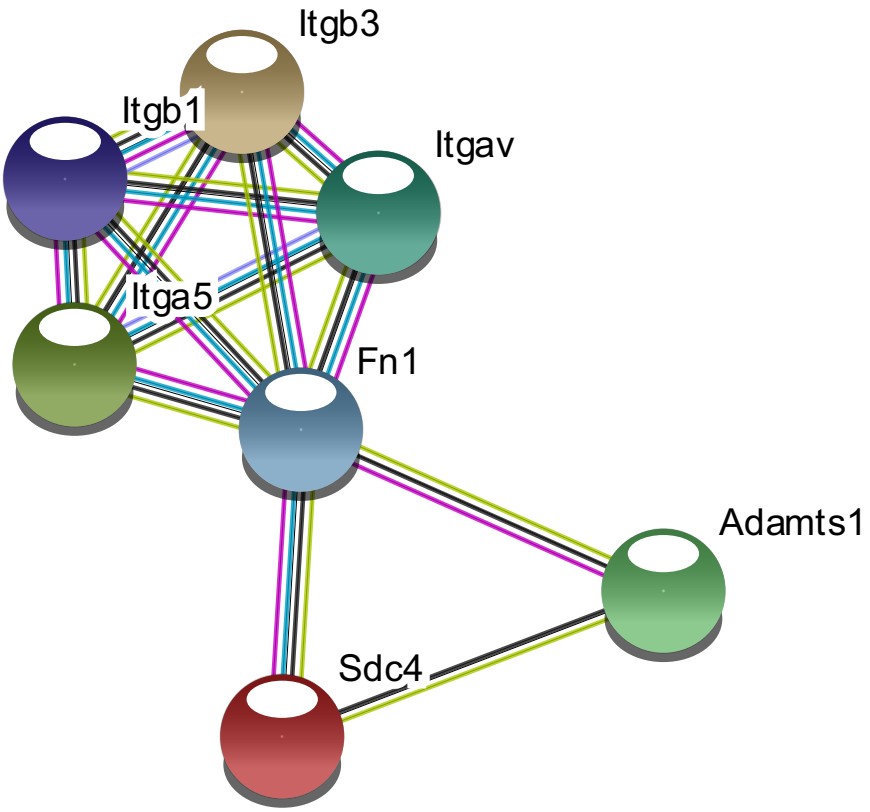


Figure 3.1 Predicted interactions between *Mus musculus* ADAMTS-1, syndecan 4, fibronectin and selected integrins. The map shows a network view of predicted associations for a group of proteins generated using STRING©. Edges represent predicted functional associations. Colours signify type of evidence used in predicting association. Green: neighbourhood evidence, Blue: co-occurrence evidence, purple: experimental evidence, black: co-expression evidence.

3.1 Characterisation of mammalian cell models

Two cell culture models were used in this work, namely 3T3 cells; fibroblasts originally isolated from swiss albino mouse embryos and spontaneously immortalised in cell culture (3T3s), and mouse lung microvascular endothelial cells (ECs); originally isolated from C57BL/6 mice followed by PyMT immortalisation (Todao and Green, 1963; Wang *et al.*, 2019).

Experiments were performed in two cell lines to assess the universality of any phenotypes. Conflicting reports exist with regards to the roles of ADAMTS-1 and syndecan 4, and this may be due to cell type specificity, or different contributing growth factors. Using two cell lines may help unpick these contradictions (Bass *et al.*, 2007; Rodríguez-Manzaneque *et al.*, 2009; Esselens *et al.*, 2010; Ham *et al.*, 2017).

As it is highly likely that any interaction between ADAMTS-1 and syndecan 4 centres around the ECM, fibroblast cells were chosen due to their high degree of ECM interaction. Fibroblasts are stromal cells and are responsible for the production and secretion of all ECM components, including collagen, fibronectin and glycosaminoglycan. Physiologically, fibroblasts play pivotal roles in ECM maintenance, wound healing, and angiogenesis (Kendall and Feghali-Bostwick, 2014).

Both *Adamts1* and *Sdc4* knockout mice show defective wound healing phenotypes; this further invites the use of fibroblast cells, as during wound healing fibroblasts become activated, secreting ECM proteins. Fibroblasts must proliferate and migrate to the site of injury in order to generate the contraction of the matrix required to seal an open wound (Gabbiani, 2003).

Fibroblasts interact closely with endothelial cells, and are important for facilitating angiogenesis; reducing fibroblast ECM synthesis results in a reduction in EC tube formation (Berthod *et al.*, 2006). Fibroblast derived matrix proteins are essential for endothelial cell lumen formation; in the absence of fibroblasts angiopoietin-1 can drive vessel sprouting but lumens do not form (Newman *et al.*, 2011). Fibroblasts can

also produce and secrete pro-angiogenic growth factors including VEGF, PDGF and TGF-β (Fukumura *et al.*, 1998).

Fibroblasts are highly motile cells, and most previous work on syndecan 4 in adhesion and migration has focused on fibroblasts isolated from the Echtermeyer *Sdc4*-/- knockout mouse, therefore utilising fibroblasts from an alternate source with siRNA knockdowns allows for comparison with current literature (Echtermeyer *et al.*, 2001).

The ECM interactive, migratory, and angiogenic properties of fibroblasts therefore make them a good cellular model for investigation of interactions between ADAMTS-1 and syndecan 4. The 3T3 fibroblast cell line was chosen, and these cells are known to be easy to work with, rapidly proliferating, highly migratory and responsive to growth factors.

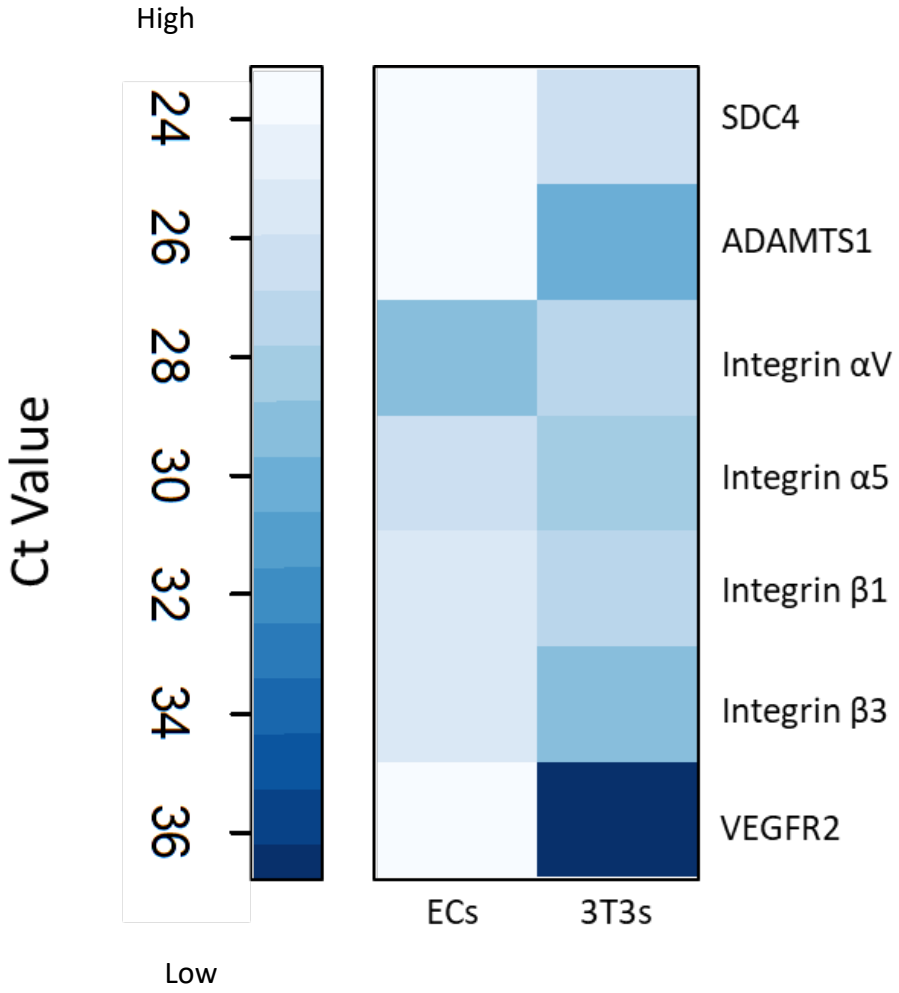
The second cell line chosen was ECs. ECs line the interior surface of blood vessels, a single cell layer covers the entire luminal surface of all vessels; they are in direct contact with circulating blood (Michiels, 2003). This interface allows ECs to participate in homeostasis, as well as pathological and physiological processes such as inflammation and angiogenesis.

Endothelial cells maintain their capacity for proliferation throughout life; this ability is essential for repair of vessel damage, and grow of new sprouts. Endothelial cell signalling, proliferation and migration in response to VEGF is necessary to direct proper angiogenesis. Endothelial cells also secrete signalling molecules such as PDGFB, needed to recruit pericytes and promote vessel maturation (Kourembanas and Faller, 1989).

As ECs are the key cellular mediators of angiogenesis, they were chosen as a good model in which to study the effects of ADAMTS-1 and syndecan 4 in angiogenesis. PyMT immortalised murine lung microvascular ECs were utilised in order to generate cell numbers required for experiments; these cells have been used extensively as models for angiogenesis research and have been demonstrated to maintain their endothelial identity (Robinson *et al.*, 2009; Ellison *et al.*, 2015).

3.1.1 Endothelial cells and fibroblasts express genes of interest at varying levels

To determine expression levels of genes of interest, including A*damts1, Adamts14, Sdc4, Kdr, Itga5, Itgav, Itgb1*, and *Itgb3*, TaqMan qPCR was utilised. Surprisingly, *Adamts15* expression was not detected in either cell line. *Sdc4* and *Adamts1* were expressed in both cell lines, although at much higher levels in ECs, hinting at their potential importance in the regulation of endothelial cell behaviour and angiogenesis. All four integrins were expressed in both cell lines at varying levels. As expected, high levels of *Kdr* were found in ECs, and very low expression in the 3T3s, representative of the relative importance of VEGF signalling in these cell lines (Figure 3.2)



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 3.2 Expression profile of genes relevant to angiogenesis and cell migration in 3T3s and ECs. RNA was isolated from cultured ECs and 3T3, RNA was reverse transcribed, and TaqMan qPCR was performed to determine gene expression levels. Recorded Ct values are represented in a heatmap. Displayed Ct values are averaged from three independently collected RNA samples.

3.1.2 siRNA mediated knockdown is effective at depleting targets in both cell lines

In order to assess the functions of ADAMTS-1 and syndecan 4, depletion of the targets in cell culture was necessary. The choice of siRNA for depletion of targets was made due to ease of use in difficult to transfect ECs, and because it generally elicits efficient short-term depletion of targets in these cells. Moreover, compensation effects confounding interpretation of results have been seen in previous studies when using constitutive syndecan 4 knockout models (Wilcox-Adelman, Denhez and Goetinck, 2002; Bass *et al.*, 2007). Therefore, short term depletion may give a more complete picture of syndecan 4's functions. Treatment with siRNA of both ECs and 3T3s in culture gave robust and reliable depletion of either ADAMTS-1 or syndecan 4, as assessed by TaqMan qPCR (Figure 3.3).

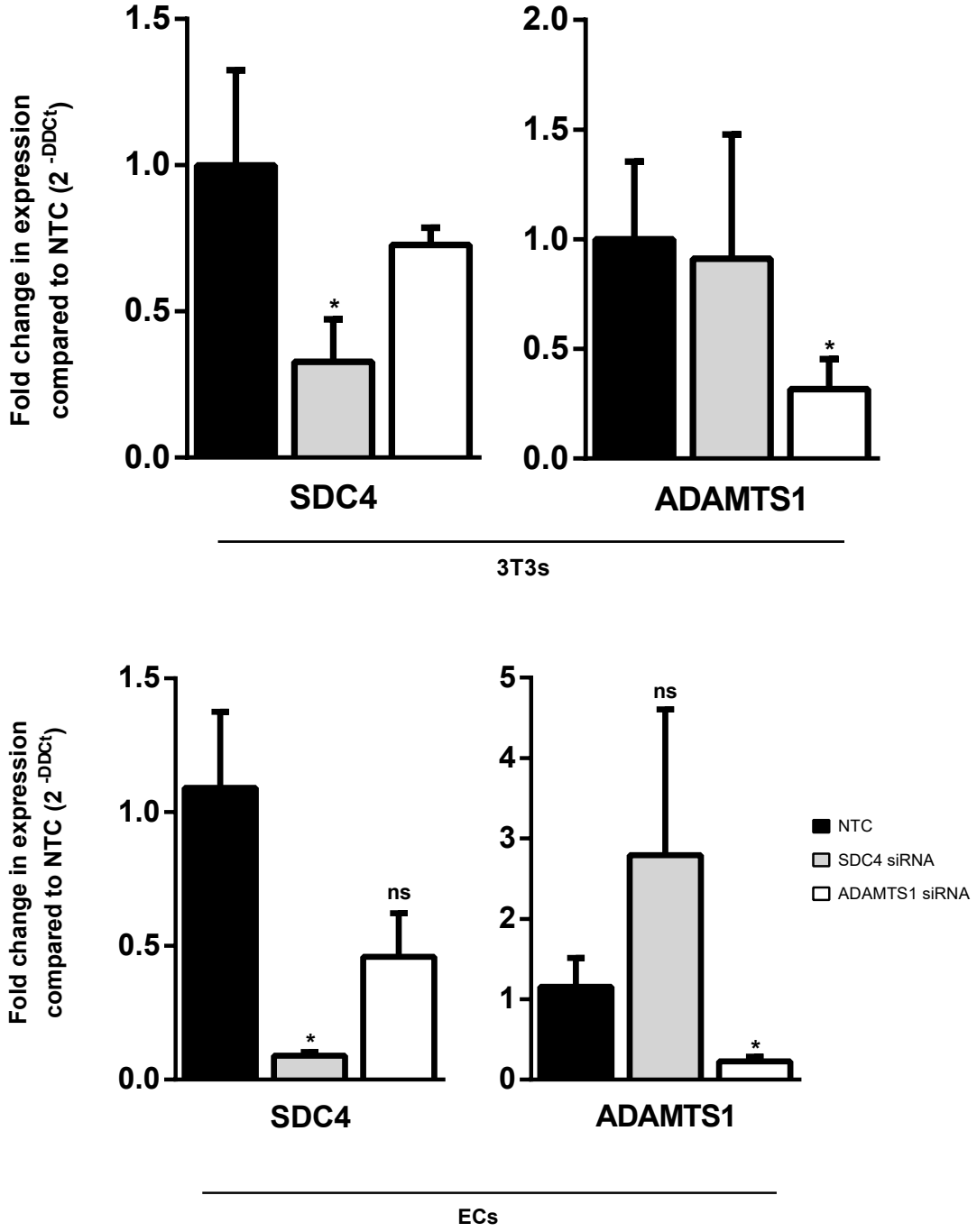


Figure 3.3 siRNA is effective at depleting targets in cell culture. 3T3s or ECs were treated with 50 nM non targeting control (NTC), *Adamts1* or *Sdc4* siRNA. RNA was collected 24 hours post transfection, and gene expression levels were determined using TaqMan qPCR. N=3, Bars represent S.E.M, *P <0.05, ns = not significant.

3.2 ADAMTS-1 depletion results in a loss of cell surface syndecan 4

As qPCR confirmed efficient knockdown of targets at the RNA level, we therefore next aimed to establish knockdown at the protein level. Unfortunately in our hands, commercially available antibodies were not able to reliably detect murine ADAMTS-1 or syndecan 4 by western blotting. As syndecan 4 is membrane bound, flow cytometry was used as an alternative.

As well as establishing knockdowns, flow cytometry provides a means to quantify cell surface syndecan 4, and therefore also allows for the effect of ADAMTS-1 on syndecan 4 expression to be evaluated in a way that qPCR could not. Syndecan 4 expression has been previously linked to ADAMTS family proteases; the expression of ADAMTS-15 in cultured MDA-MB-231 cells results in an increase of syndecan 4 surface expression (Kelwick, Wagstaff, *et al.*, 2015). ADAMTS-1 is also predicted to affect syndecan 4 surface expression as it has been shown to clip a small (6kDa) fragment from the extracellular N-terminus of syndecan 4. It was therefore predicted that the loss of ADAMTS-1 would enhance syndecan 4 surface expression (Rodríguez-Manzaneque *et al.*, 2009).

3.2.1 Optimisation of cell detachment for flow cytometry

In order to perform flow cytometry experiments, the adherent cell lines used needed to be detached from tissue culture plates. As trypsin detachment may result in cleavage of cell surface proteins, including syndecans, alternative cell detachment methods had to be investigated (Subramanian, Fitzgerald and Bernfield, 1997).

Flow cytometry experiments confirmed trypsin removed syndecan 4 from the cell surface, and that this surface syndecan 4 did not recover when cells were incubated in suspension in growth media for up to 90 minutes post trypsinisation. Treatment with TrypLE, a synthetic replacement for trypsin, also resulted in the loss of syndecan 4. Frequently EDTA is used as a non-enzymatic cell dissociation reagent for flow cytometry, however although EDTA detached 3T3s from cell culture plates and maintained cell surface syndecan 4, EDTA was not able to successfully detach ECs. An alternative non-enzymatic cell detachment method using citric saline was found to

successfully detach both cell types while preserving syndecan 4 surface expression (Figure 3.4).

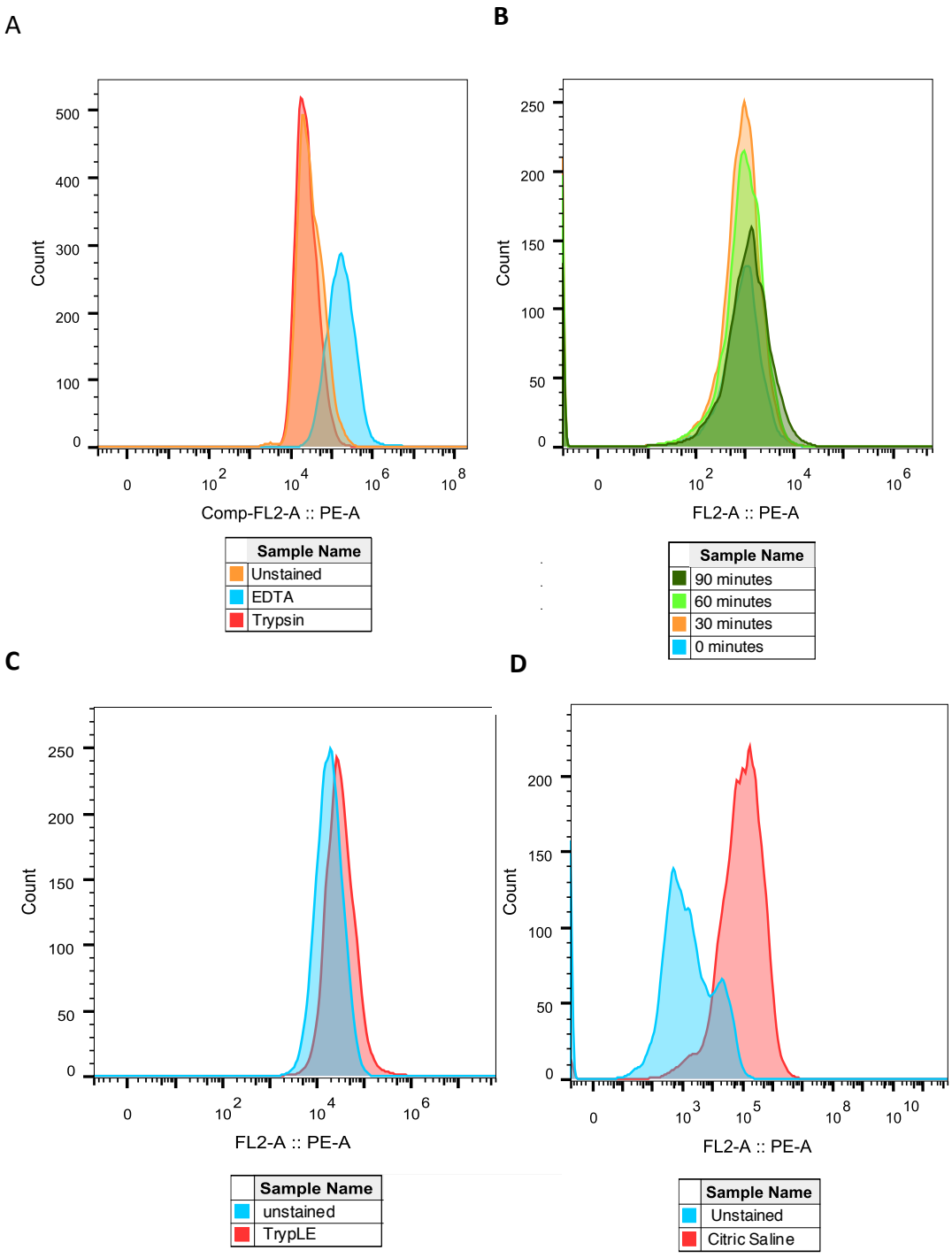


Figure 3.4. Citric saline detachment buffer retains syndecan 4 on the cell surface. 3T3s were collected using several cell detachment buffers, and levels of cell surface syndecan 4 were assessed using flow cytometry with an anti-syndecan 4 antibody (BD Bioscience) A) trypsin or EDTA detachment B) cells were detached using trypsin, followed by incubation in suspension at 37°C for indicated timepoints. C) cells detached with TrypLE D) detachment with citric saline. N=1.

Cell surface syndecan 4 expression is dependent on ADAMTS-1

To determine knockdown of syndecan 4 at the cell surface level, and begin to unpick the interaction between ADAMTS-1 and syndecan 4, the effect of siRNA target depletion on cell surface syndecan 4 was evaluated. Flow cytometry was performed on ECs and 3T3s siRNA treated for ADAMTS-1 or syndecan 4, and collected in citric saline buffer. Flow cytometry revealed that the syndecan 4 siRNA gave good depletion of the target at the protein level. Flow cytometry also revealed that contrary to the initial hypothesis, depletion of ADAMTS-1 resulted in a reduction of syndecan 4 at the cell surface (Figure 3.5).

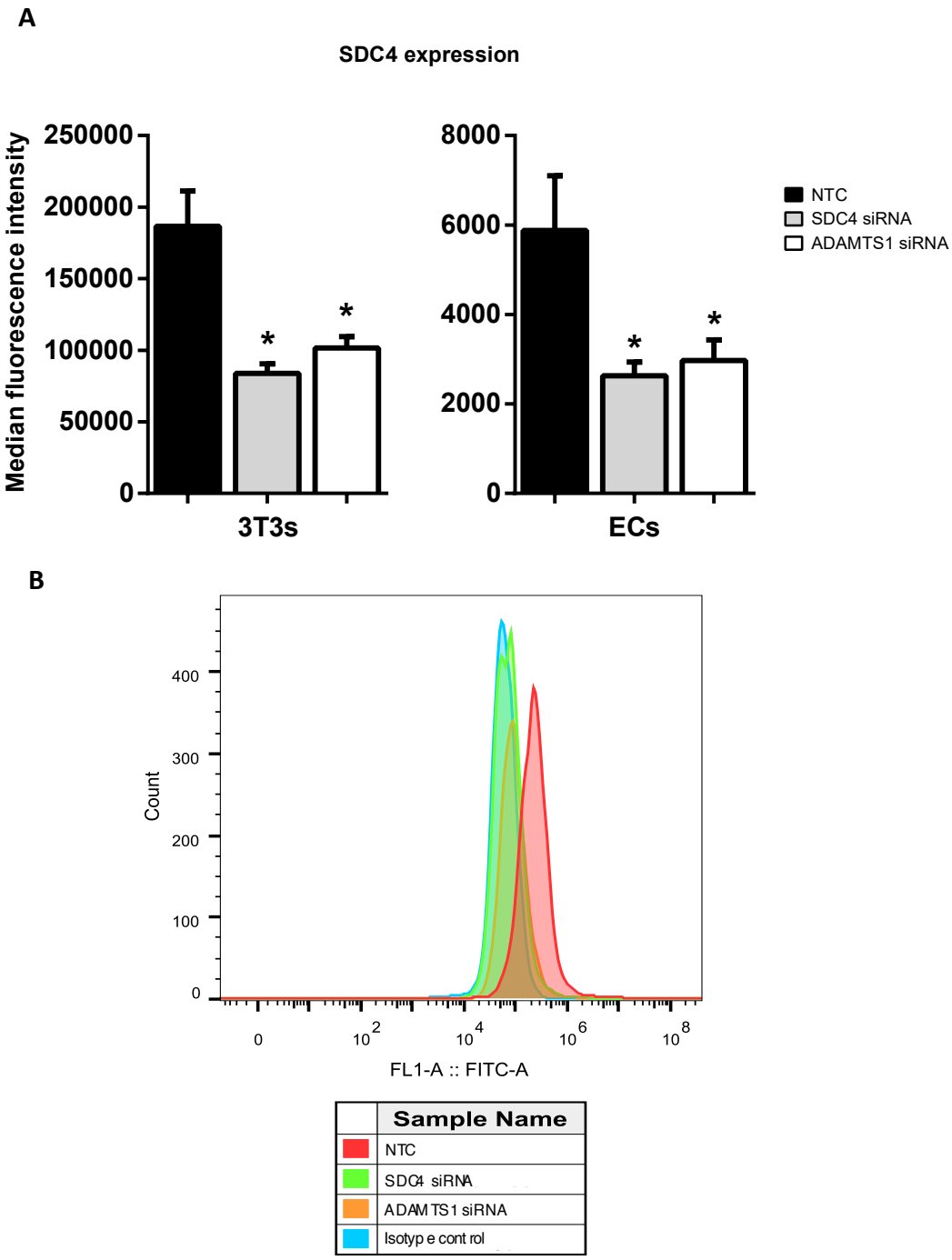


Figure 3.5. siRNA depletion of ADAMTS-1 results in a corresponding loss of syndecan 4 at the cell surface. 3T3s and ECs were siRNA treated for *Adamts1* or *Sdc4*. After 48 hours cells were detached using citric saline buffer, and levels of SDC4 were analysed using flow cytometry. A) Bar chart showing median fluorescent intensities, calculated after gating based on forward and side scatter and normalising to an isotype control, medians were averaged from three independent experiments. Error bars represent S.E.M, * = P< 0.05. B) Representative flow cytometry histogram.

In order to validate the flow cytometry data, and gain some insight into syndecan 4 localisation, immunocytochemistry was utilised. Unfortunately in our hands, antibodies directed against syndecan 4 were not successful for use in visualising syndecan 4. To overcome this, an HA-tagged syndecan 4 construct was utilised. Endothelial cells were transduced with a lentivirus containing the murine syndecan 4 cDNA mutated to contain the HA epitope tag between I32 and D33 in the extracellular region, and a sequence encoding eGFP (HA-SDC4).

Immunocytochemical visualisation of the HA-SDC4 cells using antibodies directed against the HA epitope tag resulted in a staining pattern fitting with the reported cellular localisation of syndecan 4, with syndecan 4 being primarily seen in the Golgi apparatus and at the plasma membrane (Rønning *et al.*, 2015; Uhlén *et al.*, 2015) Further validating the use of the HA-tag construct, staining of HA-SDC4 was only seen in cells which co-expressed the GFP reporter (Figure 3.6). For future experimental use, transfected cells expressing the SDC4-HA tag construct and therefore also GFP were selected for using FACS.

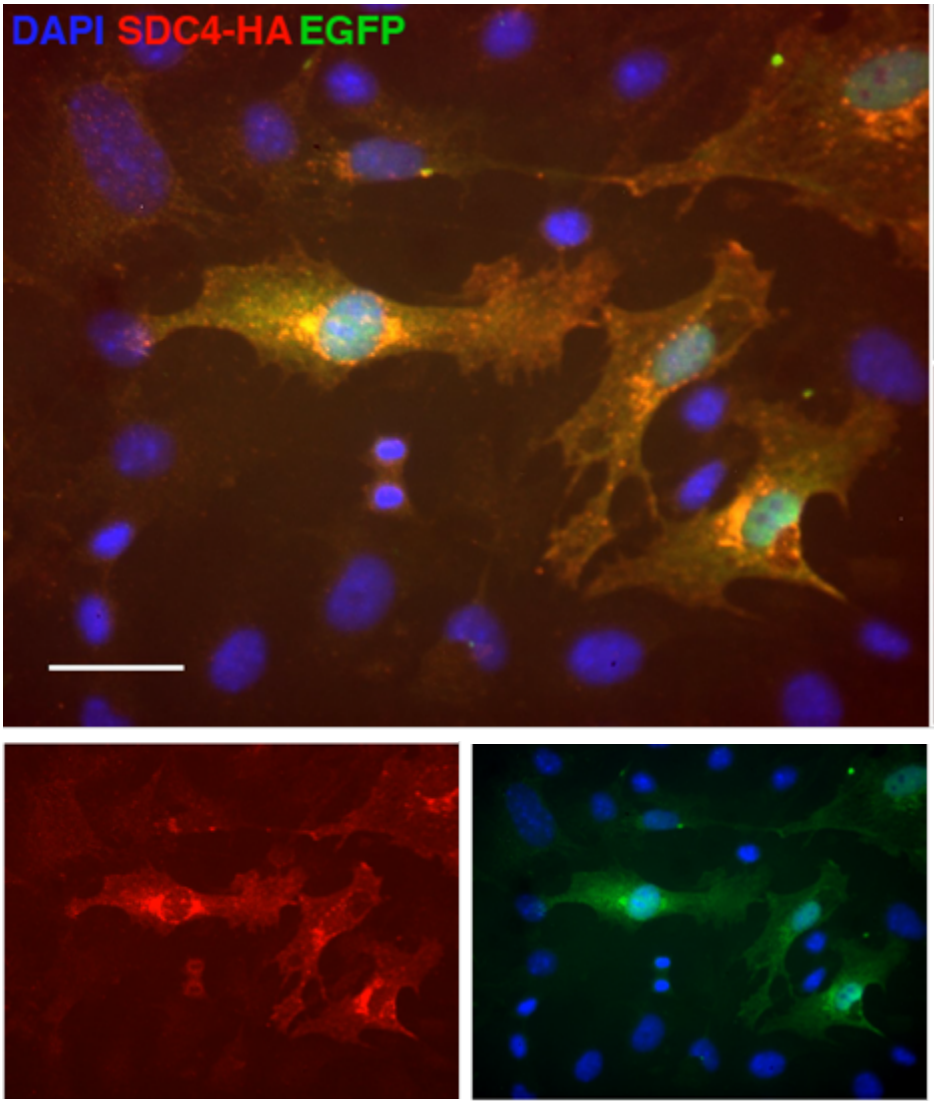


Figure 3.6 Expression of a HA-tagged syndecan 4 construct allows visualisation of syndecan 4 via immunocytochemistry. Representative image of ECs transfected to express eGFP and N-terminal HA-tagged syndecan 4. Transfected cells were seeded onto fibronectin coated coverslips (20 µg/ml). After adhering for 24 hours, cells were fixed and immunostained for HA tag (red) and DAPI (blue). Scale bar = 20 µm.

To both visualise and validate the loss of syndecan 4 seen upon ADAMTS-1 depletion via flow cytometry, HA-SDC4 transfected cells were treated with siRNA targeting *Adamts1* or NTC. Transfected cells were seeded onto fibronectin overnight, followed by fixation and immunostaining for HA-SDC4.

Supporting the flow cytometry data, immunocytochemistry revealed an altered distribution of syndecan 4 upon ADAMTS-1 knockdown. Compared to NTC cells, reduced syndecan 4 expression was seen at the cell periphery; syndecan 4 also seemed to no longer localise in focal adhesions (indicated with white arrows in the NTC panel), and instead appeared to be accumulating in vesicles or the endoplasmic reticulum (Figure 3.7).

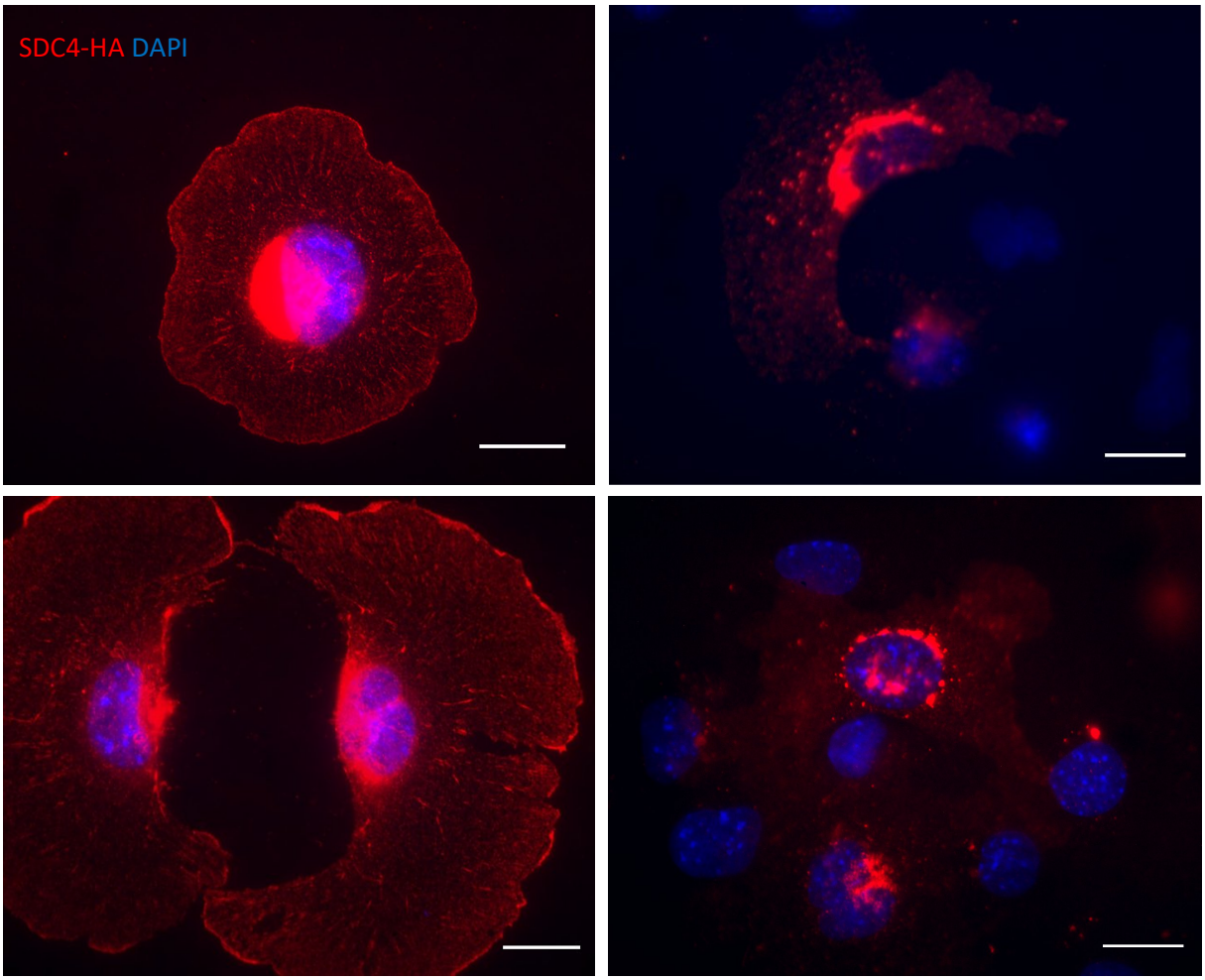


Figure 3.7 *Adamts1* siRNA treatment alters the cellular distribution of syndecan 4. Cells transfected to express HA-Syndecan 4 were treated with siRNA against *Adamts1*, or NTC. Cells were seeded onto fibronectin overnight, then fixed and stained for HA tag (red) and DAPI (blue). White arrows indicate putative focal adhesions. Images are representative from three independent experiments. Scale bar = 20 µm.

3.3 Plasma membrane trafficking of syndecan 4 is unaltered in response to ADAMTS-1 siRNA treatment

Several possibilities were considered when establishing possible causes of the syndecan 4 cell surface loss. Reduced transcription of syndecan 4 may lead to a concomitant reduction in protein expression, however syndecan 4 still appeared to be found intracellularly after ADAMTS-1 knockdown (Figure 3.7) and TaqMan qPCR revealed no significant change in syndecan 4 RNA expression after *Adamts1* siRNA treatment (Figure 3.3). Other potential mechanisms included altered transport of syndecan 4 protein to the membrane, ectodomain shedding of membrane bound syndecan 4, or altered syndecan 4 membrane trafficking.

Initially, altered membrane trafficking was selected as the most likely cause of the loss of cell surface syndecan 4 in response to *Adamts1* siRNA treatment. Adhesion receptors frequently internalise and then are either degraded, or recycled back to the cell surface. This phenomenon is well documented for integrin family members, and accumulating evidence demonstrates syndecan function can be regulated by endocytic trafficking, with functional consequences (Zimmermann *et al.*, 2005). Syndecan recycling is regulated by the PDZ domain containing protein syntenin, PIP2, and the G protein Arf6. Regulation of syndecan recycling in this way is essential for embryogenesis (Lambaerts *et al.*, 2012). In cells which cannot recycle syndecan via this pathway, syndecan becomes trapped intracellularly and inhibits cell spreading (Zimmermann *et al.*, 2005).

Cell surface biotinylation based assays were used to investigate syndecan 4 trafficking at the membrane. Endothelial cells were surface-labelled with cleavable biotin, incubated for various times to allow internalisation, then biotin remaining on the cell surface was cleaved. For the recycling assay, cells were incubated at 37°C again (i.e. after biotin cleavage), to allow biotinylated protein to recycle to the surface. Syndecan 4 internalisation was quantified by a syndecan 4 capture ELISA and biotin detection using streptavidin.

Biotinylation membrane trafficking assays revealed no change in the speed of either the internalisation or recycling of syndecan 4 in response to ADAMTS-1 siRNA, implying that altered membrane trafficking was not the mechanism by which cell surface syndecan 4 was lost (Figure 3.8).

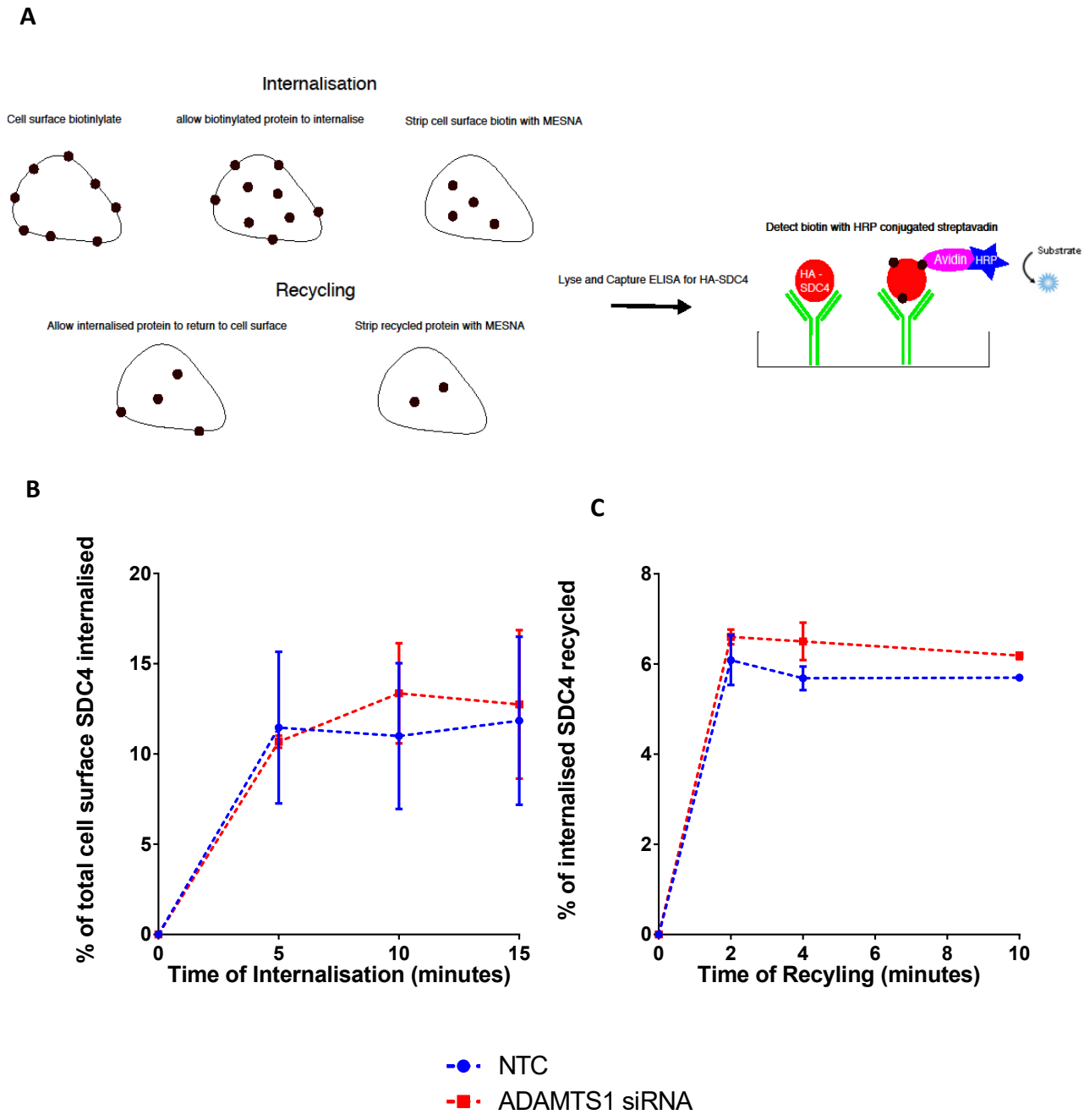


Figure 3.8 ADAMTS-1 depletion does not affect the internalisation or recycling of syndecan 4 at the plasma membrane. A) A diagram outlining the protocol of cell surface biotinylation based internalisation and recycling assays. B) Internalisation; EC surface proteins were biotinylated using a cleavable, membrane non-permeable biotin. ECs were incubated to allow internalisation, remaining surface biotin was removed using the reducing agent MESNA. A SDC4 capture ELISA was performed on lysed ECs. Streptavidin was used to detect biotinylated SDC4 (N=3, bars represent S.E.M). C) SDC4 recycling assay; after the first MesNa treatment, internalised biotin was allowed to return to cell surface. Biotinylated protein that had returned to the surface was again removed using MesNa and an ELISA was performed as in B (N=3, bars represent S.E.M).

3.4 The loss of cell surface syndecan 4 is the result of MMP activity

As Figure 3.8 demonstrated that the surface trafficking of syndecan 4 was unaffected, alternate mechanisms of syndecan 4 regulation needed to be evaluated. The next possibility to be considered was surface shedding of syndecan 4.

Ectodomain shedding is an important syndecan regulatory mechanism, altering surface receptor dynamics and generating soluble ectodomains that have potential to act as autocrine or paracrine effectors (Manon-Jensen, Itoh and Couchman, 2010). Syndecan ectodomain shedding occurs constitutively in many cell lines, and can be accelerated in response to wound healing and other diverse pathophysiological events. Cleavage of syndecans is primarily carried out by the MMPs; therefore they were the first to be considered as potential mediators of syndecan 4 cell surface loss via shedding (Manon-Jensen, Itoh and Couchman, 2010).

3.4.1 MMP inhibitors prevent the loss of syndecan 4

To establish if MMP mediated shedding was responsible for the loss of cell surface syndecan 4 seen in response to *Adamts1* siRNA treatment, cells were treated with commercially available broad spectrum MMP inhibitors, followed by flow cytometry to assess changes in levels of cell surface syndecan 4.

Endothelial cells were transfected with *Adamts1* or NTC siRNA, followed by treatment with one of the following MMP inhibitors: BB-94, CT1746, GM6001, or a DMSO vehicle control. Cells were collected and flow cytometry was used to quantify syndecan 4 expression.

In the case of NTC treated cells, treatment with MMP inhibitors resulted in an accumulation in cell surface syndecan 4, supporting an important role for MMPs in the regulation of cell surface syndecan 4 expression, however this was not statistically significant. For *Adamts1* siRNA treated cells, when a DMSO vehicle control was added, as before a statistically significant decrease in syndecan 4 was seen in comparison to NTC, however when *Adamts1* siRNA depleted cells were treated with MMP inhibitors this phenotype was lost, and a statistically significant increase in syndecan 4 was seen, with expression levels similar to those seen in NTC-treated cells (Figure 3.9).

The recovery of the phenotype when MMPs are inhibited strongly suggests that MMPs are responsible for the cell surface loss of syndecan 4 in response to loss of ADAMTS-1. The predicted mechanism is via ectodomain shedding, however unfortunately shed syndecan 4 could not be detected under any conditions.

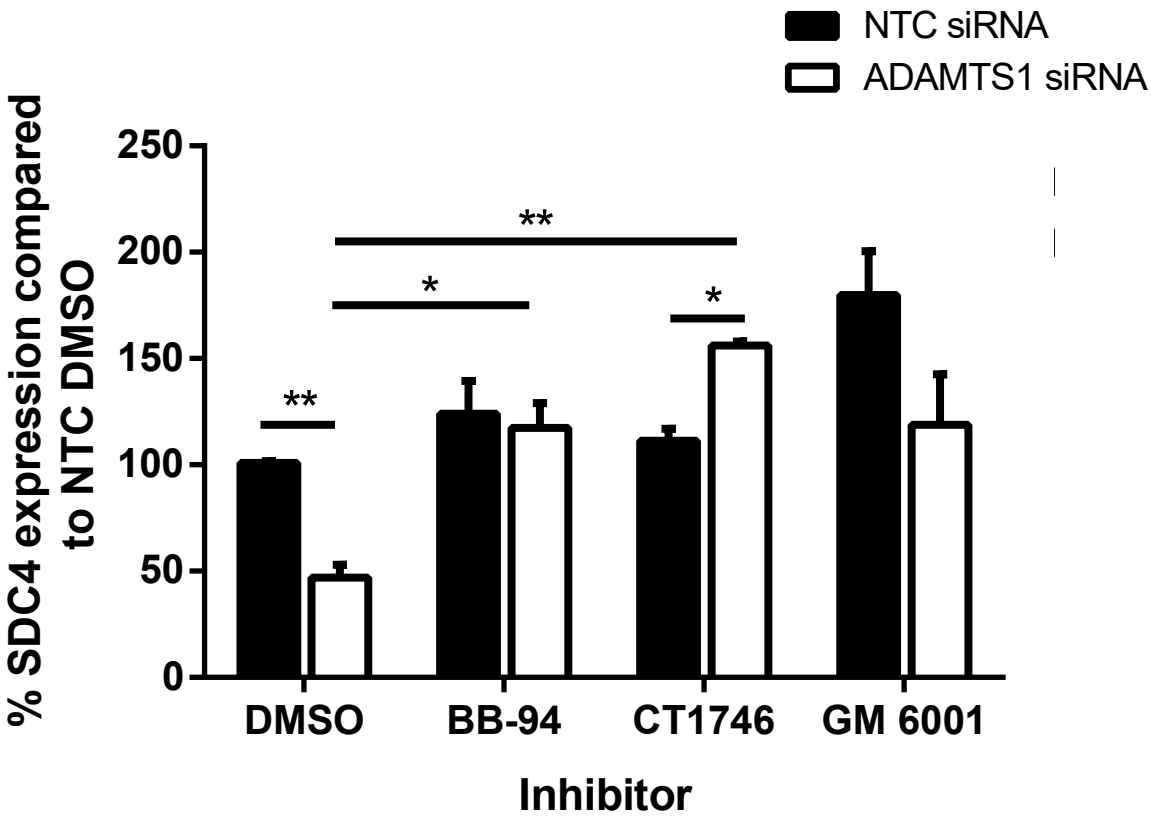


Figure 3.9 MMPs are responsible for the loss of cell surface syndecan 4 in response to ADAMTS-1 siRNA. Cells were transfected with NTC or ***Adamts1*** siRNA, followed by treatment for 18 hours with one of the following broad spectrum MMP inhibitors: 5 μM BB-94, 10 μM CT1746, 10 μM GM6001 or a DMSO vehicle control. Levels of surface syndecan 4 were assessed using flow cytometric analysis. Bar chart shows percentage of syndecan 4 expression compared to NTC cells treated with DMSO. N= 3 independent experiments, bars represent S.E.M, * P< 0.05, ** p <0.01.

3.4.2 ADAMTS-1 siRNA treatment is correlated with increased MMP-9 activity

The inhibitors used in 3.4.1 are broad spectrum, and therefore affect a wide cache of metalloproteinases, including MMPs and other related enzymes. To establish which specific protease was responsible for the loss of syndecan 4, gene expression levels of MMPs in *Adamts1* siRNA treated cells were profiled. A panel of MMPs known to be expressed in endothelial cells and predicted to cleave syndecan 4 were selected. These included MMP-2, MMP-9, ADAMTS-4 and MT1-MMP (MMP-14) (Rodríguez-Manzaneque *et al.*, 2009; Manon-Jensen, Multhaupt and Couchman, 2013; Ramnath *et al.*, 2014).

These MMPs are generally regarded as inducible, and they are often not expressed by the quiescent endothelium; instead their expression is activated as part of the 'angiogenic switch', where the balance of angiogenic factors tilts towards a pro-angiogenic outcome. This is the case for MT1-MMP, which upon activation initiates endothelial cell migration via degradation of the basement membrane, as well as MMP-9, which releases VEGF trapped in the ECM promoting pro-angiogenic signalling (Bergers *et al.*, 2000; Genís *et al.*, 2006). Although the role of MMP-2 in angiogenesis is less well defined, its expression is induced in activated endothelium, and inhibition of MMP-2 has been shown to inhibit angiogenesis and cell migration (Webb *et al.*, 2017). These MMPs have all been shown to cleave the ectodomains of syndecan 1 and syndecan 4 at two membrane proximal regions (Manon-Jensen, Multhaupt and Couchman, 2013).

The fourth protease considered was ADAMTS-4. ADAMTS-4 is expressed by endothelial cells, however as opposed to the other proteases investigated ADAMTS-4 acts in an anti-angiogenic mechanism (Hsu *et al.*, 2012). Further separating ADAMTS-4 from the other MMP candidates where cleavage is membrane proximal, ADAMTS-4 has been shown to clip a small N-terminal fragment, in the same manner as demonstrated for ADAMTS-1 (Rodríguez-Manzaneque *et al.*, 2009).

Cells were siRNA treated, RNA was collected, and TaqMan qPCR was used to determine protease gene expression levels. In the case of ECs, of the four proteases

profiled, *Mmp9* was the only target seen to be upregulated. Under NTC or syndecan 4 siRNA treated conditions *Mmp9* expression was almost undetectable, however after treatment with *Adamts1* siRNA, *Mmp9* expression spiked, indicating that *Mmp9* is selectively induced following ADAMTS-1 depletion and is potentially the protease responsible for the loss of cell surface syndecan 4 (Figure 3.10A). As both ECs and 3T3s displayed the phenotype of cell surface syndecan 4 loss, to determine if the mechanism was universal, *Mmp9* expression was also profiled in 3T3 fibroblasts. In this instance, while a trend in increased *Mmp9* expression was seen, this was not significant (Figure 3.10B).

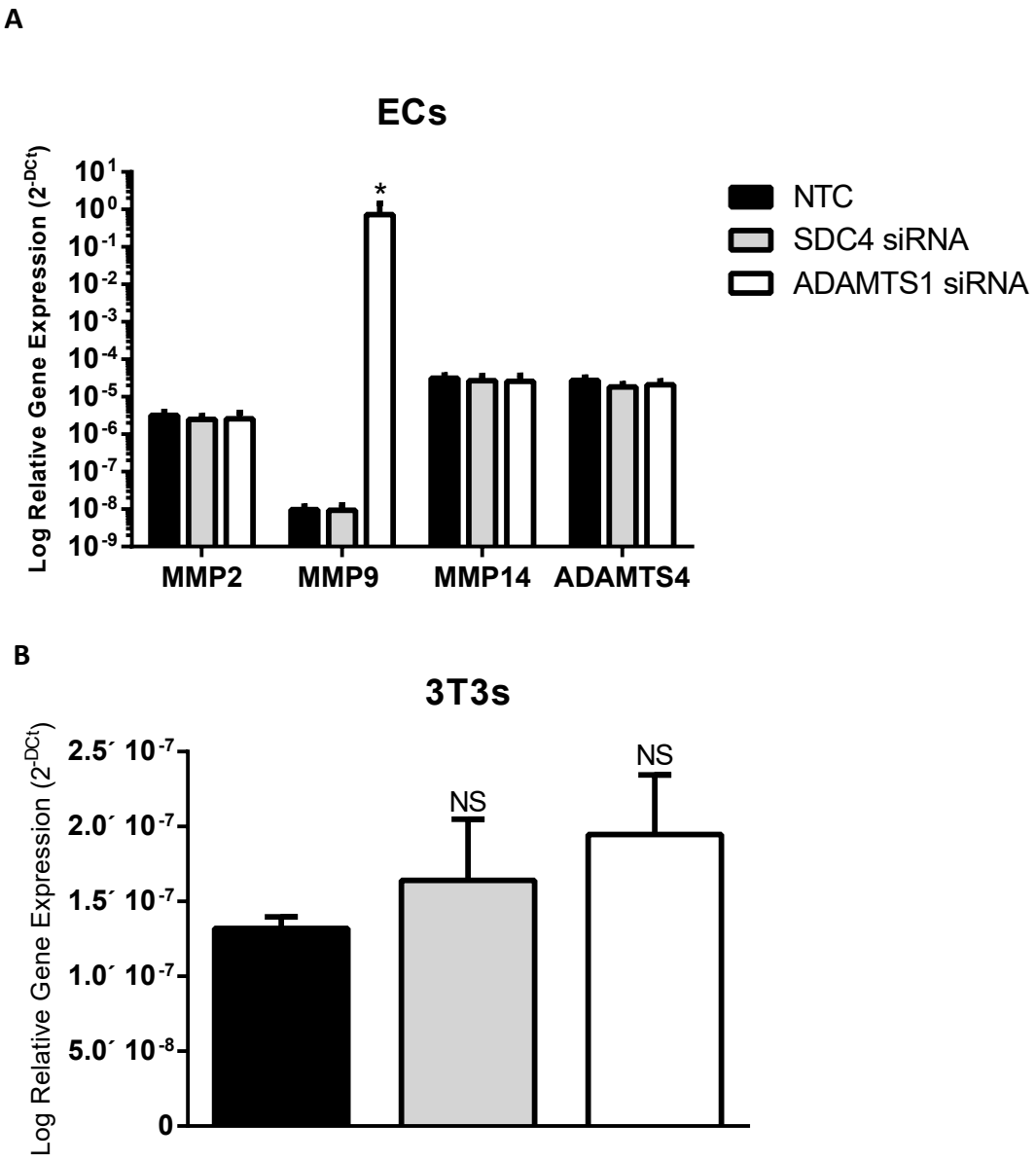


Figure 3.10 *Mmp9* transcription is upregulated in endothelial cells siRNA depleted for ADAMTS-1. A) ECs were siRNA depleted of *Adamts1*, *Sdc4* or NTC, RNA was collected 24 hours post transfection, and TaqMan qPCR was performed for proteases: *Mmp2, Mmp9, Mmp14* and *Adamts4*. or B) 3T3s were siRNA depleted of *Adamts1*,, syndecan 4 or NTC, RNA was collected 24 hours post transfection, and TaqMan qPCR was performed for *Mmp9.* Graphs display relative gene expression (2-ΔCt), values are averaged from three independent experiments. Bars = S.E.M, *P < 0.05, NS = not significant.

Although an upregulation in *Mmp9* expression was seen, the functional relevance of this was yet to be established. Total quantity of MMP protein does not always reflect the level of MMP activity, often due to the complicated balance between proteases and their endogenous inhibitors (Lowrey *et al.*, 2008). To establish whether the transcriptional increase in *Mmp9* was reflected by an increase in MMP-9 activity, gelatin zymography was performed. Gelatin zymography utilises the digestion of the major substrate of MMP-2 and -9, gelatin, as a marker of gelatinase activity.

Zymography using media samples of ECs treated with NTC, *Adamts1* or *Sdc4* siRNA revealed increased MMP-9 activity (105 kDa) in ADAMTS-1 depleted cells, as compared to NTC or syndecan 4 (Figure 3.11). No MMP-2 activity was detected (72 kDa). These data support the qPCR results, and demonstrate that the increased *Mmp9* transcription in response to ADAMTS-1 knockdown resulted in a functionally relevant increase in MMP-9 activity.

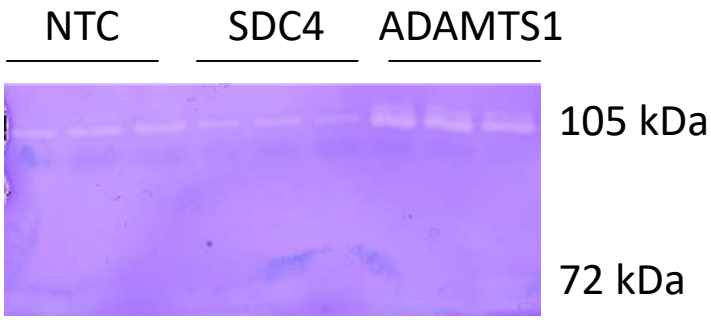


Figure 3.11 MMP-9 activity is increased in response to *Adamts1* siRNA. Cells were siRNA depleted using NTC, *Sdc4* or *Adamts1* siRNA. Serum free media was added to cells and collected after 16 hours incubation. Media samples were run on a gelatin embedded polyacrylamide gel. The gel was incubated to allow gelatinases to unfold and cleave gelatin, followed by Coomasie staining to reveal sites of digestion. Image is representative of three independent experiments.

3.4.3 MMP-9 specific inhibition prevents the loss of syndecan 4

TaqMan and zymography revealed a clear induction of MMP-9 activity in response to ADAMTS-1 knockdown. To confirm that syndecan 4 cell surface loss was dependent on the induction of MMP-9, MMP-9 was inhibited using the specific small molecule inhibitor SB-3CT or siRNA, followed by quantification of syndecan 4 using flow cytometry.

Treatment with *Mmp9* siRNA resulted in accumulation of syndecan 4 on the cell surface relative to NTC, supporting a role for MMP-9 in homeostatic regulation of syndecan 4. Treatment with *Adamts1* and *Mmp9* siRNA together did not result in a reduction in surface syndecan 4, indicating that MMP-9 is necessary for this phenotype (Figure 3.12).

Experiments with the specific inhibitor SB-3CT supported the siRNA data. The loss of syndecan 4 after treatment with *Sdc4* siRNA could not be rescued by MMP-9 inhibition, however syndecan 4 expression in *Adamts1* siRNA treated cells was massively upregulated after SB-3CT treatment (Figure 3.12). These data provide convincing evidence that the loss of syndecan 4 in response to ADAMTS-1 knockdown is dependent upon the induction and activity of MMP-9.

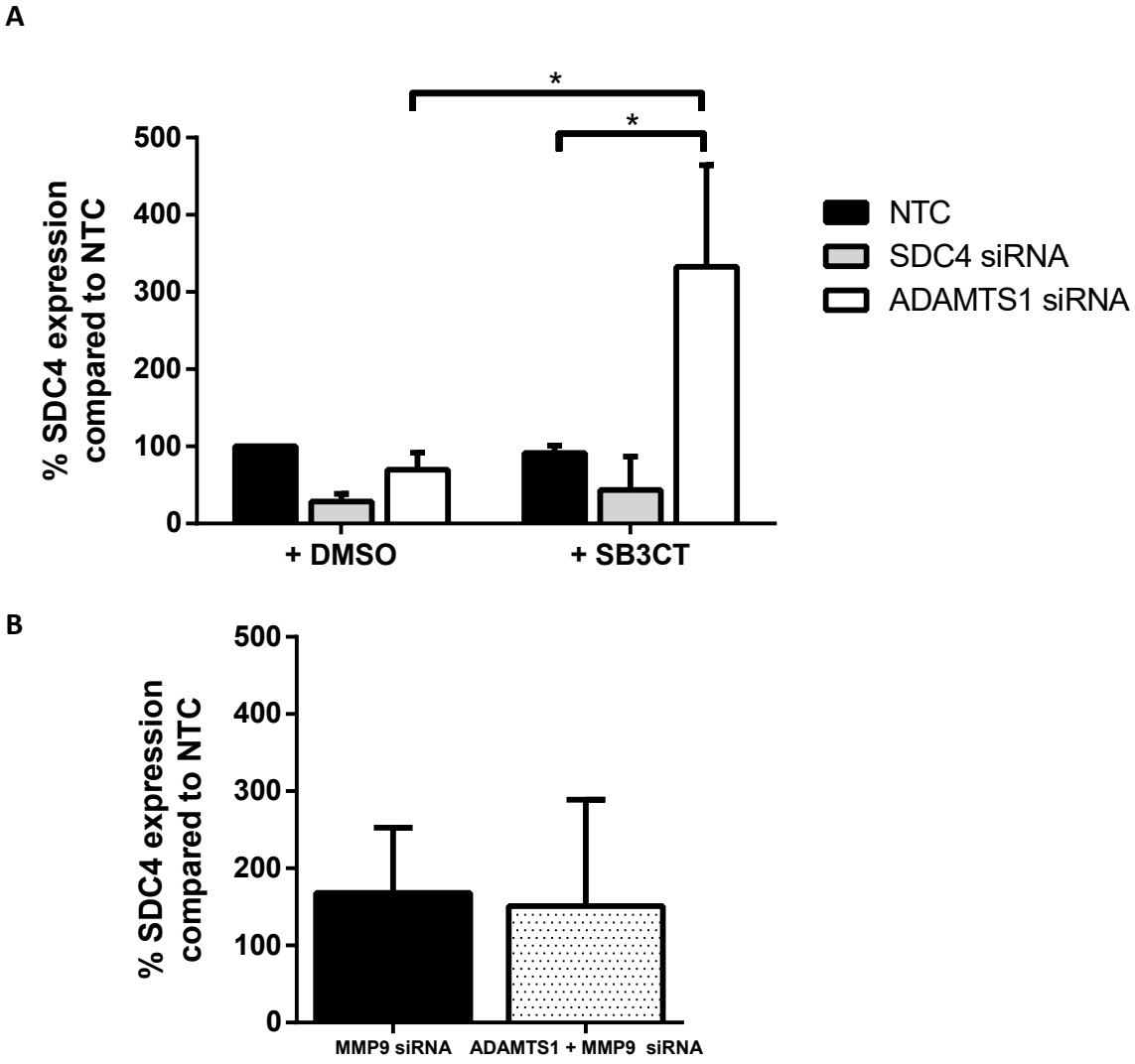


Figure 3.12 Loss of syndecan 4 in response to *Adamts1* siRNA is dependent on MMP-9. A) Cells were treated with NTC, *Sdc4* or *Adamts1* siRNA, followed by 16 hour treatment with the MMP-9 specific inhibitor SB-3CT or a DMSO vehicle control. Cells were collected and flow cytometric analysis was performed to quantify surface SDC4 levels. N = 3, Bars represent S.E.M, * = P<0.05. B) Endothelial cells were treated with NTC, *Adamts1*, *Mmp9* or *Adamts1* and *Mmp9* siRNA, cells were collected and flow cytometry for syndecan 4 was performed. Graph shows percentage expression of *Sdc4* compared to NTC.

MMP-9 can be regulated in a number of ways. Regulation of *Mmp9* transcription is one mechanism, however other factors such as signalling and TIMP activity play important roles. The TIMPs tightly regulate MMP activity, and therefore when trying to uncover the mechanism behind increased MMP-9 activity TIMPs were a likely target.

TaqMan qPCR was performed for all four mammalian *Timps* to determine their expression levels, as they have all been shown to regulate MMP-9 activity to some degree (Murphy, 2011). *Timp3* was the only TIMP whose expression could be detected in endothelial cells. There was a trend toward decreased *Timp3* expression in response to ADAMTS-1 knockdown, but this was not statistically significant (Figure 3.13)

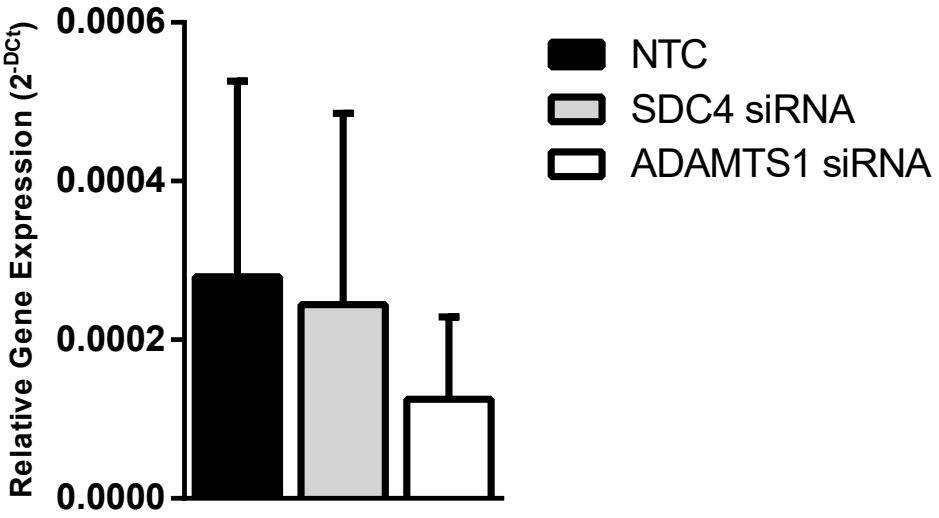


Figure 3.13 *Timp3* expression in siRNA treated ECs. Endothelial cells were treated with siRNA against NTC, *Sdc4* or *Adamts1*, 24 hours post transfection RNA was collected, and reverse transcribed. TaqMan qPCR was performed to determine levels of *Timp3* expression. Relative gene expression ($2^{-\Delta}$Ct) was averaged from three independent experiments, error bars represent S.E.M.

3.5 ADAMTS-1 overexpression results in the loss of cell surface syndecan 4

It was initially hypothesised that the loss of ADAMTS-1 would protect syndecan 4 from shedding by this protease, increasing syndecan 4 cell surface level, however our data revealed that *Adamts1* siRNA decreased syndecan 4 at the cell surface. Therefore, to build upon this data, we utilised constructs to overexpress ADAMTS-1.

3.5.1 ADAMTS-1 overexpression using plasmid vectors

3T3s were transfected with an empty vector, a myc-labelled full length human ADAMTS-1 (ADAMTS-1-myc) or a myc labelled catalytically dead human ADAMTS-1 (ADAMTS-1-Z11). Antibiotic selection was used to give stably transfected lines, and expression of constructs was validated using western blotting. Unfortunately we were unable to successfully transfect ECs with these constructs.

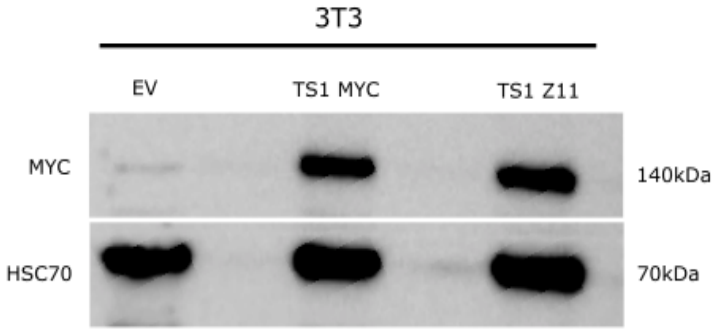


Figure 3.14 ADAMTS-1 overexpression plasmids were successfully transfected into 3T3 fibroblasts. Lipofectamine was used to transfect 3T3 fibroblasts with ADAMTS-1 plasmids, followed by antibiotic selection. Cells were lysed, and protein was western blotted for myc-tagged ADAMTS-1.

3.5.2 Overexpression of WT, but not catalytically inactive ADAMTS-1 results in a loss of cell surface syndecan 4

Fibroblasts expressing ADAMTS-1-Myc or ADAMTS-1-Z11, were collected in citric saline buffer, followed by flow cytometry for syndecan 4. Flow cytometry found that overexpression of full length, but not catalytically inactive ADAMTS-1 causes a statistically significant reduction in the level of cell surface syndecan 4 (Figure 3.15).

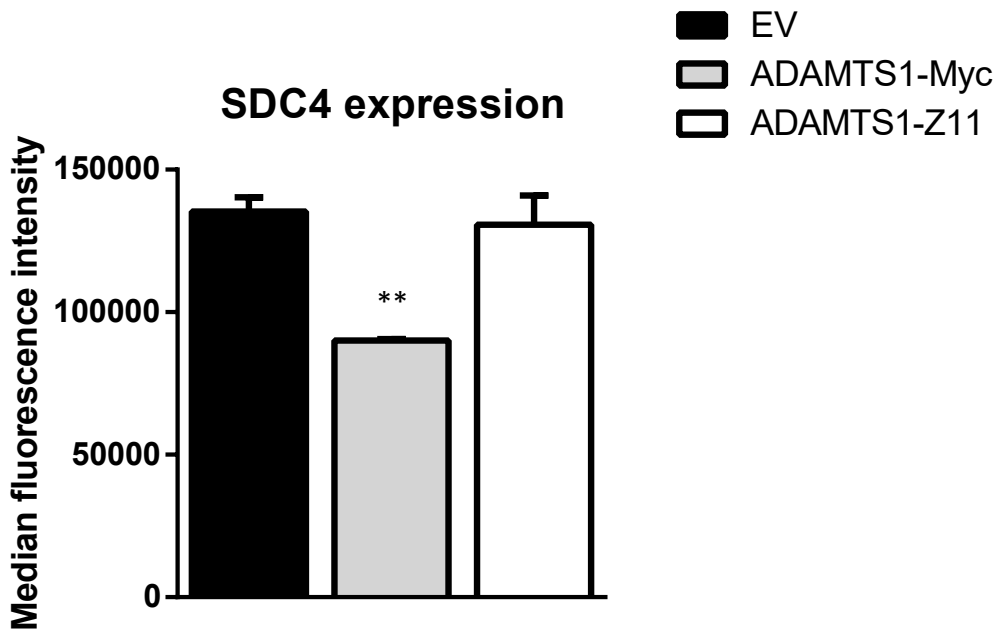


Figure 3.15 Overexpression of full length ADAMTS-1 decreases cell surface syndecan 4. 3T3s overexpressing ADAMTS-1 or a catalytically inactive mutant were collected in citric saline buffer, followed by immunolabelling of syndecan 4 and quantification via flow cytometry. Graph shows median fluorescence intensity values relative to an isotype control. Values represent means averaged from three independent experiments. Bars = S.E.M. ** = $P < 0.01$.

3.6 Discussion

It is clear from the literature that connections exist between members of the ADAMTS and syndecan families. We set out to investigate possible interconnections between ADAMTS-1 and syndecan 4 using two cell models, ECs and 3T3s.

The results of this chapter demonstrate an interdependence between ADAMTS-1 and syndecan 4. Upon siRNA depletion of ADAMTS-1, a corresponding reduction in cell surface syndecan 4 is seen. This phenotype was dependent on the action of proteases, and could be abrogated through the inhibition of MMP-9.

Previous work by Rodríguez-Manzaneque *et al.* found that ADAMTS-1 is capable of cleaving syndecan 4, clipping the N-terminal (Rodríguez-Manzaneque *et al.*, 2009). The hypothesis at the outset of this project was therefore that ADAMTS-1 knockdown would increase the surface expression of syndecan 4, however the contrary was found. In *Adamts1* siRNA treated ECs and 3T3s, a statistically significant reduction in cell surface syndecan 4 was detected by flow cytometry. The possibility of off-target effects of the siRNA was considered, however 4 different siRNAs were trialled, all of which induced this phenotype (data not shown).

The mechanism of ADAMTS-1-syndecan 4 cleavage may go some way towards explaining this result. Cleavage of syndecans is typically carried out by metzincin family proteases and occurs juxta membrane. In this manner, intact proteoglycan fragments which retain their growth factor binding ability are often generated. ADAMTS-1's cleavage of syndecan 4 is, however, atypical, as only a small 6 kDa fragment at the N- terminal is clipped from the protein. This cleavage was shown to be physiologically relevant, resulting in defects in adhesion and gain of migratory capacities. However, this cleavage mechanism does not necessarily result in loss of syndecan 4 from the cell surface, as the proteoglycan is merely adapted. It is possible that clipping of syndecan 4 in this way may protect the proteoglycan from further protease activity and shedding, but further investigation would be needed to support this hypothesis.

It is also important to note that ADAMTS-1 overexpression also resulted in a loss of syndecan 4 at the cell surface, in a mechanism that was dependent upon ADAMTS-1's catalytic activity. These data support a role for ADAMTS-1 in the shedding of syndecan 4, however we were unable to detect shed syndecan 4 in the media to support this conclusion. There are also several other difficulties when interpreting these data; firstly, the constructs overexpressed human ADAMTS-1, as these studies were conducted in mouse cells this may lead to unexpected results. Secondly, we were unable to detect murine or human ADAMTS-1 using western blotting, and therefore expression levels of ADAMTS-1 could not be validated. Finally, we were unable to successfully transfect ECs, which prohibited validating results using this second cell line.

Despite these issues, the contrasting knockdown/overexpression data highlight a need to consider 'dose dependent' effects, as it is possible that ADAMTS-1 has different functions depending upon expression level. Concentration dependent phenomena have been previously reported for ADAMTS-1; in work by Werner *et al*, low ADAMTS-1 concentrations stimulated migration, whereas high doses were inhibitory (Krampert *et al.*, 2005).

The mechanism by which syndecan 4 is lost from the cell surface in response to *Adamts1* siRNA is not completely clear, however it seems plausible that it is due to increased shedding of the proteoglycan by MMP-9. A clear increase in MMP-9 expression and activity was seen upon *Adamts1* siRNA treatment, and specific disruption of MMP-9 activity via an inhibitor or siRNA was sufficient to reverse the phenotype. MMP-9 has been reported to cleave syndecan 4 in response to TNF-α; therefore it is reasonable to conclude that the increase in MMP-9 leads to increased shedding of syndecan 4, as unlike the clipping of ADAMTS-1 by syndecan 4, MMP-9 mediated cleavage results in the shedding of intact proteoglycan ectodomains (Ramnath *et al.*, 2014). Unfortunately, in the context of this work, shed fragments of syndecan 4 could not be detected in the media under any conditions, limiting the strength of this conclusion. It is also not yet fully clear if this is mechanism is universal.

MMP-9 transcription was seen to be upregulated in fibroblast cells, but this remains inconclusive as changes were not significant.

Ectodomain shedding is an important mechanism required to properly regulate syndecan function, and the accumulation of syndecan 4 in response to MMP inhibitors supports homeostatic roles of these proteases in governing syndecan expression. Of note, specific blockade or knockdown of MMP-9 demonstrates its key role, as inhibition of this protease alone was sufficient to cause significant accumulation of cell surface syndecan 4.

As well as being part of regulated cell behaviour, syndecan shedding can be accelerated in response to pathophysiological events to alter signalling, adhesion and migration, as the ectodomain has the potential to act as a paracrine or autocrine effector, whereas the remaining membrane bound portion is unable to interact with growth factors (Manon-Jensen, Itoh and Couchman, 2010). For example, enzymes such as MMPs upregulated in glioma tumorigenesis stimulate shedding of the syndecan 2 ectodomain from endothelial cells, however the role of this ectodomain in either promoting or inhibiting angiogenesis is controversial, with differing reports existing (Fears, Gladson and Woods, 2006; Rossi *et al.*, 2014).

Further work to identify a shed syndecan 4 fragment, followed by investigation into any contributions it may make to angiogenic signalling would greatly advance this work.

As of yet, it remains unclear how the loss of ADAMTS-1 expression results in an upregulation of MMP-9 expression and activity. MMP-9 can be regulated in a number of ways. MMP-9 expression is inducible and regulated by transcription factors. An Ap-1 consensus sequence is considered the most critical binding motif for MMP transcriptional activity, yet maximal expression of the protease requires other transcription factors including Sp-1 and NF-κb (Mittelstadt and Patel, 2012). MMP expression is also tightly regulated by TIMPs, however although an increase in *Timp3* expression was seen, this was not significant.

Signalling is also important in MMP-9 expression, in particular there is a link between MMP-9 expression and PKC, with PKC inhibitors seen to suppress MMP-9 expression. This is of particular interest as syndecan 4 has been seen to regulate the localisation and activity of PKCα (Oh, Woods and Couchman, 1997b; Keum *et al.*, 2004). The PKC cascade can regulate the recruitment of MMP-9 to podosomes, and its release and activation (Xiao *et al.*, 2010). It is also not completely clear how sheddases are influenced to cleave syndecan, however it is thought to be stimulated by numerous growth factors and chemokines (Manon-Jensen, Itoh and Couchman, 2010).

Both ADAMTS-1 and MMP-9 are involved in VEGF signalling, and their regulation therefore may be dependent on a fine angiogenic signalling balance. ADAMTS-1 is anti-angiogenic, and can bind and sequester $VEGF_{165}$, whereas MMP-9 is highly pro-angiogenic, and can trigger the 'angiogenic switch', a process in tumours where the balance of pro and anti- angiogenic factors swings towards a pro-angiogenic outcome (Bergers *et al.*, 2000). MMP-9 promotes release of VEGF, primarily $VEGF_{165}$, bound in the matrix to heparan-sulfate chains through cleavage (Hawinkels *et al.*, 2008). VEGF induces MMP-9 expression which leads to elevated VEGF levels resulting in a positive feedback loop (Hollborn *et al.*, 2007). Similarly, VEGF can upregulate ADAMTS-1 expression - suggesting a negative feedback mechanism at play (Xu, Yu and Duh, 2006). It seems possible that loss of ADAMTS-1 disturbs the fine balance of VEGF signalling, resulting in the cells switching to a more angiogenic phenotype, and the upregulation of MMP-9, cleavage of syndecan 4 and other HS chains, release of VEGF and more pro-angiogenic signalling. Alterations to the VEGF signalling balance triggering MMP-9 activation may also explain why expression of MMP-9 was not as dramatic in the fibroblast cells, which are traditionally more responsive to growth factors such as FGF. Future work investigating signalling and transcriptional regulation of MMP-9 would hopefully shed some light on these unanswered questions.

3.7 Chapter summary

In conclusion, the loss of ADAMTS-1 results in a corresponding loss of syndecan 4 in an MMP-9 dependent manner, although it remains unclear how MMP-9 activity is triggered. Overexpression of ADAMTS-1 also results in a reduction of syndecan 4 at the cell surface, in a mechanism dependent upon ADAMTS-1's catalytic activity. The next chapters set out to answer questions with regards to the physiological relevance of this interdependency. As the overexpression strategy involved the use of human rather than mouse constructs, siRNA mediated knockdowns were the focus of future studies.

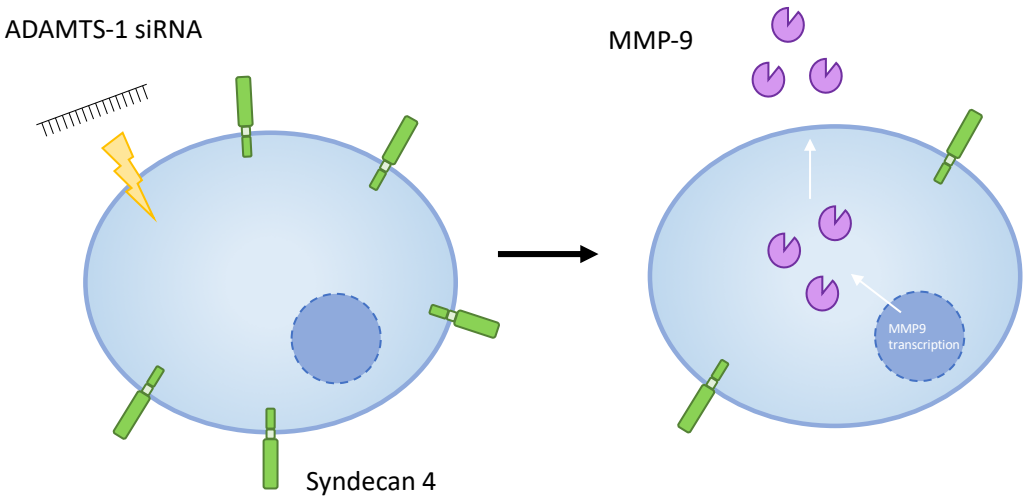


Figure 3.16 Graphical summary of chapter three findings. Knockdown of ADAMTS-1 in endothelial cells via siRNA results in a loss of cell surface syndecan 4, dependent upon increased MMP-9 transcription and activation.

4 Syndecan 4 and ADAMTS-1 sequester VEGF-A_{164} and inhibit angiogenesis

As chapter three demonstrated an interdependency between ADAMTS-1 and syndecan 4, the next stage was to therefore evaluate the functions of these two proteins, and potential collaboration within their roles. The roles of ADAMTS-1 and syndecan 4 centre around the ECM, and therefore processes which rely upon a high degree of ECM interaction were considered most likely to be affected by ADAMTS-1 and syndecan 4. The ECM plays a number of key roles in angiogenesis, it must be degraded to release ECs, growth factors, and cryptic bioactive sites, and released ECs must migrate upon the matrix (Neve *et al.*, 2014). As ADAMTS-1 is a well-defined anti-angiogenic protein, the initial focus of the roles of these proteins therefore focused upon angiogenesis.

ADAMTS-1 inhibits angiogenesis via two distinct mechanisms; firstly it can function catalytically, generating a pool of anti-angiogenic peptides by cleavage of TSP1 and 2. Secondly, ADAMTS-1 can act independent from its catalytic activity, binding and sequestering $VEGF_{165}$, preventing it from activating its major receptor VEGFR2 and thereby inhibiting pro-angiogenic signalling (Luque, Carpizo and Iruela-Arispe, 2003; Lee *et al.*, 2006).

The role of syndecan 4 in angiogenesis is less well defined, however in the case of diabetes mellitus, the shedding of syndecan 4 has been seen to inhibit angiogenesis (Li *et al.*, 2016). Syndecan 4 has also been demonstrated to function in non-VEGF induced angiogenesis; the process of angiogenesis is dependent upon multifactorial signalling pathways, and emerging evidence highlights its interconnection with inflammation. During inflammation recruited immune cells secrete pro-angiogenic factors, and the two processes are believed to be interdependent, as newly developed vasculature supports the recruitment of immune cells to sites of inflammation. PGE_2 is a pro-inflammatory lipid, which functions via activation of GPCRs, and has been seen to promote tube formation of HUVECs *in vitro* (Zhang and Daaka, 2011). Induction of angiogenesis by PGE_2 appears to rely on syndecan 4

activation of PKCα, as in *Sdc4-/-* endothelial cells PGE_2 induction of ERK is reduced (Corti *et al.*, 2013).

This chapter therefore sought to investigate the roles of these two proteins in angiogenesis, with the aim of exploring any co-operation between the them, and of clarifying the role of syndecan 4.

4.1 ADAMTS-1, syndecan 4 and VEGF-A associate *in vitro*

Several lines of evidence hint at a physical interaction between ADAMTS-1 and syndecan 4. Both proteins bind the extracellular matrix, and in the case of ADAMTS-1 this interaction is likely mediated by GAGs (Kuno and Matsushima, 1998). Glycosaminoglycans are unbranched polysaccharides consisting of a repeating disaccharide unit. When attached to a protein they from a proteoglycan, such as the ECM protein, and major substrate of ADAMTS-1, aggrecan. Core proteins are synthesised in the rough endoplasmic reticulum, they then transit the secretory pathway, and are posttranslationally modified in the Golgi by glycosylation, generating the GAG chains (Zulueta *et al.*, 2015). One source of ECM GAGs is in the HS-GAG chains covalently attached to the syndecan 4 core protein, intimating the possibility that syndecan 4 provides a linkage between ADAMTS-1 and the ECM (Woods and Couchman, 1994).

Another potential interaction between syndecan 4 and ADAMTS-1 intersects at VEGF. Syndecan 4 has been predicted to bind VEGF due to its capabilities for interaction with heparin binding growth factors (Elfenbein and Simons, 2013). ADAMTS-1 binds and sequesters VEGF; this interaction is exclusive to $VEGF_{165}$ as this isoform contains a heparin binding domain upon which the connection is dependent (Iruela-Arispe, Carpizo and Luque, 2003). Previous work by Ireula-Irispe *et al.* has suggested that heparin functions as a chaperone to facilitate the ADAMTS-1 VEGF interaction, as *in vitro*, addition of exogenous heparin increased ADAMTS-1 to VEGF binding. Interestingly, *in vivo,* the exogenous addition of heparin was not a requirement for ADAMTS-1 VEGF binding, and it was therefore hypothesised that a heparan sulfate proteoglycan, such as a member of the syndecan family, may act as a bridge to facilitate this contact (Iruela-Arispe, Carpizo and Luque, 2003).

To establish if physical connections between these proteins exists in the context of endothelial cells, and to test the hypothesis that syndecan 4 may contribute to the regulation of angiogenesis via aiding in ADAMTS-1's sequestration of VEGF, co-immunoprecipitations were performed. As reliable antibodies against mouse

syndecan 4 could not be found, human umbilical vein endothelial cells (HUVEC) were utilised. Unfortunately, neither mouse nor human antibodies were able to reliably detect ADAMTS-1. Depletion of targets in culture was achieved using lentiviral shRNAs, and successfully transfected cells were selected for using puromycin. Cells were lysed in RIPA buffer and lysates were immunoprecipitated for VEGF-A.

Immunoprecipitations for VEGF-A were able to co-precipitate syndecan 4, revealing that syndecan 4 either directly or indirectly binds VEGF-A. Lentiviral silencing of ADAMTS-1 reduced the amount of syndecan 4 co-precipitated, suggesting a functional connection dependent on both proteins (Figure 4.1). However, since chapter 3 showed that depletion of ADAMTS-1 reduced cell surface syndecan 4, this may also be a contributing factor.

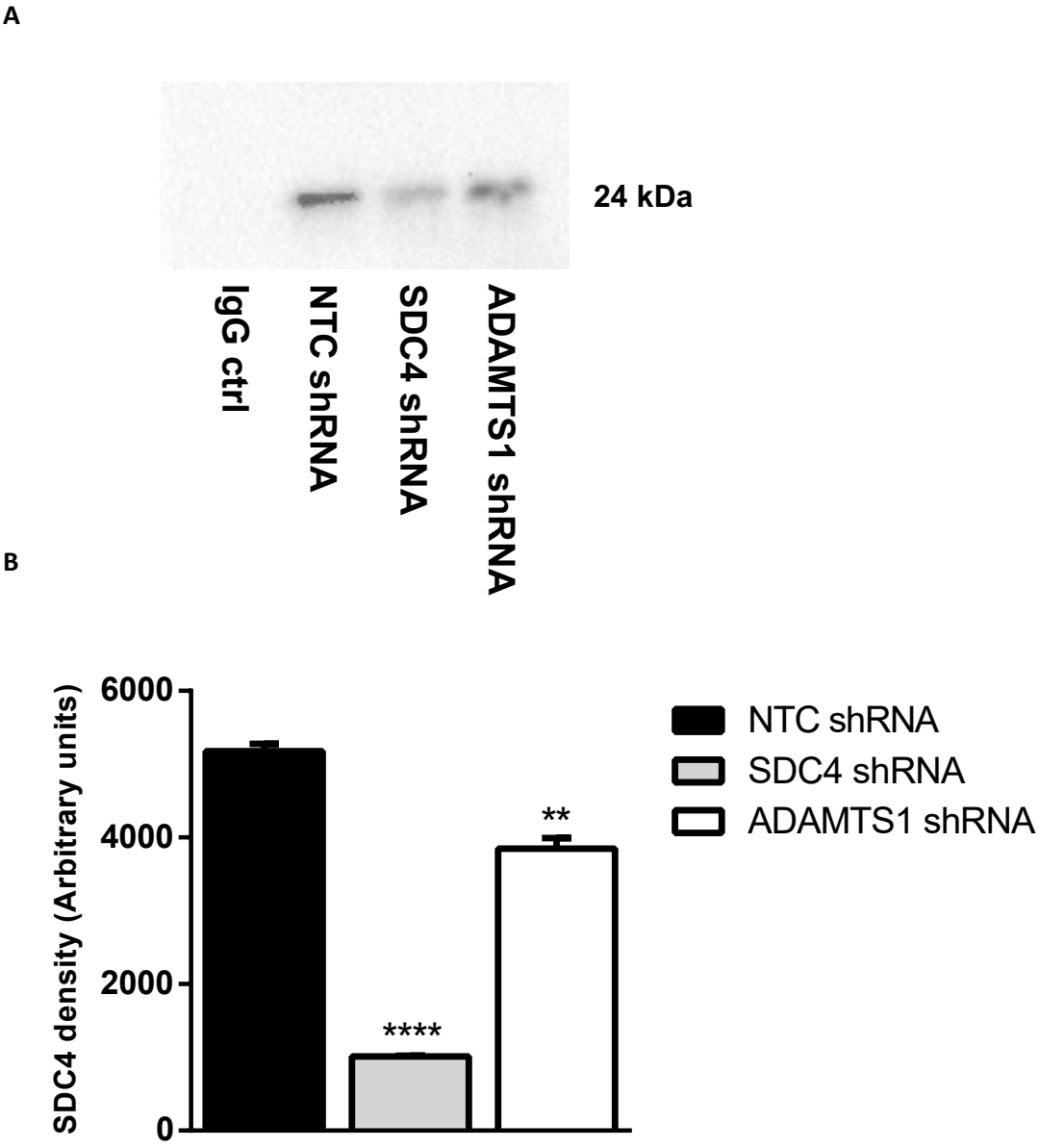


Figure 4.1 VEGF-A and Syndecan 4 form complexes in endothelial cells. Lentiviral shRNAs against NTC, *Sdc4* or *Adamts1* were used to silence targets in HUVECs. Transfected cells were lysed and protein concentrations equalised, followed by immunoprecipitations using a VEGF-A antibody. An IgG control was used to rule out non-specific binding. The presence of co-precipitated SDC4 was determined by western blotting. A) representative image of western blotting of immunoprecipitated protein, bands represent SDC4. B) Image J densitometric quantification of three independent western blots (error bars represent S.E.M, **P<0.01, ****P<0.0001).

4.2 Loss of ADAMTS-1 or Syndecan 4 increases signalling in response to VEGF-A_{164}

Immunoprecipitations clearly demonstrate that syndecan 4 is capable of binding VEGF, however the physiological relevance of this was yet to be established. The formation of Syndecan 4-VEGF complexes could imply that Syndecan 4 acts as a VEGF receptor or co-receptor as it does for other growth factors such as FGF2 (Jang *et al.*, 2012). Alternatively, syndecan 4 could be functioning with ADAMTS-1 and sequestering VEGF, a hypothesis supported by the function of other HSPGs in VEGF trapping (Tkachenko *et al.*, 2004; Kadenhe-Chiweshe *et al.*, 2008).

To gain initial insight into the role of syndecan 4 in angiogenesis, the impact of ADAMTS-1 or syndecan 4 knockdown on VEGF signalling was investigated. In the major pro-angiogenic pathway, VEGF-A activates the receptor tyrosine kinase VEGFR2, activating downstream signalling pathways controlling proliferation, survival, migration, and inhibiting apoptosis. One well described signalling response to VEGF is MAPK pathway activation, and therefore VEGFR2 and ERK phosphorylation were used as markers of pro-angiogenic signalling in response to exogenous VEGF addition.

For this work we returned to the murine endothelial cells, as $VEGF_{164}$ (the mouse equivalent of human $VEGF_{165}$) was prepared in house, and prior work had established VEGF dosing protocols for these cells (Ellison *et al.*, 2015). Endothelial cells were serum-starved for 3 hours to synchronise background signalling, then stimulated with 30 ng/mL VEGF-A_{164} in serum free media. Cells were lysed at 0, 5, 15, and 30 minutes post stimulation and western blotted to assess any changes in signalling.

Western blotting revealed increased levels of phospho-VEGFR2 and phospho-ERK in ADAMTS-1 or syndecan 4 siRNA knockdown cells, indicating increased VEGF induced pro-angiogenic signalling (Figure 4.2). These data suggest that syndecan 4 is not acting as a VEGF co-receptor, and instead support the hypothesis that ADAMTS-1 and syndecan 4 sequester VEGF-A_{164} to inhibit angiogenesis, and that when they are depleted VEGF is more readily available to activate its receptor.

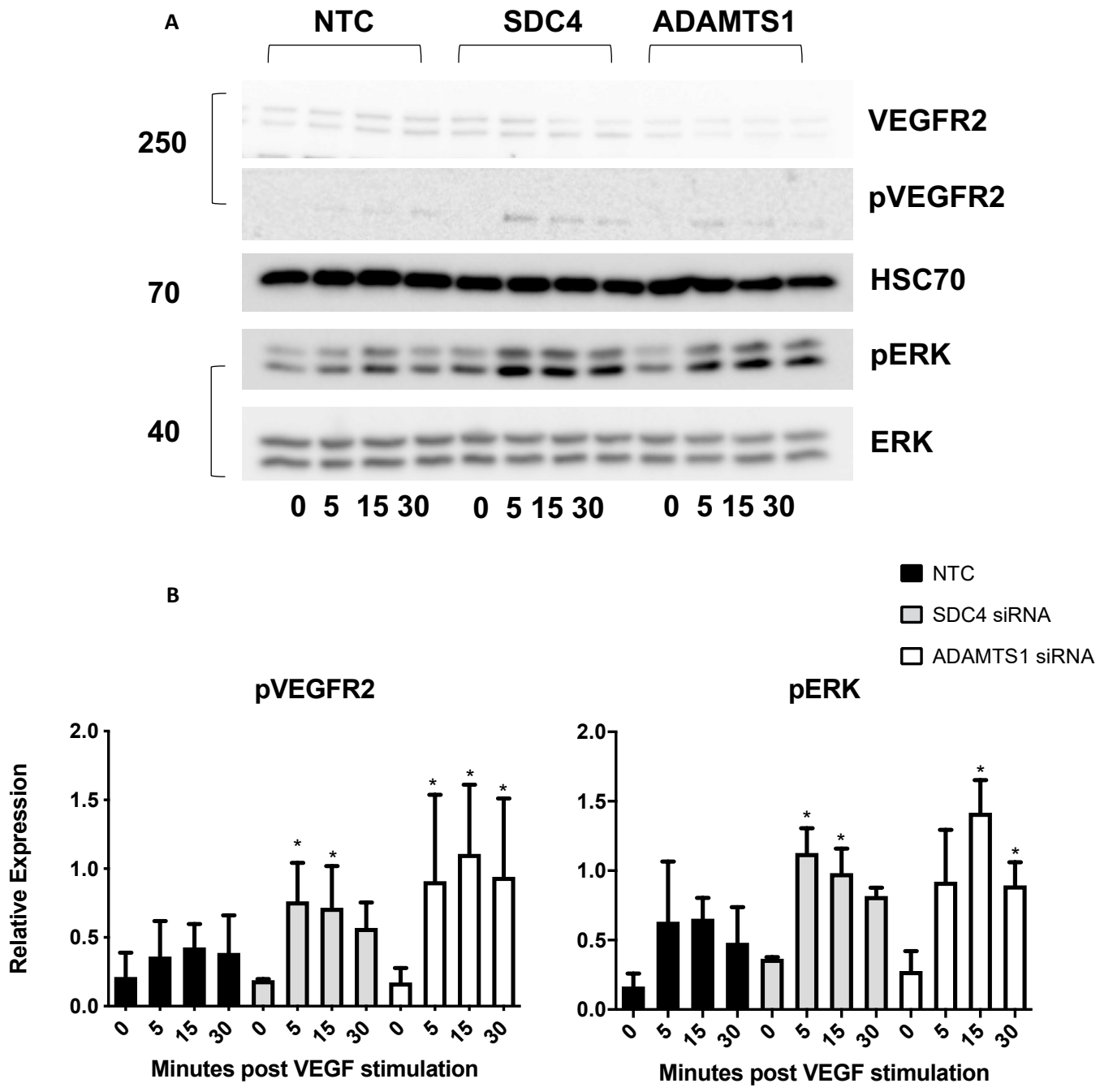


Figure 4.2 Depletion of ADAMTS-1 or syndecan 4 results in increased VEGF signalling. Endothelial cells were serum starved for 3 hours, then stimulated with **VEGF-A_{164}** and lysed at 0, 5, 15 or 30 minutes post stimulation. Western blots were performed on lysates with anti-VEGFR2, ERK, and their phosphorylated forms. HSC70 was used as a loading control. A) Representative blot from 4 independent experiments. B) densitometric quantification performed in ImageJ, levels of phosphorylated protein are shown relative to total protein, N=3 independent experiments, error bars represent S.E.M.

4.3 ADAMTS-1 and syndecan 4 sequester VEGF-A_{164}

Based on the capacity of syndecan 4 to bind VEGF-A, and the increased signalling seen upon its depletion, it was hypothesised that syndecan 4 was contributing to ADAMTS-1's sequestration of VEGF. To confirm this, the capability of endothelial cells to bind and sequester VEGF-A_{164} was assessed using an ELISA.

Endothelial cells were cooled to 4°C to inhibit signalling, 30 ng/mL VEGF-A_{164} in ice cold serum free media was then added to ECs, followed by incubation for 30 minutes at 4°C, allowing cells to bind the VEGF. The media was then recovered, and a sandwich ELISA was performed to determine the amount of VEGF-A_{164} remaining free in the media. Both *Adamts1* and *Sdc4* siRNA treatment increased the amount of free VEGF-A_{164} in the media compared to NTC cells, and when *Adamts1* and *Sdc4* siRNA treatments were given in combination, virtually all added VEGF-A_{164} could be recovered (Figure 4.3A).

This increase in free VEGF-A_{164} could reflect reduced ability of the ADAMTS-1/syndecan 4 knockdown cells to sequester VEGF-A_{164}, and could also explain the increased VEGF-A_{164} signalling seen in these cells in Figure 4.2. However, before a firm conclusion was made, other possibilities were explored. To confirm that incubation at 4°C was adequate to prevent cell signalling, and thereby uptake of VEGF-A_{164} by receptor internalisation, which would affect the concentration of VEGF in the media, western blots for VEGF-A signalling cascade proteins were performed. Western blots revealed no increase in VEGFR2 phosphorylation, indicating that signalling was not activated (Figure 4.3B). An alternate possibility was that the increase in VEGF-A_{164} seen was due to increased production and secretion in ADAMTS-1 and syndecan 4 siRNA cells. TaqMan qPCR was used to investigate this concept, and it was found that levels of *VegfA* transcription were unchanged between NTC, syndecan 4 and ADAMTS-1 siRNA treated cells. These data combined allow for the conclusion that ADAMTS-1 and syndecan 4 conspire to sequester VEGF-A_{164}.

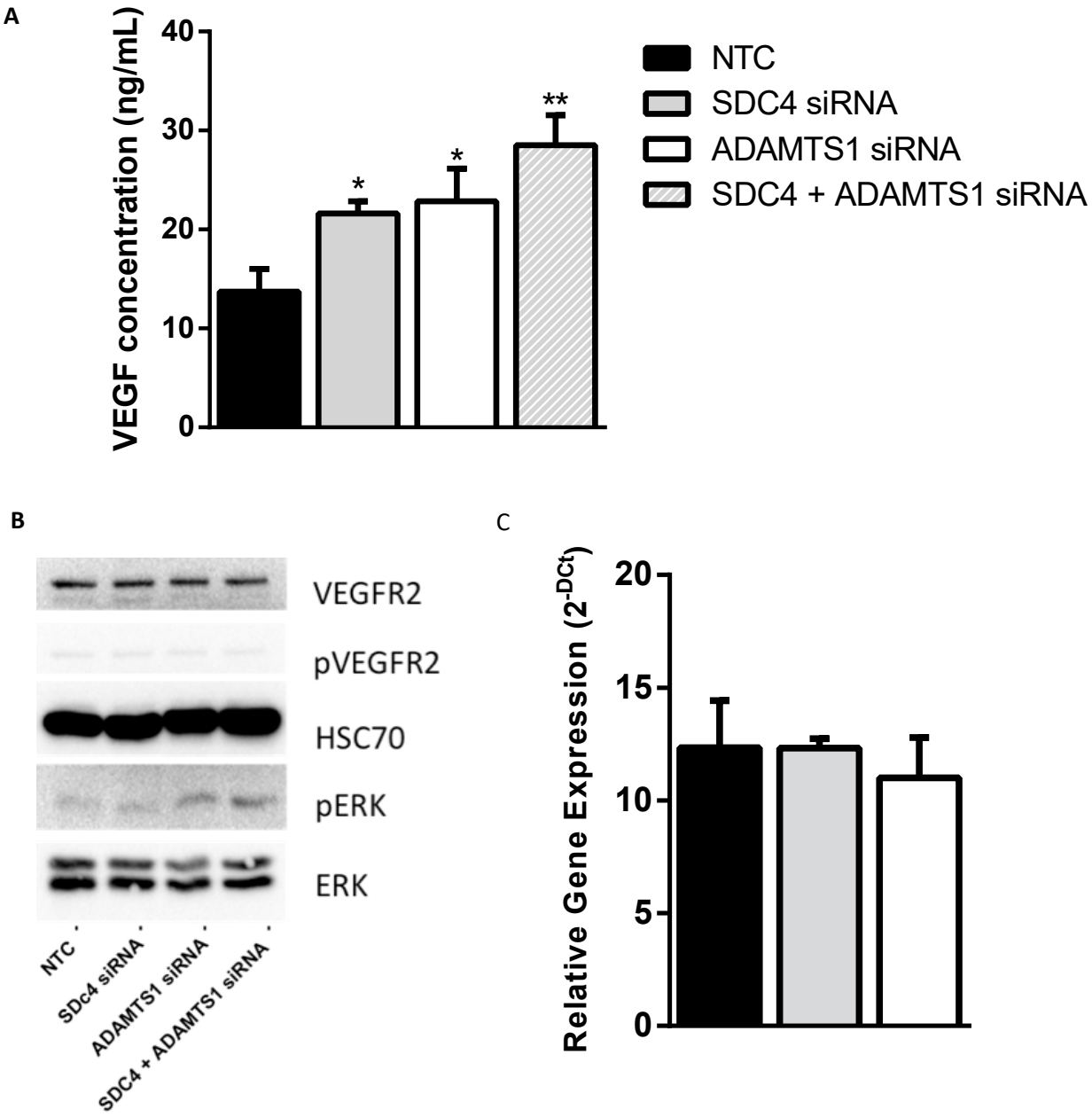


Figure 4.3 ADAMTS-1 and syndecan 4 conspire to sequester VEGF. **VEGF-A$_{164}$** (30ng/mL) in ice cold serum free media was added to ECs siRNA treated for NTC, *Sdc4* or *Adamts1*, and incubated at 4°C for 30 minutes. A) Media was recovered and a **VEGF-A$_{164}$** sandwich ELISA was performed to quantify **VEGF-A$_{164}$** remaining in the media. (N=3, bars represent S.E.M, *P<0.05, **P<0.01). B) Cells were lysed and western blot analysis performed to assess signalling pathway activation. C) TaqMan qRT-PCR was performed on RNA isolated from cells to determine levels of *VegfA* transcription.

4.4 *Adamts1* siRNA treatment increases proliferation in endothelial cells, but not fibroblasts

The physiological relevance of the sequestration of VEGF-A_{164} by ADAMTS-1 and syndecan 4 was next investigated. Increased ERK phosphorylation in response to VEGF-A_{164} treatment was seen in ADAMTS-1 and syndecan 4 knockdown cells (Figure 4.2). Activation of the RAS-MAPK kinase pathway downstream of VEGFR2 leads to phosphorylated and activated ERK, which translocates to the nucleus and transactivates transcription factors, promoting proliferation. The proliferation of endothelial cells in response to VEGF is an essential step in angiogenesis, and therefore as ERK phosphorylation was upregulated in response to ADAMTS-1 or syndecan 4 depletion it seemed likely that this would result in a corresponding increase in proliferation.

Bromodeoxyuridine (BrdU) incorporation was used to quantify cell proliferation. Endothelial cells were siRNA treated, and incubated with BrdU over a 14 hour period. To quantify the percentage of proliferating cells, they were then fixed and immunolabelled with and anti-BrdU antibody, and DAPI nuclear stain. An increase in proliferation was seen in ADAMTS-1 depleted cells, but not in syndecan 4 depleted cells. To determine if the mechanism was VEGF specific, experiments were repeated in 3T3 fibroblast cells, as these cells express little VEGFR2 and primarily respond to FGFs. No increase in proliferation was seen in 3T3 cells with either *Adamts1* or *Sdc4* siRNA treatment, and in fact a non-significant decrease in proliferation was seen (Figure 4.4).

These data support an important role for ADAMTS-1 in the mediation of VEGF signalling. The data for syndecan 4 were less clear. However, as syndecan 4 is known to interact with a number of other growth factors, this may be a result of multiple signalling pathway involvement, particularly as the fibroblasts siRNA treated for syndecan 4 show a trend for a decrease in proliferation (Harburger and Calderwood, 2008).

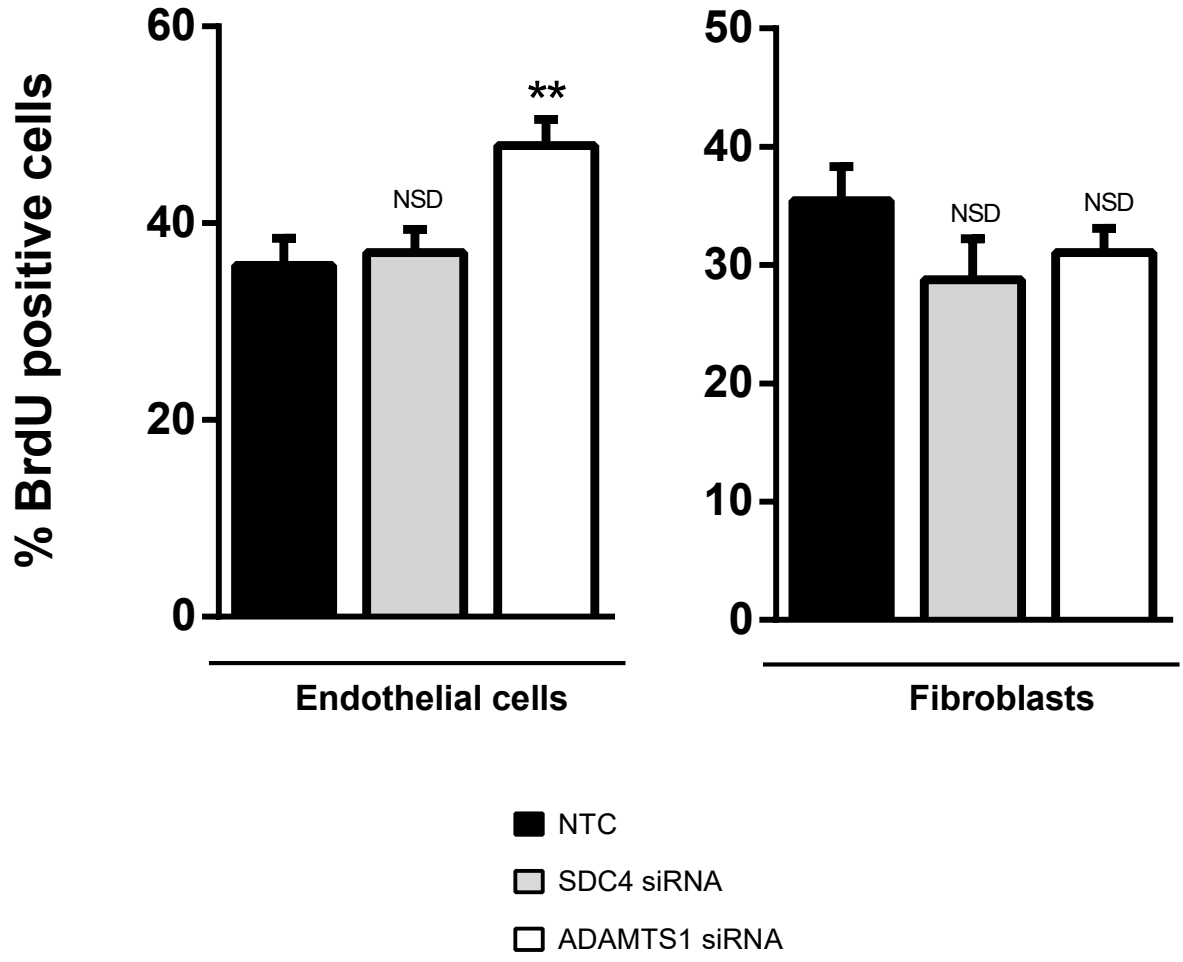


Figure 4.4 ADAMTS-1 inhibits proliferation of endothelial cells. siRNA-treated ECs and 3T3s were allowed to incorporate BrdU for 14 hours. Cells were fixed and stained for BrdU and DAPI. Total number of nuclei and BrdU positive nuclei were counted, bar chart shows the percentage of proliferating cells (N=4, **P<0.01, ns = not significant).

4.5 ADAMTS-1 or Syndecan 4 treatment increases sprouting in the mouse aortic ring assay

To assess the role of ADAMTS-1 and syndecan 4 sequestration of VEGF in a more physiologically relevant context, a mouse aortic ring assay was utilised. This *ex vivo* assay is a model of sprouting angiogenesis which takes advantage of the capacity of aortic grafts to develop *de novo* sprouts when appropriately stimulated with pro-angiogenic factors (Baker *et al.*, 2012).

Aortas were harvested from 6-8 week-old mice, the fat layer was removed and they were sliced into rings. The rings were then treated with siRNA against ADAMTS-1 or syndecan 4 overnight, followed by embedding into a collagen matrix. The rings were stimulated with VEGF-A_{164} periodically over 6 days. They were then fixed and stained with FITC-conjugated BS1-lectin, which demarcates endothelial cells. The number of sprouts that formed from each ring were counted, and this was used as a marker of angiogenesis (Baker *et al.*, 2012). Quantitative RT-PCR was used to confirm that both siRNAs successfully depleted their targets in the aortic rings (Figure 4.5A).

Both ADAMTS-1 and syndecan 4 depletion resulted in a marked increase in new vessel sprouting (Figure 4.5B,C) demonstrating a physiologically relevant contribution of ADAMTS-1 and syndecan 4 to angiogenesis. The increased vessel sprouting seen supports an anti-angiogenic role for ADAMTS-1 and syndecan 4, regulating VEGF bioavailability and signalling via sequestration.

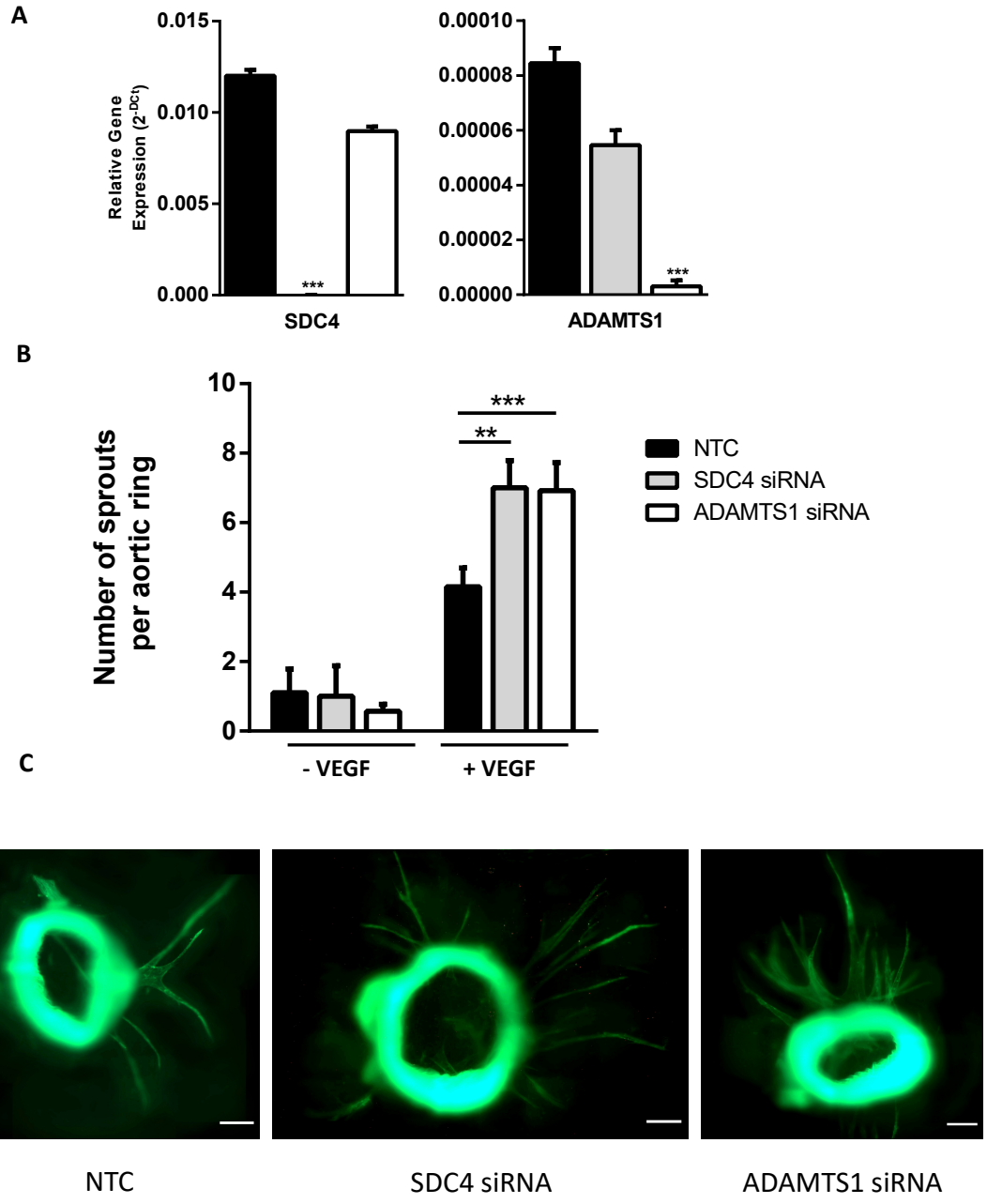


Figure 4.5 ADAMTS-1 and syndecan 4 siRNA depletion results in increased vessel sprouting in the mouse aortic ring assay. Aortas were harvested from 6-8 week old mice, the fat layer was removed, and they were cut into rings. Rings were siRNA treated and embedded in collagen and periodically treated with **VEGF-A_{164}** where indicated. A) TaqMan qPCR showing relative levels of *Sdc4* and *Adamts1* in 3 independent experiments, bars represent S.E.M, **p<0.001.B) Bar chart showing the total number of micro vessel sprouts per aortic ring 6 days post-VEGF-stimulation (n≥50 rings per condition, N = 4 independent experiments, error bars represent S.E.M, **P<0.001, ***P<0.0001). C) representative images of aortic rings stained with FITC-conjugated BS1-lectin, scale bar = 200 µm.

4.6 Discussion

We sought to explore the roles of ADAMTS-1 and syndecan 4 in angiogenesis through investigation essential angiogenic stages, including VEGF signalling and cell proliferation, with a focus on mouse endothelial cells. This chapter demonstrates that ADAMTS-1 and syndecan 4 sequester VEGF-A_{164}, inhibiting activation of VEGFR2 and preventing signalling, with functionally relevant consequences on endothelial cell behaviour and angiogenesis.

ADAMTS-1 is already well established as an angio-inhibitory protein, however the contribution of syndecan 4 to new vessel growth has remained somewhat unclear. This project worked from two contrasting hypotheses: Firstly, that syndecan 4 was acting as a bridge between ADAMTS-1 and VEGF-A aiding in its sequestration and inhibiting angiogenesis. This hypothesis was supported by evidence of the roles of HSPG as a reservoir of growth factors which can be released upon proteoglycan cleavage (Ruoslahti and Yamaguchi, 1991). The second hypothesis suggested that syndecan 4 acts as a VEGF co-receptor, acting to either promote or enhance VEGF signalling to encourage angiogenesis. This hypothesis was drawn from syndecan 4's interactions with other heparin binding growth factors; syndecan 4 has been shown to function as a co-receptor, supporting signalling. Syndecan 4 delivered in proteoliposome form can enhance the activity of PDGF-BB and FGF2 activity (Sperinde and Nugent, 2000; Jang *et al.*, 2012; Das, Majid and Baker, 2016). In the case of FGF2, syndecan 4 binding of PIP2 and downstream PKCα activation can mediate FGF2 signalling. Mutations of the syndecan 4 cytoplasmic tail which alter its affinity for PIP2 reduced migration, growth, and tube formation in endothelial cells in response to FGF2 (Horowitz, Tkachenko and Simons, 2002). Full activity of FGFs requires internalisation of the growth factor as well as receptor interaction (Goldfarb, 2001). The internalisation of FGF2 is dependent upon syndecan 4 clustering dependent activation of Rac1 and the CDC42 dependent macropinocytic pathway (Tkachenko *et al.*, 2004).

Co-Immunoprecipitations demonstrated an association between VEGF-A and syndecan 4, however VEGF-A_{164} signalling was enhanced upon syndecan 4 depletion, contrasting with the suggestion that syndecan 4 was acting as a VEGF-A co-receptor. As an increase in free VEGF-A_{164} was seen upon syndecan 4 depletion, this led to the conclusion that syndecan 4 was sequestering VEGF-A_{164}. These data, in combination with evidence that ADAMTS-1 and syndecan 4 depletion increases micro-vessel sprouting in a physiologically relevant *ex vivo* model, strongly suggest that syndecan 4 does not promote VEGF signalling, but instead alongside ADAMTS-1, sequesters VEGF-A_{164} to inhibit angiogenesis. Unfortunately antibodies were unable to detect ADAMTS-1, and therefore a direct link to the protease could not be demonstrated, however a physical connection between ADAMTS-1 and $VEGF_{165}$ has been previously demonstrated in work by Iruela-Arispe *et al* (Luque, Carpizo and Iruela-Arispe, 2003).

The function of syndecan 4 in sequestering VEGF-A_{164}, and its differing roles in VEGF and FGF2 signalling is not completely unexpected, as differential functions of syndecan 4 on different growth factors have been demonstrated *in vivo*. Mice overexpressing syndecan 4 in cardiac endothelial cells saw an augmentation of FGF2 but not VEGF-A induced NO-release (Zhang *et al.*, 2003). Syndecan 4 is an HSPG, and these complex biopolymers can regulate growth factor signalling in numerous ways, often dependent upon context. In addition to promoting signalling, HSPGs have been seen to sequester growth factors, reducing pro-proliferative signals. One example of a glycoprotein acting in both pro and anti-proliferative mechanisms is glypican, which can both promote and inhibit Wnt signalling (Steinfeld, Van Den Berghe and David, 1996; Zittermann *et al.*, 2010).

FGF-2 signalling also promotes and supports angiogenesis, and therefore future work investigating a connection between ADAMTS-1, syndecan 4 and FGF2 would hopefully provide more insight into multifunctional contributions of syndecan 4 (Cross and Claesson-Welsh, 2001). In particular, assessing the sprouting of aortic rings stimulated with FGF2 rather than VEGF may give deeper insight into syndecan 4's multifactorial roles.

Regulation of VEGF bioavailability is one important mechanism in the control of angiogenesis. The VEGF-A isoforms 189, 206 and 165 bind the ECM in an interaction mediated by HSPG, generating a pool of VEGF which can be released by MMP mediated HSPG cleavage (Houck *et al.*, 1992). Manipulation of VEGF gradients in this way can have profound effects on development and cancer (Schlieve *et al.*, 2016). In one important mechanism MMP-9 cleavage of HSPG releases VEGF, triggering the angiogenic switch, increasing circulating VEGF and inducing colorectal cancer angiogenesis (Hawinkels *et al.*, 2008). Based on this it seems highly plausible that syndecan 4 is functioning as a reservoir of VEGF, which can be released by MMPs in a pro-angiogenic context.

Although both *Adamts1* and *Sdc4* siRNA treatment increased VEGF-A_{164} initiated ERK signalling, only *Adamts1* siRNA had an effect on the proliferation of endothelial cells. Reasons for this may be multifold; as previously discussed, syndecan 4 is an important signalling nexus, and its depletion may have consequences for signalling by growth factors other than VEGF. A key example is found in FGF2. FGF2 activation of FGFR1 can induce endothelial cell migration and angiogenesis, and as syndecan 4 is important in shaping cell responses to FGF2 the loss of syndecan 4 is likely to influence FGF2s contribution to endothelial cell proliferation (Elfenbein *et al.*, 2012). The possibility that the contribution of other growth factors influences proliferation is supported by data collected in 3T3 fibroblasts. *Adamts1* a siRNA only increased proliferation in ECs, not fibroblasts, and in the case of fibroblasts *Sdc4* siRNA treated cells had a non-statistically significant decrease in proliferation. Fibroblasts only express low levels of VEGFR2 and are much more responsive to FGF2, whereas endothelial cells have high VEGFR2 levels, thus the differing actions of syndecan 4 depending on the specific growth factor may therefore explain the proliferation data, and suggest that syndecan 4 modulation of FGF signalling has a role in endothelial cells. Investigating proliferation in response to specific growth factor stimulation may help develop understanding of this phenotype.

It is also important to consider the other roles of ADAMTS-1, independent of syndecan 4. The sequestration of VEGF is just one mechanism by which it exerts its

anti-angiogenic affects. ADAMTS-1 also has important catalytically dependent functions. One such role is the cleavage of thrombospondin 1 and 2, releasing a pool of anti-angiogenic polypeptides (Lee *et al.*, 2006). In a broader sense, although syndecan 4 and ADAMTS-1 null mice share a delayed wound healing response the models do not phenocopy each other (Echtermeyer *et al.*, 2001). Whilst syndecan 4 knockout mice are relatively healthy, ADAMTS-1 knockout mice exhibit developmental issues with stunted growth and high embryonic mortality (Shindo *et al.*, 2000).

4.7 Chapter summary

In conclusion, ADAMTS-1 and syndecan 4 intersect in the sequestration of VEGF-A_{164} and inhibit angiogenesis. It is likely that they exist in an equilibrium with MMP-9 which is able to cleave syndecan and release VEGF to initiate angiogenesis. Although ADAMTS-1 and syndecan 4 functions co-operate in this instance, it remains important to consider their unique functions, an evaluation of the contribution of FGF2 in this context would hopefully give deeper insight into the mechanisms at play.

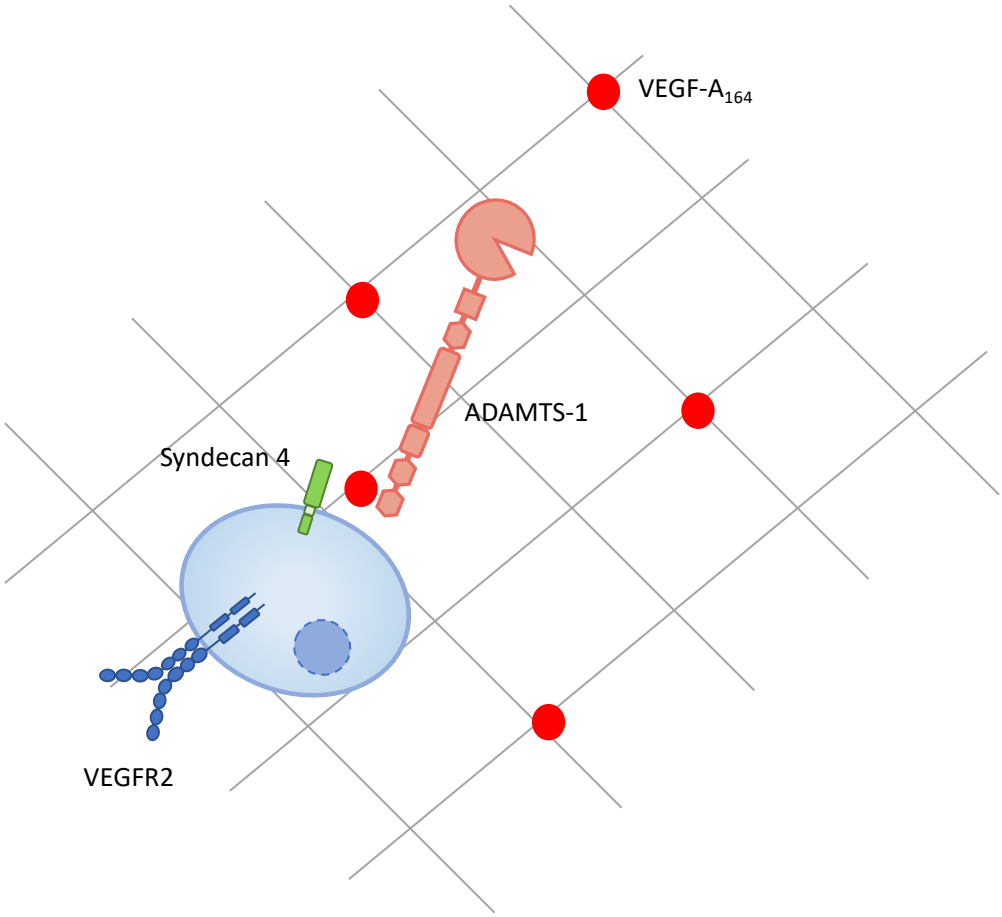


Figure 4.6 Graphical summary of the findings of chapter 4. ADAMTS-1 and syndecan 4 bind and sequester VEGF-A_{164}, preventing it from binding and activating VEGFR2, and inhibiting angiogenesis.

5 Syndecan 4 and ADAMTS-1 regulate endothelial cell adhesion and migration in an ECM dependent mechanism

Cell migration is an essential step in the process of angiogenesis; endothelial cells must migrate under the influence of VEGF to drive the formation of new vessel sprouts. As ADAMTS-1 and syndecan 4 had a profound influence on angiogenic sprouting and VEGF-A bioavailability, their contribution to cell migration was next investigated.

Both ADAMTS-1 and syndecan 4 null mice have phenotypes of delayed wound healing, attributed to altered cell migration and angiogenesis, and indeed syndecan 4 has a clear contribution to the process of cell migration (Echtermeyer *et al.*, 2001; Krampert *et al.*, 2005). It binds fibronectin in co-operation with α5 integrin, as well as regulating small G proteins to generate sustained directional migration (Bass *et al.*, 2007, 2008).

The role of ADAMTS-1 in cell migration is less well defined. ADAMTS-1 has been shown to cleave semaphorin 3C, promoting migration of breast cancer cells in a mechanism which may promote metastasis (Esselens *et al.*, 2010). Conversely, ADAMTS-1 has also been demonstrated to inhibit cell migration; in the context of breast cancer cell invasion and migration, activation of the receptor PPARδ increased ADAMTS-1 expression, inducing a marked inhibition of cell migration and TSP1 expression, and these effects could be mitigated by siRNA mediated ADAMTS-1 knockdown (Ham *et al.*, 2017).

Members of the ADAMTS family have been previously shown to regulate cell migration in a syndecan 4 dependent mechanism. ADAMTS-6 and -10 have opposing roles in focal adhesion formation: ADAMTS-6 inhibits whereas ADAMTS-10 is required for focal adhesions, since cells overexpressing ADAMTS-10 have more prominent focal adhesions, whereas those overexpressing ADAMTS-6 did not form focal adhesions. This relationship holds true in reverse, as ADAMTS-6 siRNA treatment induced prominent adhesions, and cells siRNA depleted of ADAMTS-10 formed very few focal adhesions. The functions of ADAMTS-6 and -10 were linked to

syndecan 4, as the C-termini of both proteases were shown to bind syndecan 4, and in ADAMTS-10 depleted cultures expression of syndecan-4 rescued focal adhesions (Cain *et al.*, 2016). Similar syndecan 4 dependent effects have been seen with ADAMTS-15. Addition of ADAMTS-15 to aortic rings inhibited their sprouting, and expression of ADAMTS-15 in culture inhibited the migration of MDA-MB-231 breast cancer cells on fibronectin, which could be abrogated by siRNA depletion of syndecan 4 leading to recovery of migration speed (Kelwick, Wagstaff, *et al.*, 2015).

There is thus a clear connection between ADAMTS family members and syndecan 4 in cell migration, and it is hypothesised that this extends to ADAMTS-1. Both ADAMTS-1 and syndecan 4 interact with the ECM, placing them in an ideal location to facilitate cell movement (Kuno and Matsushima, 1998; Elfenbein and Simons, 2013). A connection between ADAMTS-1 and syndecan 4 in the regulation of cell migration was demonstrated by Rodriquez-Manzeneqe *et al.* Somewhat contradictory to previous work on syndecan 4 in cell migration, they found that cleavage of syndecan 4 by ADAMTS-1 promoted cell migration, in a manner that resembled genetic deletion of the proteoglycan (Rodríguez-Manzaneque *et al.*, 2009). This essential interplay between ADAMTS-1 and syndecan 4 was supported by data from Chapter three, however these experiments demonstrated the requirement of ADAMTS-1 for syndecan 4 expression, and therefore the relative contribution of a cleavage mechanism is unclear in this context.

As of yet, investigation of ADAMTS-1 and syndecan 4 regulation of migration in an endothelial cell specific context has been limited, since work on syndecan 4 primarily focused on fibroblasts, and ADAMTS-1 in cancer cell lines. Therefore, the aim of this chapter was to investigate the roles of ADAMTS-1 and syndecan 4 in the regulation of endothelial cell migration, and how this may contribute to their angio-inhibitory functions.

5.1 ADAMTS-1 and Syndecan 4 inhibit cell migration in a fibronectin-specific manner

Given the current contradictions in the reported roles of ADAMTS-1 and syndecan 4 in cell migration, we investigated their migratory roles both in fibroblasts to allow for comparison with existing literature regarding syndecan 4, and in endothelial cells, to give context to our angiogenesis studies. To gain an initial perspective of the relative contributions of these two proteins to cell motility, random migration assays were carried out. Cells were siRNA treated and seeded onto collagen I and fibronectin matrices, and time-lapse videomicroscopy was then performed, followed by quantification of migration speed using the ImageJ plugin MTrackJ.

An increase of migration speed was seen upon ADAMTS-1 or syndecan 4 knockdown in both ECs and fibroblasts. Of note, this effect was specific to fibronectin, and in fact a decrease in migration speed was seen in *Sdc4* siRNA treated cells migrating on a collagen I matrix (Figure 5.1).

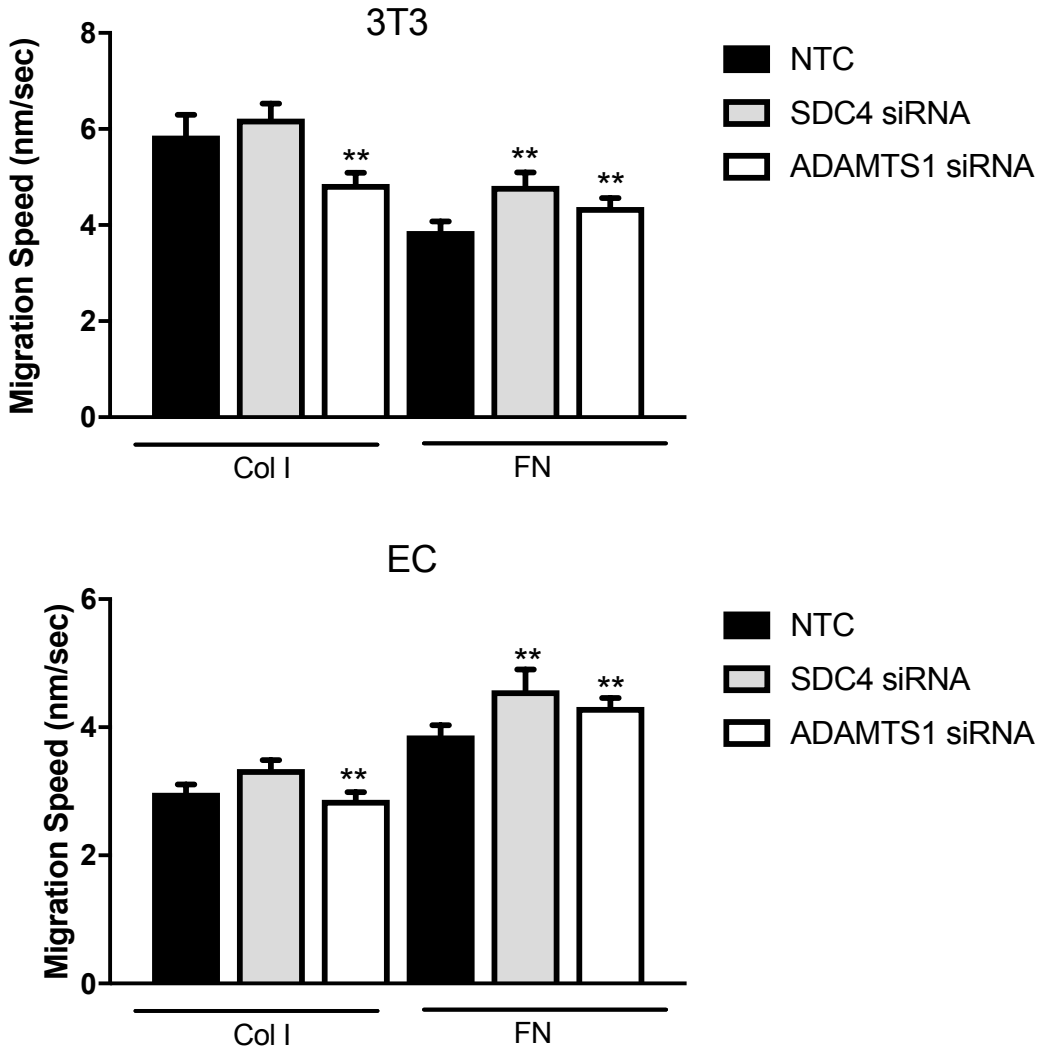


Figure 5.1 *Adamts1* or *Sdc4* siRNA depletion increases random migration speed on fibronectin matrices only. 3T3 fibroblasts or ECs were treated with siRNA, and plated onto 10 µg/mL collagen I (Col I) or fibronectin (FN) matrix and allowed to adhere overnight. Time-lapse video microscopy was used to image cells every 16 minutes, over a period of 16 hours. Migration speed (nm/sec) was calculated using the MTrackJ plugin in FIJI®. n= ≥100 cells in 4 independent experiments, error bars represent S.E.M, **P<0.001).

These data suggested that ADAMTS-1 and syndecan 4 were acting to inhibit cell migration on fibronectin. Signalling by VEGF can drive endothelial cell migration though the activation of several signalling pathways downstream of VEGFR2; two key pathways are activation of p38MAPK which drives actin polymerization, and FAK, which in-turn drives focal adhesion turnover (Abhinand *et al.*, 2016). As chapter four showed ADAMTS-1 and syndecan 4 depletion increased VEGF-A_{164} availability and signalling, this was hypothesised to be the mechanism behind the increased random migration speed.

To determine the contribution of VEGF signalling to increased migration speed, random migration experiments were repeated using ECs in growth factor free conditions. An increase in random migration speed was still seen in ADAMTS-1 and syndecan 4 siRNA depleted cells, despite the lack of VEGF (Figure 5.2). This, combined with the increased speed seen in fibroblasts, which are largely not responsive to VEGF signalling, and the specificity of the increased speed to fibronectin, suggested that a different, more complex mechanism was at play.

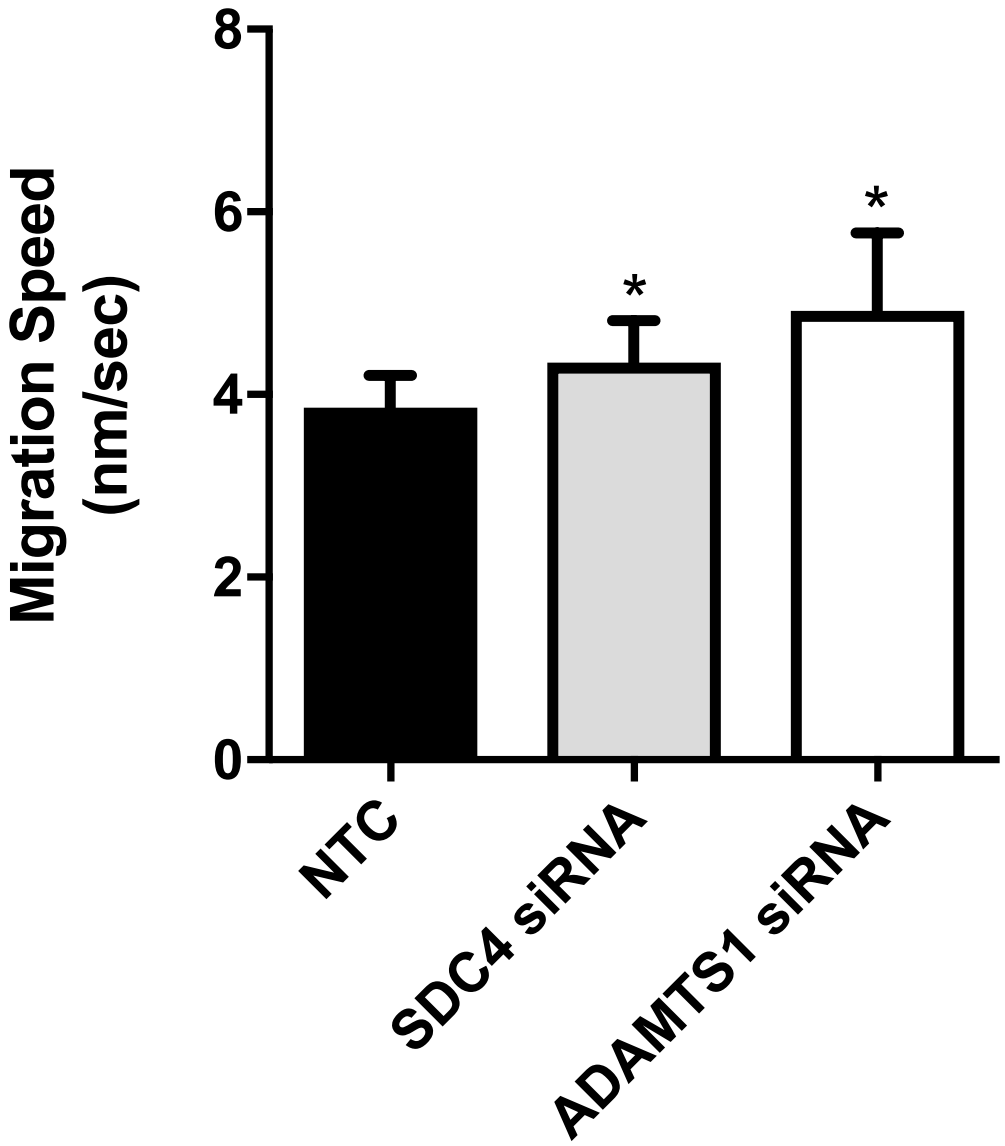


Figure 5.2 Increased random migration speed is independent of VEGF. Endothelial cells were treated with siRNA, plated onto 10 µg/mL FN matrix, and allowed to adhere overnight. Complete media was replaced with serum free OptiMem®. Time-lapse video microscopy was used to image cells every 16 minutes, over a period of 16 hours. Migration speed (nm/sec) was calculated using the MTrackJ plugin in FIJI®. n= ≥50 cells in 3 independent experiments, error bars represent S.E.M, *P<0.05.

5.3 Immunocytochemistry experiments reveal a more migratory phenotype with altered actin distribution when ADAMTS-1 or syndecan 4 are siRNA depleted

To begin to understand the phenotypes driving the increased cell migration speed, immunocytochemistry experiments were performed to visualise migrating cells.

Two essential structural components of migration, focal adhesions and F-actin were imaged. Actin forms a part of the cytoskeleton that is temporally and spatially regulated and redistributed, and is required to generate force in migrating cells (Wozniak *et al.*, 2004). Focal adhesions are sites of cytoskeleton – ECM connection and allow the cell to generate the traction required to translocate the cell body when migrating. These are highly dynamic structures that respond to the environment, and therefore imaging these two components gives a static snapshot of these two structures, and could reveal any gross changes in their morphology, providing insight into the migratory behaviour of the cells.

Endothelial cells were siRNA treated and serum starved, then stimulated with VEGF-A_{164} 10 minutes prior to fixation and staining. In quiescent endothelium, actin forms a cortical rim that interacts with both cell-cell and cell-matrix adhesion complexes. In contrast, stress fibres, actin-myosin bundles necessary for inducing cell contraction, form when cells are activated. Immunocytochemistry revealed differences in actin distribution between NTC and *Adamts1/Sdc4* siRNA depleted cells. Non-target control cells displayed mostly cortical actin with a largely heterogeneous distribution, whereas cells treated with *Adamts1* or *Sdc4* siRNA seemed to have more stress fibres, aligned along the cell axis, in a distribution fitting with a more migratory phenotype (Figure 5.3).

To quantify the images, a software for analysis of 2D and 3D biopolymer networks, SOAX, was used to calculate the radial orientation of actin fibres (Xu *et al.*, 2015). A density plot of azuthimal angles of actin filaments was generated in R (Figure 5.4). Quantification confirmed that the actin of *Adamts1* and *Sdc4* siRNA treated cells was more aligned along the cell axis, with most fibres orientated at a 0° angle.

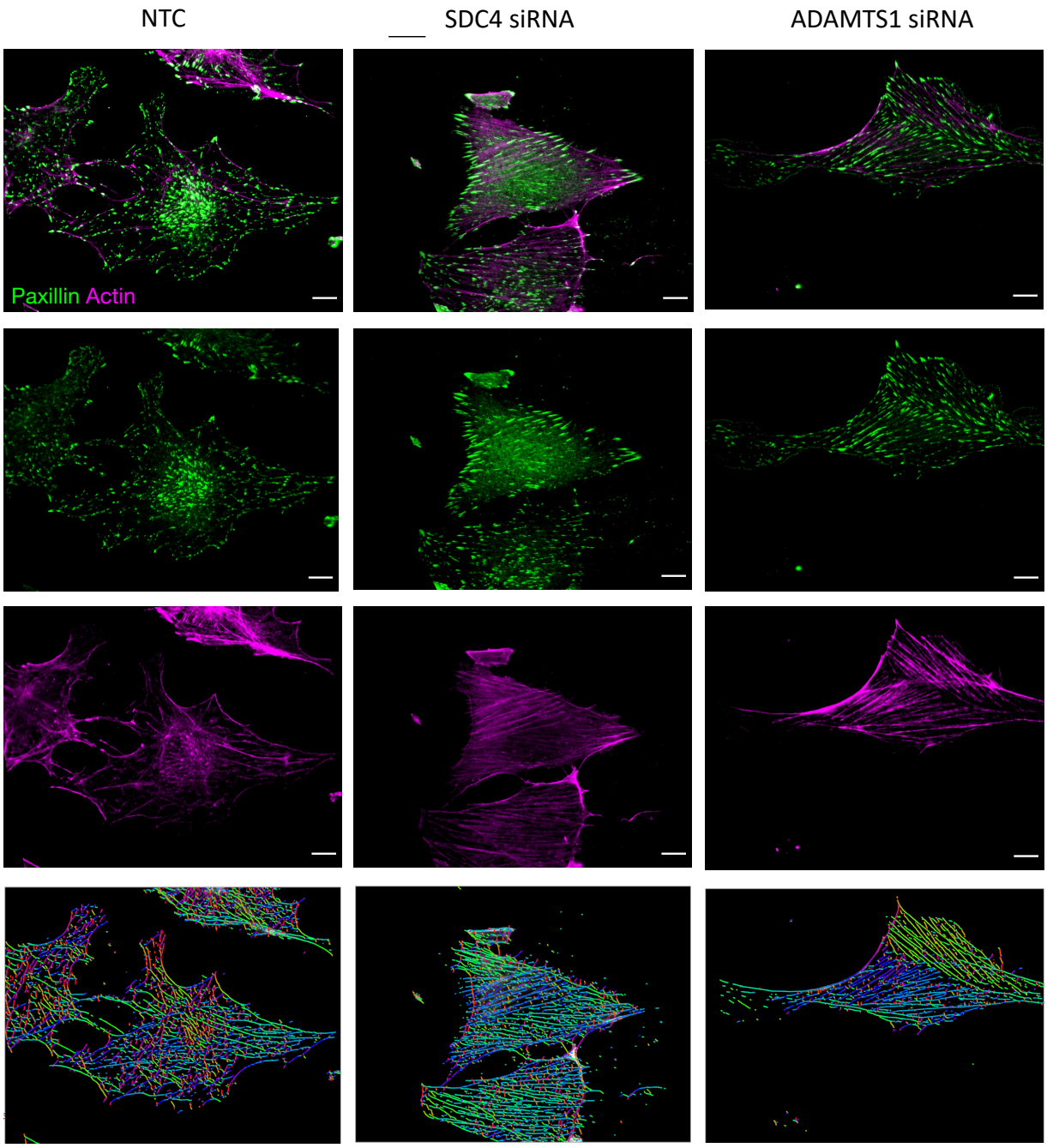


Figure 5.3 *Adamts1* or *Sdc4* siRNA alters actin distribution. ECs were siRNA treated and plated onto fibronectin overnight. Cells were then serum starved for 3 hours in OptiMem®, followed by stimulation of migration by the addition of VEGF-A_{164} (30 ng/ml). After 10 minutes of stimulation, cells were fixed in PFA and immuno-labelled for paxillin, as a marker of focal adhesions, and for F-actin. The final panel shows actin filaments coloured by their azimuthal angle, analysis was performed in SOAX (Xu *et al.*, 2015). Images are representative of three independent experiments.

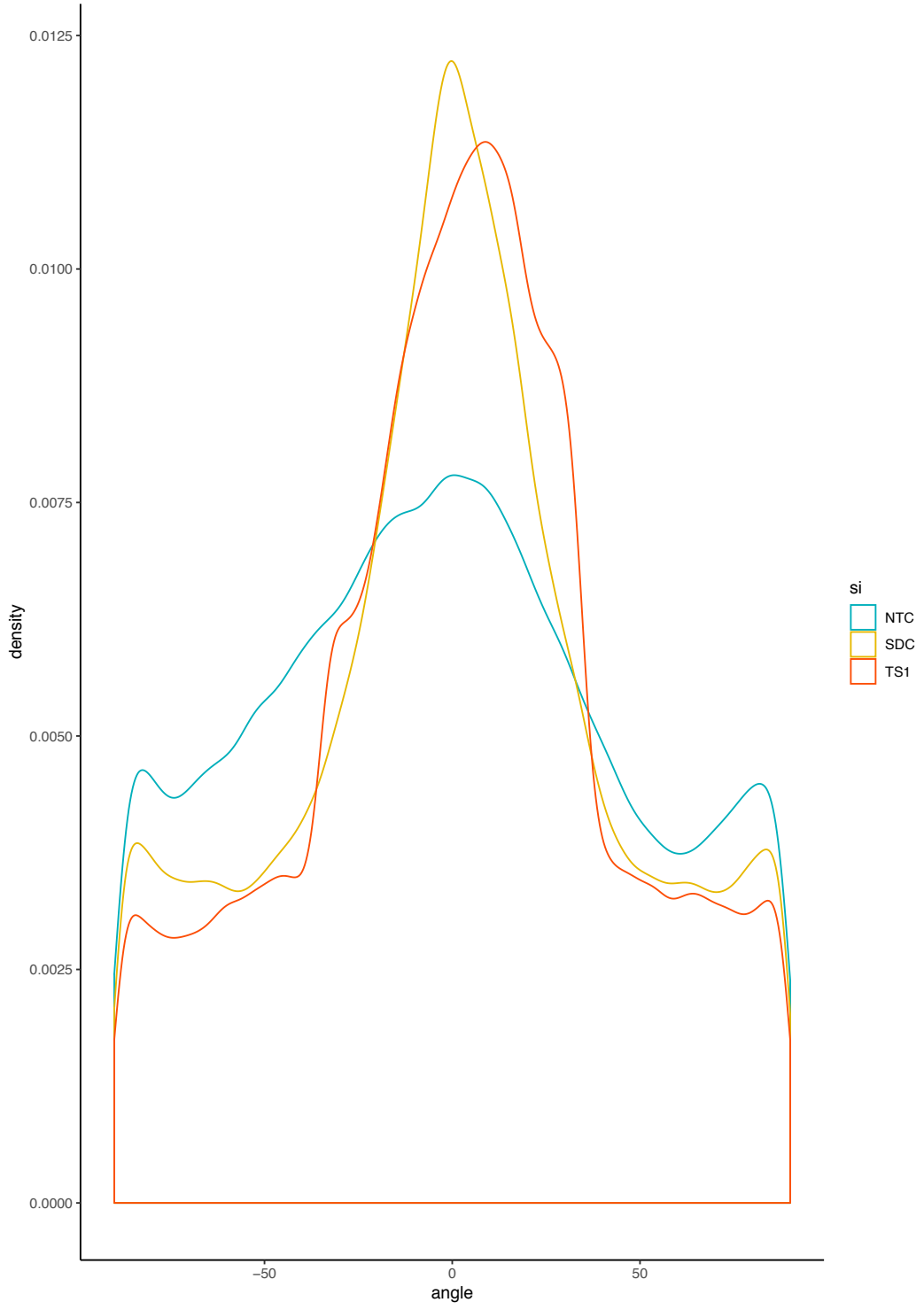


Figure 5.4 Actin filaments are more linearly arranged in *Adamts1* and *Sdc4* siRNA treated cells. ECs were siRNA treated and plated onto fibronectin overnight. Cells were then serum starved for 3 hours in OptiMem®, followed by stimulation of migration by the addition of VEGF-A_{164} (30 ng/mL). After 10 minutes of stimulation, cells were fixed in PFA and stained for F-actin. Radial orientation of actin filaments was quantified using SOAX, and azuthimal angles were plotted using R.

5.4 Focal adhesion size is altered by *Adamts1* or *Sdc4* siRNA depletion, in a fibronectin-dependent manner

Given the actin distribution typical of migratory cells seen with ADAMTS-1 and syndecan 4 depletion, we wished to gain more insight into focal adhesion formation and turnover. Cells were siRNA depleted for ADAMTS-1 or syndecan 4, and fixed after 90 or 180 minutes of adhesion to fibronectin coated coverslips. Focal adhesions were visualised using immunocytochemistry with the adaptor protein paxillin, which incorporates into focal adhesions via direct interaction with integrin β chain cytoplasmic tails, as a focal adhesion marker (Schaller *et al.*, 1995) (Figure 5.5). Computational analysis of images found that the number of focal adhesions formed was unchanged at either timepoint, however at 90 minutes of adhesion, focal adhesions in siRNA treated cells had a larger average area (Figure 5.6).

Nascent adhesions form when an initial integrin mediated cell-ECM connection occurs. Depending on the environment, this initial adhesion has the potential to mature into a focal adhesion. A more detailed breakdown of focal adhesion data revealed a higher percentage of mature focal adhesions (2-6 μm^2) in *Adamts1* and *Sdc4* siRNA treated cells as opposed to NTC cells. While only 4.2% of adhesions in NTC treated cells could be classed as focal adhesions, 8.5 and 7.2% of syndecan-4 and ADAMTS-1 adhesions fell into this category. This increase in focal adhesions was reflected in a decrease in nascent adhesions, with 95.6% of NTC, 90.9% of syndecan 4 and 92% of ADAMTS-1 adhesions classed as this type. Only a very small percentage of adhesions under any condition became super-mature adhesions within the 90 minute timeframe, with these adhesions representing 0.2%, 0.6%, and 0.7% of NTC, syndecan 4 and ADAMTS-1 adhesions respectively. The differences in focal adhesion maturity state were statistically significant, as assessed with a Chi Square test. These data suggest that the increase in focal adhesions size is due to the faster maturation of nascent adhesions into focal adhesions when ADAMTS-1 or syndecan 4 are knocked down, as opposed to the generation of extra-large adhesions (Figure 5.7).

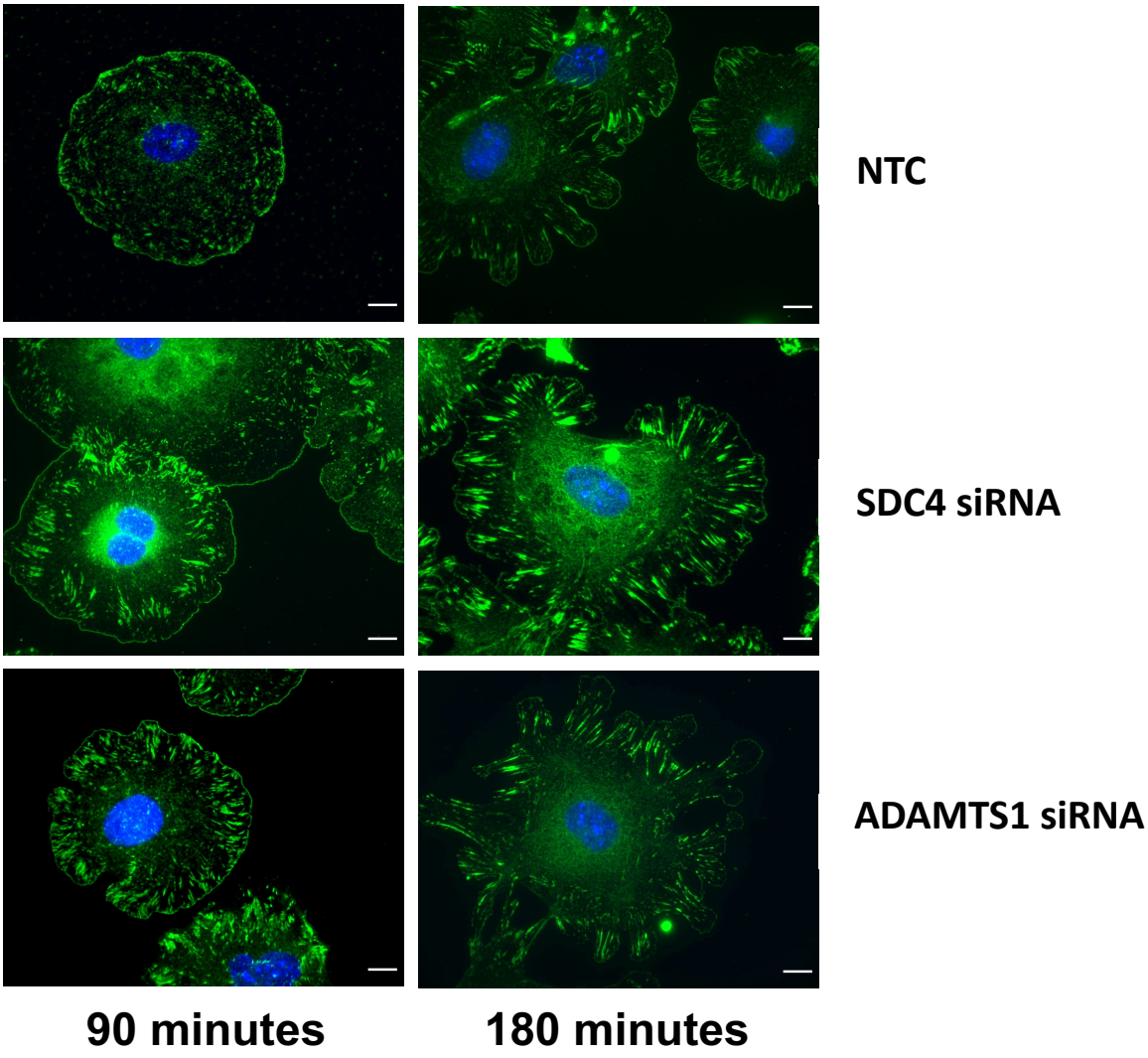


Figure 5.5 Focal adhesions are larger at 90 minutes when ADAMTS-1 or Syndecan 4 are siRNA depleted. ECs were treated with indicated siRNA, and allowed to recover for 48 hours. siRNA treated cells were then plated onto glass coverslips coated with 10 µg/mL fibronectin, and allowed to adhere for 90 or 180 minutes. Cells were then fixed and stained for paxillin as a marker of focal adhesions. Images are representative of three independent experiments, scale bar = 10 µm.

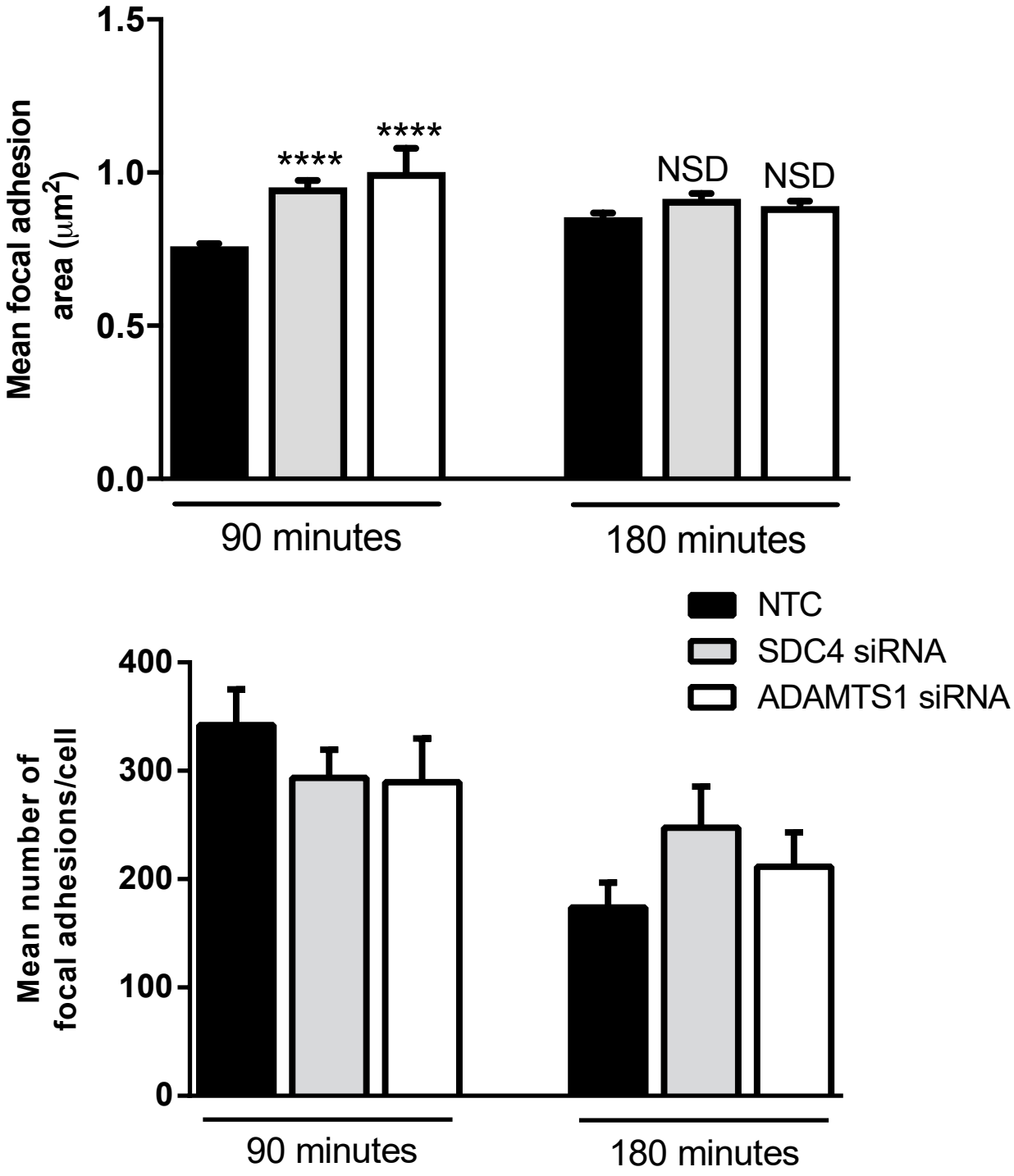
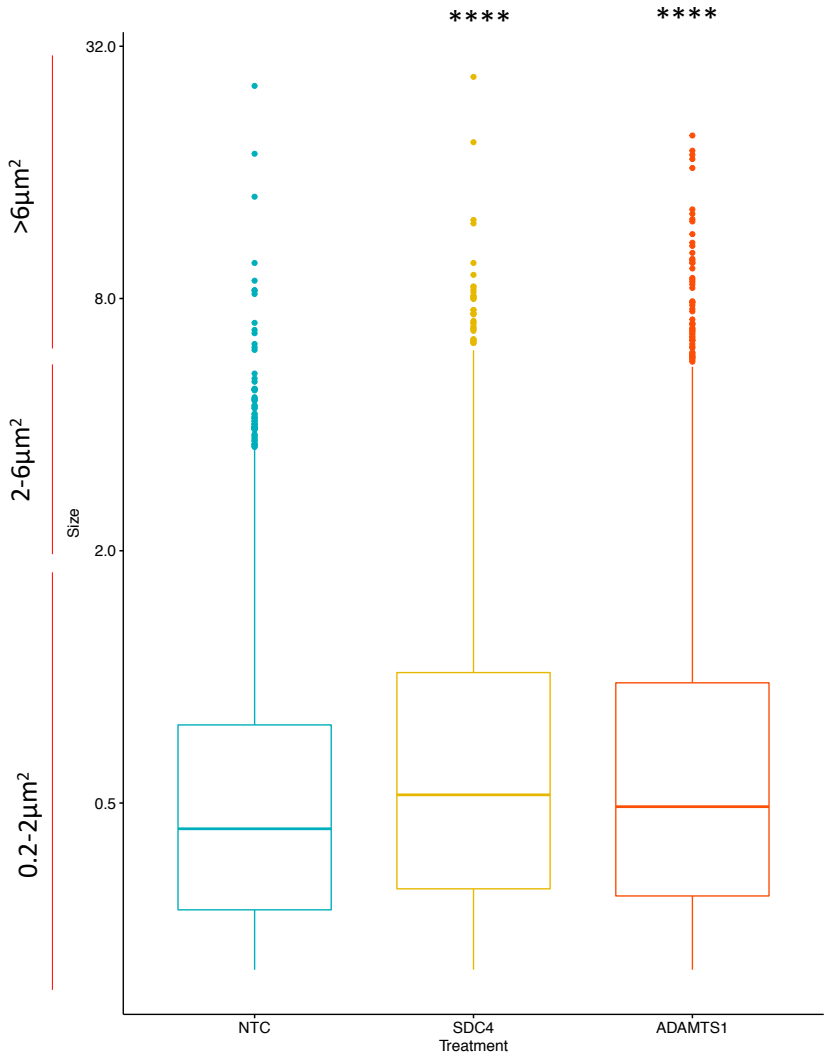


Figure 5.6 Quantification of focal adhesions revels larger average area at 90 minutes when ADAMTS-1 or syndecan 4 are siRNA depleted, but the number of adhesions is unchanged. Focal adhesion images (as in 5.5) were analysed using FIJI®. Graphs show average number of focal adhesions per cell and average area of focal adhesion. n= 20 cells from three independent experiments, statistical significance was determined using a Kruskal Wallis test, **** = P< 0.00001.



Size (um²)	Category	Percentage in range		
		NTC	SDC4 SIRNA	ADAMTS-1 SIRNA
0-2	Small nascent	95.6	90.9	92.0
2-6	Focal adhesion	4.2	8.5	7.2
6+	supermature	0.2	0.6	0.7

Figure 5.7 Categorising focal adhesions by maturity reveals *Adamts1* or *Sdc4* **siRNA increases the percentage of mature focal adhesions.** Individual adhesion areas calculated from Figure 5.5 were categorised into maturity state, with adhesions 0-2 µm² being classes as nascent, 2-6² µm as focal adhesions, and >6 µm² as super mature. ****P >0.00001, assessed using a Chi Square test, compared to NTC.

ADAMTS-1 and syndecan 4's effects on cell migration were limited to fibronectin matrices. To determine if the focal adhesion alterations were also specific to this matrix, focal adhesion imaging experiments were repeated using collagen coated coverslips. Immunocytochemical visualisation of focal adhesions followed by computational analysis revealed no changes in the size of focal adhesions in siRNA treated cells when adhering to collagen, supporting a fibronectin specific mechanism of action of ADAMTS-1 and syndecan 4 (Figure 5.8).

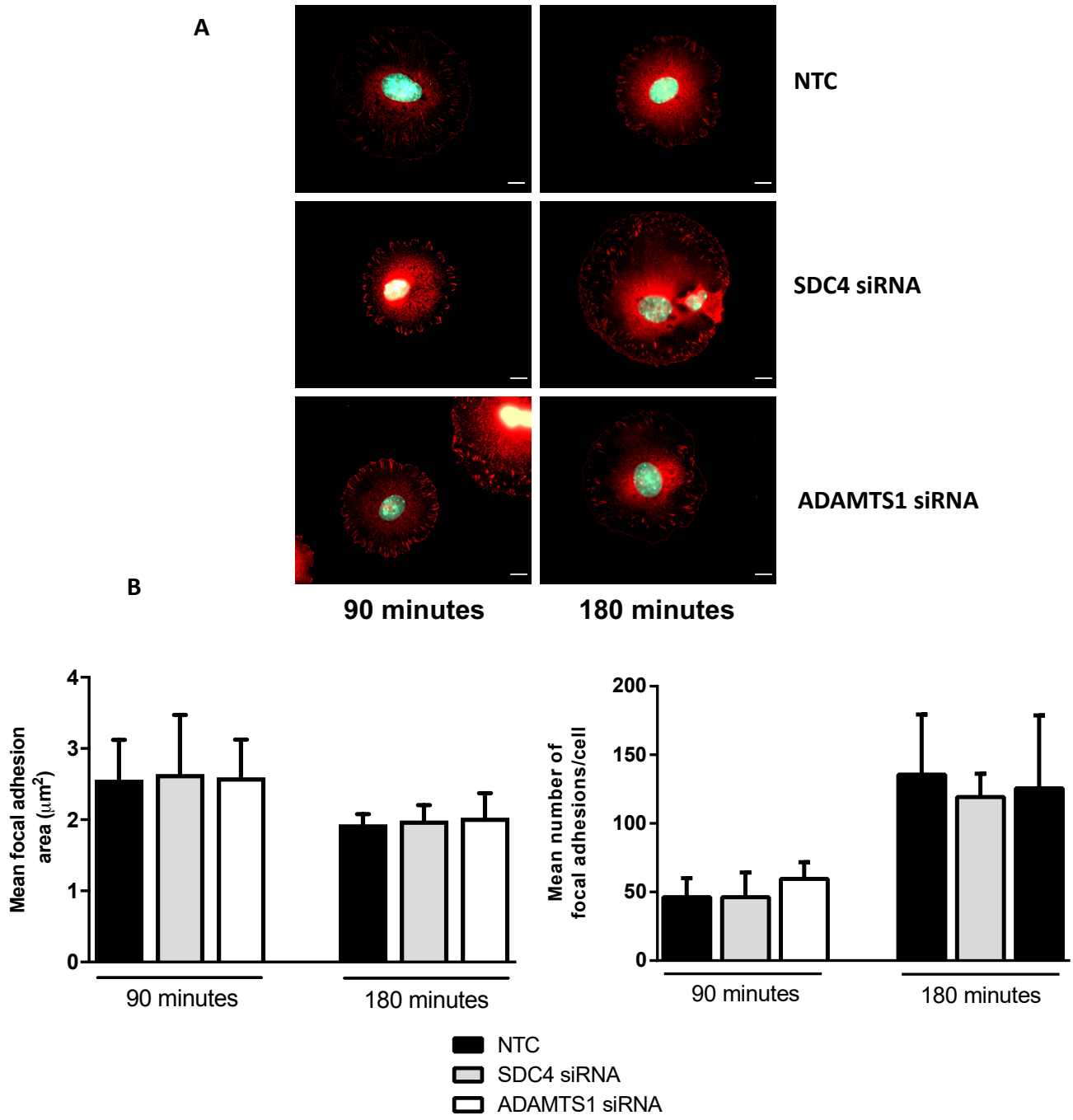


Figure 5.8 ADAMTS-1 and Syndecan 4 do not affect focal adhesion formation on collagen. ECs were treated with indicated siRNAs. After 48 hours recovery, cells were seeded onto a collagen I matrix for 90 or 180 minutes. Cells were fixed and stained for paxillin as a marker of focal adhesions. A) representative immunocytochemistry images of cells adhered to collagen, scale bar = 10 µm. B) quantification of focal adhesion area and numberusing FIJI. n= 20 cells from three independent experiments, bars represent S.E.M.

5.5 ADAMTS-1 or syndecan 4 depletion promotes pro-migratory signalling

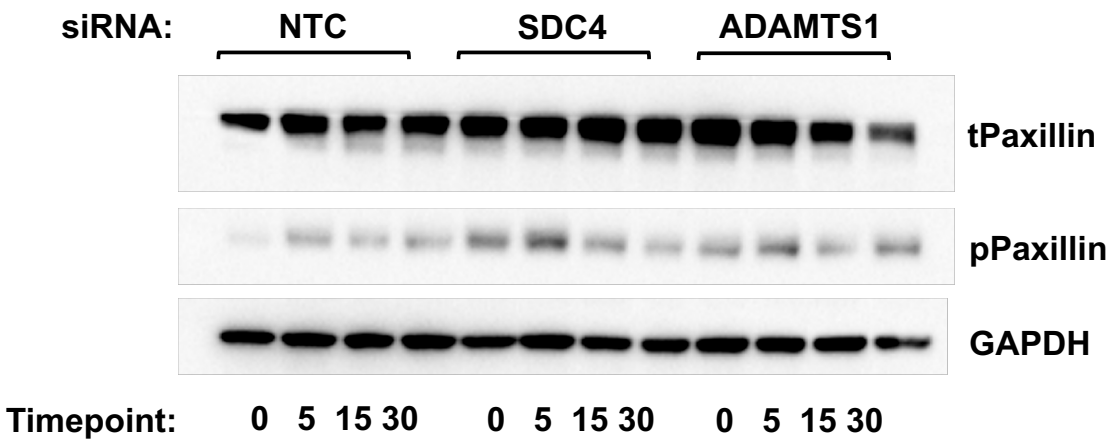
Cell signalling is important during cell migration, as well as focal adhesion formation, maturation and turnover. Paxillin functions as a scaffold, or adaptor protein, and is key in recruiting and organising kinases and other adaptors to facilitate signalling (Turner, 2000). Paxillin is a target for phosphorylation, which can occur as a result of integrin engagement with the ECM, during cell spreading, after stimulation with growth factors or cytokines or upon focal adhesion formation (Burridge, Turner and Romer, 1992). Of note, activation of VEGFR2 can lead to downstream phosphorylation of paxillin via phosphorylation and activation of FAK (Yang *et al.*, 2015).

Paxillin phosphorylation can regulate the assembly and turnover of focal adhesions, and has been associated with the formation of stress fibres and their dynamic regulation (Frame, 2004). Paxillin phosphorylation on tyrosine residues is driven by FAK, in association with Src, and occurs at two main sites, tyrosine 31 and tyrosine 118. Phosphorylation of paxillin at these residues generates two SH2 binding sites, to which adaptor proteins of the Crk family can bind. Crk can also bind p130Cas, and the association of Crk with both p130Cas and phosphorylated paxillin is important in coordinating integrin mediated cell motility (Petit *et al.*, 2000). FAK preferentially interacts with phosphorylated paxillin, and its recruitment is implicated in high FA turnover and translocation of focal adhesions. Tyrosine 119 phosphorylation of paxillin has also been shown to enhance migration in several cancer cell lines (Petit *et al.*, 2000; Iwasaki *et al.*, 2002). Due to the demonstration of increased migration speed, altered actin distribution and altered focal adhesions, it was therefore deemed important to investigate paxillin phosphorylation.

Endothelial cells were siRNA treated, seeded onto fibronectin and allowed to adhere overnight. Cells were then serum starved, followed by stimulation with VEGF-A_{164}. Cells were collected and lysed at sequential time points and western blotted for levels of Tyr118 phosphorylated paxillin (pPax). ADAMTS-1 and syndecan 4 siRNA treated cells had higher levels of pPax both prior to stimulation (0 minutes) and in

response to VEGF-A_{164} addition; concurrent with the increased migration speed and focal adhesion formation (Figure 5.9).

A



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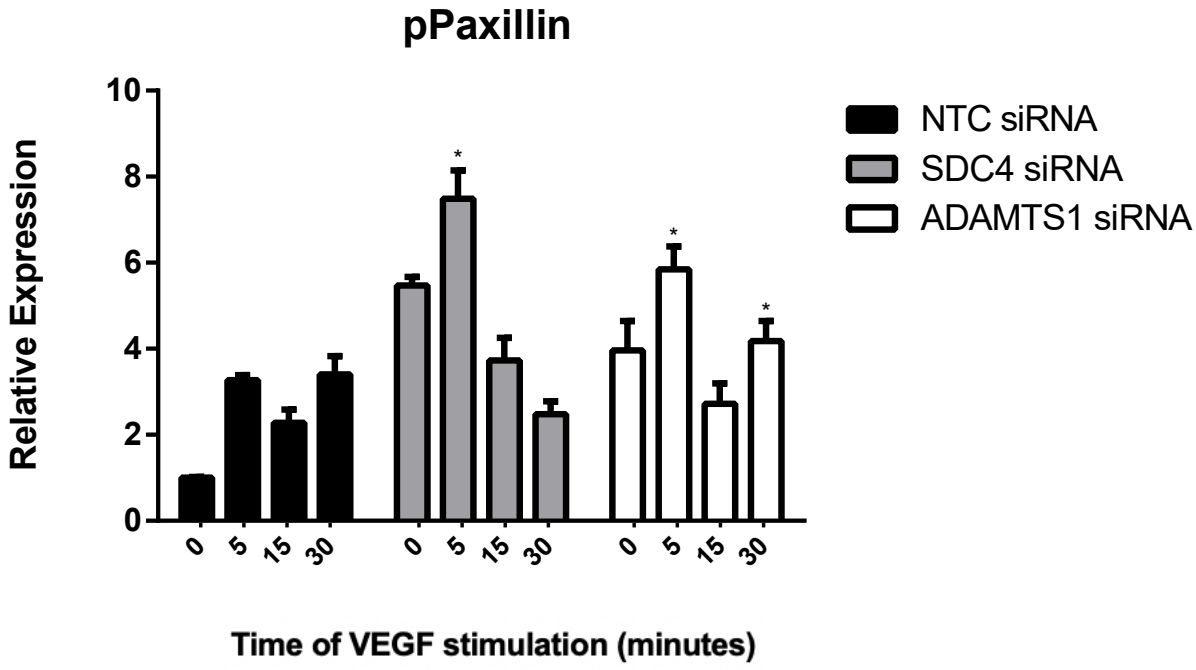


Figure 5.9 Depletion of ADAMTS-1 or syndecan 4 results in increased paxillin signalling. Western blot analysis of paxillin signalling using an anti phospho-paxillin antibody in siRNA-treated ECs adhered to fibronectin, serum starved for 3 hours then stimulated with 30 ng/mL VEGF-A_{164} for 0, 5 15 and 30 minutes. GAPDH was used as a loading control. Image is representative of 3 independent experiments. Densitometric quantification of western blots performed in FIJI®, bars represent S.E.M. *P<0.05 calculated using a Student's T test compared to timepoint control (N=3).

5.6 Levels of endothelial integrins appear unchanged

As mentioned previously, the increase in random migration speed observed with ADAMTS-1 and syndecan 4 depletion was independent of VEGF. Therefore the question of how ADAMTS-1 and syndecan 4 are regulating cell migration was raised. The initial candidate target was integrins. The original project hypothesis considered the involvement of these transmembrane receptors due to their essential roles in cell migration and angiogenesis, and strong connections to syndecan 4. Data regarding perturbation of adhesion and migration upon ADAMTS-1 or syndecan 4 depletion further hints at their involvement.

ADAMTS-1 and syndecan 4 regulation of adhesion and migration was specific to fibronectin, and therefore the RGD binding integrin heterodimers α5β1 and αvβ3 were considered potential targets for regulation. These were also good targets as syndecan 4 has been implicated in the alternate recycling of α5β1 and αvβ3. Phosphorylation of syndecan 4 by Src leads to suppression of ARF6 and recycling of αvβ3 to the membrane, at the expense of α5β1, altering adhesion and migration dynamics by promoting stabilisation of focal adhesions (Morgan *et al.*, 2013).

Changes in migration can be correlated with changes in the integrin repertoire of a cell. In particular there is a dynamic relationship between α5β1 and αvβ3. On fibronectin, integrin α5β1-integrin-mediated adhesions are more dynamic than αvβ3-mediated adhesions. The relative expression levels of the two heterodimers can therefore regulate migration and could explain how ADAMTS-1 and syndecan 4 control migration (Truong and Danen, 2009).

TaqMan qPCR and flow cytometry were used initially, to profile levels of integrin expression in both 3T3s and ECs after ADAMTS-1 or syndecan 4 knockdown. Quantitative PCR showed no changes in integrin gene expression levels (Figure 5.10), and this was reflected in the flow cytometry data which displayed no changes in cell surface levels of integrin proteins (Figure 5.11).

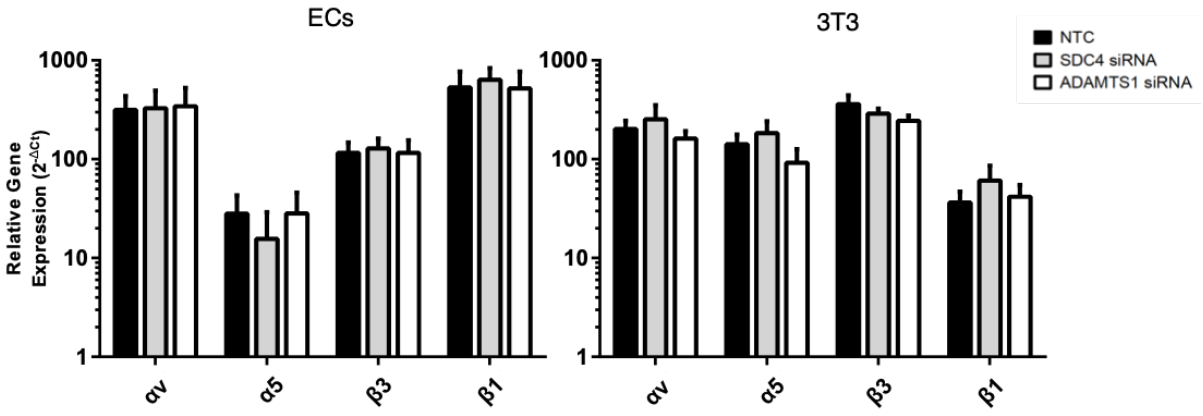


Figure 5.10 RNA expression levels of fibronectin binding integrins are unchanged. ECs or 3T3 fibroblasts were siRNA depleted for syndecan 4 or ADAMTS-1. RNA was collected 24 hours post transfection, and reverse transcribed. TaqMan qRT-PCR was used to determine relative RNA expression levels ($2^{-\Delta Ct}$). N = 3 independent experiments, bars represent S.E.M.

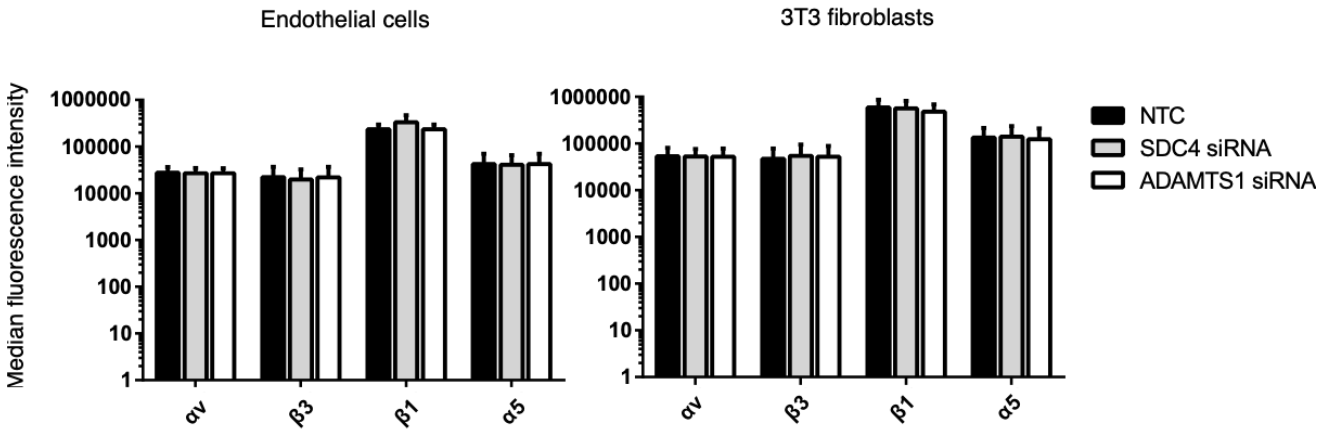


Figure 5.11 Flow cytometry reveals no change in cell surface levels of fibronectin binding integrins. ECs or 3T3 fibroblasts were siRNA depleted of ADAMTS-1 or syndecan 4 and seeded onto gelatin, 48 hours post transfection cells were collected in citric saline buffer. Flow cytometric analysis was used to determine levels of integrins. Median fluorescence intensities were averaged from three independent experiments, error bars represent S.E.M.

5.7 ADAMTS-1 and syndecan 4 play a role in α5 integrin trafficking

Although flow cytometry showed no changes in cell surface integrin expression upon ADAMTS-1 or syndecan 4 knockdown, these experiments had to be performed on cells adhered to gelatin, due to limitations in detaching cells adhered to fibronectin. Flow cytometry experiments also did not provide information about overall integrin localisation. Therefore, to get a more comprehensive understanding of integrin behaviour, immunocytochemistry was used to visualise α5 integrin. This integrin was chosen as it is the canonical fibronectin receptor, and functions co-operatively with syndecan 4 to form focal adhesions on fibronectin (Bass, Morgan and Humphries, 2007).

Immunocytochemical visualisation found striking differences in integrin α5 presentation upon ADAMTS-1 or syndecan 4 knockdown. At 90 minutes, the phenotype resembled that seen with paxillin, with larger adhesions forming in the *Adamts1* or *Sdc4* siRNA treated cells as opposed to NTC. At 180 minutes of adhesion to fibronectin, while α5 expression in NTC treated cells resembled typical EC focal adhesions, in *Adamts1* or *Sdc4* siRNA treated cells the α5 integrin formed long, fibrillar-like adhesions (Figure 5.12).

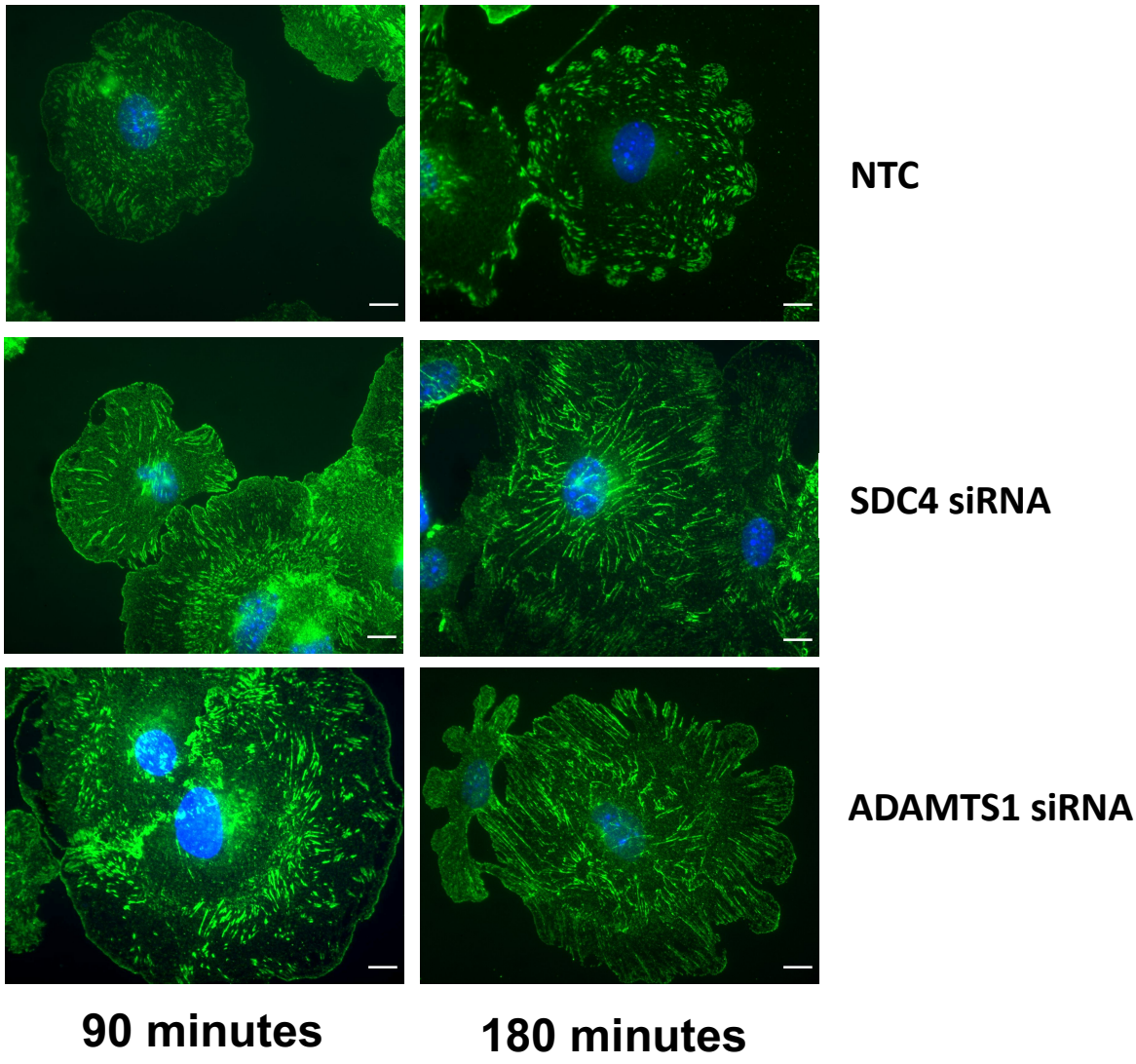


Figure 5.12 *Adamts1* or *Sdc4* siRNA treatment alters α5 integrin distribution. Endothelial cells were treated with indicated siRNA, after 48 hours recovery, they were seeded onto fibronectin coated glass coverslips and allowed to adhere for 90 or 180 minutes. Cells were then fixed and immunostained for α5 integrin. Images are representative of three independent experiments. Scale bar = 10 μm.

This altered integrin α5 distribution was predicted to be a result of altered membrane trafficking, due to the key role of syndecan 4 in integrin recycling. Accordingly, cell surface biotinylation based assays were used to assess α5 integrin internalisation rates. Cells treated with siRNA were seeded onto fibronectin and allowed to adhere. Cell surface proteins were then biotinylated via incubation with a cleavable biotin; cells were then stimulated to induce internalisation of cell surface integrins, followed by removal of cell surface biotin by incubation with the membrane impermeable reducing agent Mesna. Cells were lysed and immunoprecipitations were performed to collect internalised biotinylated protein. Lysates were then western blotted to quantify levels of α5 integrin uptake.

Internalisation assays revealed that there was an increase in total cell surface integrin α5 in *Adamts1* or *Sdc4* siRNA depleted cells, and that integrin α5 internalises at a reduced rate in these cells (Figure 5.13). These data strongly suggest that the α5 phenotype seen in immunocytochemistry is a result of reduced α5 internalisation.

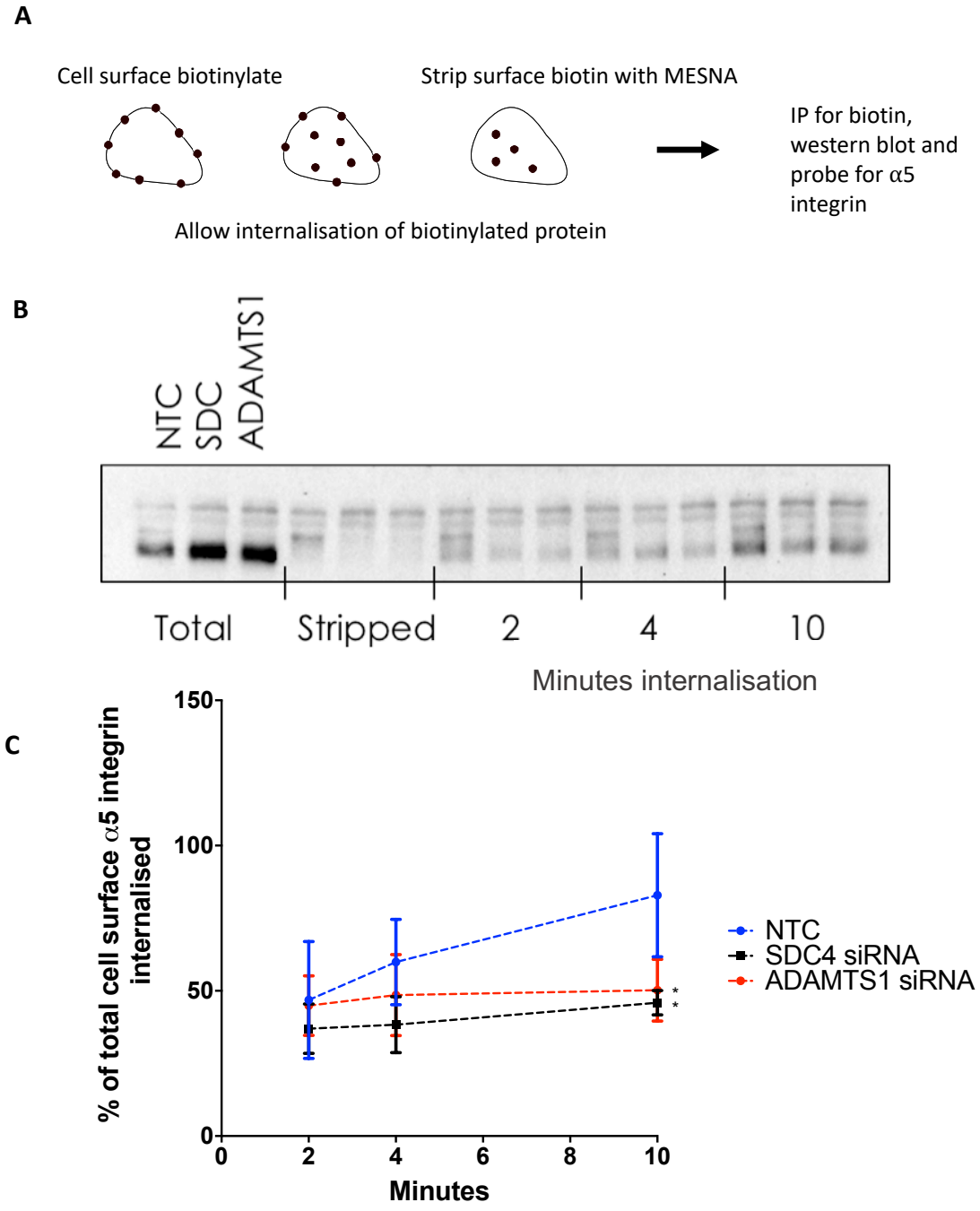


Figure 5.13 Depletion of ADAMTS-1 or syndecan 4 inhibits α5 integrin internalisation. A) schematic outlining biotinylation based internalisation assay. Cells are surface biotinylated with a cleavable biotin, then stimulated to allow internalisation, biotin remaining on the cell surface can be removed by incubation with a membrane impermeable reducing agent (Mesna). B) representative western blot of internalisation assays. C) densitometric quantification of internalisation western blots, N = 3 independent experiments, bars represent S.E.M. *P<0.05 calculated using a Student's T test, compared to NTC timepoint control.

5.8 ADAMTS-1 and Syndecan 4 phenotypes are not dependent on α5 integrin

As α5 integrin is highly important in migration, and ADAMTS-1 and syndecan 4 have profound effects on its trafficking, it seemed plausible that their effects on adhesion, migration, and potentially angiogenesis, were mediated via this integrin.

To determine the contribution of α5 integrin to ADAMTS-1/syndecan 4 depletion induced phenotypes, ECs with a Cre-recombinase mediated deletion of α5 were utilised. Experiments to determine random migration speed, focal adhesion sizes, and capability to sequester VEGF-A_{164} upon ADAMTS-1 or syndecan 4 knockdown were repeated in these α5 deleted cells. If ADAMTS-1 and syndecan 4 were modulating α5 to exert their affects, it was predicted that phenotypes would be lost in α5 null cells.

The α5 null cells displayed reduced migration as compared to wild-types, as would be expected upon loss of this important fibronectin binding integrin. Focal adhesion sizes were also slightly reduced, again expected as α5 integrin is important in initiating focal adhesion formation on fibronectin. Interestingly the loss of α5 also affected VEGF-A_{164} sequestration, with less VEGF-A_{164} found in media compared to wild-type, suggesting increased binding of VEGF-A_{164}, although this did not reach the threshold for statistical significance it is nevertheless an interesting observation.

Despite the phenotypes seen in the α5 null cells, experiments found that *Adamts1/Sdc4* siRNA treatment of these cells induced the same phenotypes as seen in wild-type cells; random migration speed was increased upon ADAMTS-1/syndecan 4 depletion, larger focal adhesions were seen, and VEGF-A_{164} availability increased (Figure 5.14). These data strongly suggested that alterations in α5 integrin behaviour were not responsible for the affects induced by *Adamts1* or *Sdc4* siRNA depletion.

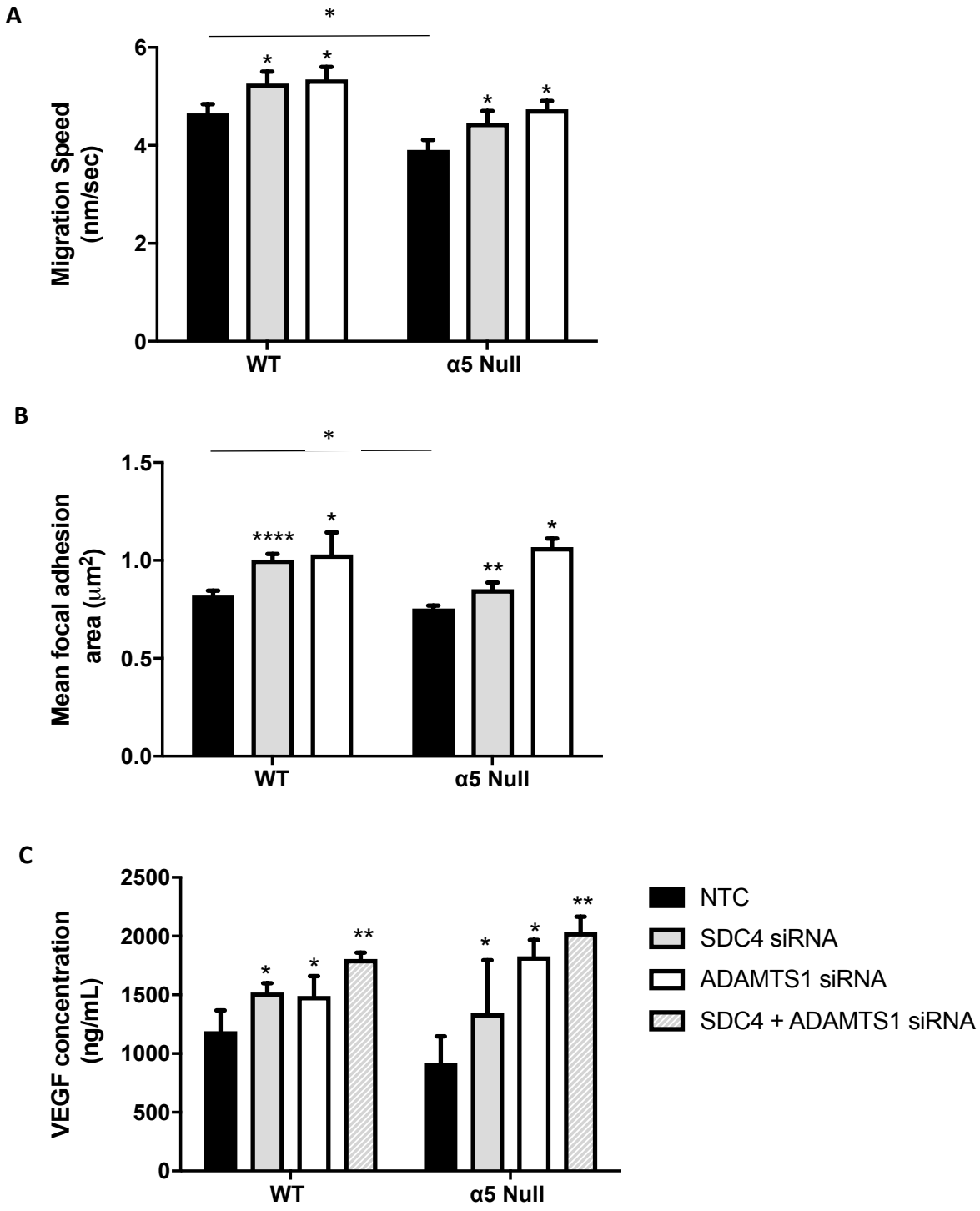


Figure 5.14 ADAMTS-1 and syndecan 4 phenotypes are maintained in α5 null cells. Experiments were repeated in ECs isolated from α5 null mice and their wildtype controls. A) VEGF-A_{164} ELISA, 30 ng VEGF-$A_{164}$was added to ECs at 4°C B) Average size of focal adhesions in cells adhered to 10 μg/mL fibronectin for 90 or 180 minutes. C) Random migration speed, cells were seeded onto fibronectin and timelapse videomicroscopy was performed over a period of 16 hours, cells were tracked using the MTrackJ plugin in FIJI. (N=3 independent experiments, error bars represent S.E.M, *P<0.05, **P<0.01, ****P<0.00001).

5.9 Active Rac1 levels are not altered by ADAMTS-1 or syndecan 4 knockdown

When assessing other mechanisms by which ADAMTS-1 and syndecan 4 may regulate cell migration, Rac1 presented as a strong candidate. Rac1 Is a small G protein, and its activation by RhoG promotes cell migration, additionally, syndecan 4 has been shown to determine directional migration via Rac1 (Katoh, Hiramoto and Negishi, 2006; Bass *et al.*, 2007).

An active Rac1 pulldown and detection kit was used to isolate active Rac1, followed by western blotting to facilitate quantification. Western blots revealed no differences in levels of active Rac1 in ADAMTS-1 or syndecan 4 conditions, suggesting the mechanism was likely Rac1 independent.

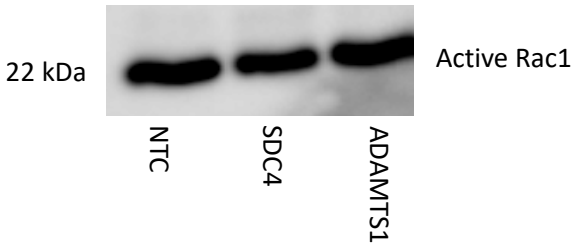


Figure 5.15 Active Rac1 levels are not affected by ADAMTS-1 or syndecan 4 knockdown. Endothelial cells were treated with *Adamts1*, *Sdc4* or NTC siRNA, then seeded on fibronectin for 90 minutes, protein was collected and active Rac1 pulldowns performed. Pulldown samples were western blotted and probed for Rac1. Image is representative of three independent repeats.

5.10 Conditioned matrix experiments reveal a contribution of the ECM to ADAMTS-1 and syndecan 4 knockdown phenotypes

As ADAMTS-1 and syndecan 4 appeared not to rely upon α5 integrin or Rac1 to exert their effects, other avenues had to be considered. Cell-ECM interactions are essential for cell motility, and the ECM can regulate cell migration, proliferation and survival, as well as function as a reservoir of growth factors, and therefore the roles of the ECM in this context were assessed.

Several lines of evidence hinted at a contribution of the ECM: both ADAMTS-1 and syndecan 4 localise at and can interact with the ECM, positioning them well to modify it. Furthermore, phenotypes were specific to fibronectin, implying the importance of this matrix protein in ADAMTS-1 and syndecan 4's regulation of cell behaviour. Therefore, it was hypothesised that changes in migration, adhesion and altered α5 integrin localisation and trafficking were occurring in response to alterations in the extracellular matrix.

Endothelial cells have the capability to synthesise and remodel the ECM. Therefore, to assess the contribution of the ECM modification to ADAMTS-1/syndecan 4 knockdown phenotypes, 'conditioned matrix' experiments using cell generated matrix were undertaken (Davis and Senger, 2005). Endothelial cells were siRNA treated, seeded onto glass coverslips, and allowed to produced matrix over a 48-hour period. Cells were removed from plates using ammonium hydroxide, and after extensive washing, untreated cells could be seeded onto this conditioned matrix (CM).

To determine if the altered α5 patterning seen was due to an altered ECM, untreated, or 'naïve' endothelial cells were seeded onto NTC, ADAMTS-1 or syndecan 4 knockdown CM, followed by immunocytochemistry to visualise α5 integrin. Much like the *Adamts1* or *Sdc4* siRNA treated cells, naïve cells plated onto a syndecan 4 or *Adamts1* siRNA CM displayed the accumulation of α5 integrin into fibrillar adhesions after 180 minutes of adhesion, whereas cells seeded onto an NTC matrix did not (Figure 5.16).

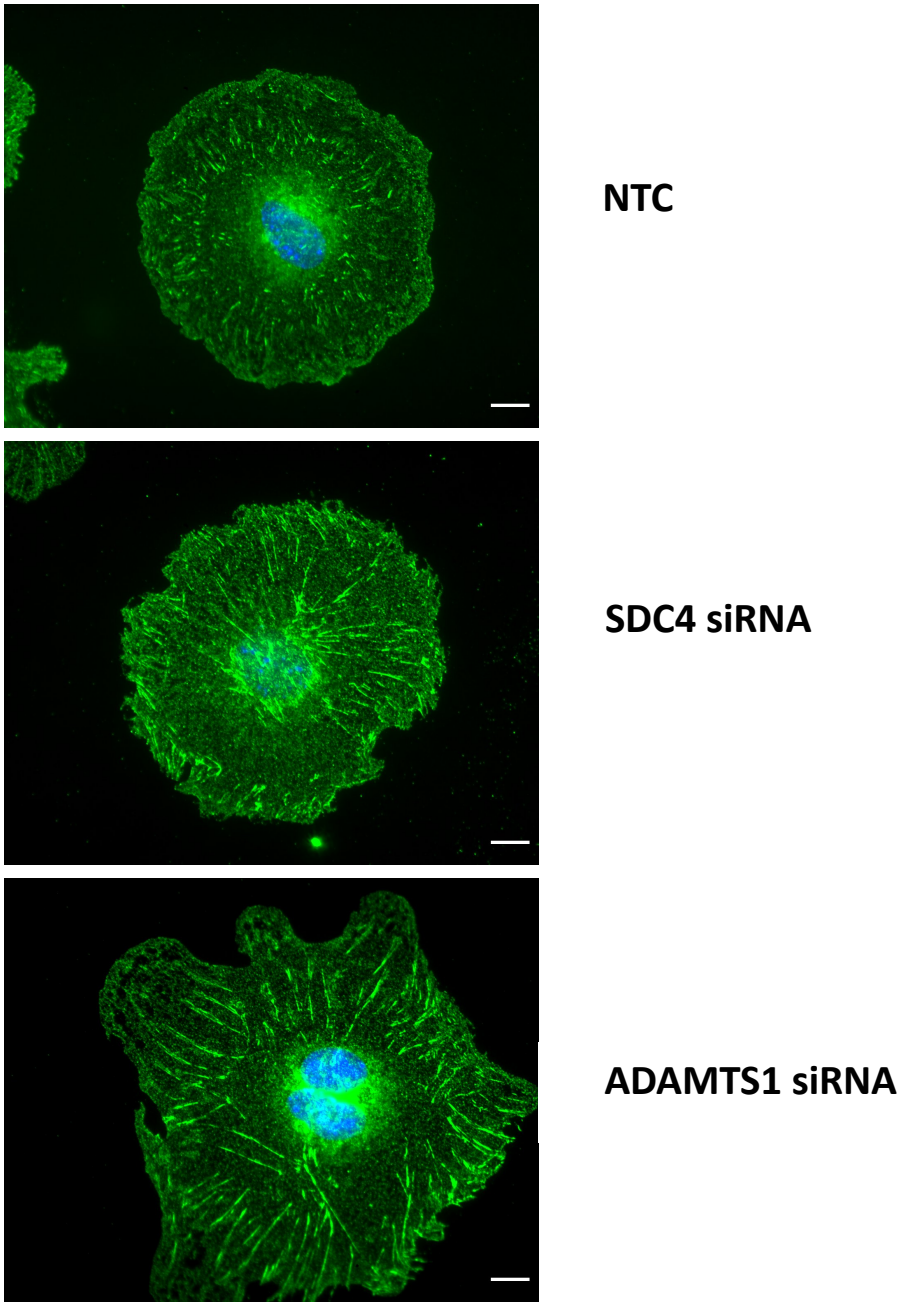


Figure 5.16 Naïve endothelial cells displayed altered α5 integrin distribution in response to conditioned matrix from ADAMTS-1 or syndecan 4 knockdown cells. Cells treated with siRNA were seeded onto uncoated coverslips and allowed to produce matrix for 48 hours, the matrix was then decellularized and after extensive washing untreated cells were plated. Images are representative of three independent experiments, scale bar = 10 μm.

From the immunocytochemistry data, it was clear that the ECM was responsible for the altered α5 integrin expression. To gain further perspective, and to assess if the ECM was contributing more widely to the phenotypes of increased migration and angiogenesis, cell signalling in response to CM was assessed. Endothelial cells were seeded onto NTC, ADAMTS-1 or syndecan 4 knockdown CM and allowed to adhere for 45, 90 or 180 minutes, at the respective timepoints cells were lysed, followed by western blotting for signalling proteins involved in migration and angiogenesis: FAK, paxillin and ERK. Cells seeded onto CM from ADAMTS-1 or syndecan 4 knockdown cells had greater signalling responses than those seeded onto NTC CM,. This was demonstrated by increased levels of phosphorylated paxillin and ERK, indicating that the altered ECM contributes to the increased cell migration and angiogenesis seen upon ADAMTS-1 or syndecan 4 depletion (Figure 5.17).

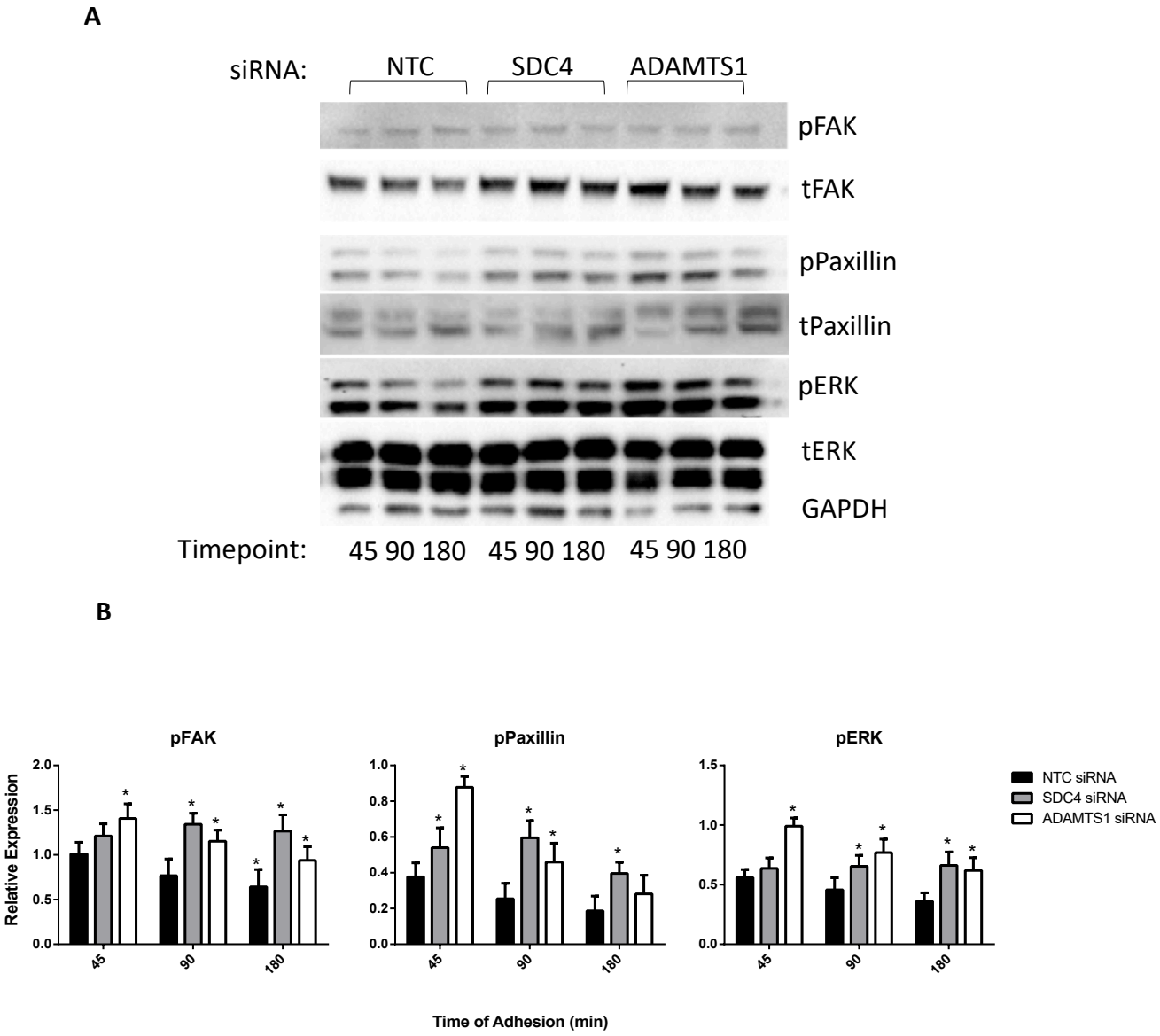


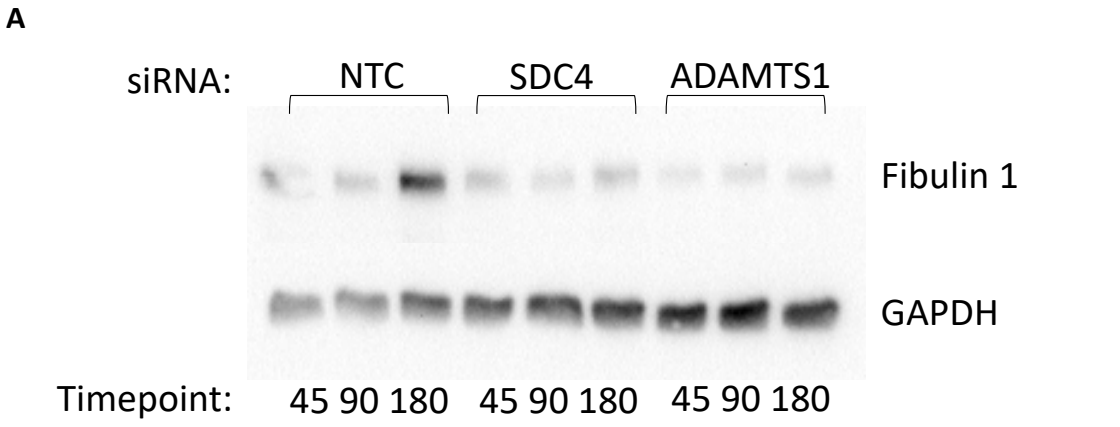
Figure 5.17 ECs have increased pro-angiogenic and pro-migratory signalling in response to seeding on ADAMTS-1 or syndecan 4 conditioned matrix. Naïve cells were seeded onto NTC, ADAMTS-1 or syndecan 4 knockdown CM for 45, 90 or 180 minutes, cells were then lysed and western blotted for signalling pathways. A) representative western blot. B) densitometric quantification performed in FIJI®, phosphorylated protein normalised to total, N = three independent experiments, bars = S.E.M. *P<0.05 calculated using a Student's T test.

5.11 ADAMTS-1 and syndecan 4 activate fibulin 1 expression

Based on conditioned matrix studies, ADAMTS-1 and syndecan 4 appeared to be altering the extracellular matrix in order to inhibit cell migration and angiogenesis, however the mechanism by which this was achieved was unclear.

Fibulin 1 was chosen as a possible target of ADAMTS-1 and syndecan 4 regulation based on extensive review of potential candidate effectors. Fibulin 1 is an extracellular matrix protein which functions as a co-factor for ADAMTS-1, enhancing its aggrecanase activity (Lee *et al.*, 2005). The inhibitory effects of ADAMTS-1 and syndecan 4 on migration are specific to fibronectin; this is in keeping with the proposition of a fibulin 1 mediated mechanism, as fibulin 1 has been shown to suppress migration in a fibronectin-dependent manner (Twal *et al.*, 2001). Fibulin 1 is frequently found in association with fibronectin *in vivo*, and it can be detected with fibronectin in focal adhesion sites an hour after seeding (Argraves *et al.*, 1989; Balbona *et al.*, 1992). Through this close interaction with fibronectin, fibulin 1 is able to modify fibronectin's important biological activities.

To assess any contribution of fibulin 1 to the phenotype, siRNA treated ECs were seeded onto a fibronectin matrix. After 45, 90 or 180 minutes of adhesion, cells were lysed and western blotting for fibulin 1 was performed. A statistically significant increase in fibulin 1 expression was seen by 180 minutes of adhesion in NTC cells, however no increase was seen in ADAMTS-1 or syndecan 4 knockdown cells (Figure 5.18). As fibulin 1 inhibits migration on fibronectin, but not on collagen, this lack of fibulin 1 expression may go some way to explain the increased random migration speed of ADAMTS-1 and syndecan 4 knockdown cells specific to fibronectin seen in Figure 5.2.



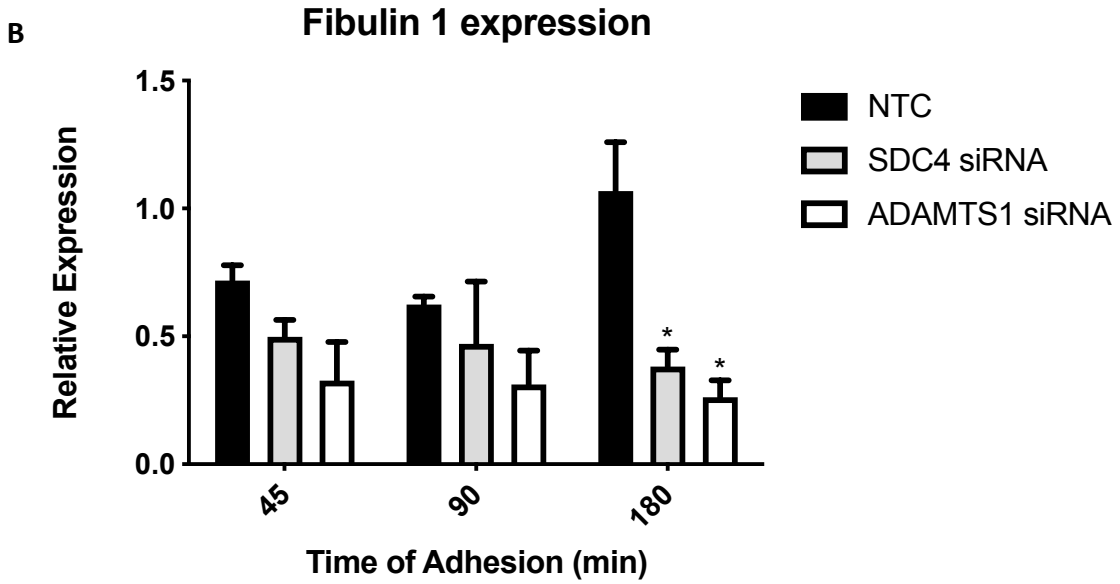


Figure 5.18 Fibulin 1 expression is upregulated in NTC but not *Adamts1* or *Sdc4* **siRNA treated cells.** Endothelial cells were treated with indicated siRNAs, cells were seeded into 10 μg/ml fibronectin coated wells. After adherence for the indicated timepoints, cells were lysed and western blotting for fibulin 1 was performed. A) representative western blot image. B) densitometric quantification performed in FIJI®, N=3 independent experiments, error bars represent S.E.M, *P<0.05 calculated using a Student's T test.

5.12 Discussion

Chapter four found that ADAMTS-1 and syndecan 4 loss drives angiogenic sprouting. As both proteins are reportedly involved in cell migration, this suggested that ADAMTS-1 and syndecan 4 interaction with the ECM may regulate cell migration, contributing to their regulation of angiogenesis. These data revealed key roles for ADAMTS-1 and syndecan 4 in endothelial and fibroblast cell migration. These proteins can activate fibulin 1 expression in the ECM, which leads to inhibition of cell migration, internalisation of α5, and inhibition of nascent adhesion maturation.

Cell migration experiments were performed on fibronectin and collagen I matrices. These matrices were chosen due to their roles in angiogenesis. Fibronectin is strongly expressed around developing vessels and interacts with many ECM components, as well as integrin receptors (Peters, Chen and Hynes, 1996). While synthesis and deposition of various collagens affects EC survival and vessel formation, type I collagen is the main ECM component to which ECs are exposed in an injured tissue.

Of note, the effects of ADAMTS-1 and syndecan 4 on cell migration and adhesion were specific to fibronectin. This specificity was not entirely unexpected; ADAMTS-15 has been shown to inhibit breast cancer cell migration in a syndecan 4 dependent mechanism on fibronectin or laminin matrices, but motility on type 1 collagen was unimpaired (Kelwick, Wagstaff, *et al.*, 2015). The profound effects on fibronectin only are also consistent with the key role of syndecan 4 in binding to the heparin binding domains of fibronectin, important in focal adhesion formation (Woods *et al.*, 2000). The specificity of the phenotype can also be explained by the involvement of fibulin 1. Fibulin 1 binds fibronectin, but not other ECM proteins such as laminin, or type I or IV collagen. The binding site for fibulin 1 in fibronectin is in the C-terminal heparin binding region, however heparin does not compete for the interaction, suggesting distinct binding sites (Balbona *et al.*, 1992). Incorporation of fibulin 1 into FN-containing type I collagen cells inhibits migration, but not in those lacking fibronectin, demonstrating the specificity of fibulin 1's actions to fibronectin (Twal *et al.*, 2001).

As ADAMTS-15 also affects migration on laminin, it would be interesting to see how fibulin 1, ADAMTS-1 and syndecan 4 affect cell migration on this substrate. Furthermore, migration and adhesion experiments were conducted in 2D environments using a single matrix coating, however this is not overly representative of the environment in which angiogenesis occurs, which is 3D and contains many interacting ECM proteins. It is also important to understand the style in which endothelial cell migration occurs: cells migrate collectively, with the tip cell leading, followed by proliferating stalk cells (Gerhardt *et al.*, 2003). Therefore, using 3D migration models more representative of the *in vivo* tissue environment could enhance understanding of ADAMTS-1 and syndecan 4 in cell migration.

The role of ADAMTS-1 in cell migration has remained somewhat controversial, with work demonstrating both pro- and anti- migratory functions (Rocks *et al.*, 2008; Esselens *et al.*, 2010; Freitas *et al.*, 2013; Ham *et al.*, 2017). The apparent contrasting roles of ADAMTS-1 may be in some part explained by the work of Werner *et al.* which demonstrates a dual role for ADAMTS-1 in cell motility; exogenous addition of low concentrations of ADAMTS-1 stimulated migration, whereas high concentrations were inhibitory (Krampert *et al.*, 2005). Furthermore, work by Lui et al, demonstrated that the catalytically active ADAMTS-1 has different functions to a fragment lacking the catalytic domain, with the full length promoting metastasis and the fragment inhibiting (Liu, Xu and Yu, 2006).

The function of ADAMTS-1 in cell migration may also be context dependent; it appears as though work showing ADAMTS-1 to be pro migratory uses overexpression systems, whereas those demonstrating antimigratory functions focused on cell expressed ADAMTS-1. Research showing ADAMTS-1 cleaves semaphorin 3C to promote cell migration utilised breast cancer cells transfected to overexpress ADAMTS-1, and the in vivo data supporting a pro-migratory role of ADAMTS-1 also overexpressed the protease in lung carcinoma cells (BZR), prior to tumour injection into mice (Rocks *et al.*, 2008; Esselens *et al.*, 2010). To contrast, in work showing ADAMTS-1 to be anti-migratory, for example in the research which described PPARδ activation resulting in expression of ADAMTS-1 to inhibit migration, the role of

endogenous ADAMTS-1 was investigated via the use of knockdowns. This was the same for *in vivo* data investigating the migratory roles of ADAMTS-1; lower levels of ADAMTS-1 protein were detected in human breast cancers as opposed to normal tissue, with a striking decrease in high-malignancy cases, which was supported by ADAMTS-1 knockdown in MDA-MB-231 cells being shown to increase migration and invasion (Freitas *et al.*, 2013; Ham *et al.*, 2017).

The syndecan 4 cell migration data seemed to contrast with the wider literature. While here syndecan 4 seems to function in an anti-migratory capacity, Bass *et al,* found that syndecan 4 was required to maintain directional migration (Bass *et al.*, 2007). Several hypotheses exist which may help to explain these contrasting data. The first regards the use of cells isolated from knockout animals. Data demonstrating syndecan 4 is needed for cell migration was performed in fibroblast cells isolated from the *Sdc4*-/- knockout mouse.

The *Sdc4*-/- knockout mouse was generated in the 129/SVJ strain, via introduction of an IRESβgeo cassette, followed by injection into the C57BL/6 line generating chimeras, which were then backcrossed onto the C57BL/6 line. Generation of knockout mice in this way gives the potential for 'passenger mutations'; the carry-over of aberrant genes which can influence the phenotype. Passenger mutations are caused by 129 embryonic stem cell derived genetic material that has remained in the genome, which is a problem as the 129 genome contains 1,089 SNPs resulting in alternative or aberrant amino acid sequences (Vanden Berghe *et al.*, 2015). Modified genes are carried over alongside the knockout, and backcrossing to the C57BL/6 line replaces 129 genetic material, however a problem arises when 129 mutant genes are close to the gene of interest. Flanking regions have a high degree of genetic linkage and therefore even extensive backcrossing may not result in complete replacement of these mutant 129 regions with C57BL/6 genetic material (Eisener-Dorman, Lawrence and Bolivar, 2009).

A Me-PaMuFind-It web tool developed to identify passenger mutations in congenic mice identified several potential passenger mutations in *Sdc4*-/- mice (Figure 5.19).

Therefore, when assessing differences between the siRNA mediated knockdown cells and the *Sdc4*-/- cells it is important to take into consideration the contribution of the 129 background.

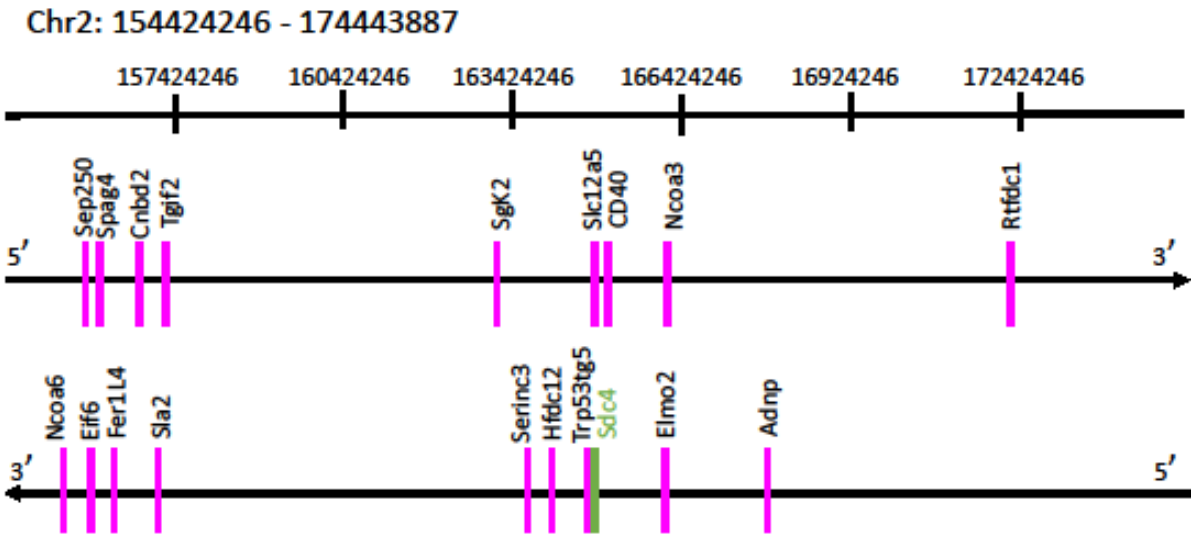


Figure 5.19 Potential passenger mutations in *Sdc4-/-* mice. Image shows gene indels/SNPs in a genomic region of 10 mbp upstream and downstream from *Sdc4*. Gene of interest is coloured in green, flanking genes with SNPs affecting their amino acid sequences are in pink (Vanden Berghe *et al.*, 2015).

Passenger mutations are not the only issue confounding interpretation of phenotypes observed in constitutive knockout models. Phenotypic differences between knockouts and knockdowns have been previously reported in the mouse. These changes may be as a result of off target effects, however they may also be a result of gene compensation (De Souza *et al.*, 2006). Genetic compensation in response to gene knockout is a widespread phenomenon; cells are highly robust and maintain their viability despite genetic perturbation (El-Brolosy and Stainier, 2017). Transcriptional adaptation to counteract the effects of the constitutive knockout may therefore affect the phenotype. Furthermore, the *Sdc4*-/- mouse is an embryonic constitutive knockout, and reduction or absence of a phenotype in germline mutants

compared to conditional or tissue specific counterparts has also been reported in mouse studies (El-Brolosy and Stainier, 2017).

The premise, therefore, is that inconsistencies between the data presented here, and that reported in the literature, can be explained by the use of knockout mouse models. This is specifically supported by the work of Cavalheiro *et al*. Using RNAi (shRNA) to induce syndecan 4 knockdown in ECs they found, much like this work, that syndecan 4 knockdown cells migrated at an increased rate (Cavalheiro *et al.*, 2017).

The generation of an inducible, and endothelial cell specific *Sdc4*-/- mouse model may go a long way in answering some of the contradictions posed by this report, by assisting in negating the effects of passenger mutations and genetic compensation which may contribute to the knockout mouse phenotype.

A further consideration regards the type of migration investigated; syndecan 4 was shown to be essential in directional migration, whereas in this thesis the focus was speed. While scratch wound assays were performed to assess directionality, no changes were seen, and experiments were discontinued due to concerns that the effect of ADAMTS-1 on proliferation would confound interpretation of results. In future research, directional migration should be examined in a more sophisticated system, perhaps in scratch wounds with the use of mitomycin C as a proliferation blocker, or to gain more physiological relevance, assays with the use of chemotactic gradients such as a Boyden chamber assay (Chen, 2005; Nishimura, 2009). Either way, despite some contradictions, syndecan 4 is clearly essential for co-ordinating cell migration and its loss has profound affects.

Actin appears to be of importance in ADAMTS-1 and syndecan 4's regulation of cell migration, as its distribution was dramatically altered upon their perturbation. Actin is essential for cell migration. Its polymerisation drives lamellipodial protrusion, and stress fibre formation and actomyosin activity are enhanced during cell migration. Reports of syndecan 4 interaction with actin exist, with the coupling of vinculin to F-actin reportedly depending on syndecan 4 (Cavalheiro *et al.*, 2017). Syndecan 4 siRNA knockdown driving an altered actin distribution has been also been reported

previously; Vuong *et al,* found that actin filaments in syndecan 4 knockdown cells became thinner in appearance, and aligned in parallel with the cell axis, as opposed to thick bundles in a more heterogenous pattern seen in control cells (Vuong *et al.*, 2015). How syndecan 4/ADAMTS-1 regulate actin organisation is unclear, however it may include the involvement of fibulin 1.

Naïve cells plated onto ADAMTS-1 and syndecan 4 cell conditioned matrix demonstrated phenotypes resembling those of ADAMTS-1 or syndecan 4 knockdown cells on fibronectin, suggesting ADAMTS-1 and syndecan 4 inhibit cell migration by modulating the ECM. These ECM dependent anti-migratory activities were attributed to activation of fibulin 1 expression. Fibulin 1 is an ECM protein, and a co-factor for ADAMTS-1(Lee *et al.*, 2005); expression of fibulin 1 has been reported to result in a fibronectin-specific inhibition of migration in a syndecan 4 dependent manner (Twal *et al.*, 2001; Williams and Schwarzbauer, 2009). As fibulin 1 expression is upregulated at 180 minutes post adhesion in NTC-treated cells, but not in *Adamts1* or *Sdc4* siRNA cells, the increase in migration speed seen upon ADAMTS-1 or syndecan-4 depletion could be attributed to the lack of fibulin 1. At present it remains unclear how fibulin 1 regulates migration, however it has been hypothesised to involve regulation of acto-myosin contractility (Twal *et al.*, 2001).

Focal adhesions were also assessed. They were visualised and the average size calculated. The size of focal adhesions is important as it can predict cell speed, independently of focal adhesion surface density and molecular composition (Kim and Wirtz, 2013). Alongside the altered actin distribution, larger focal adhesions were found in the *Adamts1* and *Sdc4* siRNA treated cells. This alteration was attributed to faster maturation of focal adhesions, due to the higher percentage of mature adhesions found in these cells as opposed to nascent adhesions. Despite this conclusion, alternative possibilities exist, such as delayed turnover or prevention of degradation, therefore to fully understand focal adhesion behaviour it would be useful to investigate focal adhesion turnover with the use of a fluorescent reporter paxillin construct and live microscopy.

The increase in random migration speed upon *Adamts1* and *Sdc4* siRNA depletion was concluded to be independent of increased VEGF signalling activity, as cells in serum-free media still displayed a statistically significant increase in random migration speed. This hypothesis was supported by western blots to quantify levels of phosphorylated paxillin; *Adamts1* and *Sdc4* siRNA depleted cells did exhibit increased phosphorylated paxillin in response to VEGF-A_{164}compared to NTC cells. However, the baseline levels of phosphorylated paxillin, prior to addition of exogenous VEGF-A_{164}, were also much higher in these cells, indicating VEGF is not required for this phenotype. In future studies, knocking down VEGFR2 expression in these cells could confirm the lack of VEGF requirement.

Integrins have some involvement in this process, although to what extent is not entirely clear. No changes in relative levels of integrins were seen via qPCR or flow cytometry. The lack of change in RNA expression is not entirely surprising as integrin dynamics must be regulated rapidly in order to respond to the environment and regulate migration. Therefore they are primarily regulated by trafficking at the cell membrane and by internalisation followed by either recycling or degradation, more rapid processes than regulation by synthesis (Caswell and Norman, 2006). In light of the immunocytochemisty and internalisation data showing clear accumulation of α5 integrin on the cell surface, the flow cytometry data showing no change in the integrin profile was more unexpected, however this may be explained by the matrix. In our hands, it was not possible to non-enzymatically detach cells from fibronectin, and therefore cells used for flow cytometry experiments had been cultured on a gelatin matrix. As the integrins profiled are fibronectin binding, and migration and adhesion phenotypes were specific to fibronectin, it is likely that no change would be seen on a gelatin matrix.

A deeper investigation into integrin α5 revealed that in *Adamts1* and *Sdc4* siRNA cells, the rate of α5 integrin internalisation was reduced. Immunocytochemistry experiments showed an altered expression pattern, with α5 incorporating into what were determined to be fibrillar adhesions, based on their morphology. Fibrillar adhesions appear as long streaks, and are developed exclusively on fibronectin when

α5β1 integrins translocate from focal adhesions along fibronectin fibrils in the ECM (Smilenov *et al.*, 1999). Despite this clear perturbation of integrin α5, phenotypes of increased random migration, increased focal adhesion size, and decreased sequestration of VEGF-A_{164} were all maintained upon *Adamts1* or *Sdc4* siRNA depletion in α5 deleted cells, strongly suggesting that the alterations to α5 integrin occur downstream, or as a response to fibulin 1 expression and alteration to the ECM, rather than acting as the driving force behind phenotypic changes. Supporting this conclusion is strong evidence that the ECM influences the formation of fibrillar adhesions (Katz *et al.*, 2000).

Integrin α5 internalisation was inhibited in *Adamts1* and *Sdc4* siRNA treated cells. As previously discussed syndecan 4 phosphorylation is a control point in integrin recycling. Upon its phosphorylation, αVβ3 recycling to the plasma membrane is promoted at the expense of α5β1. The loss of syndecan 4 can therefore explain this disrupted α5 integrin internalisation. Furthermore, differential regulation of these two integrins has been shown to regulate cell migration, with more α5 adhesions promoting focal adhesion turnover, and more αv adhesions stabilising focal adhesions (Morgan *et al.*, 2013).

Although syndecan 4 is clearly involved in integrin recycling, the connection to ADAMTS-1 is less clear. ADAMTS-1 contains a disintegrin domain, however crystallisation found this had no structural homology to integrin-interacting disintegrin domains of ADAM proteins and suggested this 'disintegrin' may be a misnomer (Gerhardt *et al.*, 2007). It therefore remains unclear if ADAMTS-1 has a direct contribution to α 5 internalisation, or if it is simply a consequence of the loss of syndecan 4 upon *Adamts1* siRNA depletion.

Although in this instance the mechanism was not mediated by integrin α5, the loss of this integrin resulted in some relevant phenotypes. Integrin α5 is essential for adhesion to and migration upon fibronectin, and therefore the smaller focal adhesions and decreased migration speed in integrin α5 null cells is not hard to

rationalise. What was more interesting was a trend towards decreased VEGF-A_{164}availability, an unexpected consequence that merits further consideration.

5.13 Chapter summary

The key findings of this chapter were that ADAMTS-1 and syndecan 4 inhibit cell migration on fibronectin via an ECM dependent, VEGF-independent mechanism, which involves expression of the ADAMTS-1 co-factor fibulin. Further work is needed to fully understand the roles and activities of fibulin 1 with regards to migration and ECM modification.

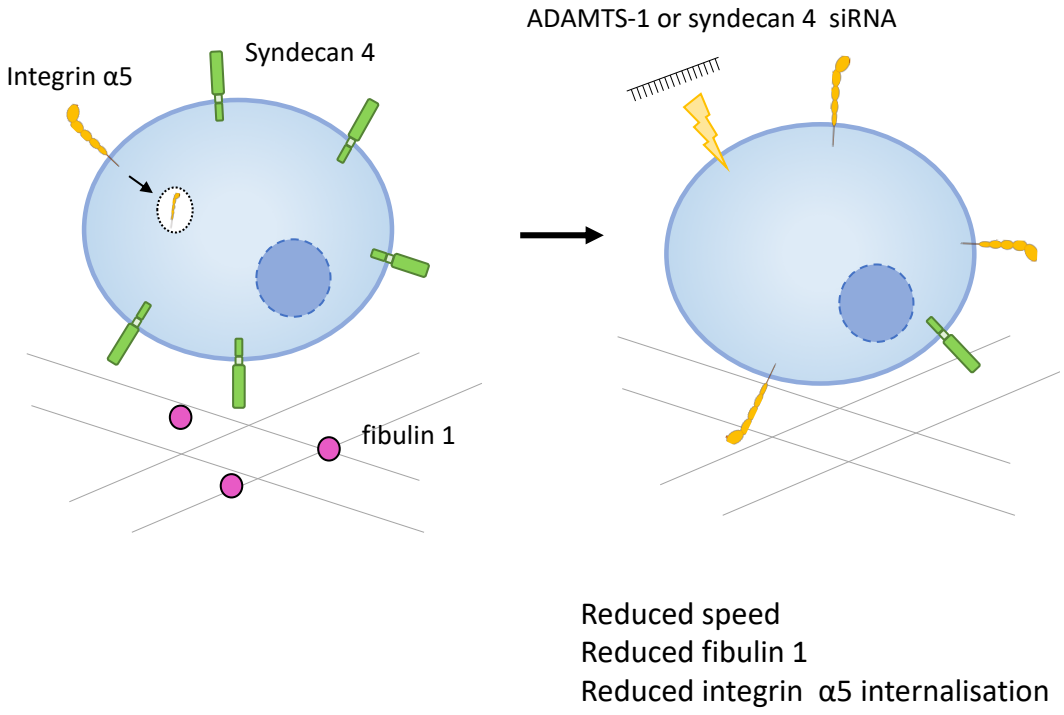


Figure 5.20 Graphical summary of chapter five results. *Adamts1* or *Sdc4* siRNA treatment reduces random migration speed and inhibits alpha 5 integrin internalisation. These changes are dependent upon the ECM, and are associated with decreased fibulin 1 expression.

6 Final discussion and future work

The extracellular matrix is a diverse and complex environment which influences every aspect of cell biology through its physical properties, and by direct interactions with cell surface receptors (Hynes, 2009). The ECM can be remodelled by proteases, in particular the zinc dependent metalloproteases of the metzincin family, which includes the 'A Disintegrin and Metalloproteinase with Thrombospondin Motifs', or ADAMTS subfamily. The ADAMTS family of matrix proteases have proved an interesting topic of research due to their diverse roles in tissue development and growth, as well as in diseases such as CVD, cancer and arthritis (Kelwick, Desanlis, *et al.*, 2015). Notably, members of the aggrecanase subgroup have displayed numerous functions in the regulation of angiogenesis, a process which relies upon remodelling of, and interaction with, the ECM. Of particular interest is ADAMTS-1, an ECM anchored member of this clade which acts as a key antiangiogenic factor(Luque, Carpizo and Iruela-Arispe, 2003).

This thesis sought to investigate how ADAMTS-1 may contribute to the regulation of cell-ECM interactions, to mediate physiological processes such as cell migration and angiogenesis. It was hypothesised that ADAMTS-1 may exert its functions through interaction with syndecan 4, a transmembrane heparan sulfate proteoglycan, and indeed whilst this thesis does not present any evidence of a direct association between ADAMTS-1 and syndecan 4, an interplay between the two was demonstrated, and experiments found that ADAMTS-1 and syndecan 4 share roles in sequestering VEGF-A_{164} to inhibit angiogenesis, as well as inhibiting cell migration via alterations to the ECM, suggesting a functional association between these two proteins.

6.1 ADAMTS and syndecans

Syndecan 4 belongs to the syndecan family of 4 transmembrane heparan sulfate proteoglycans, syndecan 1, 2, 3 and 4. All members of the syndecan family have heparan sulfate chains, allowing them to interact with a large number of ligands, which has led to debate over their specificity and roles. While almost all cells express

one or more syndecan, each family member has a distinct spatial and temporal expression patterns; these unique and developmentally regulated patterns may contribute to distinctive functions (David *et al.*, 1993; Kim *et al.*, 1994). In adult tissues, syndecan 1 is primarily expressed in epithelial cells, syndecan 2 in epithelial and mesenchymal cells, syndecan 3 is primarily expressed in the neural crest, and syndecan 4 is found ubiquitously (Teng, Aquino and Park, 2012).

Interestingly, several ADAMTS family members appear to rely on the syndecans in order to exert their functions; in particular syndecan 4, which is ubiquitously expressed and important for focal adhesion formation. Examples include ADAMTS-15, which requires syndecan 4 to inhibit cell migration, ADAMTS-6 and -10 which contrastingly regulate cell migration in a mechanism which involves syndecan 4, and in osteoarthritis, where syndecan 4 activates ADAMTS-5's cleavage of aggrecan (Echtermeyer *et al.*, 2009; Kelwick, Wagstaff, *et al.*, 2015; Cain *et al.*, 2016).

Syndecan 4 was chosen as the initial target of investigation due to reported connections to ADAMTS-1, specifically, ADAMTS-1 has been reported to clip syndecan 4, leading to increased migration (Rodríguez-Manzaneque *et al.*, 2009).

In order to investigate a potential relationship between ADAMTS-1 and syndecan 4, the targets were siRNA depleted in two cell types key to interactions with the ECM, which are both migratory and important for angiogenesis: fibroblasts and ECs. Flow cytometry found that loss of ADAMTS-1 resulted in a concomitant loss in syndecan 4, in contrast with data showing ADAMTS-1 cleaves syndecan 4, yet supporting a collaborative role for the two proteins (Rodríguez-Manzaneque *et al.*, 2009). Despite this, due to difficulty in antibody based detection of ADAMTS-1, a direct connection between syndecan 4 and ADAMTS-1 could not be demonstrated. However, ADAMTS-1 has been previously shown to co-immunoprecipitate with VEGF-A, and this thesis was able to demonstrate an interaction between VEGF-A and syndecan 4 using co-immunoprecipitation. As both proteins are capable of interacting with VEGF, it is therefore likely that, at least under certain conditions, ADAMTS-1 and syndecan 4 co-localise.

6.2 MMP-9

The loss of syndecan 4 upon ADAMTS-1 depletion was dependent upon an upregulation of MMP-9 activity. A large increase in *Mmp9* transcription was seen in ADAMTS-1 knockdown cells, and specific inhibition of MMP-9 was sufficient to prevent the loss of cell surface syndecan 4. As MMP-9 is a known syndecan 4 sheddase, this suggests that an upregulation in MMP-9 is responsible for increased syndecan 4 shedding, thereby reducing the cell surface levels (Ramnath *et al.*, 2014; Reine *et al.*, 2019). How MMP-9 transcription is upregulated, and how MMP-9 is further involved in the ADAMTS-1 syndecan 4 interaction is as yet unclear, leading to many new avenues for exploration.

The regulation of MMPs is complex and multilevel; dysregulation of MMPs is a hallmark of many diseases, thus strict spatiotemporal regulation, primarily achieved by transcriptional control, is essential. Signalling pathways that activate transcription factors that bind to cis regulatory elements in MMP promoters facilitate this (Murphy, 2011). Posttranslational modifications such as acetylation and methylation can also serve to regulate MMP expression, and MMP activity is further regulated at the protein level; pro-MMPs are secreted as zymogens which must be activated, and TIMPs inhibit the activity of active MMPs (Fanjul-Fernández *et al.*, 2010). Although this thesis found a small, non significant, change in *Timp3* expression, a striking upregulation of *Mmp9* mRNA transcription in response to ADAMTS-1 siRNA treatment was seen, suggesting *Mmp9* transcriptional regulation is where future research should focus when attempting to understand how the loss of ADAMTS-1 triggers MMP-9 mediated loss of cell surface syndecan 4.

MMP-9 is highly inducible, and its expression, driven by the MMP-9 promoter, is triggered in contexts such as wound healing, tumorigenesis and angiogenesis. Several of the MMP promotors are highly similar in structure, and all contain multiple cis-elements, allowing for precise regulation of gene expression. In the case of the MMP-9 promotor, it contains, relative to the transcription start site, a TATA box at around -30, and an AP-1 site at roughly -70 (Yan and Boyd, 2007). Although the AP-1 site is the major mediator of MMP transcription, it does not act alone and relies upon

several other regulatory elements and composite site within the promote, which also allow for specificity of responses (Benbow and Brinckerhoff, 1997). In the case of the MMP-9 promoter, there is a PEA-3 biding motif upstream proximal to the AP-1 site, as well as Sp1 and NF-κB binding sites (Clark *et al.*, 2008).

Expression of MMPs is driven by a variety of cytokines and growth factors including interleukins, interferon, EGF, HGF, bFGF, VEGF, PDGF, TNF-α and TGF-β. They function to trigger cell signalling involving MAPK, NF-κB and Smad-dependant pathways, culminating in trans-activation of MMP promoters. These signals depend at least partially on the AP-1 site as transcriptional increase in MMPs is preceded by an increase in Fos and Jun protein components of AP-1, yet the other regulatory elements are often essential (Benbow and Brinckerhoff, 1997). Activation of the *Mmp9* promotor by TNFα requires co-operation of the AP-1 and PEA3 sites with an NFkB site at -600 bp and the Sp1 site at 558 bp. In another example, the MAPK pathway serves to upregulate Ets transcription factors for PEA3, as well as AP-1 transcription factors, and these act synergistically with NF-κB, SP1, and AP1 binding sites when cells are activated by Ras (Gum *et al.*, 1996). Notably, signals originating from ECM proteins also converge upon the AP-1 element; engagement of integrins activates FAK, transmitting signals resulting in increased transcriptional activity of AP-1 (Yan and Boyd, 2007).

It is clear that *Mmp9* transcription is upregulated in response to ADAMTS-1 depletion, yet the question remains as to which transcription factors are responsible for the spike in *Mmp9* transcript, and how they are induced to do so. Due to the complex nature of *Mmp9* regulation, several areas of research would be required to understand the mechanisms leading to its upregulation.

When beginning to understand the mechanism that leads to the upregulation of MMP-9 it is important to consider the roles of these proteins in angiogenesis. Investigating the roles of ADAMTS-1 and syndecan 4 using the aortic ring assay found that these proteins were anti-angiogenic, and investigation of VEGF-A_{164} signalling revealed the mechanism behind this was sequestration of VEGF-A_{164}. Depletion of

ADAMTS-1 or syndecan 4 thereby increased VEGF-A_{164} dependent angiogenic signalling, demonstrated by increased ERK phosphorylation. The ability of these proteins to influence signalling provides potential opportunities for downstream activation of MMP-9.

While ADAMTS-1 and syndecan 4 act in an angioinhibitory fashion, sequestering VEGF, MMP-9 is a highly pro-angiogenic protein, and as part of the 'angiogenic-switch' it cleaves HSPG in the ECM, releasing trapped VEGF and triggering angiogenesis (Bergers *et al.*, 2000). Based upon this, we can form a hypothesis that a syndecan 4 – ADAMTS-1 – MMP-9 axis in the ECM regulates angiogenesis: in the quiescent vasculature ADAMTS-1 and syndecan 4 complex with VEGF sequestering it, however in activated vessels, or when ADAMTS-1 is lost, MMP-9 expression is initiated, MMP-9 then cleaves syndecan 4, a HSPG, disrupting the sequestration complex, releasing the trapped VEGF and initiating a pro-angiogenic cascade.

These signalling pathways provide numerous opportunities for downstream activation of MMP-9. Both ADAMTS-1 and MMP-9 are involved in VEGF signalling feedback loops and it is possible that the fine angiogenic signalling balance is disrupted when ADAMTS-1 is lost, which triggers MMP-9 expression(Xu, Yu and Duh, 2006; Hollborn *et al.*, 2007). As previously mentioned, upon ADAMTS-1 siRNA depletion, an increase in ERK signalling is seen, ERK is known to drive MMP-9 expression, however complicating this syndecan 4 depletion also resulted in an increase in ERK but no increase in MMP-9 (Yang *et al.*, 2014). The TNFα pathway should also be considered, as TNFα induces MMP-9 to shed syndecan 4, as well as having roles in angiogenesis (Sainson *et al.*, 2008).

Specific inhibition of signalling pathway components may help resolve how *Mmp9* transcription is upregulated. Furthermore, profiling transcription factors may give clues as to which upstream pathways are affected, and how they are exerting their functions. Blockers of specific signalling pathways, for example those inhibiting inhibitory kappa b kinases (IKKs), regulators of the NF-κB cascade, and those which target the MAPK pathway are commercially available, as are small molecule inhibits

that target AP-1 transcription factors (Burkhard and Shapiro, 2010; Gamble *et al.*, 2012; Ye *et al.*, 2014)

Another possibility to be considered regards the catalytic activity of ADAMTS-1. Inhibitory factors in serum have been reported to negatively regulate MMP-9 expression, and as a protease ADAMTS-1 has the potential to cleave and activate these factors, or release them from inhibitory complexes or sequestration (Lee, Tran and Quang, 2009). Several factors have been shown to inhibit *Mmp9* transcription, examples include suppression of TNF-α induced *Mmp9* transcription by TGF-β (Vaday *et al.*, 2001). Alongside IFN-gamma, TGF-β can also inhibit MMP transcription in response to LPS (Xie, Dong and Fidler, 1994). Another example is suppression of MMP-9 by cortisol via the glucocorticoid receptor (Rae *et al.*, 2009). Advancement of this work requires broadening our understanding of ADAMTS-1, possibly with the use of proteomic techniques, which will be discussed in greater detail later.

Furthermore, as *Mmp9* transcription is influence by epigenetic mechanisms, for example an inverse correlation is seen between level of MMP-9 promotor methylation and level of *Mmp9* expression, our understanding could be advanced by analysis of protein-DNA interactions through methods such as ChIP-sequencing (Chicoine *et al.*, 2002).

6.3 Syndecan shedding

Further work is needed to support the conclusion that MMP-9 sheds syndecan 4 as part of an angiogenic balancing act, particularly as we were unable to detect a shed fragment of syndecan 4. There are also several unanswered questions with regards to ADAMTS-1's role in syndecan 4 shedding; ADAMTS-1 had been previously shown to cleave syndecan 4, clipping a small N-terminal fragment (Rodríguez-Manzaneque *et al.*, 2009). This is somewhat contradictory with the work of this thesis which demonstrates that ADAMTS-1 is necessary for syndecan 4 cell surface expression. One potential explanation is that clipping of syndecan 4 by ADAMTS-1 does not shed the proteoglycan, and is instead protective against shedding by other proteases, therefore when ADAMTS-1 is lost syndecan 4 can be shed by MMP-9. A protective

effect of ADAMTS protease on syndecan shedding has been seen previously with ADAMTS-15, giving potential weight to this hypothesis (Kelwick, Wagstaff, *et al.*, 2015).

Finally, shed syndecan ectodomains have biological roles, and it is therefore necessary to assess the functions of a shed syndecan 4 ectodomain in this context. It remains largely unclear what exact roles the shed syndecan ectodomain fulfils, however shed syndecan ectodomains can be detected in humans, particularly in disease states, suggesting it has roles (Bollmann *et al.*, 2017). Importantly, due to the migration effect seen, the syndecan 4 ectodomain has been reported to modulate RhoA and Rac1 activation (Kim, Roshanravan and Dryer, 2015)

6.4 Syndecan 4 in angiogenesis and cell migration

ADAMTS-1 is already well established as an antiangiogenic protein, however the description of syndecan 4 as antiangiogenic is novel, and somewhat unexpected. Previous research into syndecan 4 has demonstrated roles in maintaining directed cell migration, and in perpetuating signalling by FGFR (Horowitz, Tkachenko and Simons, 2002; Bass *et al.*, 2007). Therefore, it could be hypothesised that in the context of angiogenesis its loss would inhibit cell migration and growth factor signalling, inhibiting angiogenesis, however the contrary was found; loss of syndecan 4 resulted in increased cell migration and increased angiogenesis.

While attempting to resolve these contrasting sets of data it is highly important to consider the models used. Previous work on syndecan 4 has focused on the use of *Sdc4*-/- mice, and cells isolated from these, whereas the work in this thesis was conducted using siRNA depletion of targets. The choice to use siRNA was made due to the speed and simplicity of siRNA transfection. Initial experiments with CRIPSR cas9 were also performed to give long term knockouts. The CRISPR deletion of targets was successful in fibroblasts, and showed the same phenotypes with regards to cell migration, however as the angiogenesis aspect of the project developed the use of endothelial cells was primarily required, and these proved difficult to transfect with the CRISPR construct. There were also concerns with regards to off-target effects,

reported to be a problem with CRISPR, with some publications reporting frequencies as high as 50% (Zhang *et al.*, 2015). Off target affects are also possible using siRNA, but to minimise these effects, Dharmacon onTarget-plus siRNAs were chosen, which are chemically modified to reduce off target effects. The minimum dose required to give effective knockdown was also established and used henceforth, as siRNA off target effects are often concentration dependent. Furthermore, an initial pool of 4 siRNAs was used, and using any of the 4 *Adamts1* siRNAs individually induced the loss of cell surface syndecan 4.

Differences between the knockout mouse model and cells siRNA depleted for syndecan 4 suggest interesting differences between the developmental lack of syndecan 4, as opposed to acute loss. This invites consideration of compensation mechanisms, and consideration of other syndecan family members.

The data presented in this thesis connect ADAMTS-1 and syndecan 4, and support an essential interplay between ADAMTS family proteases and syndecans, whether the functions of ADAMTS-1 extend to other syndecans is yet to be determined, but may prove an interesting area for future work. The other syndecans also have roles in cell migration and angiogenesis, particularly syndecan 1 and 2, which can also be shed by MMP-9 (Brule *et al.*, 2006; Fears, Gladson and Woods, 2006; Manon-Jensen, Multhaupt and Couchman, 2013).

Syndecan 1 binds to many mediators of disease pathogenesis, and through these interactions mediates cancer cell proliferation and invasion, as well as angiogenesis and matrix remodelling(Teng, Aquino and Park, 2012). Supporting an important role for syndecans in matrix remodelling, *Sdc1*-/- mice have abnormal infarct healing, associated with assembly of a disorganised matrix with smaller and fragmented collagen fibres (Vanhoutte *et al.*, 2007). Syndecan 1 has also exacerbates cardiac fibrosis in an angiotensin II induced mouse model of cardiac fibrosis (Schellings *et al.*, 2010).

In the context of cell ECM interaction and migration, syndecan 1 promotes adhesion to the ECM and inhibits cell migration. Similar to the interaction of syndecan 4 and α5β1 in binding fibronectin, syndecan 1 functions as a collagen 1 co-receptor alongside α2β1 integrin, regulating Rac1 and RhoA to control filopodial and lamellipodial formation (Ishikawa and Kramer, 2010). The shed syndecan ectodomain can also contribute to cell migration, however whereas cell surface syndecan 1 inhibits migration, the shed ectodomain is pro-migratory (Endo *et al.*, 2003).

Syndecan 1 has clear links to ADAMTS proteases. The activation of ADAMTS-4 involves both cleavage by MT4-MMP, and binding of ADAMTS-4 to the CS and HS chains of syndecan 1 (Gao *et al.*, 2004). This interaction is supported by studies regarding the treatment of OA, since the cannabinoid win-55,212-2 inhibits ADAMTS-4 activity via inhibiting expression of syndecan 1 (Kong *et al.*, 2016). This invites consideration of an ADAMTS-1/syndecan 1 connection, as ADAMTS-1 and ADAMTS-4 are closely related members of the aggrecanase subgroup, and have been previously shown to function similarly: ADAMTS-4 shares ADAMTS-1's capability to cleave syndecan 4 (Rodríguez-Manzaneque *et al.*, 2009).

Syndecan 2 is also of high interest, as it has been shown to be essential for angiogenic sprouting (Chen, Hermanson and Ekker, 2004). Furthermore, the shed syndecan 2 ectodomain functions to inhibit angiogenesis via inhibition of endothelial cell migration (Rossi *et al.*, 2014). Syndecan 2 has been previously linked to ADAMTS-4 and -5, as siRNA knockdown of syndecan 2 results in a loss of ADAMTS-4 and -5 expression (Yan *et al.*, 2018).

Syndecan 3 should also not be forgotten, despite being primarily expressed in the nervous system and less well studied than the others, as the syndecan 3 extracellular core protein is able to inhibit angiogenesis by reducing the migratory potential of ECs (De Rossi and Whiteford, 2013).

It is also important to consider cell-non-autonomous effects. With the exception of aortic rings assays, this thesis primarily profiled 3T3s or ECs in isolation, however a more global picture may result in different outcomes. Therefore, it would be of

interest to assess how universal these phenotypes are: while both 3T3s and endothelial cells showed the same adhesion and migration phenotypes in response to ADAMTS-1 or syndecan 4 knockdown, it can be assumed that the angiogenesis linked VEGF dependent phenotypes are limited to the ECs. Supporting this, increased proliferation in response to ADAMTS-1 knockdown was only seen in the ECs and not fibroblasts, suggesting this mechanism is specific to the VEGF responsive ECs. With regards to induction of MMP-9, while fibroblasts did see increased *Mmp9* transcription when ADAMTS-1 was depleted, this did not reach the threshold for statistical significance and therefore needs further investigation. Syndecan 4 is ubiquitously expressed, although at varying levels, whereas ADAMTS-1 shows a more restricted distribution, expressing in brain, fibroblasts, endothelium, renal, and breast and female reproductive tissues. It would be interesting to repeat experiments in a wider range of cell types, to determine whether the roles of ADAMTS-1 and syndecan 4, as well as the MMP-9 connection, is universal. Particularly interesting cell types would be other vascular cells such as VSMCs, further investigation of the human endothelial cells (HUVEC), as well as cancer cell lines – particularly as the ADAMTS-15/syndecan 4 link has been reported there. It is plausible that the roles of syndecan 4 are dependent on which growth factors the cell type is responsive to, and syndecan 4 may function differently depending on whether or not it is co-expressed with ADAMTS-1. Alternate interactions may also be found between ADAMTS-1 and other syndecan family members, and as cell lines have different syndecan profiles this may greatly affect the role of ADAMTS-1 in these cells.

In the future, our understanding of syndecan 4 in angiogenesis and cell migration would also be greatly advanced by the development of conditional and endothelial specific knockout mice, where the role of this protein could be investigated in a physiological environment, without the limitations posed by traditional knockout models. This also may help understand the universality of the phenotype, as well as giving depth to the consideration of physiological impact. For example the upregulation of MMP-9 in endothelial cells may affect syndecan 4 in other proximal cell types, secreted MMP-9 can diffuse and therefore there is scope to hypothesise

that epithelium, mural cells, or even tumour cells in proximity would have varying responses (Collier *et al.*, 2011).

6.5 ADAMTS-1 and syndecan 4 as key modifiers of the ECM

Upon *Adamts1* or *Sdc4* siRNA depletion, the migratory and adhesive properties of cells on fibronectin matrices were altered. ADAMTS-1 or syndecan 4 knockdown cells migrated faster, and α5 integrin internalisation was inhibited. When initially exploring how ADAMTS-1 and syndecan 4 may effect cell migration, α5 integrin was hypothesised to be the mediator, as it is the fibronectin receptor, it has multiple reported links to syndecan, and it showed changes in its distribution and trafficking. To investigate the contribution of α5 integrin, endothelial cells were isolated from the lungs of α5 null mice. When syndecan 4 or ADAMTS-1 was depleted in these cells an increase in migration speed was seen, as with the wild type cells, implying that ADAMTS-1 and syndecan 4 regulate cell migration in a pathway independent of α5 integrin.

Although α5 integrin was not the mediator of the migratory phenotypes, it was clearly affected by the loss of ADAMTS-1 or syndecan 4. These effects, as well as the increase in migration were attributed to alteration of the ECM, as the plating of untreated cells on matrix derived from *Adamts1* and *Sdc4* siRNA depleted cells was sufficient to induce α5 integrin accumulation, and upregulate pro-migratory and pro-angiogenic signalling. These data suggest roles for ADAMTS-1 and syndecan 4 in maintaining ECM homeostasis, and highlight the high importance of the ECM in regulating cell migration. These data also serve as an example of how the environment in which a cell exists can be as important as the cell itself in controlling behaviour.

The extracellular matrix is known to regulate cell migration, particularly in 3D environments. Physiological process such as angiogenesis are highly dependent on their 3D extracellular matrix surrounding, cells must migrate through the complex macromolecular structure, of which the content is dependent upon tissue. In these situations, structural features of the matrix such as stiffness play important roles

(Pathak and Kumar, 2012). Cell-ECM interactions are also far more complex, and proteases are usually required to degrade the extracellular environment. As this work was primarily conducted in 2D, the investigation of cell migration in more physiologically relevant 3D models could greatly advance our understanding of how ADAMTS-1 and syndecan 4 modulate the ECM biochemically and structurally.

When regarding cancer cell migration, spheroid models are often used to evaluate 3D migration and invasion (Vinci, Box and Eccles, 2015). More sophisticated customisable systems have also been developed, one such model is synthetic hydrogels. Hydrogels have highly tuneable compositions and elastic properties, and recent advances in hydrogels have enabled the design of improved 3D scaffolds and microenvironments, which have shown that migratory trends on 2D can be significantly different in 3D (Vu *et al.*, 2015; Dietrich *et al.*, 2018). Synthetic hydrogels have been used to develop a model to study angiogenesis, constructed of moulded tubular channels seeded with endothelial cells inside the hydrogel, which can be submitted to chemokine gradients. Using this model Trappmann *et al,* demonstrated how matrix crosslinking influences angiogenic sprouting, as does the degradability of the matrix (Trappmann *et al.*, 2017). This is just one example of how newer technologies can provide insight into previously unexplored areas, and using such models could help in our understanding of ADAMTS-1 and syndecan 4.

The adhesion and migration phenotypes reported here were only seen in cells plated onto a fibronectin, not collagen type 1 matrix, suggesting that ADAMTS-1 and syndecan 4 modify the ECM through interaction with fibronectin. Matrix specific effects have previously been seen with the ADAMTSs, and as ADAMTS-15 expression inhibits migration on laminin and fibronectin, but not type 1 collagen, this suggests it would be of interest to follow up these studies using laminin matrices. The lack of phenotype on collagen may be due to differential preferences of syndecans for ECM ligands. While syndecan 4 and integrin α5β1 co-operate in adhesion and migration on fibronectin, it appears that syndecan 1 and α1β1 fill this niche for type 1 collagen. Syndecan 1 promotes adhesion and inhibits migration on collagen, much like as is

reported here for syndecan 4 and fibronectin, reiterating the need to consider other syndecan family members when moving forwards (Endo *et al.*, 2003).

6.6 Fibulin 1

The ECM driven phenotypes of altered α5 integrin adhesion and promigratory signalling were accompanied by a reduction in expression of fibulin 1 in syndecan 4 or ADAMTS-1 siRNA depleted cells. Fibulin 1 is an ECM protein, and a co-factor for ADAMTS-1. The incorporation of fibulin 1 into matrices inhibits cell migration in a fibronectin dependent manner. We therefore hypothesise a mechanism where ADAMTS-1 and or syndecan 4 activate fibulin 1 expression and secretion into the ECM, inhibiting cell migration and promoting α5 integrin internalisation.

In future, it is essential to confirm that fibulin 1 is responsible for the phenotypes seen with regards to α5 integrin and migration. This could be achieved via siRNA depletion of fibulin 1, to see if it gives similar phenotypes as ADAMTS-1 and syndecan 4 depletion, as well as using exogenous fibulin 1 in matrices, to see if this can rescue the ADAMTS-1 and syndecan 4 knockout phenotypes.

If the mechanism is indeed fibulin 1 dependent, further questions are raised, including how fibulin 1 in the ECM influences both integrin expression and cell migration. Although fibulin 1 has been reported to inhibit migration, the mechanism by which this occurs is not entirely clear, yet does not involve the perturbation of interactions between integrin $\alpha_5\beta_1$ or heparan sulfate proteoglycans with FN. Pre-incubation of FN conjugated beads with fibulin 1 did not affect the level of integrin β_1 subunit that bound to the beads, and an ELISA to evaluate binding of $\alpha_5\beta_1$ to fibronectin found no difference in the binding affinity in the presence of fibulin 1. In the case of HSPGs, cells treated with chlorate to inhibit GAG sulfation of proteoglycans still responded to fibulin 1's motility suppressive effects. Interestingly, fibulin 1 modulated signalling associated with actin-myosin complex assembly, and could inhibit FN mediated activation of ERK (Twal *et al.*, 2001). This notion, that fibulin 1 regulates actomyosin contraction, is in keeping with the altered actin distribution seen when ADAMTS-1 and syndecan 4 are siRNA depleted, thus further

researching regarding actin, including live microscopic visualisation of the cytoskeleton may therefore advance our understanding of fibulin 1.

The involvement of fibulin 1 further supports the need to investigate varying cell types. The role of ADAMTS-1 in cell migration has been somewhat debated, and there is a possibility that this is due to fibulin 1. A molecular interaction between ADAMTS-1 and fibulin 1 contributes to breast cancer biology, expression of ADAMTS-1 alone increased proliferation and invasion of MDA-MB-231 and MCF-7 cell lines, but expression of both impaired effects, therefore whether or not a cell expresses fibulin 1 could alter the role of ADAMTS-1 (Mohamedi *et al.*, 2019).

The contribution of fibulin 1 to other phenotypes is yet to be investigated, and therefore its involvement may not be limited to adhesion and migration. Fibulins have demonstrated contributions to angiogenesis; basement membrane derived fibulin-1 and fibulin-5 function as angiogenesis inhibitors and suppress tumour growth, although the mechanism by which this is achieved has not been elucidated (Xie *et al.*, 2008). It would also be of interest to understand if fibulin 1 contributes to the closely related ADAMTS-15s syndecan 4 dependent regulation of cell migration, as the mechanism here remained ambiguous.

6.7 Proteases in cancer and angiogenesis

Angiogenesis, proliferation and cell migration are processes important in cancer development and metastasis, and therefore it would be of interest to see how ADAMTS-1 and syndecan 4 regulation of these processes contribute to cancer, particularly as the ADAMTS-15/syndecan 4 link has been implicated in this context.

Proteases , particularly MMPs, have been traditionally regarded as pro-tumorigenic, driving cancer growth and supporting processes such as metastasis and angiogenesis, however after MMP inhibitors failed to improve prognosis of cancer patients in clinical trial the roles of MMPs were re-evaluated (Winer, Adams and Mignatti, 2018). This led to accumulating evidence of MMPs functioning in anti-tumorigenic

mechanisms, which are of great interest in cancer research, and this interest extends into the wider metzincin family.

ADAMTS-15 is one such anti-tumorigenic protein; via protease dependent and independent mechanisms it functions to inhibit breast cancer cell migration and angiogenesis and influenced metastasis. Based on the similarities both in structure and functions of ADAMTS-1 and ADAMTS-15, and their shared syndecan 4 connection it would be of interest to investigate the contributions of ADAMTS-1 in a cancer context. This work could begin with investigation of cancer cell lines, then extend into more sophisticated experiments such as the use of mouse cancer models, either using ADAMTS-1 or syndecan 4 knockdown cancer cell injections, or implantation of tumours into ADAMTS-1 or syndecan 4 null mice. These experiments would allow for assessment of the contribution of ADAMTS-1 and syndecan 4 to tumour growth, metastasis, and angiogenesis from both a stromal and tumoral standpoint (Cheon and Orsulic, 2011).

The protease web in general is highly important for cancer, and loss of ADAMTS-1 or syndecan 4 clearly perturbs this network as the large increase in MMP-9 is seen. MMP-9 has been widely found to relate to the pathology of cancers(Huang, 2018), tumour cell derived MMP-9 drives malignant progression and metastasis of breast cancer (Mehner *et al.*, 2014), and breast cancer cells have been seen to induce stromal fibroblasts to express MMP-9. As ADAMTS-1 depletion results in increased expression of MMP-9 the implications of this for cancer would be interesting to consider, particularly as decreased expression of ADAMTS-1 in breast tumours has been seen to stimulate migration and invasion (Freitas *et al.*, 2013).

6.8 Moving forward with ADAMTS-1

Whilst the connection between ADAMTS-1 and syndecan 4 is interesting, it is likely only one function of ADAMTS-1. Although cell lines with ADAMTS-1 and syndecan 4 depletion show phenotypic similarity here, *Adamts1*-/- mice present with many severe developmental defects not seen in syndecan 4 null mice. ADAMTS-1 is highly important in development; versican cleavage by the protease is essential for cardiac

development. Interestingly, fibulin 1 is expressed very early in embryogenesis, and is expressed prominently in the endocardial cushion in the heart, and this colocalisation, combined with fibulin 1's role as an ADAMTS-1 cofactor suggests their functions may be linked in multiple contexts (Bouchey, Argraves and Little, 1996; Lee *et al.*, 2005).

The loss of syndecan 4 upon ADAMTS-1 depletion make it somewhat difficult to pick apart the individual roles of these proteins, and as syndecan 4 is ubiquitously expressed, it is not simple to study ADAMTS-1 in a syndecan 4-less system. However, independent roles of ADAMTS-1 are demonstrated by the upregulation of MMP-9 and increase in proliferation in ADAMTS-1 knockdowns alone. Moving forward, experiments to establish the ADAMTS-1 interactome may highlight new roles for ADAMTS-1. Proteins which bind to ADAMTS-1, either directly or indirectly could be established using IP mass-spectrometry; one limitation is the lack of a reliable ADAMTS-1 antibody, and therefore the ADAMTS-1-Myc construct, or similarly tagged mutant would have to be utilised.

Another consideration is catalytic vs non catalytic functions of ADAMTS-1. An avenue that was not followed up, as it involved expression of human ADAMTS-1 in mouse cells, was data which showed that overexpression of ADAMTS-1, but not a catalytically dead mutant resulted in the loss of cell surface syndecan 4. This suggests a 'Goldilocks' effect, where a just right balance of ADAMTS-1 and syndecan 4 is necessary. It also suggests roles for ADAMTS-1's catalytic activity in this context. To resolve the question, and shed some light onto the function of ADAMTS-1, CRISPR ADAMTS-1 knockouts could be made in human cells, followed by re-expression of the catalytically dead ADAMTS-1, if this reverses the phenotype then clearly these roles of ADAMTS-1 do not require proteolysis, however if it does not, it would demonstrate a need for ADAMTS-1's catalytic functions.

Catalytically dead ADAMTS-1 mutants can have different roles to the active protease; the overexpression of ADAMTS-1 promoted metastasis and angiogenesis, whereas the proteinase-dead version inhibits these events (Liu, Xu and Yu, 2006). These data

showing a role for overexpression of ADAMTS-1 support the notion that ADAMTS-1 has 'Goldilocks-type effects', and that too much or too little ADAMTS-1 can disrupt normal cell-ECM homeostatic relationships.

Although ADAMTS-1 is an aggrecanase, it only has weak aggrecan degrading activity, and ADAMTS-5 is regarded as the major aggrecanase (Stanton *et al.*, 2005). This suggests that ADAMTS-1 may have other catalytic functions. Proteomic techniques such as terminal amine isotopic labelling of substrates (TAILS) have been developed to identify novel substrates of proteases; this technique uses isotopic labelling of primary amines in intact proteins to identify novel cleavage sites. Samples are combined with or without a protease, N-terminals are then labelled, samples are trypsinised, unlabelled peptides remove and mass spectrometry performed to identify protease generated neo-N-termini (Prudova *et al.*, 2016; Madzharova, Sabino and Auf dem Keller, 2019). Identifying substrates and binding partners of ADAMTS-1 may give clues to its functions, and mechanisms.

6.9 Final conclusions and schematic

In conclusion, we present a model of a functional interplay between ADAMTS-1 and syndecan 4 in the extracellular matrix, where they sequester VEGF-A_{164} inhibiting angiogenesis, and activate fibulin 1 expression resulting in inhibition of cell migration and internalisation of α5 integrin. This work highlights the importance of ECM proteins in physiological processes, and provides several exciting avenues of future research (Figure 6.1).

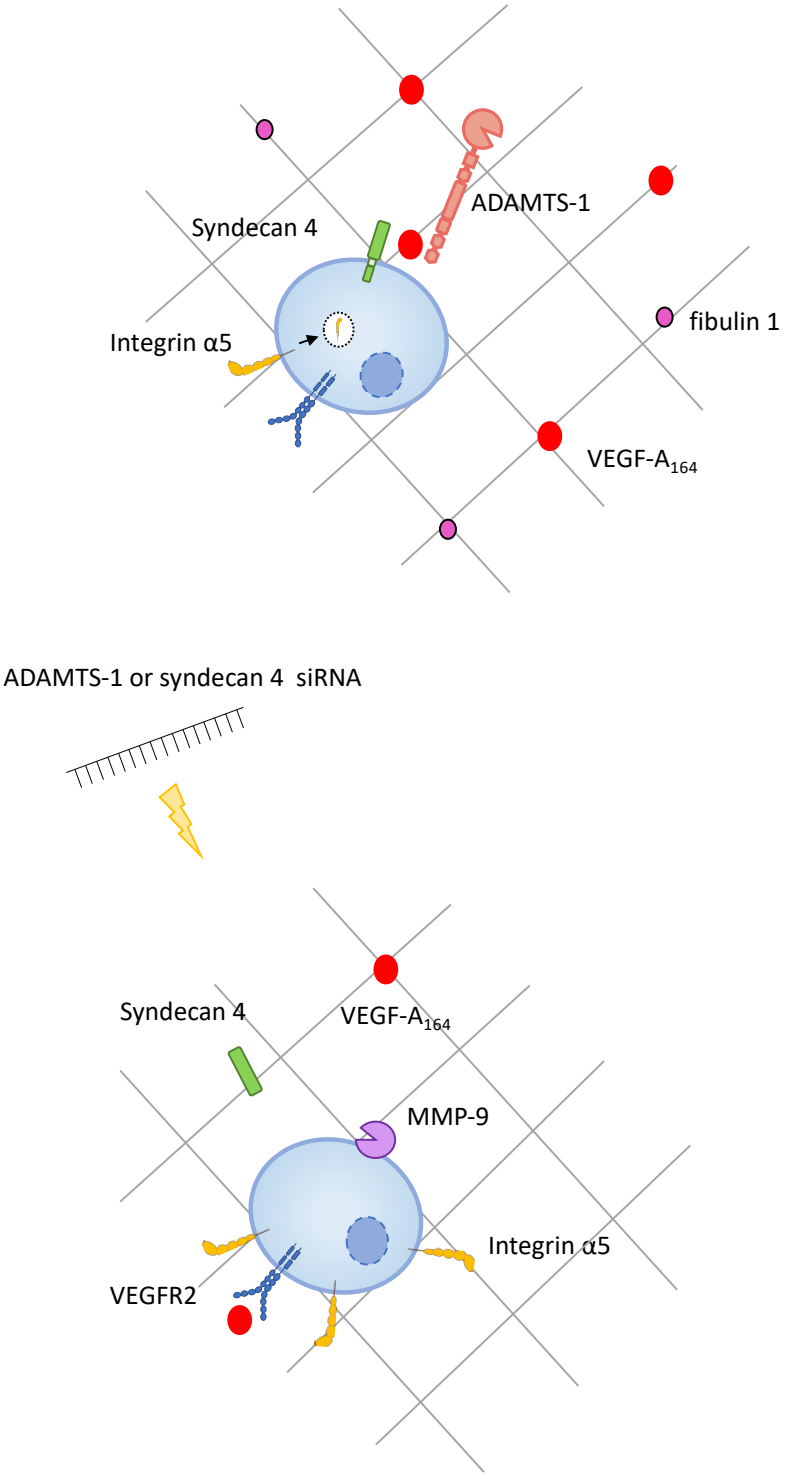


Figure 6.1 Proposed model of ADAMTS-1 and syndecan 4's functions in endothelial cells. ADAMTS-1 and syndecan-4 sequester VEGF-A_{164}, inhibiting its signalling, and promote expression of fibulin 1, inhibiting cell migration and promoting α5 integrin internalisation.

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