

Characterisation of ADAMTS-4 endocytosis by chondrosarcoma cells and chondrocytes

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A thesis submitted for the degree of Doctor of Philosophy to
Imperial College London

September 2009

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Abstract

Aggrecan is one of the most abundant components of the articular cartilage matrix and its degradation by aggrecanases is considered to be a key early event in the development of osteoarthritis. This study investigates whether the extracellular level of ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs-4), one of the most studied aggrecanases, can be regulated by receptor-mediated endocytosis.

Exogenously-added ADAMTS-4 disappeared from the medium of HTB94 cells and TCA-soluble degradation products of ADAMTS-4 increased in the medium over time. Fluorescent-labeled ADAMTS-4 could be detected within the cell by confocal microscopy. ADAMTS-4 clearance by HTB94 cells and chondrocytes could be inhibited by more than 70 % by heparin and another sulfated glycan, calcium pentosan polysulfate, suggesting that the cellular uptake of ADAMTS-4 was via a mechanism involving cell surface heparan sulfate proteoglycans (HSPGs). GM6001 also inhibited ADAMTS-4 clearance by 50 %, suggesting that cell surface metalloprotease-mediated degradation played a contributory role. However, ADAMTS-4 clearance was reduced by 50 % in a HSPG-deficient cell line compared to wild-type cells, further supporting a HSPG-dependent mechanism of ADAMTS-4 cellular uptake and preliminary studies using *Timp-3*-null fibroblasts suggest that ADAMTS-4 may be endocytosed in a complex with TIMP-3. In HTB94 cells and chondrocytes, the appearance of TCA-soluble degradation products was also inhibited by at least 50 % by receptor-associated protein (RAP), an antagonist for members of the low-density lipoprotein receptor (LDL-R) family. However, siRNA knockdown of LDL-receptor-related protein (LRP-1), a member of the LDL-R family, in HTB94 cells and the use of an LRP-1-deficient cell line had no effect on the clearance of ADAMTS-4. These results suggest that another member of the LDL-R family may be involved. Taken together, this research has revealed a novel RAP-sensitive and/or HSPG-sensitive mechanism of ADAMTS-4 regulation which may have a role in the degradation of the cartilage matrix.

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Abbreviations

α_2 M	α_2 -macroglobulin
α_2 M*	activated α_2 -macroglobulin
β -Me	β -mercaptoethanol
ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin type I motifs
APS	ammonium persulfate
AP	alkaline phosphatase
APP	amyloid precursor protein
BACE	β site of APP-cleaving enzyme
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CaPPS	calcium pentosan polysulfate
CHO	Chinese hamster ovary
Cm-Tf	carboxymethylated transferrin
COMP	cartilage oligomeric protein
ConA	Concanavalin A
CS	chondroitin sulfate
CTGF	connective tissue growth factor
CUB	complement subcomponent C1r/C1s/embryonic sea urchin protein Uegf (urchin epidermal growth factor)/bone morphogenic protein-1
DDSH	dyssegmental dysplasia, Silverman-Handmaker type
DMEM	Dulbecco's modified Eagle's medium
DMF	N, N-Dimethylformamide
DMSO	dimethyl sulfoxide
DS	dermatan sulfate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
FCS	fetal calf serum
G418	neomycin
GAG	glycosaminoglycan
GEP	granulin-epithelin precursor
GST	glutathione-S-transferase
GM6001	Ilomastat
HA	hyaluronic acid
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSPG	heparan sulfate proteoglycan
HUCF	human uterine cervical fibroblasts
IGD	interglobular domain
IL-1	interleukin-1

KS	keratan sulfate
kDa	kilo Daltons
LDL	low-density lipoprotein
LDL-R	low-density lipoprotein receptor
LP	link protein
LpL	lipoprotein lipase
LRP	low-density lipoprotein receptor-related protein
LRR	leucine-rich repeat
mDab1	mammalian Disabled
MMP	matrix metalloproteinase
MT-MMP	membrane-type MMP
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NOF	neck of femur
N-TIMP	N-terminal domain of tissue inhibitor of metalloproteinase
OA	osteoarthritis
PACE	paired basic amino acid converting enzyme
PAI-1	plasminogen activator inhibitor type-1
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	proprotein convertases
PLAC	protease and lacunin
PVDF	polyvinylidene difluoride
RAP	receptor-associated protein
RT	room temperature
SDS	sodium dodecyl sulfate
si	short interfering
sh	short hairpin
SLRPs	small leucine-rich proteoglycans
SNP	sodium nitroprusside
$t_{1/2}$	half-life
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
TGF	transforming growth factor
TIMPs	tissue inhibitor of metalloproteinases
TM	transmembrane domain
TNF- α	tumour necrosis factor-alpha
tPA	tissue-type plasminogen activator
TTP	thrombocytic thrombocytopenic purpura
UL-vWF	ultra large von Willebrand factor
uPA	urinary-type plasminogen activator
uPAR	urinary-type plasminogen activator receptor
vWF	von Willebrand factor
VEGF	vascular endothelial growth factor

Acknowledgements

I would like to thank my supervisor Professor Hideaki Nagase for his insight, direction, and encouragement throughout my studies. I would also like to thank Dr Linda Troeberg for her co-supervision, day-to-day advice and guidance over the last 4 years.

I would also like to acknowledge the BBSRC and AstraZeneca for funding my work and thank Dr Andrew Parker, AstraZeneca for his co-supervision.

I would also like to thank the Kennedy Institute of Rheumatology, in particular everyone in Matrix Biology for making the lab such a stimulating and fun place to work. I would also like to acknowledge Dr Brit Wolters and Lesley Rawlinson for their advice on confocal and radioisotope work, respectively.

I would like to thank all my family and friends for their relentless support, understanding and love. Finally, I would like to thank my boyfriend Greg for always being there to make me smile.

Declaration

This dissertation is a result of my own work. All collaborations have been acknowledged in the appropriate place within the text.

Chapter 1

Introduction

Introduction

The ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are a group of 19 secreted proteases. Since their discovery in 1997, the function of many of these proteases remains largely unknown, although several members of the ADAMTS family have been named aggrecanases due to their ability to cleave the proteoglycan aggrecan at several sites. Aggrecan is one of the most abundant components of the articular cartilage matrix and its degradation is considered to be a key early event in the development of osteoarthritis. The activity of ADAMTS-4, one of the most studied aggrecanases, can be regulated in several ways, including transcriptional regulation, prodomain removal, C-terminal processing and by endogenous tissue inhibitor of metalloproteinase-3 (TIMP-3). A further mechanism which may serve to control ADAMTS-4 activity is receptor-mediated endocytosis. Endocytosis of ADAMTS-4 would reduce its activity in the matrix by promoting intracellular degradation. The aim of this thesis was to investigate whether the extracellular level of ADAMTS-4 is regulated by endocytic clearance. This work may identify a further mechanism regulating ADAMTS-4 activity in the cartilage matrix which could also be applicable to other substrates of ADAMTS-4.

This chapter reviews the structure and function of the cartilage matrix before describing the enzymes involved in cartilage matrix degradation and the mechanisms known to regulate their activity.

1.1. Articular cartilage composition

Cartilage is a tissue which is aneural, avascular and alymphatic (Mankin and Lippiello, 1970). There are 3 types of cartilage found within the body: elastic, hyaline and fibrocartilage. Each type of cartilage is classified according to its exact biochemical composition. Elastic cartilage is rich in elastin and is found in the ear and epiglottis, where it provides a rigid but flexible framework. Fibrocartilage is very rigid due to the abundance of type 1 collagen and is found within intervertebral discs and where tendons and ligaments connect to bones.

Hyaline cartilage is found in the nose, larynx, between the ribs and the sternum and also in articular cartilage lining the surface of synovial joints such as the knee or hip. The role of articular cartilage is to provide a framework for bone deposition and a smooth surface for the frictionless movement of articulating joints. Additionally, articular cartilage has the ability to compress and in doing so can effectively absorb and distribute loads.

Articular cartilage consists of chondrocytes and an extensive extracellular matrix (ECM). The chondrocytes are relatively sparse in number, constituting less than 2 % of its total volume. They are the only cell type found in cartilage and they are responsible for synthesizing and degrading components of the ECM. The matrix consists of 65-80 % water, 10-30 % collagen, 5-10 % proteoglycans (Walsh *et al.*, 1997). Type II collagen is the most abundant collagen and its function is to provide articular cartilage with tensile strength. Aside from type II collagen, collagens VI, IX, X and XI are also cartilage specific (Martel-Pelletier *et al.*, 2008). Proteoglycans consist of a core protein covalently attached to one or more glycosaminoglycan (GAG) chain. GAG chains are long unbranched polymers consisting of repeating disaccharides; an amino sugar N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) and an uronic acid (glucuronic acid, GlcA, or iduronic acid, IdoA) (Varki *et al.*, 2002). The major GAGs are chondroitin sulfate (CS), keratan sulfate (KS), heparin, heparan sulfate (HS), dermatan sulfate (DS) and hylauronan (HA). Each GAG differs in the type and arrangement of their disaccharide units. For example, CS is composed of alternating GalNAc and GlcA disaccharides, whereas the most common disaccharide unit within HS is composed of GlcNAc and GlcA. At least one of the sugars in the repeating unit has a negatively charged sulfate or carboxylate group and therefore each GAG chain bears many negative charges. It is this feature of cartilage which draws in water from the surrounding areas to maintain a hydrated matrix and enable it to withstand compression. The major proteoglycan found in cartilage is aggrecan, although the matrix also contains other proteoglycans which play important roles in its assembly and function.

Aggrecan is composed of a core protein with KS and CS polysaccharide chains attached (Figure 1). The core protein is approximately 230 kDa and contains 3 globular domains: G1, G2 and G3 (Doege *et al.*, 1991). Two globular domains (G1 and G2) are located towards the N-terminus and a third globular domain (G3) is located towards the C-terminus. Up to 100 aggrecan monomers can bind to hyaluronan via the G1 domain of aggrecan (Watanabe *et al.*, 1997) and the interaction between aggrecan and hyaluronan is stabilized by link protein, a small glycoprotein homologous with the G1 domain of aggrecan (Hardingham, 1979). Hyaluronan binds to chondrocytes via the CD44 cell surface receptor (Knudson, 1993). This interaction anchors proteoglycan aggregates within the pericellular matrix and they become trapped within the network of collagen fibres (Hardingham and Muir, 1972). The G2 domain of aggrecan has no hyaluronan binding activity (Watanabe *et al.*, 1997) and its function is unknown whereas the G3 domain of aggrecan mediates binding to other ECM molecules such as tenascin-C, via its C-type lectin module (Day *et al.*, 2004; Thompson *et al.*, 2009). The G1 and G2 domains of aggrecan are separated a region known as the interglobular domain (IGD). The G2 domain is followed by a long region where KS and CS side chains are attached (Doege *et al.*, 1991). There are 2 CS-rich regions; CS-1 and CS-2, and the number of CS chains in the CS-1-rich region of human aggrecan can vary due to a polymorphism in the aggrecan gene (Doege *et al.*, 1997).

Other proteoglycans of the cartilage matrix include decorin, biglycan, lumican and fibromodulin. These proteoglycans are members of the small leucine-rich proteins (SLRPs). They contain multiple leucine-rich repeat (LRR) motifs which are flanked by two conserved cysteine-rich regions (Hocking *et al.*, 1998). Decorin consists of a core protein of 38 kDa together with one CS or DS side chain attached towards the N-terminus. Aside from binding to type I and type II collagen (Hedbom and Heinegard, 1989; Vogel *et al.*, 1984), decorin binds to other molecules of the cartilage matrix including type VI collagen (Bidanset *et al.*,

1992), fibronectin (Gendelman *et al.*, 2003), C1q (Krumdieck *et al.*, 1992) and TGF- β (Hildebrand *et al.*, 1994). Decorin is essential for proper collagen fibrillogenesis as decorin-null mice present with fragile skin and tendons and abnormalities in both collagen size and morphology (Danielson *et al.*, 1997). The core protein of biglycan is approximately 43 kDa with two CS or DS side chains attached towards the N-terminus. Biglycan also binds a number of molecules including type I collagen (Schonherr *et al.*, 1995) and TGF- β (Hildebrand *et al.*, 1994; Yamaguchi *et al.*, 1990) and is thought to play a role in neuronal survival (Kappler *et al.*, 1997). Although normal at birth, biglycan deficient mice have a reduced growth rate and develop an osteoporosis-like phenotype of decreased bone mass (Xu *et al.*, 1998). These results suggest that biglycan regulates bone formation and bone mass. Lumican and fibromodulin are KS proteoglycans. Lumican-null mice present with defects in corneal transparency and skin fragility and have larger than normal collagen fibrils (Chakravarti, 2002), indicating that lumican is involved in the regulation of collagen fibril growth. Fibromodulin carries up to four KS chains and binds type I and type II collagen with high affinity (Hedbom and Heinegard, 1989). Fibromodulin-null mice have increased susceptibility to OA and their tendons contain an increased frequency of small diameter collagen fibrils compared to wild-type mice (Chakravarti, 2002), showing that fibromodulin is involved in collagen fibrillogenesis.

A number of heparan sulfate proteoglycans (HSPGs) are found in cartilage, including perlecan and members of the syndecan family of cell surface receptors. Perlecan was originally isolated from a mouse Engelbreth-Holm-Swarm (EHS) tumour (Hassell *et al.*, 1980). It is approximately 470 kDa and in addition to being expressed in cartilage is also expressed in almost all basement membranes (Clement *et al.*, 1989; Handler *et al.*, 1997; Hayashi *et al.*, 1992; SundarRaj *et al.*, 1995). Perlecan is a CS/HS hybrid proteoglycan and is essential for normal cartilage development as perlecan-null mice are embryonic lethal and present with severe skeletal defects (Arikawa-Hirasawa *et al.*, 1999). Mutations in the human perlecan gene result in a skeletal disorder known as

Schwartz-Jampel syndrome and dyssegmental dysplasia, Silverman-Handmaker type (DDSH) (Arikawa-Hirasawa *et al.*, 2002; Arikawa-Hirasawa *et al.*, 2001). Members of the transmembrane family of syndecans as well as the glycosyl phosphatidylinositol-linked HSPGs, glypicans are also expressed on the surface of chondrocytes (Knudson and Knudson, 2001).

Other proteins found in the cartilage matrix include versican, chondroadherin, matrilins, thrombospondins such as cartilage oligomeric protein (COMP) and fibronectin.

1.2. Articular cartilage degradation

Proteolytic degradation of the cartilage ECM is an early and important event in the development of osteoarthritis (OA). OA is a disease characterized by progressive loss of cartilage from the surface of articulating joints and cartilage erosion eventually leads to loss of joint function (Felson and Zhang, 1998). Degradation of the cartilage matrix involves cleavage of both aggrecan and collagen fibres and aggrecan is one of the first components of the ECM to be degraded in arthritic disease (Mankin and Lippiello, 1970). Aggrecan degradation precedes collagen degradation in cartilage cultured with the proinflammatory cytokine interleukin-1 (IL-1) (Kozaci *et al.*, 1997) and the presence of aggrecan in the cartilage matrix may even protect against collagen degradation (Pratta *et al.*, 2003b).

Members of the matrix metalloproteinase (MMP) family which can cleave collagen at neutral pH include MMP-1, MMP-8, MMP-13, MMP-2 and MMP-14/MT1-MMP (see Nagase *et al.*, 2006 for review). Whilst MMP-13 seems to have a significant role the degradation of collagen in OA (Billinghurst *et al.*, 1997; Dahlberg *et al.*, 2000; Mitchell *et al.*, 1996), there is debate as to which enzyme(s) are responsible for aggrecan degradation *in vivo*. Aggrecan can be cleaved by a number of matrix metalloproteinases *in vitro*, including MMP-2,

MMP-3, MMP-7 and MMP-9, although the activity of MMP-9 against aggrecan is very weak (Fosang *et al.*, 1991; 1992). MMPs can cleave aggrecan at a number of sites within the IGD and CS-2 regions (Figure 1) although they primarily cleave within the IGD domain between Asn³⁴¹-Phe³⁴² (Flannery *et al.*, 1992; Fosang *et al.*, 1991; Sandy *et al.*, 1991). Additionally, the IGD of aggrecan can be cleaved by the cysteine protease, cathepsin B (Fosang *et al.*, 1992).

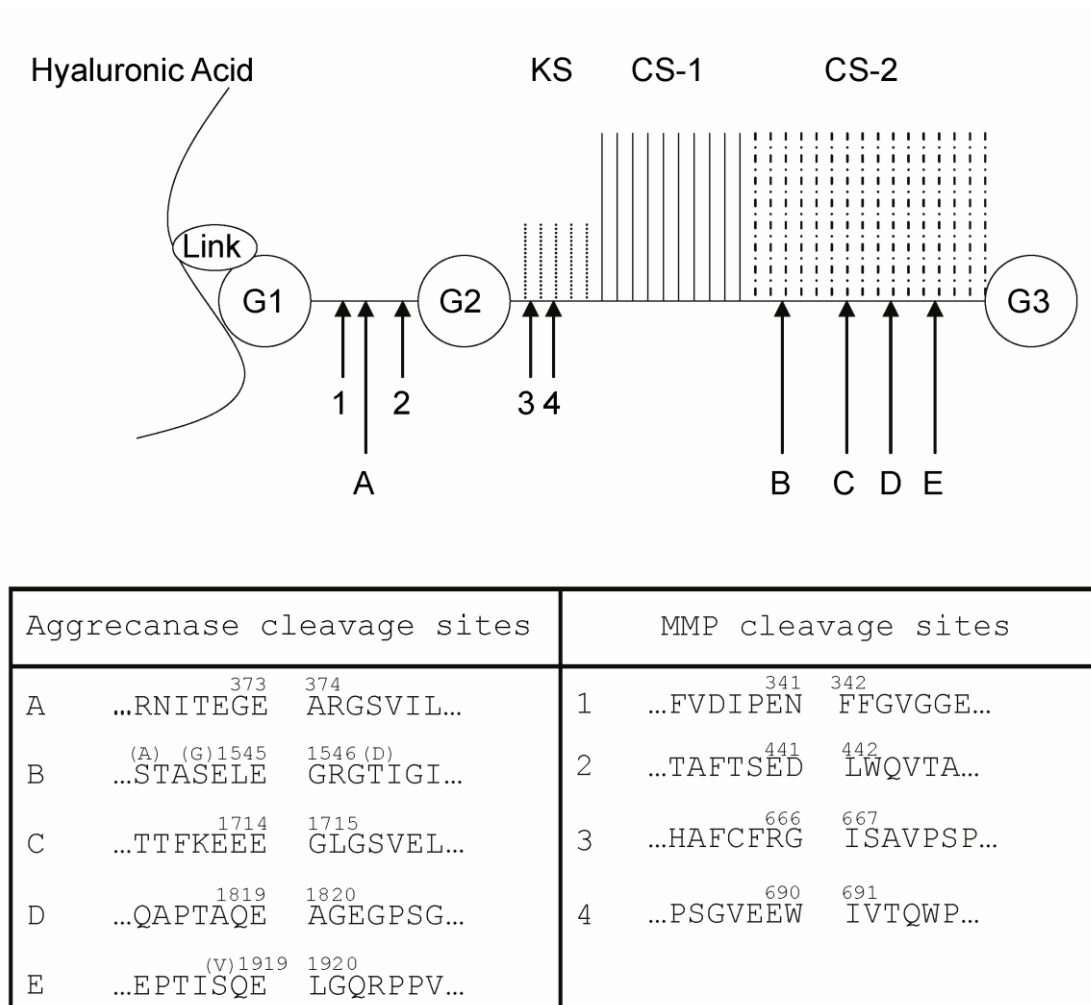


Figure 1: The domain structure of aggrecan with MMP and aggrecanase cleavage sites shown.

Aggrecan contains 3 globular domains – G1, G2 and G3. The N-terminal G1 domain interacts with hyaluronan and is stabilised by link protein. Aggrecanase cleavage sites are indicated by letter (A-E), whilst MMP cleavage sites are indicated by number (1-4). Amino acids in brackets indicate bovine sequence. KS - keratan sulfate-rich region, CS-1- 1st chondroitin sulfate-rich region, CS-2 – 2nd chondroitin sulfate-rich region. Adapted from Nagase and Kashiwagi (2003).

Another cleavage site within the IGD domain of aggrecan between Glu³⁷³-Ala³⁷⁴ was identified by N-terminal sequencing of fragments derived from bovine cartilage explant cultures stimulated with IL-1 (Sandy *et al.*, 1991). The unidentified enzyme(s) responsible for cleavage at the Glu³⁷³-Ala³⁷⁴ bond were termed 'aggrecanases'. Subsequently, aggrecanase-1 (ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs-4)) (Tortorella *et al.*, 1999) and aggrecanase-2 (ADAMTS-5) (Abbaszade *et al.*, 1999) were purified from IL-1 α -stimulated cartilage explants and were the first members of the ADAMTS family shown to cleave the Glu³⁷³-Ala³⁷⁴ bond. They also cleave at a number of sites in the KS-rich and CS-2-rich regions of aggrecan (Figure 1) (Tortorella *et al.*, 2000). Cleavage within the IGD is thought to be of greater functional significance than cleavage in the C-terminal KS-rich and CS-rich regions of aggrecan because cleavage in the IGD liberates the extensive GAG attachment region from the tissue and reduces its ability to draw in water.

Aggrecan fragments resulting from cleavage between residues Glu³⁷³-Ala³⁷⁴ are detected in the synovial fluid of OA patients (Lohmander *et al.*, 1993; Sandy *et al.*, 1992). However, at high concentrations, MMP-8 and MMP-14 can also cleave at Glu³⁷³-Ala³⁷⁴ (Buttner *et al.*, 1998; Fosang *et al.*, 1994) and in addition to cleavage at the Glu³⁷³-Ala³⁷⁴ site, ADAMTS-4 has been shown to cleave aggrecan at the Asn³⁴¹-Phe³⁴² site, although at a much slower rate (Westling *et al.*, 2002). Furthermore, both MMP- and aggrecanase-generated fragments were detected by immunohistochemistry in cartilage from OA patients, indicating that both MMPs and aggrecanases cleave aggrecan *in vivo* (Lark *et al.*, 1997).

1.3. The ADAMTS family members

The ADAMTSs belong to the M12 family in the MEROPS database (Rawlings *et al.*, 2008). They are a subfamily of the adamalysin family, which includes the snake venom metalloproteinases (reprolysins) and ADAMs (a disintegrin and metalloproteinase). The adamalysins along with other related families including

the MMPs, astacins, serralysins, snapalysins, leishmanolysins and pappalysins (Bode *et al.*, 1993; Gomis-Ruth, 2009; Hooper, 1994; Kaushal and Shah, 2000; Stocker *et al.*, 1995) all require zinc for catalytic activity and each contain a zinc binding motif (H-E-X-X-H-X-X-G-X-X-H/D) within their catalytic domain. They are all classified as metzincins as they also contain a conserved methionine residue which supports the catalytic site (Bode *et al.*, 1993). This residue is known as the 'Met turn' and is located downstream of the zinc binding motif.

1.3.1. ADAMTSs domain structure

ADAMTSs are a group of 19 secreted enzymes (see Porter *et al.*, 2005 for review) which generally consist of a signal peptide, pro-domain, catalytic metalloproteinase domain, disintegrin-like domain, a thrombospondin type I-like domain, a cysteine-rich domain and a spacer domain. Unlike the ADAMs, they are soluble proteinases due to the absence of a transmembrane domain. All ADAMTSs, except ADAMTS-4, have a second thrombospondin type 1-like motif of variable length towards the C-terminal. The functions of each domain are discussed below. The domain composition of the entire ADAMTS family and known substrates to date are detailed in Table 1.

The signal peptide is a hydrophobic sequence that directs a protein to be secreted across the ER membrane (Blobel *et al.*, 1979). The signal peptide is absent from mature proteins because it is cleaved by a signal peptidase on the ER membrane.

ADAMTSs are synthesized as inactive zymogens and most ADAMTSs require proteolytic cleavage and removal of the prodomain for activity. ADAMTSs contain a conserved furin consensus cleavage site between the prodomain and the catalytic motif (R-K-K-R²³⁵) suggesting that furin and/or other proprotein convertases (PCs) are responsible for prodomain cleavage (Rodriguez-Manzaneque *et al.*, 2000).

The mechanism of ADAMTS catalytic activity is thought to be the same as that described for the MMPs (Spurlino *et al.*, 1994; Stocker *et al.*, 1995). The catalytic domain of all MMPs contain the zinc binding motif H-E-X-G-H-X-X-G-X-X-H and the 3 histidines within this motif co-ordinate a Zn^{2+} ion. The glutamic acid polarizes a zinc-bound water molecule whereas the conserved methionine residue downstream of the zinc binding domain, a second Zn^{2+} ion and two to three Ca^{2+} ions stabilize the structure. When a substrate binds to the catalytic domain, the polarized water (OH^-) promotes a nucleophilic attack on the carbonyl carbon of the scissile peptide bond.

The exact function of the disintegrin-like domain is not known. One possible role of this domain is in mediating cellular adhesion, although there have been no reports to date that ADAMTSs associate with integrins. However, the disintegrin-like domain of related proteinases, the ADAMs has been implicated in cellular adhesion events such as sperm-egg fusion (Schlondorff and Blobel, 1999). Additionally, the disintegrin-like domain of both ADAM7 and ADAM28 have been reported to interact with $\alpha 4\beta 1$, $\alpha 4\beta 7$ and $\alpha 9\beta 1$ integrins whereas the disintegrin-like domain of ADAM33 interacts only with $\alpha 9\beta 1$ integrin (Bridges *et al.*, 2005; Bridges *et al.*, 2002). The disintegrin domain of ADAMTS-1 is required for protease activity against α_2 -macroglobulin (α_2 -M) (Wei *et al.*, 2002) whilst the disintegrin domain of ADAMTS-4 and ADAMTS-5 enhances their activity against a variety of substrates compared to the catalytic domain alone (Gendron *et al.*, 2007; Kashiwagi *et al.*, 2004).

The precise function of the thrombospondin type-1 like domain of the ADAMTSs is also unknown. The thrombospondin type-1 like domain of ADAMTS-1 contains a heparan sulfate binding motif W-G-P-W-G-P-W and the potential CD36 cell surface binding sequence C-S-(R/K)-T-C-G (Iruela-Arispe *et al.*, 1999; Kuno and Matsushima, 1998; Lawler, 2000). The thrombospondin type-1 like domain has been shown to be involved in the adhesion of ADAMTS-1 to the cell membrane and/or the ECM (Kuno and Matsushima, 1998).

The cysteine-rich and spacer domains are involved in ADAMTS localization and activity. In the case of ADAMTS-1, both domains are required for binding to the ECM (Kuno and Matsushima, 1998). The spacer domain of ADAMTS-4 interacts with the cell surface and ECM (Kashiwagi *et al.*, 2004). Removal of the spacer domain of ADAMTS-4 liberates ADAMTS-4 from the matrix and reduces its activity towards aggrecan but increases its ability to cleave other substrates such as carboxymethylated transferrin (Fushimi *et al.*, 2008; Kashiwagi *et al.*, 2004). Additionally, the spacer domain of ADAMTS-4 binds to the C-terminal domain of fibronectin and this interaction inhibits ADAMTS-4 activity (Hashimoto *et al.*, 2004) whilst the catalytic activity of ADAMTS-13 requires both the spacer and cysteine rich domains (Soejima *et al.*, 2003; Zheng *et al.*, 2003).

The C-terminal thrombospondin repeats of ADAMTS-7 are separated by a mucin-like domain rich in chondroitin sulfate. The addition of the mucin-like domain renders ADAMTS-7 a proteoglycan and may affect its cellular localization and substrate specificity. The mucin-like domain is also predicted to exist in ADAMTS-12 (Somerville *et al.*, 2004).

The protease and lacunin (PLAC) domain is found at the C-terminus of ADAMTS-2, -3, -7, -10, -12, -14, -17 and -19. This domain was first identified in lacunin, an extracellular matrix protein found in the moth *Manduca sexta* (Nardi *et al.*, 1999). Removal of the PLAC domain from ADAMTS-2 increased its N-procollagen I peptidase activity 3.5-fold, suggesting that the PLAC domain is a negative regulator of N-terminal procollagen processing (Colige *et al.*, 2005).

ADAMTS-20 and the long isoforms of ADAMTS-9 each have a *gon-1*-like domain. This domain was first described in GON-1, a secreted metalloproteinase which controls gonadal development in *Caenorhabditis elegans* (Blelloch and Kimble, 1999). However, the function of the *gon-1*-like domain in members of the ADAMTS family has not been determined.

Unlike other family members, ADAMTS-13 contains two C-terminal CUB [complement subcomponent C1r/C1s/embryonic sea urchin protein Uegf (urchin epidermal growth factor)/bone morphogenic protein 1] domains of unknown function. The CUB domain is found in many proteins involved in developmental processes such as embryogenesis and organogenesis (Bork and Beckmann, 1993). The CUB domain may be involved in substrate recognition as the CUB domain of procollagen C-proteinase-2 can bind immobilized procollagen type I (Sieron *et al.*, 2000).

1.3.2. ADAMTS functions

Substrates of several members of the ADAMTSs have been identified, including the N-propeptide of procollagen, von Willebrand factor, aggrecan and COMP. However, there are a number of ADAMTSs whose proteolytic activities have not yet been discovered.

1.3.2.1. Procollagen N-proteinases (ADAMTS-2, -3 and -14)

Collagens I, II and III are synthesized as procollagens with both NH₂ and COOH terminal propeptides which must be removed before mature collagen fibrils can be formed (Olsen, 1995). The C-terminal propeptide is cleaved by procollagen C-proteinases such as BMP-1 (Kessler *et al.*, 1996; Lee *et al.*, 1997; Li *et al.*, 1996) whilst ADAMTS-2, -3 and -14 cleave the N-terminal propeptide (Colige *et al.*, 1997; 2002; Fernandes *et al.*, 2001). Mutations within the ADAMTS-2 gene, resulting in a premature stop codon, cause Ehlers-Danlos syndrome type VII C in humans. This disease is characterized by symptoms including severe joint hyperextensibility, stretchable and easily bruised skin, short stature and a depressed nasal bridge (Colige *et al.*, 1999). Mutations within the ADAMTS-2 gene of cattle and sheep cause dermatosparaxis, a connective tissue disease similar to human Ehlers-Danlos syndrome type VII C.

Table 1: Domain composition of ADAMTSs and their known substrates.

ADAMTS	Domain Composition												Known Substrates
	SS	Pro	Cat	Dis	TS-1	Cys	Sp	TS-1 rp	Muc	PLAC	<i>gon-1</i>	CUB	
ADAMTS-1	+	+	+	+	+	+	+	2	-	-	-	-	Aggrecan, versican, Thrombospondin -1, Thrombospondin-2, α_2 M
ADAMTS-2	+	+	+	+	+	+	+	3	-	+	-	-	Procollagen I,II and III N-propeptides
ADAMTS-3	+	+	+	+	+	+	+	3	-	+	-	-	Procollagen II N-propeptides
ADAMTS-4	+	+	+	+	+	+	+	-	-	-	-	-	Aggrecan, versican, brevican, fibromodulin, decorin, fibronectin, COMP, biglycan, gelatin, Cm-Tf, casein
ADAMTS-5	+	+	+	+	+	+	+	1	-	-	-	-	Aggrecan, decorin, biglycan, versican Cm-Tf, fibronectin, gelatin, brevican
ADAMTS-6	+	+	+	+	+	+	+		-	-	-	-	-
ADAMTS-7	+	+	+	+	+	+	+	7	+ *	+	-	-	α_2 M, COMP
ADAMTS-8	+	+	+	+	+	+	+	1	-	-	-	-	Aggrecan
ADAMTS-9	+	+	+	+	+	+	+	14	-	-	+	-	Aggrecan, versican
ADAMTS-10	+	+	+	+	+	+	+	4	-	+	-	-	-
ADAMTS-12	+	+	+	+	+	+	+	7	+ *	+	-	-	COMP
ADAMTS-13	+	+	+	+	+	+	+	6	-	-	-	2	Von Willebrand factor
ADAMTS-14	+	+	+	+	+	+	+	3	-	+	-	-	Procollagen I N-propeptide
ADAMTS-15	+	+	+	+	+	+	+	2	-	-	-	-	Aggrecan
ADAMTS-16	+	+	+	+	+	+	+	5	-	-	-	-	Aggrecan
ADAMTS-17	+	+	+	+	+	+	+	4	-	+	-	-	-
ADAMTS-18	+	+	+	+	+	+	+	5	-	-	-	-	Aggrecan
ADAMTS-19	+	+	+	+	+	+	+	4	-	+	-	-	-
ADAMTS-20	+	+	+	+	+	+	+	14	-	-	+	-	-

Abbreviations - SS, signal peptide; Pro, pro-domain; Cat, metalloproteinase domain; Dis, disintegrin domain; TS-1, thrombospondin type-1 motif; Cys, cysteine-rich domain; Sp, spacer domain; TS-1 rp, C-terminal thrombospondin type 1 repeats; Muc, mucin-like domain; PLAC, protease and lacunin motif; *gon-1*, *gon-1*-like motif; CUB, complement subcomponent C1r/C1s/embryonic sea urchin protein Uegf (urchin epidermal growth factor)/bone morphogenic protein 1; Cm-Tf, carboxymethylated transferrin; COMP, cartilage oligomeric matrix protein; α_2 M, α_2 -macroglobulin. * mucin domain resides between 3rd and 4th C-terminal TS-1 repeats.

1.3.2.2. von Willebrand factor cleaving proteinase (ADAMTS-13)

von Willebrand factor (vWF) is a large plasma glycoprotein present in plasma and produced constitutively in platelets and vascular endothelial cells. Its role is to support platelet aggregation and platelet adhesion to damaged blood vessels by forming a bridge between platelet surface glycoproteins and the damaged endothelium. Von Willebrand factor is synthesized by vascular endothelial cells and assembles into homodimers and 'ultra large' multimers (UL-vWF) through the formation of disulfide bonds. Under conditions of shear stress in the circulation, UL-vWF becomes more susceptible to proteolysis and is cleaved between residues Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ in the A2 domain of vWF by ADAMTS-13 (vWF cleaving proteinase) (Fujikawa *et al.*, 2001; Soejima *et al.*, 2001; Zheng *et al.*, 2001). The absence or inactivation of ADAMTS-13 causes thrombocytic thrombocytopenic purpura (TTP), a condition characterized by the accumulation of UL-vWF multimers which form platelet-rich microthrombi in the small vessels of multiple organs. These clots eventually lead to organ ischemia and hemolytic anemia. Genetic mutations in ADAMTS-13 cause TTP (Levy *et al.*, 2001). Additionally, auto-antibodies against ADAMTS-13 cause acquired TTP (Furlan *et al.*, 1998).

1.3.2.3. Aggrecanases (ADAMTS-1, -4, -5, -8, -9 -15, -16 and -18)

As previously mentioned, ADAMTS-4 and ADAMTS-5 both cleave the IGD of aggrecan between residues Glu³⁷³-Ala³⁷⁴ although ADAMTS-5 is a 22-33-fold more active aggrecanase than ADAMTS-4 (Abbaszade *et al.*, 1999; Gendron *et al.*, 2007; Kashiwagi *et al.*, 2004; Tortorella *et al.*, 1999). In addition, several other members of the ADAMTS family have been reported to have aggrecanase activity *in vitro*: ADAMTS-1 (Rodriguez-Manzanique *et al.*, 2002), ADAMTS-8 (Collins-Racie *et al.*, 2004), ADAMTS-9 (Somerville *et al.*, 2003), ADAMTS-16 (Zeng *et al.*, 2006) and ADAMTS-18 (Zeng *et al.*, 2006). According to a European patent approved in 2004, ADAMTS-15 is also an aggrecanase (Yamaji *et al.*, 2004). However, aside from ADAMTS-4 and ADAMTS-5, the

aggrecanases have very weak activity against aggrecan and the significance of this activity *in vivo* is not understood.

ADAMTS-1 was the first member of the ADAMTS family to be identified and was initially discovered as a gene selectively expressed in cachexigenic colon 26 adenocarcinoma cells (Kuno *et al.*, 1997). ADAMTS-1-null mice present with renal abnormalities and female infertility (Shindo *et al.*, 2000) although they are not protected from aggrecan degradation in an inflammatory model of arthritis or in an *in vitro* model of early cartilage degradation (Little *et al.*, 2005).

ADAMTS-5 knockout mice, but not ADAMTS-4 knockout mice, are protected from cartilage degradation upon challenge in either inflammatory or surgically induced models of arthritis (Glasson *et al.*, 2004; Majumdar *et al.*, 2007; Stanton *et al.*, 2005). However, Malfait *et al* showed that both ADAMTS-4 and ADAMTS-5 were expressed in human OA tissue and that inhibition of these enzymes using a synthetic aggrecanase inhibitor blocked aggrecan degradation *in vitro* (Malfait *et al.*, 2002). Additionally, knockdown of either ADAMTS-4, ADAMTS-5 or both enzymes simultaneously by small interfering RNA (siRNA) reduced aggrecan degradation in both OA and normal human cartilage stimulated with a combination of TNF α and oncostatin M (Song *et al.*, 2007). This suggests that both ADAMTS-4 and ADAMTS-5 may contribute to aggrecanolysis in humans. Together, these results show that ADAMTS-5 is the major aggrecanase in mouse cartilage and infer that the specific aggrecanase(s) responsible for aggrecan cleavage *in vivo* may differ between species.

A recent paper suggested that another aggrecanase, induced by retinoic acid but not IL-1 α , caused aggrecan catabolism in cartilage explants derived from mice deficient in both ADAMTS-4 and ADAMTS-5 (TS-4/TS-5 Δ -cat) (Rogerson *et al.*, 2008). A low level of cleavage at SELE¹²⁷⁹⁻¹²⁸⁰GRG was detected upon stimulation of TS-4/TS-5 Δ -cat cartilage with retinoic acid. In addition, a major cleavage at FREEE¹⁴⁶⁷⁻¹⁴⁶⁸GLG was detected, although the level of the FREEE¹⁴⁶⁷ neoepitope detected in the tissue immediately *ex-vivo* was minor

compared to the amount detected in wild-type tissue. Nonetheless, this suggests that even in the mouse, aggrecan degradation may be more complex.

1.3.2.3.1. Other substrates of ADAMTS-1, -4, and -5

Several of the aggrecanases have activity towards other substrates. For example, ADAMTS-1 and ADAMTS-4 can additionally cleave versican V1 in the human aorta between the Glu⁴⁴¹-Ala⁴⁴² bond (Sandy *et al.*, 2001) whilst ADAMTS-1 has been reported to cleave thrombospondin-1 and thrombospondin-2 (Lee *et al.*, 2006). Recently, ADAMTS-5 has also been shown to cleave versican (Longpre *et al.*, 2009). ADAMTS-4 and ADAMTS-5 can cleave the brain specific protein, brevican between residues Glu³⁹⁵-Ser³⁹⁶ and both are overexpressed in human glioblastomas compared to normal brain tissue (Held-Feindt *et al.*, 2006; Matthews *et al.*, 2000; Nakada *et al.*, 2005). COMP has also been reported to be cleaved by ADAMTS-4 *in vitro* (Dickinson *et al.*, 2003).

Other substrates of ADAMTS-4 and ADAMTS-5 include decorin, biglycan, fibronectin, fibromodulin and gelatin, although fibronectin and gelatin are degraded at slow rates (Gendron *et al.*, 2007; Kashiwagi *et al.*, 2004).

1.3.2.3.2. Other roles of ADAMTS-1, -8, -9, -15 and -18

In addition to their ability to cleave aggrecan, both ADAMTS-1 and ADAMTS-8 inhibit angiogenesis as well as endothelial cell proliferation (Vazquez *et al.*, 1999). The anti-angiogenic action of ADAMTS-1 was shown to be due to the binding of its C-terminal region to vascular endothelial growth factor (VEGF) 165 (Luque *et al.*, 2003). Additionally, ADAMTS-1-mediated cleavage of thrombospondin-1 releases fragments with anti-angiogenic properties (Lee *et al.*, 2006). The expression of ADAMTS-8 is downregulated in brain tumours (Dunn *et al.*, 2006) whereas hypermethylation of the promoter region of the ADAMTS-9 gene was detected in 15 out of 16 esophageal carcinoma cell lines (Lo *et al.*,

2007). This suggests that ADAMTS-9 can be epigenetically silenced in tumours. Similarly, ADAMTS-1 (Lind *et al.*, 2006) and ADAMTS-18 (Jin *et al.*, 2007) genes have been reported to be hypermethylated in colorectal carcinomas. A recent study reported that the presence of ADAMTS-15 in human colorectal tumours showed a negative correlation with the differentiation grade of the tumour (Viloria *et al.*, 2009). These studies suggest that ADAMTS-1, -9, -15 and -18 may function as tumour suppressor genes.

1.3.2.4. COMP is cleaved by ADAMTS-7 and ADAMTS-12

Both ADAMTS-7 and ADAMTS-12 degrade COMP *in vitro* (Liu *et al.*, 2006a; 2006b) and their activity towards COMP can be inhibited by α_2 M (Luan *et al.*, 2008) and granulins-epithelin precursor (GEP), a chondrogenic growth factor (Liu, 2009). The expression of ADAMTS-12 was found to be significantly upregulated in cartilage from patients with OA compared with normal cartilage (Kevorkian *et al.*, 2004).

1.3.2.5. Other ADAMTSs (ADAMTS-6, -10, -17, -19 and -20)

The function of these remaining ADAMTSs remains largely unknown. However, ADAMTS-17 expression is upregulated in OA synovium compared with fractured neck of femur (NOF) cartilage (Davidson *et al.*, 2006) whereas ADAMTS-6 and ADAMTS-20 are upregulated in human breast carcinoma (Porter *et al.*, 2004). Mutations in the catalytic domain of the ADAMTS-10 gene, each coding for a premature stop codon have been described in three families with the autosomal recessive form of Weill-Marchesani syndrome (Dagoneau *et al.*, 2004). Weill-Marchesani syndrome is a connective tissue disorder characterized by short stature, joint stiffness, shortness of the fingers and toes (brachydactyly), eye defects, including severe myopia and glaucoma and occasionally heart defects. This suggests that ADAMTS-10 may have an important role in the growth and development of organs including the eye and heart.

1.4. Regulation of ADAMTS activity

ADAMTSs are subject to various levels of regulation, including gene expression, activation and inhibition. These mechanisms are discussed in further detail below, with emphasis on the regulation of ADAMTS-4.

1.4.1. Transcriptional Regulation

Although aggrecanase activity was first identified in bovine articular cartilage treated with IL-1 (Sandy *et al.*, 1991), aggrecanase activity can also be induced by other inflammatory cytokines including TNF- α , retinoic acid (Caterson *et al.*, 2000), TGF- β (Moulharat *et al.*, 2004) and IL-17 (Cai *et al.*, 2001). Additionally, a 45 kDa collagen binding fragment of fibronectin has been shown to stimulate the release of aggrecanase-generated aggrecan fragments from cartilage explants into the conditioned medium (Stanton *et al.*, 2002)

The effect of IL-1 on the expression of ADAMTSs remains debatable. Several studies report that the mRNA expression of ADAMTS-4 is increased by IL-1 in human chondrocytes (Bau *et al.*, 2002), bovine chondrocytes (Curtis *et al.*, 2000), bovine articular cartilage (Curtis *et al.*, 2000; Tortorella *et al.*, 2001) and porcine articular cartilage (Little *et al.*, 2002). However, another study reports that the expression of ADAMTS-1, ADAMTS-2, ADAMTS-3, ADAMTS-4, and ADAMTS-5 mRNA in human articular cartilage stimulated with IL-1 β did not change although aggrecanase activity was enhanced (Flannery *et al.*, 1999). This suggests that another enzyme aside from the ADAMTSs investigated may be responsible for aggrecan cleavage or that the activity of the ADAMTSs mediating degradation is not regulated at the level of expression. Similarly, in bovine cartilage and chondrocytes, the activity of ADAMTS-4 was shown to be induced by IL-1 with no detectable change in the amount of ADAMTS-4 protein. This suggests that ADAMTS-4 is constitutively produced by chondrocytes and is activated by an unknown factor (Pratta *et al.*, 2003a).

A study by Bau *et al* (2002) showed that although the expression of ADAMTS-4 was induced by IL-1 β in human chondrocytes, the expression of ADAMTS-5 was not enhanced. However, a recent study reported that IL-1 α induced both ADAMTS-4 and ADAMTS-5 in human articular chondrocytes although ADAMTS-4 was more strongly induced than ADAMTS-5 (Sawaji *et al.*, 2008). In addition, the expression of ADAMTS-5 was significantly increased at the 4 h time point but was similar to unstimulated chondrocytes at 24 h, suggesting that ADAMTS-5 expression in response to IL-1 α is transient.

Expression profiling studies have reported that the expression of ADAMTS-1, ADAMTS-5, ADAMTS-9 and ADAMTS-15 are significantly down-regulated whilst the expression of ADAMTS-2, ADAMTS-12, ADAMTS-14 and ADAMTS-16 are significantly increased in OA cartilage (Kevorkian *et al.*, 2004). In a separate study, ADAMTS-1, ADAMTS-4, ADAMTS-5 and ADAMTS-9 were down-regulated whilst ADAMTS-2, ADAMTS-10 and ADAMTS-16 were upregulated in both OA cartilage and synovium tissues compared to control samples (Davidson *et al.*, 2006). The decrease in expression of key aggrecanase genes in OA tissue is contrary to the dogma that they are involved in cartilage degradation *in vivo* although this could be due to the fact that OA cartilage samples used in these studies were from patients with end-stage disease where most of the aggrecan has already been lost from the tissue. Alternatively, it may indicate that *in vivo*, ADAMTSs activity is not regulated at the level of gene expression but is regulated by other post-translational modifications such as prodomain removal or C-terminal processing.

1.4.2. Pro-domain removal

Many proteins, including a variety of hormones, growth factors, receptors and proteases are synthesized as inactive precursor proteins and are proteolytically processed into their mature and functionally active forms by the action of proprotein convertases (PCs) (see Seidah *et al.*, 2008 for review). PCs are a family of Ca^{2+} -dependent subtilisin-type serine proteases. Seven PCs cleave after basic amino acids: PC1/3, PC2, PC4, PC5/6 (exists as 2 isoforms A and B), PACE4 (paired basic amino acid converting enzyme-4), PC7 and furin. PCs such as furin mainly reside in the trans-Golgi network but can also be found on the cell surface or as a soluble form secreted into the extracellular milieu (Klimpel *et al.*, 1992; Mayer *et al.*, 2004; Thomas, 2002). Once cleaved by PCs, the exposed carboxyl-terminal basic residues of the substrate are removed by metallocarboxypeptidases.

PCs have been shown to activate several metalloproteases *in vitro*, including MMP-11 (Pei and Weiss, 1995), MT1-MMP (Yana and Weiss, 2000) and ADAM12 (Loechel *et al.*, 1998). It is likely that N-terminal processing by furin or other PCs also activates ADAMTSs as they also contain a conserved furin consensus cleavage site between the prodomain and the catalytic motif (R-K-K-R²³⁵) and several members of the ADAMTS family have been shown to be processed upon secretion, including ADAMTS-1 (Kuno *et al.*, 1997; 1999; Rodriguez-Manzaneque *et al.*, 2000), ADAMTS-5 (Abbaszade *et al.*, 1999) and ADAMTS-9 (Somerville *et al.*, 2003). The prodomain of ADAMTS-4 can also be cleaved (Gao *et al.*, 2004; Kashiwagi *et al.*, 2004; Tortorella *et al.*, 1999; Wang *et al.*, 2004a) with the mature form secreted from the cell. It has also been reported that a 100 kDa form of ADAMTS-4 appears on the cell surface of JJ012 chondrosarcoma cells with the prodomain still intact (Gao *et al.*, 2004). However, this result could be an artifact of transient transfection where the overexpressed protein is not processed correctly due to the large amount of protein being translated.

Further evidence for furin processing of the ADAMTSs arises from studies in which a single amino acid substitution in the furin cleavage site of ADAMTS-1 (R²³² > A²³²) blocked prodomain release and inhibited its proteolytic activity against aggrecan (Rodriguez-Manzaneque *et al.*, 2002). In addition to furin, PACE4 and PC5/6A cleave proADAMTS-4 *in vitro* (Malfait *et al.*, 2008; Tortorella *et al.*, 2005; Wang *et al.*, 2004a). PACE4 also cleaves pro-ADAMTS-5 *in vitro* (Malfait *et al.*, 2008). Furthermore, the addition of recombinant PACE4 to human OA tissue increased the release of ARGS-containing fragments, suggesting that cartilage matrix contains proADAMTS-4 and/or proADAMTS-5, which become catalytically active upon addition of recombinant PACE4 (Malfait *et al.*, 2008). Recently, it was reported that proADAMTS-5 was activated extracellularly by furin, although the authors of this study also stated that they can not exclude the possibility that pro-ADAMTS-5 may be processed late in the secretory pathway, just prior to fusion with the plasma membrane (Longpre *et al.*, 2009).

The mechanism by which ADAMTSs are preserved in their latent proform is not thoroughly understood. In MMPs, a cysteine residue within the conserved sequence P-R-C-G-V/N-P-D-V/L-A/G of the prodomain co-ordinates with the catalytic Zn²⁺ in the active site (Van Wart and Birkedal-Hansen, 1990). This mechanism is known as the 'cysteine switch' and serves to retain the enzyme in an inactive state until the propeptide is cleaved by a PC or other MMPs. However, only 6 ADAMTSs (ADAMTS-1, -6, -7, -10, -12 and -15) contain a cysteine residue in their prodomain, suggesting that the prodomain of the ADAMTSs may not function in the same way as reported for MMPs (Porter *et al.*, 2005). Additionally, mutation within the furin cleavage site or deletion of its propeptide does not affect the secretion or activity of ADAMTS-13 (Majerus *et al.*, 2003). Similarly, ADAMTS-7 can also be catalytically active with its prodomain still intact (Somerville *et al.*, 2004).

1.4.3. C-terminal processing

In addition to furin-mediated removal of the prodomain, secondary processing of the mature ADAMTS protein can also occur. For example, active 64 kDa and 50 kDa fragments of ADAMTS-5 were isolated from conditioned medium collected from IL-1-stimulated bovine nasal cartilage (Abbaszade *et al.*, 1999). In addition, a 46 kDa fragment of ADAMTS-4 was identified in IL-1 α -treated porcine cartilage explants (Kashiwagi *et al.*, 2004).

There have also been several reports that ADAMTS-4 requires cleavage within the C-terminal region by MMP-17 in order to cleave aggrecan at the Glu³⁷³-Ala³⁷⁴ bond and that furin-mediated cleavage to generate the p68 form is a prerequisite for C-terminal processing by MMP-17 (Gao *et al.*, 2002; 2004; Patwari *et al.*, 2005). However, the p68 form, the result of prodomain removal alone, is able to cleave within the C-terminal CS-2-region of aggrecan (Kashiwagi *et al.*, 2004).

Our group has also reported that the full-length form of ADAMTS-4 has little activity against the Glu³⁷³-Ala³⁷⁴ bond in the IGD of aggrecan whereas removal of the spacer domain increases the ability of ADAMTS-4 to cleave aggrecan at this site (Gendron *et al.*, 2007; Kashiwagi *et al.*, 2004). In contrast, full-length ADAMTS-4 (ADAMTS4-1) and the spacer domain-deleted form (ADAMTS4-2) have been shown to have equal activity against the Glu³⁷³-Ala³⁷⁴ bond of aggrecan, suggesting that the spacer domain is not required for full catalytic activity (Hashimoto *et al.*, 2004).

In contrast to previous reports by our laboratory, we recently reported that full-length ADAMTS-4 is more active than the spacer domain-deleted form of ADAMTS-4 at both the IGD and CS-2-rich region cleavage sites of aggrecan but is still a 22-33 fold weaker aggrecanase than full-length ADAMTS-5 (Fushimi *et al.*, 2008). However, the activity of ADAMTS-4 against non-aggrecan substrates such as carboxymethylated transferrin and fibromodulin is enhanced when the C-terminal spacer domain is removed (Fushimi *et al.*, 2008). The discrepancy

between the results of our two studies was likely due to heparin contamination within the preparation of ADAMTS4-1 used in the initial study. As the spacer domain of ADAMTS4-1 interacts with the cell surface and ECM (Kashiwagi *et al.*, 2004), it therefore seems that processing of the spacer domain of ADAMTS-4 liberates ADAMTS-4 from the matrix. Removal of the spacer domain reduces ADAMTS-4 activity towards aggrecan but increases its ability to cleave other substrates.

Removal of the spacer domain of ADAMTS-5 reduces aggrecanolytic activity but has no effect on its general proteolytic activity. Removal of both CysR and Sp domains of ADAMTS-5 further reduces its activity against aggrecan (Gendron *et al.*, 2007) but has little effect on its general proteolytic activity. However, the catalytic domain of either ADAMTS-4 or ADAMTS-5 alone has little proteolytic and aggrecanolytic-activity (Gendron *et al.*, 2007; Kashiwagi *et al.*, 2004).

1.4.4. Endogenous inhibition

The tissue inhibitor of metalloproteinase (TIMP) family consists of 4 members: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMPs bind tightly and are endogenous inhibitors of most MMPs (Murphy and Willenbrock, 1995). In addition, TIMP-3 can inhibit ADAMTS-4 and ADAMTS-5 *in vitro*, with K_i values of 3.3 nM and 0.66 nM, respectively (Kashiwagi *et al.*, 2001) and is the only TIMP to protect against cartilage aggrecan breakdown in explant cultures stimulated with IL-1 or retinoic acid (Gendron *et al.*, 2003). TIMP-3 also differs from other members of the TIMP family, in that it binds tightly to the ECM (Kishnani *et al.*, 1995; Staskus *et al.*, 1991). It has been reported that TIMP-3 interacts with the ECM via both its N- and C-terminal domains and the residues involved in ECM binding are all basic amino acids (Lee *et al.*, 2007).

Additionally, the activity of ADAMTS-4 and ADAMTS-5 can be irreversibly inhibited by the general plasma proteinase inhibitor α_2 -macroglobulin (Tortorella *et al.*, 2004).

1.5. Endocytosis

Endocytosis is a further mechanism by which the extracellular level of proteases can be regulated. Although there are no reports demonstrating the endocytosis of any member of the ADAMTS family, clearance of these enzymes from the extracellular milieu would serve to reduce their activity in the matrix by promoting intracellular catabolism. In support of this potential mechanism, the endocytosis of many proteinases and proteinase/inhibitor complexes have been reported, including the endocytosis of MMP-2 (Emonard *et al.*, 2004; Yang *et al.*, 2001), MMP-9 (Hahn-Dantona *et al.*, 2001) and MMP-13 (Barmina *et al.*, 1999).

1.5.1. Clathrin-dependent endocytosis

Clathrin-mediated mechanisms of endocytosis have been well studied. The process involves the formation of clathrin invaginations around cell surface receptors. The clathrin-coated invaginations bud from the plasma membrane and form a clathrin-coated vesicle (Kirchhausen, 2000). Some receptors are constitutive, i.e. they are endocytosed both in the presence and absence of ligands, whilst others require ligand binding to initiate the formation of the clathrin-coated vesicle. Once endocytosed, the clathrin-coated vesicle is targeted to the early endosome. In the early endosome, the ligand dissociates from the receptor due to acidic pH (~pH 5). Endocytosed proteins and receptors can be recycled to the plasma membrane through recycling endosomes or are degraded by hydrolases following transport to late endosomes and lysosomes (Figure 2). Receptors can be recycled back to the cell surface and so the turnover of the receptor and its level of expression on the cell surface are factors which can regulate the rate of endocytosis of a particular ligand. Cells possess many types of cell surface receptors, each of which is able to bind and

endocytose a variety of ligands. For example, all cell types contain transferrin receptors which deliver iron to cells by endocytosis of receptor-bound ferrotransferrin (Jandl and Katz, 1963). However, the receptor also endocytoses apotransferrin and receptor internalisation also proceeds in the absence of transferrin (Dautry-Varsat *et al.*, 1983; Watts, 1985). In the early endosome, iron dissociates from the transferrin-receptor complex and remains in the cell whilst transferrin is not degraded but is secreted intact into the medium (Jandl and Katz, 1963; Klausner *et al.*, 1983). The low-density lipoprotein receptor (LDL-R) family is another class of receptors which mediate clathrin-dependent endocytosis of a variety of structurally and functionally unrelated ligands (see Hussain *et al.*, 1999 for review). In the case of the LDL-R, the bound ligand dissociates from the receptor in the early endosome and is transported to the lysosome for degradation. Members of the LDL-R family are constitutively recycling receptors although they bind their ligands with high affinity.

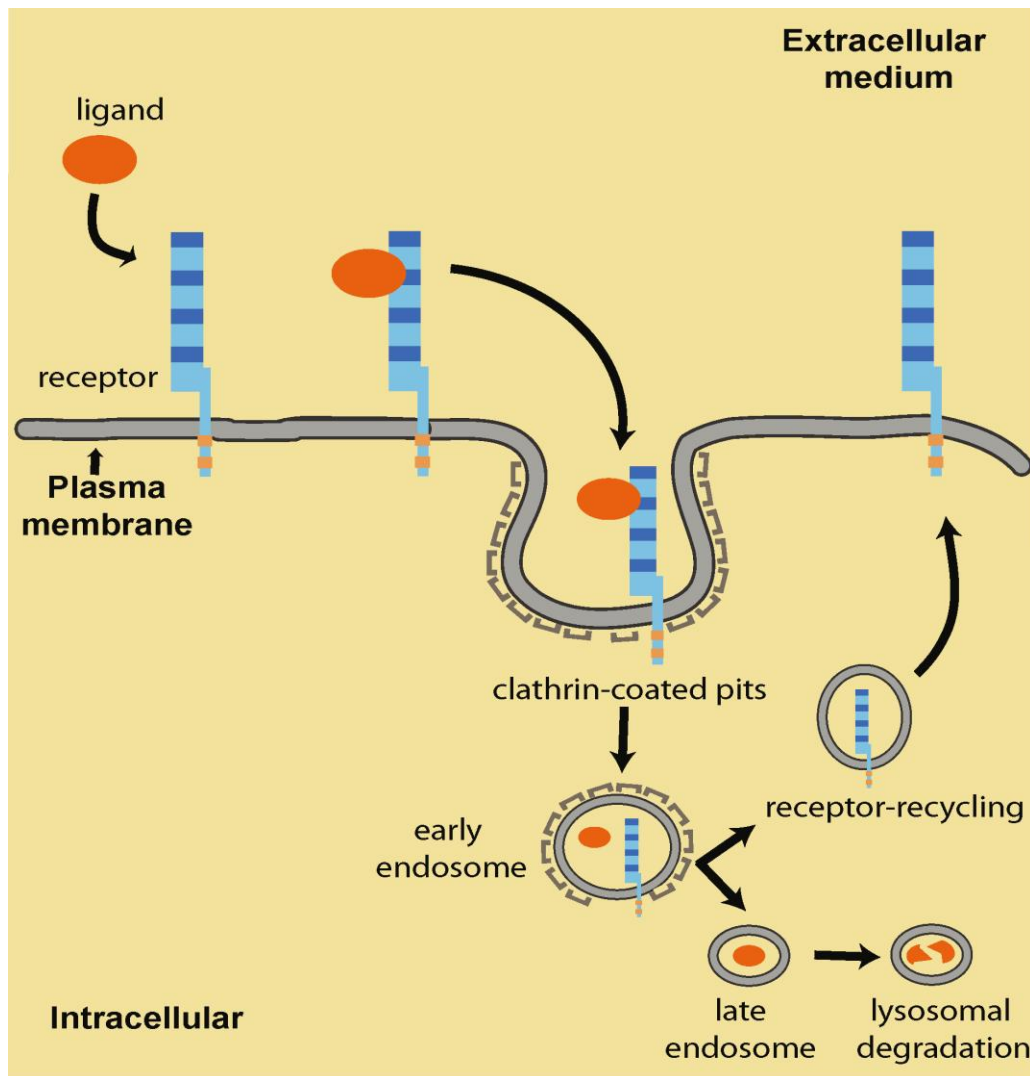


Figure 2: *Clathrin-mediated endocytosis.*

Ligands are internalised at the plasma membrane in clathrin-coated pits. Following transfer to the early endosome, endocytosed proteins and receptors are recycled to the plasma membrane through recycling endosomes or transported to late endosomes and then lysosomes. In the lysosomes they are degraded by hydrolases.

1.5.1.1. LDL-R Family

The LDL-R family is comprised of several structurally homologous receptors, 7 of which are very closely related: LDL receptor (LDL-R), LDL-R-related protein-1 (LRP-1), LRP-1b, megalin/LRP-2, very-low density lipoprotein receptor (VLDL-R), LRP-4/MEGF7 (multiple EGF-like domain) and LRP8/apolipoprotein E receptor 2 (apoER2) (Figure 3). The LDL-R family also includes members which are more distantly related such as sorting protein-related receptor containing LDL-R class

A repeats (SorLa/LRP11), LRP-5 and LRP-6. All members of the LDL-R family are type 1 transmembrane receptors and consist of an extracellular N-terminal ligand binding domain, containing cysteine-rich ligand-binding repeats, epidermal growth factor (EGF)-like repeats and β -propeller domains, a single transmembrane domain and all except LRP-5/-6 contain NPXY motifs in their cytoplasmic tail (Marzolo and Bu, 2009). The NPXY motifs mediate binding of the receptor to coated pits and have been shown to bind cytosolic adaptor proteins such as mammalian Disabled (mDab1) and FE65. This indicates that they may activate intracellular signaling (Trommsdorff *et al.*, 1998; 1999).

1.5.1.1.1. LDL-R

The founder member of the receptor family, LDL-R was identified through studies demonstrating that fibroblasts bound [¹²⁵I]-LDL with high affinity and specificity (Brown and Goldstein, 1974). The LDL-R, unlike other members of this family appears to function exclusively in lipoprotein metabolism, mediating the internalisation and degradation of apoB100-containing lipoproteins as well as apoE-containing lipoproteins from plasma (Hussain *et al.*, 1999). Mutations within the LDL-R gene cause familial hypercholesterolemia (see Hobbs *et al.*, 1990 for review). In this condition, the clearance of lipoproteins from the bloodstream is impaired which results in increased LDL levels in the circulation. Physical signs include cholesterol deposits in the tendons (tendon xanthomas) and yellow patches around the eye (arcus cornea). Deposition of cholesterol in the arteries leads to atherosclerosis and early onset cardiovascular disease.

1.5.1.1.2. VLDL-R

The structure of VLDL-R differs from that of LDL-R only by an additional ligand-binding repeat at the N terminus. It is expressed in heart, muscle, brain and adipose tissue but not in liver (Hussain *et al.*, 1999). The VLDL-R endocytoses proteins involved in lipoprotein metabolism such as apoE and VLDL as well as several protease/inhibitor complexes including protease/anti-thrombin III and

protease/PAI-1 complexes (Hussain *et al.*, 1999). A polymorphism in the VLDL-R, due to the absence of an 84 base pair O-glycosylation motif, has been described, although this does not affect ligand binding (Martensen *et al.*, 1997; Sakai *et al.*, 1994; Webb *et al.*, 1994). No human disorders have been linked with VLDL-R dysfunction. However, mutations in the chicken homolog of VLDL-R result in female sterility and severe hyperlipidemia (Bujo *et al.*, 1995).

1.5.1.1.3. LRP-1

LRP-1, also known as CD91, was first described as the receptor for activated α_2 -macroglobulin (α_2 -M*) (Hanover *et al.*, 1983). In 1988, Herz *et al.* screened a mouse lymphocyte cDNA library and identified a cell surface receptor with sequence similarity to the LDL-receptor, naming it LDL-receptor related protein (LRP-1) (Herz *et al.*, 1988). Sequencing of both the α_2 M receptor and LRP-1 identified them as the same molecule (Strickland *et al.*, 1990). LRP-1 is synthesized as a 600 kDa precursor which is cleaved in the trans-Golgi by a furin-like protease to produce a non-covalently associated receptor of 85 kDa and 515 kDa subunits (Willnow *et al.*, 1996b). The 85 kDa subunit contains a small ectodomain, single transmembrane domain and cytoplasmic tail and the 515 kDa extracellular subunit contains 4 ligand-binding clusters (Herz *et al.*, 1990; Willnow *et al.*, 1996b). LRP-1 expression is widespread; it is found abundantly in the placenta, liver and brain and is highly expressed in several cell types including hepatocytes, fibroblasts and macrophages (Moestrup *et al.*, 1992; Zheng *et al.*, 1994). Aside from α_2 -M*, LRP-1 binds and internalizes a variety of other ligands, including the matrix molecules connective tissue growth factor (CTGF) (Segarini *et al.*, 2001), decorin (Brandan *et al.*, 2006) and fibronectin (Salicioni *et al.*, 2002). Many proteases or molecules associated with regulating protease activity are also LRP-1 ligands including tissue-type plasminogen activator (tPA) (Bu *et al.*, 1992), urinary-type plasminogen activator (uPA) (Kounnas *et al.*, 1993), tPA or uPA bound to plasminogen activator inhibitor type I (PAI-1) (Nykjaer *et al.*, 1992; Orth *et al.*, 1994), lactoferrin (Willnow *et al.*, 1992),

MMP-2 (Emonard *et al.*, 2004; Yang *et al.*, 2001), MMP-9 (Hahn-Dantona *et al.*, 2001) and MMP-13 (Barmina *et al.*, 1999). LRP-1 is also the endocytic receptor for thrombospondin-1 and -2 (Chen *et al.*, 1996; Mikhailenko *et al.*, 1995), *Pseudomonas* exotoxin A (Kounnas *et al.*, 1992b) and the transcriptional transactivator HIV-1-Tat (Liu *et al.*, 2000c). In addition, LRP-1 internalises ApoE-enriched lipoproteins and lipoprotein lipase (LpL) (Lillis *et al.*, 2009). Another important ligand is receptor associated protein (RAP), a 39 kDa protein which co-purifies with LRP-1 (Ashcom *et al.*, 1990). RAP binds to LRP-1 with a K_d of 3.3 nM (Iadonato *et al.*, 1993) and inhibits the binding and/or uptake of all known LRP-1 ligands (Bu *et al.*, 1992; Herz *et al.*, 1992; Nykjaer *et al.*, 1992).

LRP-1 has 4 ligand binding clusters (I-IV) (Figure 3). The majority of LRP-1-ligands bind to ligand-binding clusters II and IV and ligands including apoE and RAP bind to either domain with equal affinity which suggests functional duplication within the gene (Springer, 1998). Furthermore, high affinity ligand binding requires interaction with multiple ligand binding repeats.

1.5.1.1.3.1. α_2 M endocytosis

α_2 M is found at relatively high levels in the plasma (2-4 mg/ml) and is a tetramer of 4 identical 185 kDa subunits linked in pairs by disulfide bonds. The two dimers are associated non-covalently (Barrett *et al.*, 1979). Each subunit has a 'bait region' which is susceptible to cleavage by proteinases. Once cleaved, α_2 M undergoes a rapid conformational change and forms an irreversible α_2 M-proteinase complex which entraps the proteinase without blocking its active site. Although the trapped protease remains active towards low molecular mass substrates, protease activity towards large molecular weight substrates is inhibited (Barrett and Starkey, 1973; Harpel, 1973). Early studies reported that complexes of α_2 M with proteinases and not the native molecule were taken up preferentially by human fibroblasts (Van Leuven *et al.*, 1979). This supports observations that upon complex formation, α_2 M undergoes a substantial

conformational change, exposing a receptor binding site within $\alpha_2\text{M}$. This form of $\alpha_2\text{M}$ is known as activated- $\alpha_2\text{M}$ ($\alpha_2\text{M}^*$). $\alpha_2\text{M}^*$ can also be generated by reaction with methylamine, a small nucleophile which attacks the internal thioester bond within the molecule and generates four free thiol groups (Barrett *et al.*, 1979). $\alpha_2\text{M}^*$ endocytosis is inhibited by nickel (Hussain *et al.*, 1995; Kancha and Hussain, 1997; Odom *et al.*, 1997) although the internalisation of other LRP-1 ligands including RAP and lactoferrin are not inhibited by nickel. This suggests that Ni^{2+} is a specific inhibitor of $\alpha_2\text{M}^*$ endocytosis.

1.5.1.1.3.2. MMP endocytosis

MMP-2, -9 and -13 are endocytosed by LRP-1 (Barmina *et al.*, 1999; Emonard *et al.*, 2004; Hahn-Dantona *et al.*, 2001; Yang *et al.*, 2001). Pro-MMP2 can be internalised by LRP-1 in a complex with thrombospondin-2 (Yang *et al.*, 2001) or in a complex with TIMP-2 (Emonard *et al.*, 2004). Binding of pro-MMP-2-TIMP-2 to the surface of HT1080 cells was not inhibited by RAP, whereas endocytosis and lysosomal degradation of pre-bound complex was inhibited by RAP (Emonard *et al.*, 2004). This suggests that an unknown RAP-insensitive co-receptor may be involved in the initial binding of pro-MMP-2-TIMP-2 to the cell surface, followed by transfer to and endocytosis by LRP-1. Blocking the interaction of thrombospondin-1 with either LRP-1 or pro-MMP-2 partially inhibited LRP-1-mediated endocytosis of pro-MMP-2 but had no effect on the endocytosis of the pro-MMP-2-TIMP-2 complex (Emonard *et al.*, 2004). This suggests that complexing pro-MMP2 with thrombospondin-1 can mediate proMMP-2 endocytosis whereas endocytosis of pro-MMP-2-TIMP-2 complex is via a two step process which is independent of thrombospondin-1. Similar to pro-MMP2, MMP-9 can be internalised by LRP-1 alone or in a complex with TIMP-1 (Hahn-Dantona *et al.*, 2001) although another member of the LDL receptor family, megalin has also been shown to internalise MMP-9 (Van den Steen *et al.*, 2006). The internalisation of MMP-13 occurs after transfer of the proteinase to LRP-1 from an unknown Ca^{2+} -sensitive 170 kDa receptor (Barmina *et al.*, 1999).

Furthermore, LRP-1-mediated catabolism of MMP-13 was shown to be impaired in human osteoarthritic chondrocytes *in vitro* (Walling *et al.*, 2003), indicating that LRP-1-mediated endocytosis may be an important mechanism regulating the extracellular level of MMP-13 in the cartilage matrix. In this study, the mRNA level of LRP-1 was not significantly altered between control and OA chondrocytes. This suggests that other factors such as a change in cell surface expression of LRP-1 or altered expression of the unidentified 170 kDa receptor may account for the reduced endocytosis of MMP-13 by OA chondrocytes.

1.5.1.1.3.3. Other properties of LRP-1

LRP-1 can itself be cleaved by proteases on the cell surface. For example, malignant cells down-regulate the expression of LRP-1 and in malignant cells MT1-MMP has been shown to cleave the ligand binding domain of LRP-1 into several fragments, impairing the internalisation of the ¹²⁵I-labeled LRP-1-specific antibody, 8G1 (Rozanov *et al.*, 2004). Furthermore, a soluble form of LRP-1, arising from cleavage within the 85 kDa subunit of LRP-1, was detected in human plasma and is a competitive inhibitor of LRP-1 endocytosis (Quinn *et al.*, 1997; 1999). The ectodomain of LRP-1 can also be shed by BACE (β site of APP-cleaving enzyme) (von Arnim *et al.*, 2005) and tPA (Polavarapu *et al.*, 2007). ADAM-12 has been implicated as the sheddase responsible for cleaving LRP-1 in the endometrium, inhibiting endocytosis of MMP-2 and MMP-9. The resulting increase in the extracellular concentration of MMP-2 and MMP-9 facilitates ECM remodelling during the menstrual cycle (Selvais *et al.*, 2009).

Deletion of the LRP-1 gene is embryonic lethal as LRP-1-null mice die at around day 10 in embryo (Herz *et al.*, 1992; 1993). In addition to its role in fetal development, LRP-1 is emerging as having an important role in the development of Alzheimer's disease, a neurodegenerative disorder characterized by neuronal loss and the accumulation of extracellular β -amyloid plaques and intracellular neurofibrillary tangles. The main component of amyloid plaques is amyloid- β

(A β) peptide, which is a cleavage product of the β -amyloid precursor protein (APP). A study by Ulery *et al* showed that transfection of APP and LRP-1 in LRP-1-deficient cells led to a 3-fold increase in A β , demonstrating that LRP-1 promotes APP endocytosis and APP processing to A β (Ulery *et al.*, 2000). Furthermore, LRP-1 endocytoses APP, apoE, lactoferrin, A β and α_2 M, all of which have been genetically associated with Alzheimer's disease (Herz and Bock, 2002). Studies have also indicated that polymorphisms within the LRP-1 gene are associated with Alzheimer's disease and the levels of LRP-1 decrease with age (Kang *et al.*, 1997; 2000; Kolsch *et al.*, 2003).

LRP-1 is also emerging as an important signalling molecule. For example, since each α_2 M* molecule contains four receptor binding sites, α_2 M* can simultaneously bind to several LRP-1 receptors on the cell surface. This causes LRP-1 aggregation and induces a calcium influx via N-methyl-D-aspartate (NMDA) receptors in primary neurons (Bacsikai *et al.*, 2000). In addition, binding of extracellular ligands may promote secretase-mediated LRP-1 cleavage. Cleavage of LRP-1 and the release of the intracellular domain has been shown to inhibit Jun N-terminal kinases (JNK) (Hoe *et al.*, 2005) and may also directly modulate gene transcription by colocalising with the transcription modulator, Tip60, in the nucleus (Kinoshita *et al.*, 2003). A recent paper reported that ligand binding to LRP-1 in neurons resulted in LRP-1 phosphorylation, activation of Src family kinase (SFK) and SFK-dependent Trk receptor transactivation (Shi *et al.*, 2009).

1.5.1.1.4. LRPb

LRP1b shares 59 % identity with LRP-1. In contrast with LRP-1, LRP1b contains one additional ligand-binding repeat within its fourth ligand-binding domain and also has a unique 33 amino acid sequence within its cytoplasmic tail (Liu *et al.*, 2000b). LRPb is highly expressed in the brain, thyroid and salivary gland and can bind and internalise many LRP-1 ligands including uPA, tPA and PAI-1

although it has a slower rate of endocytosis ($t_{1/2} \sim 8$ min) compared to LRP-1 ($t_{1/2} \sim 0.5$ min) (Liu *et al.*, 2001). LRP1b was originally named LRP-deleted in tumours (LRP-DIT) because it was found to be totally deleted or to contain inactivating mutations in 40 % of non small cell lung cancer cell lines (Liu *et al.*, 2000a). This suggests that LRPb1 may be a tumour suppressor.

1.5.1.1.5. Megalin/LRP-2

Megalin is found in many absorptive epithelia and is expressed in a variety of tissues including the intestinal brush border (Yammani *et al.*, 2001), uterus and oviduct (Argraves and Morales, 2004) and the male reproductive tract (Van Praet *et al.*, 2003). LRP-1 ligands including MMP-9 (Van den Steen *et al.*, 2006), lactoferrin and PAI-1 complexes also bind megalin (Willnow *et al.*, 1992). Megalin is also expressed in the kidney and is required for the reabsorption of vitamins and vitamin/protein complexes including retinol (Christensen *et al.*, 1999), vitamin B (Moestrup *et al.*, 1996) and vitamin D (Nykjaer *et al.*, 1999). In addition, thyroxine (Sousa *et al.*, 2000) as well as androgens and estrogens (Hammes *et al.*, 2005) are endocytosed by megalin. Circulating antibodies to both megalin and RAP cause Heymann nephritis, a rat model of autoimmune kidney disease (Kerjaschki and Farquhar, 1982; Orlando *et al.*, 1992; Pietromonaco *et al.*, 1990).

Megalin is thought to be required for normal cholesterol transport in the developing embryo. Megalin knockout mice present with developmental abnormalities in the lung, kidney and central nervous system and die shortly after birth due to respiratory insufficiency (Willnow *et al.*, 1996a).

1.5.1.1.6. MEGF7/LRP4

MEGF7/LRP4 was identified as a member of the LDL-R family in a motif trap screen of large EGF repeat transcripts expressed in mammalian cells (Nakayama *et al.*, 1998). MEGF7/LRP4-null mice present with growth

retardation as well as fusion and duplication of the digits (polysyndactyl) (Johnson *et al.*, 2005). MEGF7/LRP4 inhibits Wnt signalling *in vitro* suggesting that it may have a role in modulating signaling pathways controlling limb development in the embryo.

1.5.1.1.7. LRP8/apoE receptor 2

LRP8, also known as apoE receptor 2 was cloned from cDNA libraries of humans, mice and chickens (Kim *et al.*, 1996; Novak *et al.*, 1996). The human receptor contains 7 ligand binding repeats and is expressed in the brain, ovaries, testis and placenta (Stockinger *et al.*, 1998). It has broad ligand binding ability, similar to that of VLDL-R. The mouse and chicken receptors contain 8 ligand binding repeats, possibly due to alternative splicing (Brandes *et al.*, 1997; Schneider *et al.*, 1997).

1.5.1.1.8. SorLa/LRP11

SorLa/LRP11 is a 250 kDa receptor which is expressed in human brain, spinal cord and testis (Jacobsen *et al.*, 1996). In addition to ligand-binding repeats, SorLa/LR11 also possesses a Vps10p domain and is thus classified as a member of the Vsp10p-domain receptor family (Hermey, 2009). SorLa/LRP11 ligands include RAP, apoE and lipoprotein lipase although it has a slower rate of endocytosis compared to LRP-1 (Jacobsen *et al.*, 1996).

1.5.1.1.9. LRP-5 and LRP-6

LRP-5 and LRP-6 are distantly related members of the LDL-R family and are closely related to each other in structure and sequence (Herz and Bock, 2002). Unlike other members of the LDL-R family, their cytoplasmic domain does not contain an NPXY motif. Both LRP-5 and LRP-6 are likely to function as co-receptors in the Wnt signalling pathway (Mao *et al.*, 2001a; 2001b).

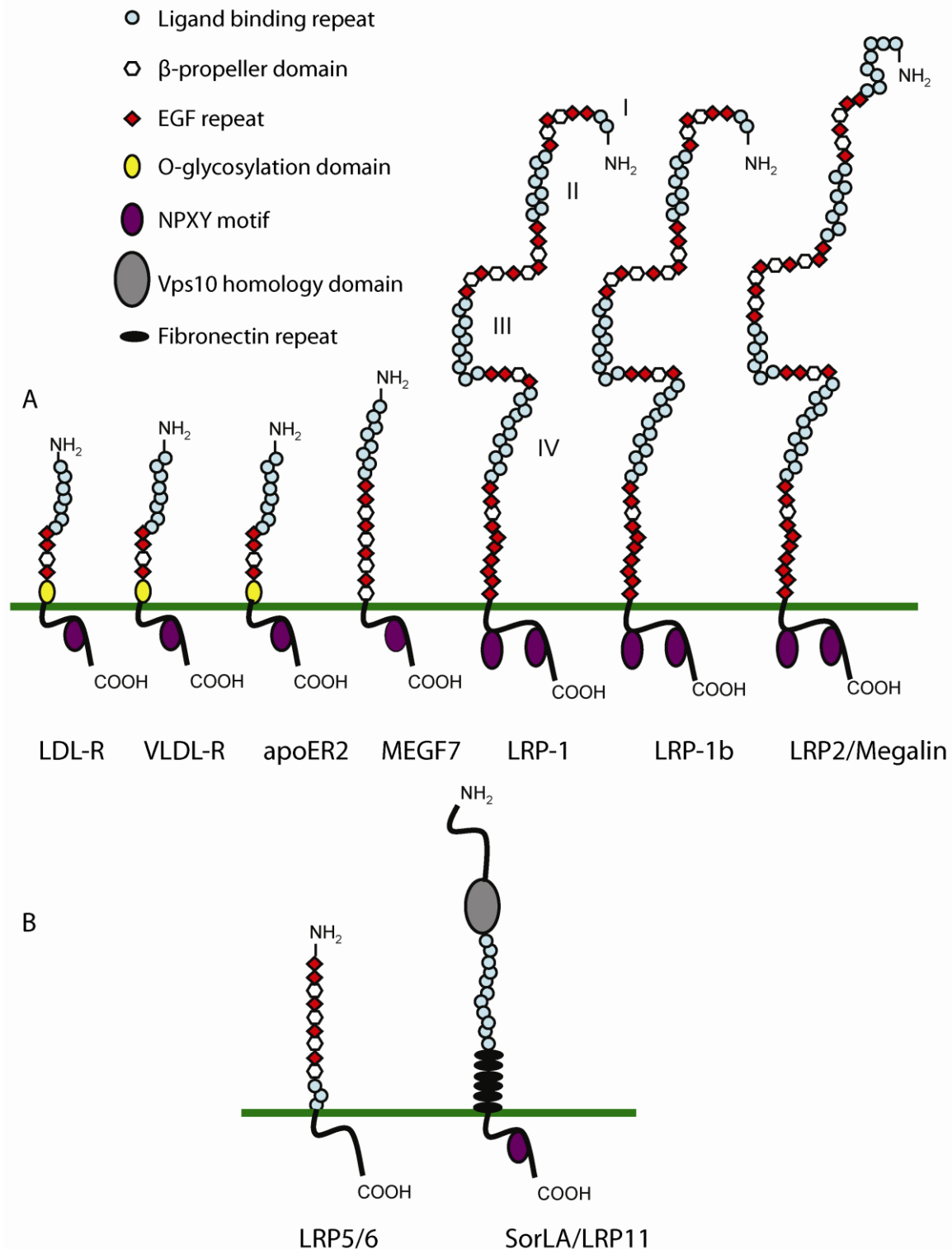


Figure 3: Structure of LDL receptor family members.

A, Core members of the LDL-receptor family. In LRP-1, the ligand binding clusters are numbered I-IV. **B**, members of the extended family. Adapted from Lillis *et al* (2008) and Herz and Bock (2002).

1.5.1.2. Other functions of RAP

Aside from its ability to bind LRP-1 and consequently inhibit the endocytosis of all its known ligands, RAP also binds to other members of the LDL receptor family with high affinity ($K_d \sim 1\text{-}10\text{ nM}$) including megalin (Kounnas *et al.*, 1992a), VLDL-R (Battey *et al.*, 1994), apoER2 (Stockinger *et al.*, 1998) and sorLA/LR11 (Jacobsen *et al.*, 1996). In addition, RAP also binds LDL-R with low affinity ($K_d \sim 250\text{ nM}$) (Medh *et al.*, 1995). RAP is found in the endoplasmic reticulum (ER), where it acts as a molecular chaperone, preventing the premature interaction of co-expressed ligands with their receptor and/or improper folding (Bu *et al.*, 1995; Bu and Rennke, 1996; Obermoeller *et al.*, 1997; Willnow *et al.*, 1996c). It is expressed in all human tissues examined to date, with very high expression in the kidney and brain (Bu *et al.*, 1994; Zheng *et al.*, 1994). Early binding studies showed that each LRP-1 molecule had 2 distinct RAP binding sites (Williams *et al.*, 1992). However, studies using truncated soluble minireceptors of LRP-1 indicated that 5 independent RAP binding sites are present on LRP-1; 2 on each of the second and fourth ligand binding domains of LRP-1 and one on the third (Bu and Rennke, 1996). It has been suggested that RAP may inhibit endocytosis by direct ligand competition. Additionally, the binding of RAP to LRP-1 may induce a conformational change in the receptor so that ligand binding sites are masked (Horn *et al.*, 1997; Neels *et al.*, 1999).

The ability of RAP to compete with the binding of many diverse ligands is likely due to its small size and presence of multiple binding sites. RAP can also inhibit the binding of the same ligand to several members of the LDL-R family, suggesting a level of redundancy between these receptors. For example, RAP inhibits the binding of lactoferrin to both LRP-1 and megalin/LRP-2 (Willnow *et al.*, 1992). The ability of RAP to act as a universal ligand antagonist to the LDL-R family makes it a useful experimental tool to identify new ligands of these receptors.

In addition to members of the LDL-R family, RAP has been shown to bind and/or inhibit other receptors, including lipolysis-stimulated receptor (Troussard *et al.*, 1995), cubilin (Birn *et al.*, 1997) and sortilin (Petersen *et al.*, 1997; Tauris *et al.*, 1998). In hepatocytes, the lipolysis-stimulated receptor clears chylomicron remnants (Yen *et al.*, 1994), whereas in the kidney, cubilin facilitates endocytosis of intrinsic factor-vitamin B12 (Birn *et al.*, 1997). Sortilin, related to SorLa/LRP11, is a receptor from the Vsp10p domain gene family that lacks LDL-A repeats and it endocytoses apolipoprotein A-V (Nilsson *et al.*, 2008).

Ligand blotting experiments using ^{125}I -Labeled Fluoresceinamine-Heparin (^{125}I -F-Hep) have showed that RAP binds directly to heparin. Unlabeled heparin effectively competed for the binding of ^{125}I -F-Hep to RAP whereas chondroitin sulfate had no effect, indicating that heparin binding was specific and not due to non-specific charge interactions (Orlando and Farquhar, 1993). In subsequent studies using recombinant RAP fusion proteins, the heparin binding site within RAP was mapped to the C-terminus between amino acids 261 and 323 (Orlando and Farquhar, 1994). This suggests that RAP could bind to cell surface HSPGs.

1.5.1.3. HSPGs as co-receptors

HSPGs have been identified as co-receptors for the endocytosis of several ligands of the LDL-R family. It is hypothesised that HSPGs may concentrate ligands on cell surface and facilitate their internalisation and degradation by presenting them to a specific receptor. The availability of HSPGs on the cell surface can be regulated by proteolytic cleavage of the core protein as well as by cleavage of the HS chains by extracellular heparanase (Vlodavsky *et al.*, 2002).

LDL can be dissociated from the LDL-R by treatment with heparin and other sulfated GAGs (Goldstein *et al.*, 1976) whilst the binding of uPA-PAI-1 complexes to LRP-1 is also heparin-sensitive (Nykjaer *et al.*, 1992). Similarly, cell surface HSPGs are required for LRP-1-mediated binding and degradation of

thrombospondin-1 and thrombospondin-2 (Mikhailenko *et al.*, 1995; Murphy-Ullrich *et al.*, 1988; Yang *et al.*, 2001), suggesting that cell surface HSPG binding is a prerequisite for internalisation. However, a later study showed that the LRP-1 binding site of thrombospondin-1 differed from its heparin binding domain (Wang *et al.*, 2004b). This suggests that HSPG binding is not necessary for thrombospondin-1 to bind LRP-1 at the cell surface but that subsequent endocytosis requires high affinity heparin binding (Wang *et al.*, 2004b).

Our laboratory has shown that RAP, heparin and another sulfated glycan, calcium pentosan polysulfate (CaPPS), cause accumulation of TIMP-3 in the conditioned medium of HTB94 human chondrosarcoma cells (Troeborg *et al.*, 2008). These agents also increase TIMP-3 accumulation by chondrocytes and matrix of porcine cartilage explants suggesting that a member of the LDL-R family may mediate TIMP-3 internalisation and that HSPGs may act as a co-receptor for ligand transfer. TIMP-3 has been reported to sequester to the ECM by association with HSPGs (Yu *et al.*, 2000). Perlecan may be the HSPG involved in TIMP-3 binding to the matrix since in a review article, Knox and Whitelock quote an unpublished study where TIMP-3 reportedly binds perlecan and is localized pericellularly (Knox and Whitelock, 2006).

1.5.1.4. HSPGs as independent receptors

There have been several studies proposing that HSPGs may also function as ligand receptors by mechanisms independent to their function as a co-receptor. Any molecule bound to HSPGs on the cell surface could be internalised with the HSPG as part of normal HSPG turnover on the cell surface. Following internalisation, HSPGs are degraded rapidly by the lysosomal pathway or recycled back to the cell surface (Egeberg *et al.*, 2001). On the cell surface, HSPGs typically have a half-life of 3 – 8 h (Egeberg *et al.*, 2001; Yanagishita and Hascall, 1992)

HSPGs have been shown to sequester BMP2 at the cell surface and mediate its internalisation in C2C12 myoblasts (Jiao *et al.*, 2007). In addition to basic fibroblast growth factor (bFGF) internalisation via FGF receptor 1 (FGFR-1) or FGF receptor 2 (FGFR-2) (Dionne *et al.*, 1990), bFGF is also internalised independently through its interaction with cell surface HSPGs (Roghani and Moscatelli, 1992). The heparan sulfate pathway of bFGF internalisation was shown to be heparin-sensitive and binding was not efficiently competed by unlabeled bFGF, indicating that bFGF bound to cell surface HSPGs with low affinity. Further studies using electron microscopy revealed that the FGFR-independent mechanism of FGF internalisation was by calveolae, flask-shaped non-coated membrane invaginations (Gleizes *et al.*, 1996). Additionally, fibronectin matrix turnover occurs through a calveolin-1-dependent mechanism (Sottile and Chandler, 2005) although soluble fibronectin has been shown to be endocytosed by an LRP-1-dependent mechanism in mouse embryonic fibroblasts (Salicioni *et al.*, 2002).

The syndecan family of proteoglycans have been reported to mediate the binding and internalisation of lipoprotein lipase (LpL)-enriched low density lipoprotein (LDL) via an LRP-1-independent pathway (Fuki *et al.*, 1997). Endocytosis of LpL-enriched LDL was triggered by ligand clustering and was sensitive to cytochalasin B. Syndecan-mediated internalisation of LpL-enriched LDL was reported to be independent of coated pits and was found to be mediated via detergent-insoluble, cholesterol-rich membrane rafts (Fuki *et al.*, 2000b). A further study by the same group reported that endocytosis of LpL-enriched LDL can be independent of both LRP-1 and syndecan, proceeding with a slower half-life ($t_{1/2}$) of internalisation of 6 h, compared to 1 h via the syndecan pathway (Fuki *et al.*, 2000a). Cells expressing perlecan but no other proteoglycans bound and internalised LpL-enriched LDL (Fuki *et al.*, 2000a). Internalisation was unaffected by cytochalasin D but was inhibited by the tyrosine kinase inhibitor, genistein. Perlecan has also been reported to mediate the uptake of HIV-1 Tat, a transcriptional transactivator of the HIV-1 virus, although only a small amount of

HIV-1 Tat was degraded by lysosomes. This suggests that perlecan uptake of HIV-1 Tat is not a major cellular clearance mechanism for this protein (Argyris *et al.*, 2004).

1.6. Aims of thesis

Our laboratory recently suggested that HTB94 chondrosarcoma cells and primary chondrocytes may endocytose TIMP-3 (Troeberg *et al.*, 2008). This work raised the hypothesis that TIMP-3-sensitive enzymes such as ADAMTS-4 may also be endocytosed. The aim of this thesis is to determine whether ADAMTS-4 can be cleared from the medium of HTB94 chondrosarcoma cells and primary chondrocytes via cellular uptake and intracellular degradation. Investigating whether the extracellular level of ADAMTS-4 could be regulated by endocytic clearance may identify a further mechanism regulating ADAMTS-4 activity in the matrix.

Chapter 2

Materials and Methods

2.1. Reagents

2.1.1. Cells/Tissue

Human chondrosarcoma cells (HTB94) and CHO-K1 cells were from American Culture Type Collection (Manassas, VA, USA). psgA-745 CHO cells, deficient in both chondroitin sulfate and heparan sulfate proteoglycans (Esko *et al.*, 1985) were a kind gift from Professor Jeffrey Esko (San Diego, USA). Human epithelial kidney (HEK-293/EBNA) cells were from Invitrogen (Paisley, UK). HEK-293/EBNA cells transfected with pCEP4-ADAMTS-4 constructs were obtained from frozen laboratory stocks prepared by Dr Christine Gendron. A schematic diagram of full-length ADAMTS-4 and the domain deletion mutants used in this study are shown in Figure 4.

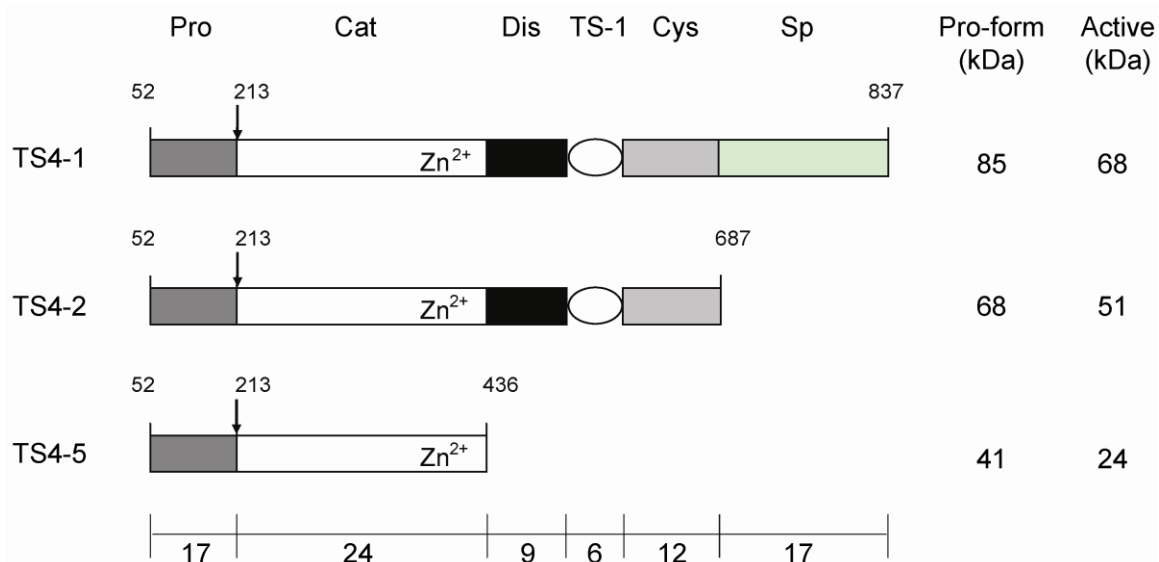


Figure 4: Schematic diagram of full-length ADAMTS-4 and domain deletion mutants used in this study.

The predicted molecular mass is based on the amino acid composition. Each protein contains a C-terminal FLAG sequence epitope (D-K-Y-D-D-D-D-K). The arrow indicates the position of the potential furin cleavage site. Abbreviations - Pro, pro-domain; Cat, catalytic metalloproteinase domain; Dis, disintegrin domain; TS-1, thrombospondin type 1 motif; Cys, cysteine-rich domain; Sp, spacer domain. Adapted from Gendron *et al* (2007).

MEF-1, PEA-10 (heterozygous LRP-1-deficient) and PEA-13 cells (homozygous LRP-1 deficient) cells were a kind gift from Professor D Strickland (Maryland, USA) and were isolated by repeated selection of mouse embryonic fibroblasts heterozygous for a disrupted LRP-1 allele with the LRP-1 ligand *Pseudomonas* exotoxin A (Kounnas *et al.*, 1992b; Willnow and Herz, 1994). Porcine metacarpophalangeal joints of 3-9 month-old-pigs were from Fresh Tissue (London, UK) and were used within 24 hours of slaughter. Human articular cartilage was obtained with informed patient consent and ethical approval from the Royal National Orthopedic Hospital (Stanmore, UK). Human chondrocytes were isolated by Mr Brendan Thomas at the Kennedy Institute of Rheumatology and were a kind gift. Wild-type and *Timp-3*-null mouse dermal fibroblasts were obtained from frozen laboratory stocks prepared by Dr Linda Troeberg.

2.1.2. Cell and tissue culture reagents

Materials were from the following sources: Dulbecco's modified eagle's medium (DMEM), DMEM without L-glutamine or phenol red, L-glutamine, HEPES and penicillin/streptomycin from Biowhittaker (Berkshire, UK); Ham's F12 medium, fetal calf serum (FCS), G418, hygromycin B and trypsin-versene from PAA Laboratories (Somerset, UK); amphotericin B from Gibco (Paisley, UK); DMEM without L-glutamine, cysteine, methionine or cystine from MP Biomedicals (CA, USA); Pronase E from BDH (Dorset, UK); lactalbumin enzymatic hydrolysate, type 1A bacterial collagenase, heparin, de-N-sulfated heparin, chondroitin sulfate, cytochalasin D, Protease Inhibitor Cocktail for mammalian cell and tissue extracts, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), GM6001 and L-trans-epoxysuccinyl-leucyl amido(4-guanidino)butane (E-64) from Sigma-Aldrich (Dorset, UK); CT1746 from Celltech (Slough, UK); dermatan sulfate from Calbiochem (Nottingham, UK); calcium pentosan polysulfate from Arthroparm (Sydney, Australia). AEBSF and each of the GAGs were dissolved in serum-free DMEM to a stock concentration of 100 mM and 50 mg/ml, respectively. Cytochalasin D, E-64, GM6001, CT1746 and phosphoramidon

were all dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM.

2.1.3. SDS-PAGE and Western blotting reagents

Materials were from the following sources: Polyvinylidene difluoride membrane (PVDF), ECL Plus, ECL Advance Western Blotting Detection System and Reblot Plus Strong Solution from Chemicon (CA, USA); photographic Hyperfilm from GE Healthcare (Buckinghamshire, UK); pre-stained and unstained Precision Protein Standards Precision Protein StandardsTM from Bio-Rad (Hemel Hempstead, UK); 30 % (w/v) acrylamide/bis-acrylamide from Severn Biotech (Worcestershire, UK); 2-amino-2-methyl-1,3-propanediol (ammediol) and Tween20 from Sigma-Aldrich (Dorset, UK); Western Blue stabilized substrate for alkaline phosphatase (AP) from Promega (Southampton, UK).

2.1.4. Proteins and antibodies

Materials were from the following sources: anti-mouse AP-linked antibody and anti-rabbit AP-linked antibody from Promega (Southampton, UK); anti-rabbit horseradish peroxidase (HRP)-linked antibody and anti-mouse HRP-linked antibody from GE Healthcare (Buckinghamshire, UK); mouse anti-low-density lipoprotein receptor-related protein-1 (LRP-1) antibody (8G1) and Azocoll > 100 mesh from Calbiochem (Nottingham, UK); mouse monoclonal anti-FLAG M2-antibody and anti-FLAG M2-agarose from Sigma-Aldrich Ltd (Dorset, UK); TIMP-3 monoclonal antibody 183551 from R&D Systems (Minneapolis, USA); FLAG peptide (D-K-Y-D-D-D-D-K) from Advanced Biotechnology Centre (Imperial College, London, UK); recombinant glutathione S-transferase (GST)-tagged interglobular domain (IGD) aggrecan substrate (GST-IGD-FLAG) and N-TIMP-3 from Dr Linda Troeberg in this laboratory; ADAMTS4-1 from Dr Kasu Fushimi in this laboratory, ADAMTS4-2 (for iodination) from Dr Christine Gendron in this laboratory.

The rabbit anti-ADAMTS-4 antibody was raised in this laboratory against the purified recombinant catalytic domain of human ADAMTS-4. Rabbit anti-LRP-1 antibody (2629) was a kind gift from Professor Dudley Strickland (Maryland, USA). Recombinant His-tagged receptor-associated protein (RAP) was a kind gift from Dr H  rve Emonard (Christian de Duve Institute of Cellular Pathology and Universit   Catholique de Louvain, Brussels, Belgium). Human α_2 -macroglobulin (α_2 M) was a kind gift from Professor Jan Enghild (University of Aarhus, Denmark).

2.1.5. Radiochemical Reagents

Materials were from the following sources: RedivueTM Pro-MixTM L-[³⁵S] *in vitro* Cell Labeling Mix from GE Healthcare (Buckinghamshire, UK); Goldstar Multipurpose Liquid Scintillation Cocktail from Meridian (Surrey, UK); Iodine-125 was from MP Biomedicals (CA, USA); IODO-BEADS Iodination Reagent from Pierce Biotechnology (Illinois, USA).

2.1.6. Molecular Biology Reagents

Materials were from the following sources: LipofectamineTM, RNAiMAX and Optimem 1 + GlutaMax medium from Invitrogen (Paisley, UK); siRNAs (double-stranded RNA targeting LRP-1 (forward, GACUUGCAGCCCCAAGCAGUU; reverse, CUGCUUGGGGCUGCAAGUCUU) and control) (Dedieu *et al.*, 2008) were kind gifts from Dr H  rve Emonard (Christian de Duve Institute of Cellular Pathology and Universit   Catholique de Louvain, Brussels, Belgium).

2.1.7. Fluorescent Microscopy Reagents

Materials were from the following sources: Alexa Fluor 488 and Alexa Fluor 568 Succinimidyl Esters and ProLong Gold antifade reagent from Molecular Probes, Invitrogen (Oregon, USA); concanavalin A and N, N-Dimethylformamide (DMF) from Sigma-Aldrich (Dorset, UK).

2.1.8. Other reagents

Materials were from the following sources: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Molecular Probes, Invitrogen (Oregon, USA); Vivaspin Ultrafiltration Spin Columns (MWCO 3000) from Sartorius Ltd (Epsom, UK); Slide-A-Lyzer Dialysis Cassettes (MWCO 3500) from Thermo Scientific (Illinois, USA); PD-10 Desalting Columns, G-50 Micro Columns and Sephacryl S-200 from GE Healthcare (Buckinghamshire, UK); Sephadex G-50 from Pharmacia Fine Chemicals (Uppsala, Sweden); methylamine-hydrochloride, Brij35 solution 30 % (w/v) and sodium nitroprusside from Sigma-Aldrich (Dorset, UK).

2.2. Cell culture

2.2.1. Cell line culture

Cells (HEK-293/EBNA, HTB94, MEF-1, PEA-10, PEA-13, wild-type and *Timp-3*-null mouse dermal fibroblasts) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % (v/v) FCS. HEK-293/EBNA cells transfected with pCEP4-ADAMTS-4 constructs were cultured with the addition of 250 µg/ml G418 and 100 µg/ml hygromycin B. Wild-type and psgA-745 CHO cells were grown in Ham's F12 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % (v/v) FCS. Cells were passaged every 3-5 days and detached from the culture dish by incubation with trypsin-versene.

2.2.2. Porcine chondrocyte isolation and culture

Porcine metacarpophalangeal joints were dissected into DMEM containing 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mg/ml fungizone and 10 mM HEPES. The cartilage was washed thrice with medium to remove synovial fluid. Cartilage (1 g) was incubated overnight with 10 ml DMEM containing 0.25 mg/ml type 1A bacterial collagenase (37 °C with shaking). The chondrocytes were

isolated from undigested material by passing the solution through a cell strainer into a 50 ml Falcon tube. The medium was centrifuged at 327 g for 5 min and the cell pellet was washed with 10 ml of DMEM. The wash step was repeated and the chondrocytes were re-suspended in 10 ml of DMEM. Cell yield and viability was determined by diluting a sample with an equal volume of trypan blue and counting the number of live and dead cells under a light microscope. The isolated chondrocytes were plated in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % (v/v) FCS and rested for 2-3 days before use.

2.3. SDS-PAGE and Western blots

2.3.1. SDS-PAGE

Proteins were resolved by SDS-PAGE with reduction using a modification of the ammediol/glycine/HCl buffer system of Wyckoff *et al.* (Wyckoff *et al.*, 1977) or the Tris-glycine buffer system according to Laemmli (Laemmli, 1970). Polyacrylamide gels were made with 6-12 % total acrylamide, depending on the size of the proteins to be separated. Samples were diluted with an equal volume of 2 x reducing sample buffer [42 mM ammediol or 0.125 mM Tris-HCl, 0.01 % (w/v) sodium azide, 2 % SDS, 0.1 % (w/v) bromophenol blue, 50 % (w/v) glycerol, 1 % β-mercaptoethanol] and boiled for 5 min prior to SDS-PAGE. Typically, ammediol gels were run at 150 V for 45-60 min and Tris-glycine gels were run at 150 V for 90 min.

2.3.2. Gel staining

2.3.2.1. Coomassie Brilliant Blue R-250 staining

SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 stain [0.1 % (w/v) Coomassie Brilliant Blue R-250, 50 % (v/v) methanol and 20 % (v/v) acetic acid] for 30 min before several 30 min washes with destaining solution [30 % (v/v) methanol and 1 % (v/v) formic acid].

2.3.2.2. Silver staining

SDS-PAGE gels were stained using silver stain (Shevchenko *et al.*, 1996). Gels were incubated with fixing solution [50 % (v/v) methanol and 5 % (v/v) acetic acid] for 20 min and washed for 10 min with 50 % (v/v) methanol. Next, gels were washed for at least an hour (usually overnight) with dH₂O and then incubated with sensitizer [0.02 % (w/v) sodium thiosulfate] for 1 min. After washing twice with dH₂O, gels were incubated with cold silver reagent [0.1 % (w/v) silver nitrate] for 30 min at 4 °C. Following this, gels were washed twice with dH₂O and protein bands were detected by addition of developer [0.04 % (v/v) formalin and 2 % (w/v) sodium carbonate]. The developer was discarded and replaced as soon as it turned yellow so that protein bands were developed in an absolutely transparent solution. Once protein bands could be detected, the developing reaction was stopped using 5 % (v/v) acetic acid.

2.3.3. Western blotting

Proteins separated by SDS-PAGE were analysed by Western blotting using Novex Mini-cell (Invitrogen) or Mini-Trans-Blot cell (BIO-RAD) systems. The proteins were transferred to PVDF at 200 mA for 2.5 h (Invitrogen) or 350 mA for 1 h (BIO-RAD) in transfer buffer [20 % (v/v) methanol, 12.6 mM Tris, 96 mM glycine and 0.1 % (w/v) SDS]. Following transfer, the membranes were blocked with either 5 % BSA in TBS or 5 % dry skim milk in TBS for 1 h at room temperature. In general, membranes were incubated overnight with primary antibodies in either 1 % BSA-TBS or 1 % dry milk-TBS at 4 °C. Antibody dilutions were as follows: anti-ADAMTS-4-catalytic 1:500, anti-FLAG M2 1:1000, anti-LRP-1 (2629) and anti-LRP-1 (8G1) 1:5000, anti-TIMP-3 (183551) 1:2500. Membranes were then washed thrice with TBS containing 0.1 % Tween 20 and incubated with AP-linked secondary antibody (1:5000) or HRP-linked secondary antibody (1:5000) for 1 h at room temperature. Membranes were subsequently washed thrice with TBS containing 0.1 % Tween 20 before development. However, in the case of LRP-1 detection using the mouse anti-LRP-1 antibody

(8G1), membranes were blocked with 5 % dry skim milk in 20 mM Tris (pH 7.5), 0.5 M NaCl, 0.05 % Tween 20 for 2 h at room temperature. They were also washed extensively with 20 mM Tris (pH 7.5), 0.5 M NaCl, 0.05 % Tween 20 and incubated with both primary and secondary antibodies in 0.5 % milk in 20 mM Tris (pH 7.5), 0.5 M NaCl, 0.05 % Tween 20. Western blots were developed as follows: AP Western blots developed with Western Blue AP substrate until bands appeared. The AP reaction was then stopped with AP stop solution [20 mM Tris-HCl (pH 7.5), 10 mM EDTA]; HRP Western blots were incubated according to the manufacturers instructions (GE Healthcare) with either ECL-Plus or ECL-Advance chemiluminescence substrate for 1 or 5 min, respectively. Protein bands developed by HRP were detected by exposure to photographic film.

2.3.4. Densitometry

Gels and Western blots were scanned using a BioRad GS-710 imaging densitometer. The pixel intensity of the protein bands were quantified using Phoretix 1D software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). Unmodified 16-bit TIFF images were analysed and the background pixel intensity was subtracted using the 'rolling ball' method.

2.4. Protein purification

2.4.1. ADAMTS4-2 purification

HEK-293/EBNA cells stably transfected with pCEP4-ADAMTS4-2 were grown to confluence in the presence of hygromycin B (200 µg/ml) and G418 (250 µg/ml). Upon reaching confluence, the cells were washed once with serum-free DMEM to remove serum and then incubated in serum-free DMEM containing 0.2 % lactalbumin enzymatic hydrolysate. The conditioned medium was collected after 3-5 days and centrifuged to remove cell debris.

The conditioned medium was applied to a 2 ml anti-FLAG M2-agarose column at room temperature. The column was washed with 10 column volumes of 1 M NaCl buffer [50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10 mM CaCl₂, 0.02 % sodium azide, 0.02 % Brij-35] to remove non-specifically bound proteins. The column was then washed with 5 column volumes of Tris-buffered-saline (TBS, pH 7.4). The protein was eluted with 200 µg/ml FLAG peptide in TNC-Brij buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 0.02 % sodium azide, 0.02 % Brij-35]. Fractions were run on SDS-PAGE and proteins were silver stained.

Fractions containing the protein were pooled and concentrated to a volume of 2.5 ml using a Vivaspın ultrafiltration spin column (MWCO 3000). The protein was applied to a Sephacryl S-200 gel filtration column (1.6 cm X 100 cm) equilibrated with TNC-Brij. This step removed the FLAG peptide from the protein. Fractions were run on SDS-PAGE and proteins were silver stained. Fractions containing the protein were pooled, divided into aliquots and stored at -80 °C.

2.4.2. Metabolic labeling and purification of recombinant ADAMTS-4 constructs

HEK-293/EBNA cells stably transfected with pCEP4-ADAMTS4-2 or pCEP4-ADAMTS4-5 constructs were grown to confluence in a 150 cm² flask, washed once with serum-free DMEM without L-glutamine, cysteine, methionine or cystine to remove serum and then incubated in serum-free DMEM without L-glutamine, cysteine, methionine or cystine containing 0.2 % lactalbumin enzymatic hydrolysate and [³⁵S]Met/[³⁵S]Cys (25 µCi/ml). The conditioned medium was collected after 3-5 days and centrifuged to remove cell-debris. Conditioned media was applied to a 2 ml anti-FLAG M2-agarose column and eluted with FLAG peptide as described above.

2.4.3. Titration of ADAMTS4-2 using GST-IGD-FLAG substrate

The concentration of active ADAMTS4-2 was determined by titration with N-TIMP-3, using a recombinant GST-IGD-FLAG substrate. The GST-IGD-FLAG substrate was designed in-house by Dr Masa Kashiwagi and characterized by Dr Ngee Han Lim. The substrate is based on the IGD sequence of aggrecan core protein with a GST-tag at the N-terminus and a FLAG-tag at the C-terminus (Figure 5) and contains both MMP and aggrecanase cleavage sites. ADAMTS4-2 (2.5 μ l) was incubated with 2.5 μ l of N-TIMP-3 (0-400 nM) for 1 h at 37 °C. The enzyme and inhibitor complex was incubated with 2.4 mg/ml GST-IGD-FLAG substrate (5 μ l) for 90 min at 37 °C. The reaction was stopped with 2 x reducing sample buffer containing 10 mM EDTA (10 μ l). Each sample (10 μ l) was analysed by SDS-PAGE (10 % acrylamide) and Coomassie Brilliant Blue R-250 staining.



Figure 5: Schematic diagram of recombinant aggrecan IGD substrate.

The recombinant substrate was constructed in this laboratory by Dr Masa Kashiwagi and is composed of the IGD domain of aggrecan with a GST-tag at the N-terminus and a FLAG-tag at the C-terminus. The substrate contains both the MMP and aggrecanase cleavage sites. It is expressed in *E.coli* and purified using glutathione Sepharose. Figure adapted from Christine Gendron's thesis (2005).

2.4.4. Determination of ADAMTS4-5 concentration using BSA standard curve

ADAMTS4-5 has essentially no aggrecanase activity (Kashiwagi *et al.*, 2004). Therefore, to determine the approximate concentration of [³⁵S]ADAMTS4-5, the amount of protein was determined by standard curve against BSA. Increasing amounts of BSA (ng) and an aliquot of [³⁵S]ADAMTS4-5 (5 μ l) were analysed by SDS-PAGE and silver staining. The gel was scanned and the pixel volume of

each band was determined using Phoretix software. The concentration of [³⁵S]ADAMTS4-5 was calculated from the standard curve using the equation $y = mx + c$.

2.5. Assays to monitor ADAMTS-4 clearance/cellular uptake

2.5.1. Western blot assay

Cells were plated at a density of 1×10^6 cells/well (6-well plate). After resting overnight, cells were washed thrice with serum-free DMEM before incubation for 1 h at 37 °C with DMEM containing 0.1 % BSA with or without GM6001 (10 µM), E-64 (10 µM), AEBSF (100 µM), heparin (5-500 µg/ml), chondroitin sulfate, de-N-sulfated heparin or dermatan sulfate (all at 100 µg/ml). Following this, conditioned medium, containing ADAMTS4-2 (1.5 nM), was added and incubated for up to 24 h in a final volume of 2 ml. After incubation, the conditioned medium was removed and the protein was precipitated with TCA (3.3 %) overnight at 4 °C. The TCA-soluble fraction was separated from the TCA-insoluble fraction by centrifugation at 14170 g for 15 min at 4 °C and the TCA-insoluble fraction was re-suspended in 60 µl of 2 x reducing sample buffer. In order to detect the enzyme within the cell, the cell layer was washed with ice-cold PBS and lysed with 60 µl of 2 x reducing sample buffer. The wash fractions were also precipitated with TCA (3.3 %) as described above. All samples (15 µl) were analysed by SDS-PAGE (7.5 % acrylamide) and Western blotting using the anti-ADAMTS-4 catalytic domain antibody.

2.5.2. Radioactivity-based assay

Cells were plated at a density of 1×10^6 cells/well (6-well plate) or 0.5×10^6 cells/well (12-well plate). After resting overnight, cells were washed thrice with serum-free DMEM before incubation for 1 h at 37 °C with DMEM containing 0.1 % BSA with or without the following agents: GM6001 (1-50 µM), CT1746 (1-20 µM), E-64 (10 µM), AEBSF (100 µM), phosphoramidon (10 µM), cytochalasin D

(10 μ M), heparin (5-500 μ g/ml), chondroitin sulfate, de-N-sulfated heparin, dermatan sulfate, calcium pentosan polysulfate or hylauronan (all at 250 μ g/ml), RAP (500 nM). As a control for agents diluted in DMSO, the equivalent dilution of DMSO was added to separate wells. Following this, [^{35} S]ADAMTS-4 constructs or [^{125} I]- α_2 M* (see section 2.11.) were added and cultured for up to 24 h in a final volume of 2 ml (6-well plate) or 1 ml (12-well plate). After incubation, the conditioned medium was removed and the protein was precipitated with TCA (3.3 %) overnight at 4 $^{\circ}$ C. The TCA-soluble fraction was separated from the TCA-insoluble fraction by centrifugation at 14170 g for 15 min at 4 $^{\circ}$ C. The cells were placed on ice and washed thrice with ice-cold PBS. The wash fractions were transferred into a scintillation vial and counted directly. The cell layer was incubated with 0.1 % pronase in DMEM for 10 min on ice. The cells were then pipetted into an Eppendorf tube. Each well was then washed once with DMEM and the wash was added to each cell suspension. The surface bound (pronase-sensitive) fraction was separated from the intracellular fraction (pronase-resistant) fraction by centrifugation at 755 g for 5 min at 4 $^{\circ}$ C. Both the TCA-insoluble pellet and the cell pellet were dissolved in 1 M NaOH (500 μ l). In some experiments, the surface bound and intracellular fractions were harvested together (cell-associated fraction) by lysis with 1 M NaOH. Radioactivities in each fraction were counted as follows: [^{35} S] using a 1410 Liquid Scintillation Counter (Wallac, Pharmacia) and [^{125} I] using a 1282 COMPUGAMMA Universal Gamma Counter (Wallac, Pharmacia). The amount of TCA-soluble radioactivity in the preparation of labeled protein prior to incubation with cells was subtracted from the amount of TCA-soluble radioactivity in the sample at the time of harvest. This corrected for any TCA-soluble radioactivity in the starting material.

2.6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for measuring cell viability

HTB94 cells were plated at a density of 1×10^6 cells/well (6-well plate) or 0.5×10^6 cells/well (12-well plate). After resting overnight, cells were washed thrice with serum-free DMEM. To examine cell viability, cells were incubated in DMEM

containing 0.1 % serum and treated with either GM6001 (100 μ M), heparin (500 μ g/ml), cytochalasin D (10 μ M) or RAP (500 nM) or DMSO carrier control at the appropriate dilution for up to 24 h. Sodium nitroprusside (2 mM) was used as a positive control for cell death in all experiments (Del Carlo and Loeser, 2002). Cell viability was then determined by the MTT assay, described by Mosmann *et al.* (Mosmann, 1983). The conditioned medium was removed and the cells were washed once with phenol red-free DMEM before incubation with 975 μ l phenol red-free DMEM and 25 μ l of MTT reagent (5 mg/ml in PBS) for 45 min at 37 °C. One ml of 10 % SDS in 10 mM HCl was added to solubilise the formazan crystals. After overnight incubation at 37 °C, 200 μ l of each well was transferred to a 96-well plate and the absorbance was read at 574 nm using a FLUOstar Omega (BMG Labtech) plate reader.

2.7. siRNA knockdown of LRP-1 protein expression in HTB94 cells

siRNAs (double-stranded RNA targeting LRP-1 or control) were transiently transfected into HTB94 cells using Lipofectamine RNAiMAX (Invitrogen). Throughout the transfection procedure, penicillin and streptomycin were not added to the medium because these antibiotics reduce transfection efficiency. HTB94 cells were plated overnight at a density of 1.5×10^4 cells/well (12-well plate) in DMEM containing 10 % FCS. Immediately before transfection, the medium on the cells was replaced with 1 ml of serum-free DMEM. For each transfection, siRNA (50 – 300 pmol) was diluted in serum-free OptiMem to a final volume of 100 μ l. In a separate tube, 2 μ l of RNAiMAX was diluted in 98 μ l serum free OptiMem. The siRNA and RNAiMAX solutions were combined and incubated for 20 min at room temperature. The RNAiMAX-siRNA mixture (200 μ l) was then gently pipetted into each well to ensure an even distribution of the siRNA in the medium. After overnight culture, the medium was removed and the cells were washed once with serum-free DMEM. The medium was replaced with 1 ml of DMEM containing 10 % FCS and the cells were cultured for 3 days. After 3 days in culture, the cells were confluent. The medium was removed and the cells washed with ice-cold PBS. The cells were lysed with CHAPS buffer [10 mM

CHAPS, 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl₂], supplemented with protease inhibitor cocktail (1:40) and incubated for 5 min on ice. The cell lysate was then scraped into an Eppendorf tube, diluted 1:1 with non-reducing sample buffer [0.125 mM Tris-HCl, 0.01 % (w/v) sodium azide, 2 % SDS, 0.1 % (w/v) bromophenol blue, 50 % (w/v) glycerol] and briefly sonicated on ice. Equal volumes of each sample were analysed by SDS-PAGE using the Tris-Glycine buffer system (6 % acrylamide).

2.8. Microscopy studies

2.8.1. Protein labeling

2.8.1.1. Alexa Fluor 488 labeling of ADAMTS4-2

After purification, 400 µl of a 2.4 µM solution of ADAMTS4-2 was exchanged into HEPES-Brij buffer [20 mM HEPES (pH 8.2), 150 mM NaCl, 10 mM CaCl₂, 0.02 % sodium azide, 0.02 % Brij-35] by dialysis at 4 °C using a Slide-A-Lyzer dialysis cassette (MWCO 3500). Dialysis was necessary to exchange ADAMTS4-2 from TNC-Brij into a buffer free from ammonium ions or primary amines as these groups react with the fluorophore and reduce the efficiency of the labeling reaction. Alexa Fluor 488 was diluted in DMF (1 mg/ml). ADAMTS4-2 was incubated with Alexa Fluor 488 for 1 h at room temperature. The amount of Alexa Fluor 488 in the final reaction was 110 µg/ml. After incubation, glycine was added to a final concentration of 50 mM to quench the reaction and the solution was passed over a PD10 column equilibrated with TNC-Brij to ensure the removal of unreacted Alexa Fluor 488 fluorophore. Alexa Fluor 488-labelled ADAMTS4-2 was analysed by SDS-PAGE with silver staining. The gel was also visualized using a Fujifilm FLA-200 phosphoimager (resolution 50, sensitivity F1000, Fluor 473, filter 7520). The fluorescence intensity was also determined using a SpectraMax Gemini plate reader (ex. 495 nm, em. 519 nm).

2.8.1.2. Alexa Fluor 568 labeling of Concanavalin A

Concanavalin A was used to visualize the cell surface as it binds specifically to mannosyl and glucosyl residues of polysaccharides and glycoproteins (Goldstein *et al.*, 1965). Concanavalin A was diluted to a concentration of 1 mg/ml with HEPES-Brij buffer [20 mM HEPES (pH 8.2), 150 mM NaCl, 10 mM CaCl₂, 0.02 % sodium azide, 0.02 % Brij-35] and Alexa Fluor 568 was diluted in DMF (1 mg/ml). Concanavalin A (1 ml) was incubated with Alexa Fluor 568 for 1 h at room temperature. The amount of Alexa Fluor 568 in the final reaction was 48 µg/ml. After incubation, Alexa Fluor 568-concanavalin A was purified using a PD10 column as described above for Alexa Fluor 488-ADAMTS4-2. However, 10 mM manganese was added to the TNC-Brij buffer because concanavalin A requires a transition metal ion, such as manganese, plus calcium for binding (Sumner and Howell, 1936).

2.8.2. Confocal microscopy

To visualize ADAMTS4-2 within the cell, HTB94 cells were seeded on glass coverslips (1x10⁵ cells in 12-well plate), rested overnight and washed thrice in phenol red-free, serum-free DMEM. Cells were then incubated at 37 °C in phenol red-free DMEM containing 0.1 % BSA with or without 250 µg/ml heparin. After 1 hour, Alexa Fluor 488-ADAMTS4-2 (15 nM in final volume of 500 µl), with a fluorescent reading of ~ 1300 fluorescent units (FU) was added. As a negative control to demonstrate that unbound fluorophore in the Alexa Fluor 488-ADAMTS4-2 preparation could not be detected, ~ 1300 FU of free Alexa Fluor 488 diluted in TNC-Brij was added to another well.

Cells were incubated overnight with Alexa Fluor 488-ADAMTS4-2. The medium was then removed and the cells washed thrice with ice-cold PBS. The cells were fixed with 4 % PFA at room temperature for 20 min. After fixation, the cells were washed with PBS and incubated with 35 µg/ml Alexa Fluor 568-concanavalin A in

PBS for at room temperature for 10 min. Following this, the cells were washed with PBS before mounting with ProLong Gold reagent.

Each sample was viewed using a Nikon Eclipse TE2000-U confocal laser scanning microscope. To ensure that the intensity of each image could be compared, each sample was scanned on the same day with lasers of the same intensity and identical exposure times. The data were collated using Volocity software (Improvision).

2.9. Heparin-binding affinity of ADAMTS4-2 and ADAMTS4-1

The heparin-binding affinity of ADAMTS4-2 and ADAMTS4-1 was analyzed by heparin-Sepharose affinity chromatography. ADAMTS4-2 or ADAMTS4-1 (250 ng) diluted in TNC-Brij (500 μ l) was passed over a column of heparin-Sepharose under gravity flow. The flow-through was collected and bound protein was eluted with a step-wise gradient of 0.15-1 M NaCl in 50 mM Tris-HCl (pH 7.5), 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35. Elutions (500 μ l) were TCA-precipitated (3.3 %) overnight at 4 °C. The TCA-soluble fraction was separated from the TCA-soluble fraction by centrifugation at 14170 g for 15 min at 4 °C. The TCA-insoluble fraction was resuspended in 50 μ l of 2 x SDS-reducing SDS-PAGE buffer and 15 μ l of each sample were analysed by SDS-PAGE (7.5 % acrylamide) and Western blotting using the anti-ADAMTS-4-catalytic domain antibody.

2.10. α_2 M preparation

2.10.1. Azocoll assay

Azocoll was suspended in 50 mM Tris-HCl (pH 7.8), 1 mM CaCl_2 , to a concentration of 5 mg/ml. Trypsin was diluted to a concentration of 10 - 100 μ g/ml with TNC-Brij and incubated 1:1 vol with TNC-Brij, α_2 M or α_2 M* for 15 min at room temperature prior to addition of 490 μ l Azocoll suspension. The samples were rotated at room temperature. At the time intervals indicated, the reaction

was stopped by centrifugation at 14170 g for 5 min at 4 °C. The supernatant was removed and 150 µl aliquots transferred to a 96-well plate. The absorbance was read in triplicate at 550 nm using a FLUOstar Omega (BMG Labtech) plate reader.

2.10.2. Methylamine-HCl treatment of α_2 M

α_2 M (6 µM) was incubated with 264 mM methylamine-HCl in 100 mM Tris-HCl (pH 8) at room temperature for 1 h. A separate incubation of α_2 M in 100 mM Tris-HCl (pH 8) served as a negative control. The samples were exchanged into TNC-20 % glycerol buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 20 % glycerol] using a G-50 Sepharose (50 µl Micro Column) and activity against trypsin assayed using the Azocoll assay described above. Two µl aliquots of α_2 M or α_2 M* were incubated with 8 µl of 10 µg/ml trypsin for 15 min prior to the addition of 490 µl Azocoll substrate.

2.10.3. Heat induced fragmentation of α_2 M.

α_2 M or α_2 M* (2.2 mg/ml) was incubated with 35 µl of 0.05 M Tris-HCl (pH 9.5), 2 % SDS and 28 mM DTT and heated at 100 °C for 30 min. Twenty µl of 75 % glycerol/ H_2O /bromophenol blue was added and 40 µl was analysed by SDS-PAGE (6 % acrylamide) and stained with Coomassie Brilliant Blue R-250.

2.11. Iodination of α_2 M*

Iodination of α_2 M* was performed using iodination beads, according to the manufacturer's instructions. Two iodination beads were washed with 1000 µl PBS to remove any loose particles and then dried on filter paper. The beads were then added to the carrier-free Na^{125}I in PBS (1 mCi/37 MBq in 100 µl). The beads and carrier-free Na^{125}I were incubated at room temperature for 5 min before addition of α_2 M* (100 µg). The reaction was incubated for 15 min. The reaction was stopped by transferring the solution to a fresh Eppendorf tube so

that the protein was no longer in contact with the iodination beads. Excess Na^{125}I or unincorporated ^{125}I was separated from iodinated $\alpha_2\text{M}^*$ by gel filtration in TNC-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35] using SephadexG-50 (5 ml column). Elutions (200 μl) were collected and 5 μl aliquots of each elution was analysed by reducing SDS-PAGE (6 % acrylamide) and silver staining.

2.12. Determination of $\alpha_2\text{M}^*$ concentration using $\alpha_2\text{M}$ standard curve

To determine the approximate concentration of ^{125}I - $\alpha_2\text{M}^*$, the amount of protein was estimated using a standard curve constructed relative to known amounts of $\alpha_2\text{M}$. Increasing amounts of $\alpha_2\text{M}$ (ng) and aliquots of ^{125}I - $\alpha_2\text{M}^*$ (5 μl and 10 μl) were analysed by SDS-PAGE and silver staining. The gel was scanned and the pixel volume of each band was determined using Phoretix software. The concentration of ^{125}I - $\alpha_2\text{M}^*$ was calculated from the standard curve using the equation $y = mx + c$.

2.13. Statistical analysis

Experiments were generally conducted in triplicate ($n = 3$). Statistical significance ($P = \leq 0.05$) was determined using GraphPad Prism software (GraphPad Software Inc, CA, USA). Data were analysed by one way ANOVA with a Dunnett's multiple comparison post test.

Chapter 3

Preliminary investigation of ADAMTS-4 clearance by HTB94 cells

3.1. Introduction

Our laboratory has recently reported an increase in endogenous TIMP-3 levels in cartilage explants and in the conditioned medium of porcine chondrocytes and HTB94 chondrosarcoma cells upon treatment with calcium pentosan polysulfate or heparin (Troeberg *et al.*, 2008). In addition, TIMP-3 accumulation was observed when cells were treated with cytochalasin D, an agent which blocks endocytosis by disrupting actin polymerization (Flanagan and Lin, 1980) and also when cells were treated with receptor-associated protein (RAP), a ligand antagonist to members of the low-density lipoprotein receptor (LDL-R) family (Hussain *et al.*, 1999). This work suggested that TIMP-3 may be endocytosed and raised the hypothesis that TIMP-3-sensitive enzymes such as ADAMTS-4 and ADAMTS-5 may also be endocytosed. This is considered because TIMP-1 and TIMP-2 can be endocytosed alone or in a complex with MMP-9 and MMP-2, respectively (Emonard *et al.*, 2004; Hahn-Dantona *et al.*, 2001).

The hypothesis that ADAMTS-4 could be endocytosed by HTB94 cells was investigated by: i) monitoring the disappearance of exogenous ADAMTS4-2 from the conditioned medium by Western blot analysis and ii) detecting the appearance of Alexa Fluor488-labeled ADAMTS4-2 within the cell using confocal microscopy.

3.2. Results

3.2.1. Monitoring ADAMTS-4 disappearance from the medium of HTB94 cells by Western blot analysis

3.2.1.1. Expression of ADAMTS4-2 by HEK-293/EBNA cells

To study ADAMTS-4 disappearance, a truncated form of full-length ADAMTS-4 lacking the C-terminal spacer domain (ADAMTS4-2) was used. Full-length ADAMTS-4 (ADAMTS4-1) was not used in this study because the spacer domain mediates binding to the ECM and cell surface (Kashiwagi *et al.*, 2004) and the

purification of ADAMTS4-1 requires the addition of heparin to the medium in order to release the enzyme from the matrix. Additionally, about 80 % of the protein is also C-terminally processed in culture and does not bind to the anti-FLAG M2-column (Kashiwagi *et al.*, 2004). This makes the purification of sufficient quantities of ADAMTS4-1 for experimental purposes problematic. Although ADAMTS4-1 is 18-fold more active against the Glu³⁷³-Ala³⁷⁴ bond of aggrecan than ADAMTS4-2 and is 20-fold more active against the CS-2 region of aggrecan, ADAMTS4-2 has a higher activity towards general substrates such as fibromodulin and carboxymethylated transferrin than ADAMTS4-1 (Fushimi *et al.*, 2008).

HEK-293/EBNA cells transfected with FLAG-tagged ADAMTS4-2 were grown to confluence and incubated in serum-free DMEM. After 4 days, the conditioned medium (200 ml) was collected, aliquotted and 1 ml was TCA-precipitated (3.3 %). The TCA-insoluble pellet was re-suspended in 2 x reducing sample buffer and the expression of ADAMTS-4-2 was analysed by Western blotting with either the anti-ADAMTS-4 catalytic domain antibody or anti-FLAG M2-antibody. As shown in Figure 6, both antibodies were specific, detecting predominantly the active form of the enzyme (51 kDa) in the conditioned medium. The anti-ADAMTS-4 catalytic domain antibody was used in all further experiments to detect ADAMTS-4 in the conditioned medium by Western blotting. This ensured that the protein could still be detected even if the FLAG-tag underwent proteolysis during culture.

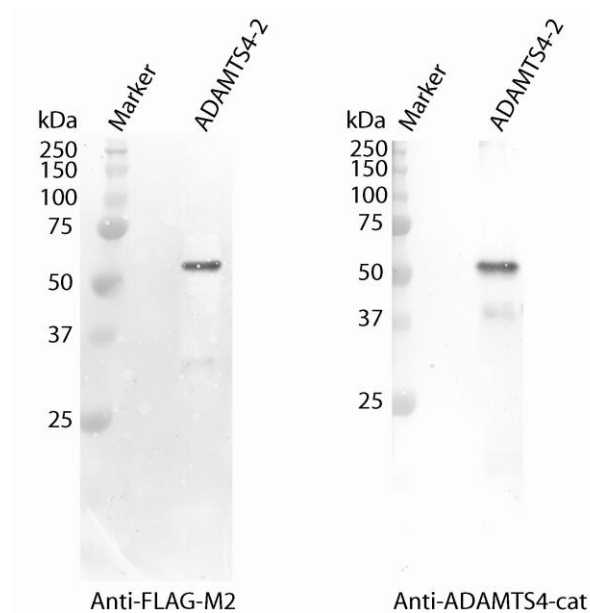


Figure 6: Western blot of conditioned medium containing ADAMTS4-2.

Conditioned medium (200 ml) from HEK-293/EBNA cells transfected with ADAMTS4-2 construct was harvested after 4 days of culture in serum-free DMEM. Conditioned medium (1 ml) was precipitated with TCA (3.3 %) and analysed by Western blotting using either the anti-FLAG M2-antibody or anti-ADAMTS-4 catalytic domain antibody.

3.2.1.2. Activity of ADAMTS4-2 as determined by GST-IGD-FLAG substrate

The concentration of active ADAMTS4-2 in the conditioned medium was determined by titration with N-TIMP-3, using a recombinant GST-IGD-FLAG substrate, designed in-house by Dr Masa Kashiwagi and characterized by Dr Ngee Han Lim. The substrate is composed of the interglobular domain (IGD) of aggrecan with a glutathione S-transferase (GST)-tag at the N-terminus and a FLAG-tag at the C-terminus. The substrate contains both the MMP and aggrecanase cleavage sites. N-TIMP-3 inhibits ADAMTSs in a 1: 1 molar ratio (Nagase and Brew, 2003) and the cleavage of GST-IGD-FLAG is completely inhibited when the number of ADAMTS molecules is equal to the number of TIMP-3 molecules (where the line of inhibition intercepts the x-axis). To determine the concentration of ADAMTS4-2 in the conditioned medium, an aliquot of conditioned medium was diluted 5-fold with DMEM. The diluted conditioned medium was then incubated with various concentrations of N-TIMP-3

for 60 min. The ADAMTS-4-2/N-TIMP-3 complex was incubated with GST-IGD-FLAG substrate for 90 min at 37 °C and the reaction was stopped by addition of 2 x reducing sample buffer containing 10 mM EDTA. Samples were analysed by SDS-PAGE and Coomassie staining (Figure 7A). The gel was scanned and the pixel volume of the cleaved 21 kDa product was determined using Phoretix software. The concentration of N-TIMP-3 was plotted against the pixel volume of the cleaved product (Figure 7B). As the medium was diluted 5-fold with DMEM before titration, the concentration of ADAMTS-4-2 in the medium was 200 nM (10.3 µg/ml). The conditioned medium was frozen in aliquots at -20 °C and used as a source of ADAMTS4-2 for all experiments within this chapter unless otherwise stated.

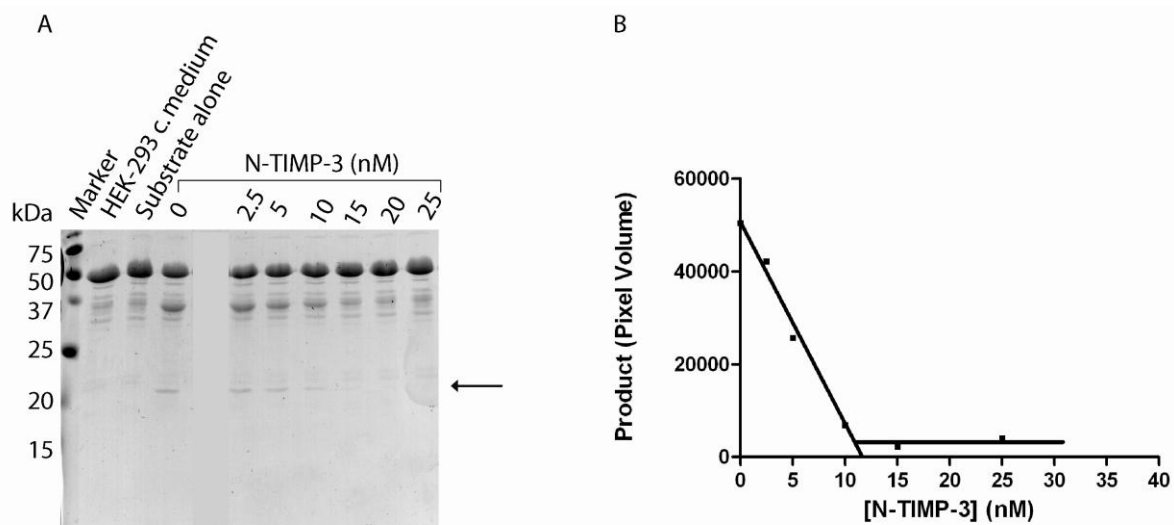


Figure 7: *N-TIMP-3 titration of conditioned medium containing ADAMTS4-2.*

The concentration of active ADAMTS4-2 in the conditioned medium was determined by titration with known concentrations of N-TIMP-3 using GST-IGD-FLAG substrate. The medium containing ADAMTS4-2 was diluted 5-fold with DMEM before titration. As a negative control, conditioned medium collected from non-transfected HEK-293/EBNA cells (HEK-293/EBNA c. medium) was incubated with substrate. **A**, The samples were analysed by reducing SDS-PAGE and stained with Coomassie Brilliant Blue R250. **B**, Pixel volume of the 21 kDa product band (arrow) was plotted against N-TIMP-3 concentration. [N-TIMP-3] on the graph represents the concentration of N-TIMP-3 in the final reaction volume (1:4 dilution). To determine the concentration of ADAMTS4-2 in the conditioned medium, the value where the line intercepts the x-axis was multiplied by 4, then by 5 (dilution factor of conditioned medium).

3.2.1.3. Time course of ADAMTS4-2 disappearance from the medium of HTB94 cells

To investigate whether ADAMTS-4 could be endocytosed, the amount of recombinant ADAMTS4-2 remaining in the medium of HTB94 cells over 24 h was examined. HTB94 cells were plated overnight (1×10^6 cells/well) in DMEM containing 10 % FCS. The next day, the cells were washed with serum-free DMEM and incubated with conditioned medium containing ADAMTS4-2 (1.5 nM). Cells were cultured for up to 24 h and the conditioned medium was harvested at the time points indicated. The cells were washed with PBS and subsequently lysed with 2 x reducing sample buffer (60 μ l). The conditioned medium and wash fractions were precipitated with TCA (3.3 %). The TCA-soluble fraction was separated from the TCA-insoluble fraction by centrifugation at 14170 g for 15 min and the TCA-insoluble fraction was re-suspended in 60 μ l of 2 x reducing sample buffer. Equal volumes of all samples were analysed by Western blotting using the anti-ADAMTS-4 catalytic domain antibody.

Figure 8A shows that the amount of intact ADAMTS4-2 in the medium of HTB94 cells reduced over a 24 h time period. To determine the half-life of ADAMTS4-2 disappearance from the medium of HTB94 cells, the pixel volume of each band in Figure 8A was quantified using Phoretix software and the amount of ADAMTS4-2 remaining in the medium at each time point was calculated as % of the amount of ADAMTS4-2 at 0 h (Figure 8B). In this experiment, the half-life ($t_{1/2}$) of ADAMTS4-2 disappearance was 2 h. However, the enzyme could not be detected in either the wash fractions (Figure 8C) or within the cells (Figure 8D).

Time course experiments to determine the amount of ADAMTS4-2 remaining in the medium after incubation with HTB94 cells were repeated twice more. Graphs of pixel volume of ADAMTS4-2 in the medium against time are shown in Figure 9. The $t_{1/2}$ of ADAMTS4-2 disappearance from the medium was 6 h in Figure 9A and 8 h in Figure 9B.

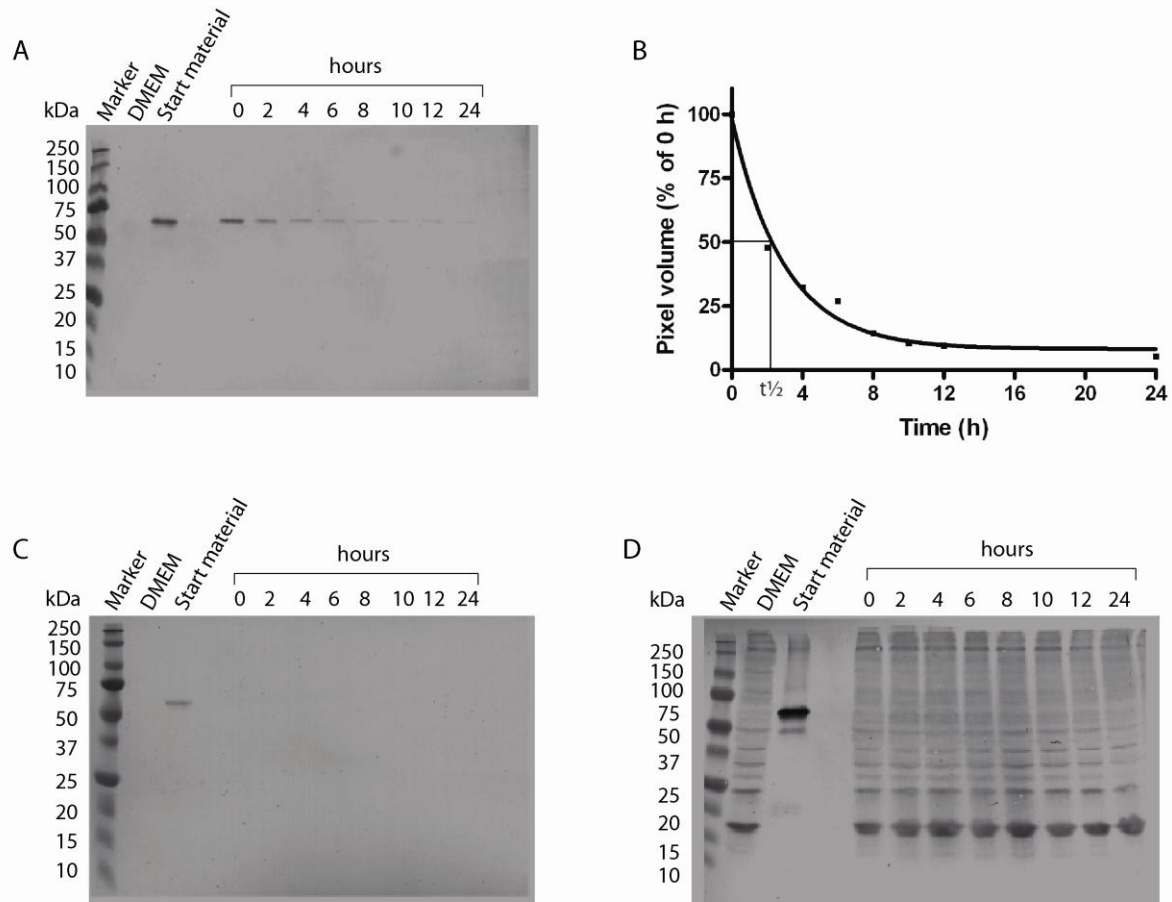


Figure 8: ADAMTS4-2 disappearance from the medium of HTB94 cells (i).

Conditioned medium containing ADAMTS4-2 (1.5 nM) was added to HTB94 cells and the conditioned medium was harvested at the time points indicated. The cells were washed with PBS and subsequently lysed with 2 x reducing sample buffer. The conditioned medium and wash fractions were precipitated with TCA (3.3 %). All fractions were analysed by Western blotting using the anti-ADAMTS-4 catalytic domain antibody. **A**, Western blot of TCA-precipitated conditioned medium. **B**, The pixel volume of each band was quantified using Phoretix software and the amount of ADAMTS4-2 remaining in the medium at each time point was calculated as % of the amount of ADAMTS4-2 at 0 h. **C**, Western blot of TCA-precipitated wash fraction. **D**, Western blot of lysed cell fraction.

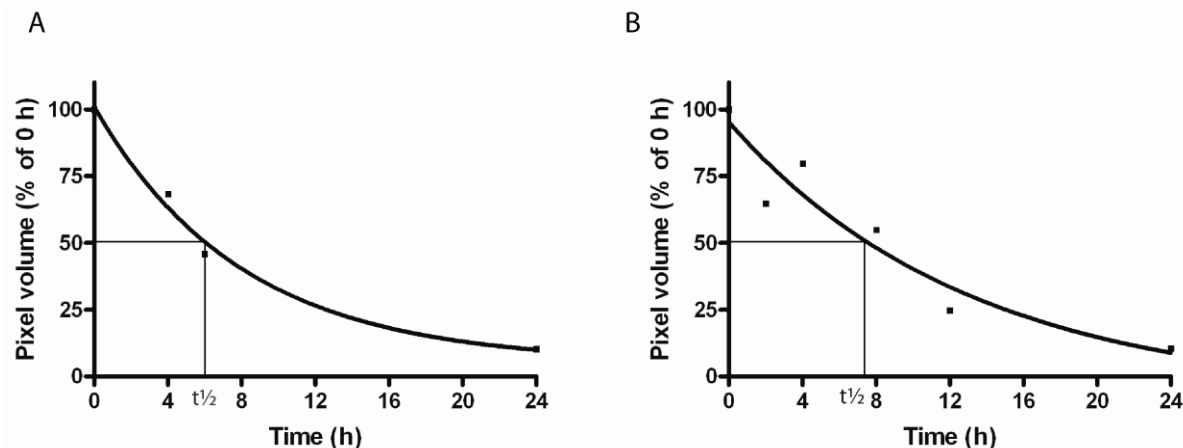


Figure 9: ADAMTS4-2 disappearance from the medium of HTB94 cells (ii).

Conditioned medium containing ADAMTS4-2 (1.5 nM) was added to HTB94 cells and the conditioned medium was harvested at the time points indicated. The conditioned medium was precipitated with TCA (3.3 %). All fractions were analysed by Western blotting using the anti-ADAMTS-4 catalytic domain antibody. The pixel volume of each band was quantified using Phoretix software and the amount of ADAMTS4-2 remaining in the medium at each time point was calculated as % of the amount of ADAMTS4-2 at 0 h. **A**, Repeat 1. **B**, Repeat 2.

3.2.1.4. ADAMTS4-2 is not autodegraded or degraded by soluble proteinases during culture

ADAMTS4-2 was observed to disappear over time from the medium of HTB94 cells. The disappearance of ADAMTS4-2 could be due to autodegradation during incubation or degradation by proteases produced by HTB94 cells during culture. To investigate these possibilities, conditioned medium containing ADAMTS4-2 was diluted in conditioned medium collected from HTB94 cells over 24 h at 37 °C. Alternatively, conditioned medium containing ADAMTS4-2 was diluted in serum-free DMEM. Each sample was incubated for a further 24 h at 37 °C. The mixture was then TCA precipitated (3.3 %) and the TCA-insoluble pellet was analysed by Western blotting with anti-ADAMTS4-catalytic domain antibody.

Upon incubation at 37 °C for 24 h, ADAMTS4-2 diluted in serum-free DMEM showed a minor degradation fragment at approximately 45 kDa whilst ADAMTS4-2 diluted in conditioned medium collected from HTB94 cells remained intact and does not appear to be significantly reduced (Figure 10). This

suggested that factors in the conditioned medium collected from HTB94 cells may even protect against ADAMTS4-2 degradation and shows that the disappearance of ADAMTS4-2 from the medium of HTB94 cells (Figure 8 and Figure 9) was not a result of autodegradation or proteolysis by soluble proteinases during culture. This result was investigated further in Chapter 4.

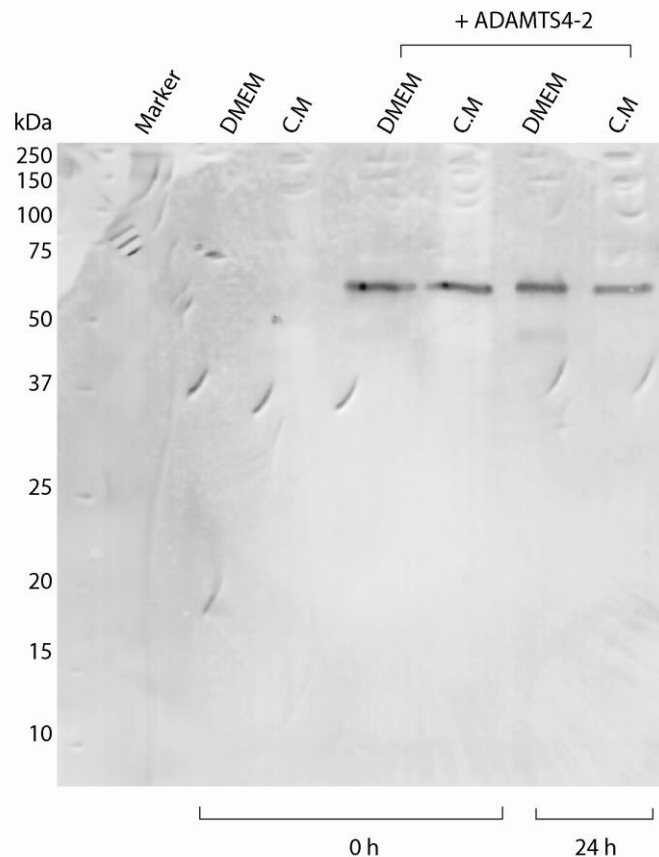


Figure 10: *Conditioned medium containing ADAMTS4-2 was not autodegraded or degraded by soluble proteinases in HTB94 conditioned medium.*

Conditioned medium containing ADAMTS4-2 was diluted to 1.5 nM with serum-free DMEM or with conditioned medium collected from HTB94 cells (C.M). The medium was precipitated with TCA (3.3. %) at 0 h or incubated at 37 °C for 24 h before TCA-precipitation. The TCA-insoluble pellet was analysed by Western blotting using the anti-ADAMTS4-catalytic domain antibody.

3.2.1.5. The effect of protease inhibitors on ADAMTS4-2 disappearance from the medium of HTB94 cells

To investigate whether ADAMTS4-2 underwent proteolytic degradation on the cell surface, HTB94 cells were incubated with a variety of protease inhibitors or

DMSO carrier solution for 1 h prior to the addition of conditioned medium containing ADAMTS4-2. Figure 11 shows that the disappearance of ADAMTS4-2 from the medium of HTB94 cells was not affected by the cysteine protease inhibitor E-64 (10 μ M) or the serine protease inhibitor AEBSF (100 μ M) but was partially blocked by the metalloproteinase inhibitor GM6001 (10 μ M). This indicates that some metalloproteinase-mediated breakdown of ADAMTS4-2 may occur on the cell surface. These results are investigated in further detail in Chapter 4.

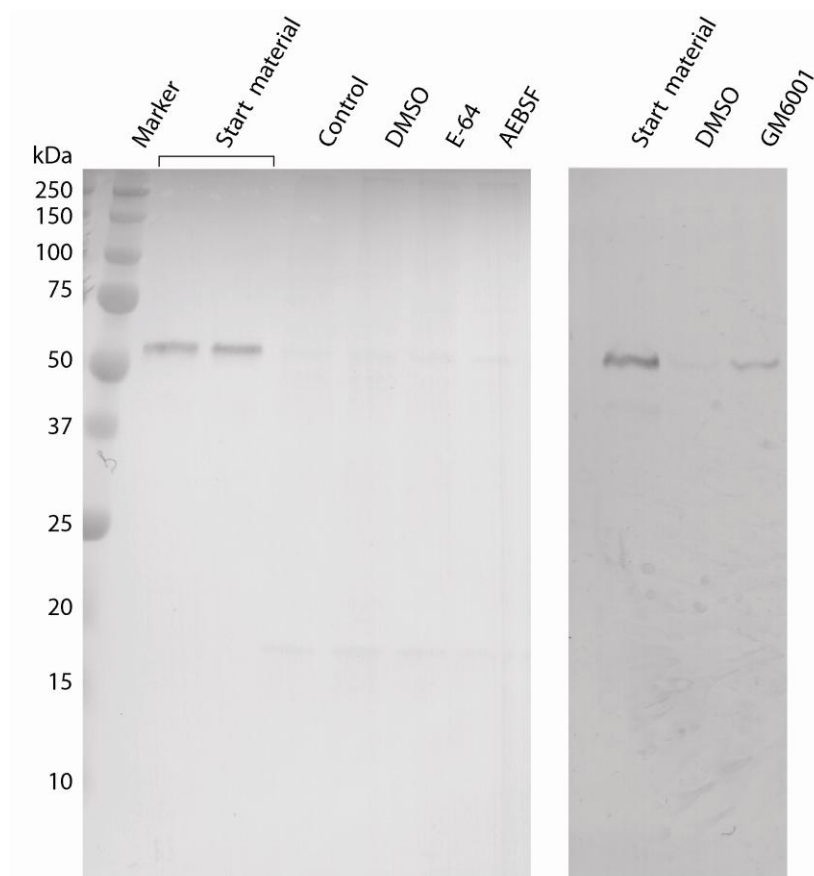


Figure 11: *The effect of protease inhibitors on ADAMTS4-2 disappearance from the medium of HTB94 cells.*

HTB94 cells were incubated at 37 °C in DMEM containing 0.1 % BSA with or without protease inhibitors: E-64 (10 μ M), AEBSF (100 μ M), GM6001 (10 μ M) or DMSO carrier (1/1000). After 1 h, conditioned medium containing ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was TCA-precipitated and analysed by Western blotting using the anti-ADAMTS-4 catalytic domain antibody.

3.2.1.6. Linearity of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

To determine whether the protection against ADAMTS4-2 disappearance in the presence of GM6001 was due to compromised cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to examine cell viability in the presence of this agent. The MTT assay, first described by Mosman (1983) is based on the cleavage of the water-soluble tetrazolium salt MTT into insoluble formazan crystals by metabolically active cells. The formazan is then solubilised and the concentration of formazan is measured by optical density at 574 nm.

To first demonstrate the dependence of the MTT assay on cell number, HTB94 cells were seeded at various densities ($0-3 \times 10^6$ cells) in a 6-well plate and grown overnight. Cells were then incubated with DMEM containing 0.1 % BSA with or without 2 mM sodium nitroprusside (SNP) for 24 h at 37 °C. The medium was replaced with phenol red-free DMEM and MTT reagent was added for 10 min before the addition of 10 % SDS in 10 mM HCl to stop the reaction. As shown in Figure 12A, the absorbance reading reached saturation at a density of 1×10^6 cells. Sodium nitroprusside (SNP) was used at a positive control for cell death (Del Carlo and Loeser, 2002) and incubation with SNP (2 mM) reduced cell viability at all cell densities. The assay was subsequently refined so that less reagents were required by plating 0.5×10^6 cells in a 12-well plate and reducing the volume of MTT reagent added to each well. The linearity of the MTT assay was determined for HTB94 cells by plotting the time incubated with MTT reagent against the absorbance at 574 nm. Figure 12B shows that under the conditions used, the assay was linear up to 0.6 absorbance units. As a result, MTT reagent was added for 45 min before solubilisation of the formazan product to ensure that the absorbance obtained fell within the linear range.

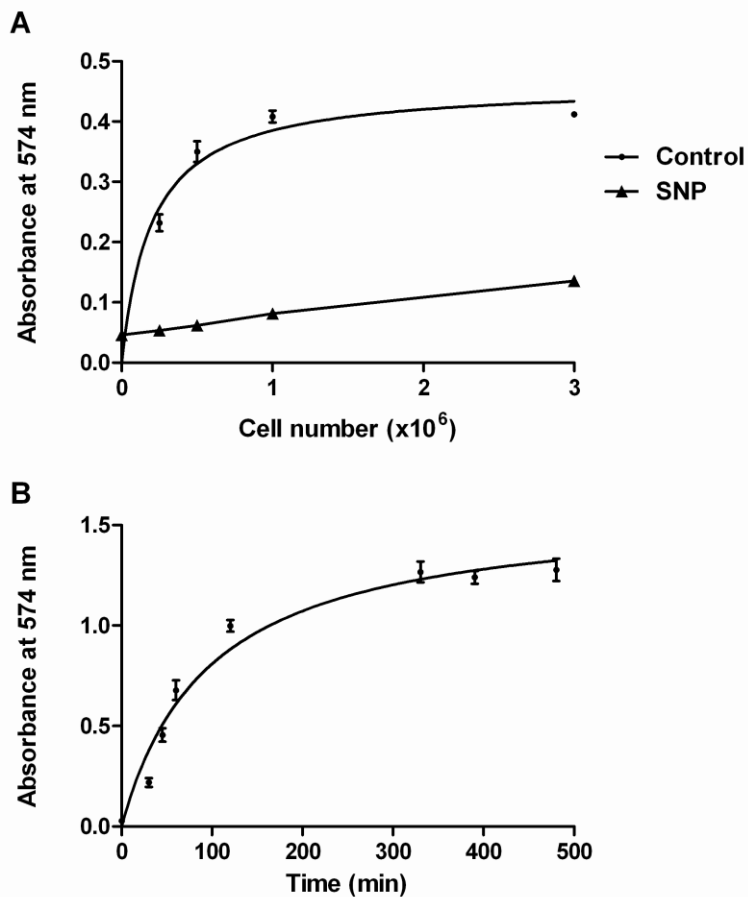


Figure 12: Linearity of MTT assay.

A, HTB94 cells were seeded in a 6-well plate at various densities (0 - 3×10^6 cells), grown overnight and treated with SNP for 24 h. Cells were washed once with phenol red-free DMEM before incubation with 900 μ l phenol red-free DMEM and 100 μ l of MTT reagent (5 mg/ml in PBS). After 10 min, 1 ml of 10 % SDS in 10 mM HCl was added to solubilise the formazan crystals. The samples were incubated overnight at 37 °C and 200 μ l of each well was transferred to a 96-well plate. The absorbance was read at 574 nm. Results are an average of triplicate wells $\pm$ S.D. **B**, HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and washed once with phenol red-free DMEM before incubation with 975 μ l phenol red-free DMEM and 25 μ l of MTT reagent (5 mg/ml in PBS). At the time points indicated, 1 ml of 10 % SDS in 10 mM HCl was added to solubilise the formazan crystals. The samples were incubated overnight at 37 °C and 200 μ l of each well was transferred to a 96-well plate. The absorbance was read at 574 nm. Results are an average of triplicate wells $\pm$ S.D.

3.2.1.7. Viability of HTB94 cells in the presence of GM6001

HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and then incubated with DMSO carrier (1/100), GM6001 (100 μ M) or SNP (2 mM) for 24 h. The cells were then assessed for cell viability using MTT.

There was no apparent difference in cell viability when cells treated with DMSO were compared to cells treated with GM6001 (Figure 13). SNP caused a 90 % reduction in cell viability.

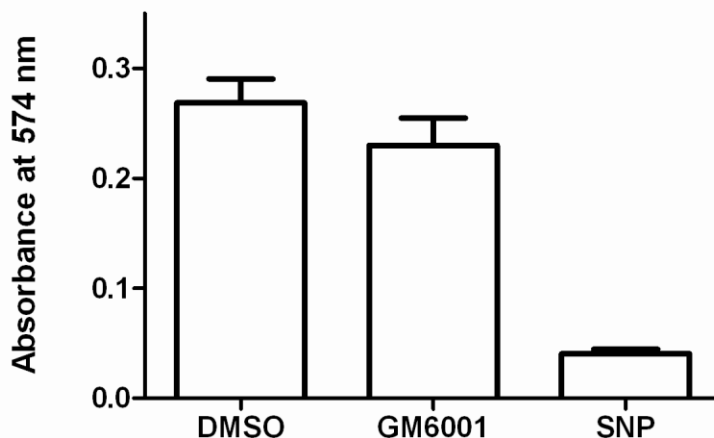


Figure 13: MTT assay of HTB94 cell viability upon incubation with GM6001.

HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and then incubated with DMSO carrier (1/100), GM6001 (100 μ M) or SNP (2 mM) for 24 h. The medium was removed and the cells were washed once with phenol red-free DMEM before incubation with 975 μ l phenol red-free DMEM and 25 μ l of MTT reagent (5 mg/ml in PBS). After 45 min, 1 ml of 10 % SDS in 10 mM HCl was added to solubilise the formazan crystals. The samples were incubated overnight at 37 °C and 200 μ l of each well was transferred to a 96-well plate. The absorbance was read at 574 nm. Results are an average of triplicate wells $\pm$ S.D.

3.2.1.8. Heparin protects against ADAMTS4-2 disappearance from the medium of HTB94 cells

TIMP-3 accumulates in the medium of HTB94 cells upon treatment with heparin (Troeberg *et al.*, 2008). To test the effect of heparin on ADAMTS4-2 disappearance, cells were incubated with or without heparin (250 μ g/ml) for 1 h before conditioned medium containing ADAMTS4-2 was added. At the time points indicated, the medium was harvested and TCA-precipitated as previously described. Equal volumes of each TCA-insoluble fraction were analysed by Western blotting with the anti-ADAMTS4-catalytic domain antibody. Figure 14

shows that the amount of ADAMTS4-2 was reduced in the medium after 24 h culture compared to 0 h. When cells were pre-treated with heparin (250 µg/ml) for 1 h prior to the addition of ADAMTS4-2, the amount of intact ADAMTS4-2 in the medium after 24 h was increased compared to control cells. This shows that heparin protected against ADAMTS4-2 disappearance from the medium. ADAMTS4-2 did not disappear from the medium of cells which had been fixed with 4 % PFA (30 min, RT) prior to incubation. In addition, the TCA-insoluble fraction recovered from fixed cells with or without heparin was comparable showing that treatment with heparin did not affect the recovery of the TCA-insoluble fraction of ADAMTS4-2 from the medium.

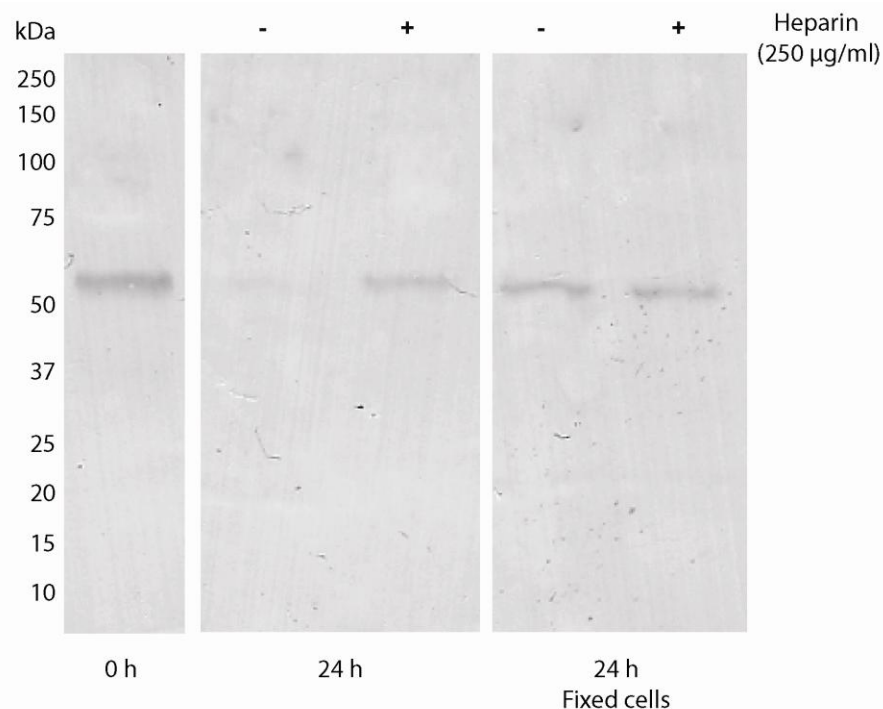


Figure 14: Heparin protects against ADAMTS4-2 disappearance from the medium of HTB94 cells.

Live cells or cells fixed with 4 % PFA were incubated with or without heparin (250 µg/ml) for 1 h prior to addition of conditioned medium containing ADAMTS4-2. After 24 h, the conditioned medium was harvested and precipitated with TCA. Samples were analysed by Western blotting using anti-ADAMTS-4 catalytic domain antibody. The 0 h and 24 h samples were loaded on the same blot as the 24 h fixed cell samples. However, the blot was cropped and is shown as 3 separate images.

3.2.1.9. Viability of HTB94 cells in the presence of heparin

HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and then incubated with or without heparin (500 $\mu\text{g/ml}$) for 24 h. The cells were then assessed for cell viability using MTT. The MTT assay showed that there was no significant difference in cell viability when cells were treated with heparin compared to control (Figure 15). SNP reduced cell viability by more than 90 %.

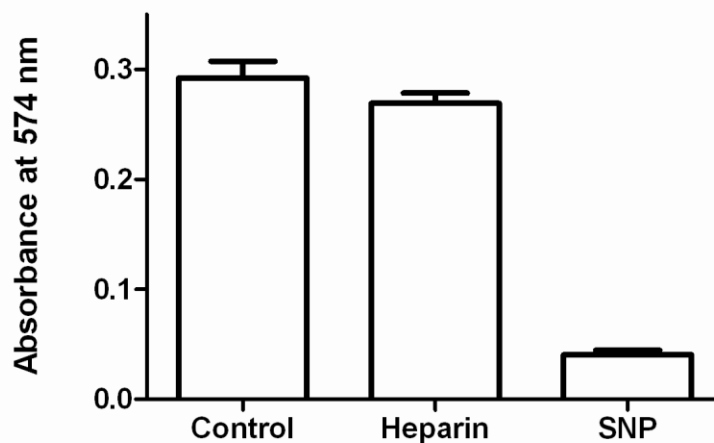


Figure 15: MTT assay of HTB94 cell viability upon incubation with heparin.

HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and then incubated with or without heparin (500 $\mu\text{g/ml}$) or SNP (2 mM) for 24 h. The medium was removed and the cells were washed once with phenol red-free DMEM before incubation with 975 μl phenol red-free DMEM and 25 μl of MTT reagent (5 mg/ml in PBS). After 45 min, 1 ml of 10 % SDS in 10 mM HCl was added to solubilise the formazan crystals. The samples were incubated overnight at 37 °C and 200 μl of each well was transferred to a 96-well plate. The absorbance was read at 574 nm. Results are an average of triplicate wells $\pm$ S.D.

3.2.1.10. Heparin dose-dependently inhibits ADAMTS4-2 disappearance from the medium of HTB94 cells

Since cell viability was not adversely affected by heparin treatment, the protective effect of heparin on ADAMTS4-2 disappearance from the medium of HTB94 cells was investigated in more detail in a heparin dose-response experiment. HTB94

cells were treated for 1 h with 0 – 400 µg/ml heparin prior to the addition of conditioned medium containing ADAMTS4-2 (1.5 nM). The medium was removed and precipitated with TCA as previously described and analysed by Western blotting using the anti-ADAMTS-4 catalytic domain antibody (Figure 16). The Western blot shows that heparin dose-dependently increased the amount of ADAMTS4-2 remaining in the medium during the 24 h incubation with HTB94 cells. The pixel volume of each lane was measured using Phoretix software and the disappearance of ADAMTS4-2 from the medium was expressed as % of ADAMTS4-2 in the medium at 0 h (start material) (Figure 16B). Fifty percent of ADAMTS4-2 remained in the medium when cells were treated with 50 µg/ml heparin. A proportion of the medium was also immunoblotted using the TIMP-3 antibody (Figure 16C). There are several non-specific bands at high molecular weight (>75 kDa) on the Western blot. However, two bands are visible at approximately 25 and 20 kDa, corresponding to the glycosylated and unglycosylated forms of TIMP-3, respectively (Apte *et al.*, 1995). The pixel intensity of these TIMP-3 bands were measured using Phoretix software, added together and plotted as TIMP-3 accumulation against heparin concentration (Figure 16D). These data are in agreement with published data from our laboratory showing TIMP-3 accumulation in the medium of HTB94 cells upon treatment with heparin (Troeberg *et al.*, 2008)

3.2.1.11. The effect of other GAGs on ADAMTS4-2 disappearance from the medium of HTB94 cells

To determine which GAGs other than heparin could protect against ADAMTS4-2 depletion, cells were treated with other GAGs, including chondroitin-4-sulfate, de-N-sulfated heparin and dermatan sulfate (all at 100 µg/ml) for 1 h prior to the addition of conditioned medium containing ADAMTS4-2 (1.5 nM). After 24 h, the medium was precipitated with TCA and analysed by Western blotting with the anti-ADAMTS-4-catalytic domain antibody. In this experiment, the amount of

intact ADAMTS4-2 remaining in the medium was increased by heparin. However, the other GAGs tested were not as protective (Figure 17).

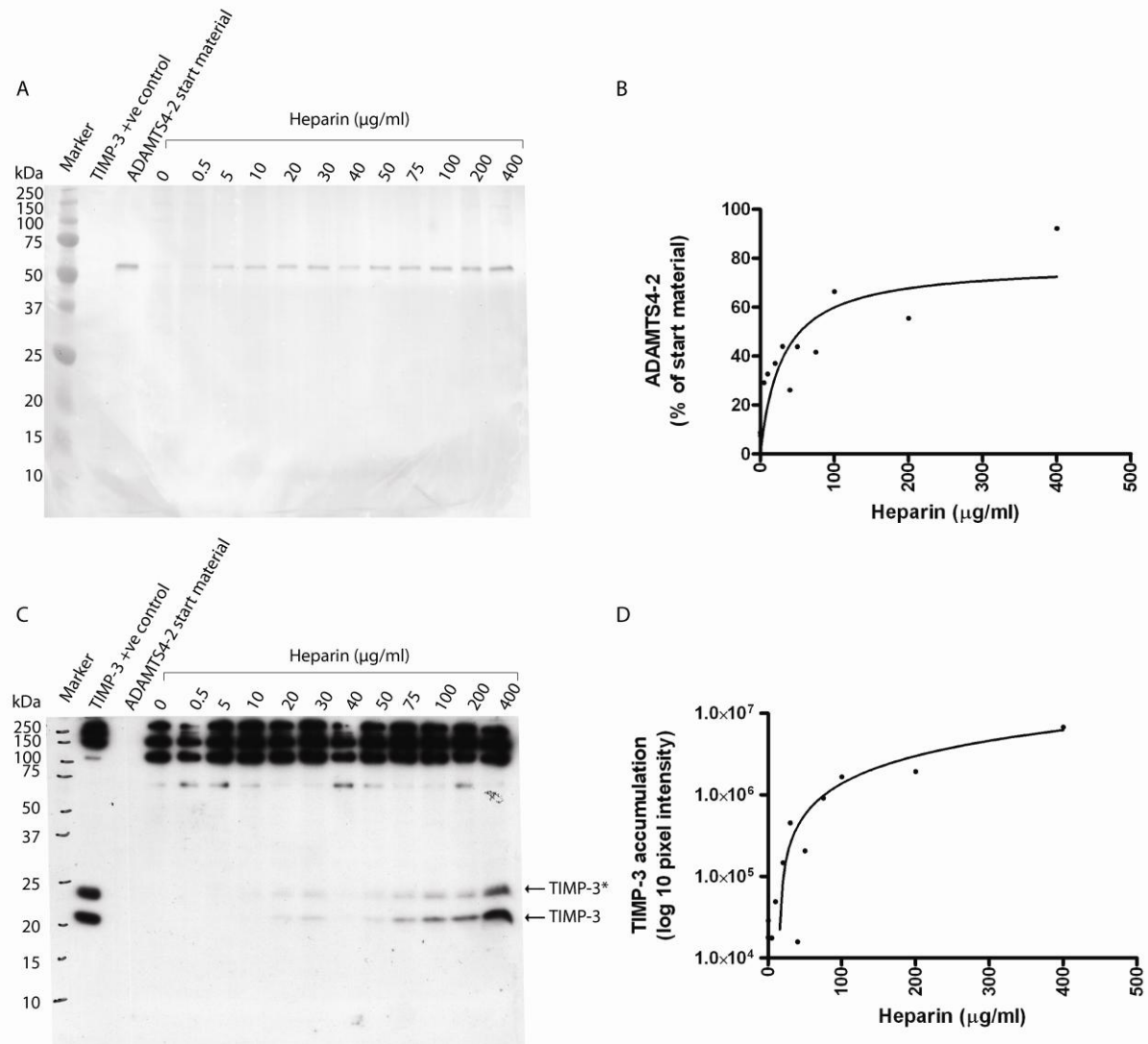


Figure 16: Heparin dose-dependently inhibits ADAMTS4-2 disappearance from the medium of HTB94 cells.

HTB94 cells were incubated with heparin (0-400 µg/ml) for 1 h prior to addition of conditioned medium containing ADAMTS4-2 (1.5 nM final). After 24 h, the conditioned medium was harvested and precipitated with TCA (3.3 %). **A**, Each sample was analysed by Western blotting using anti-ADAMTS-4 catalytic domain antibody. **B**, The pixel volume of each ADAMTS-4 band was measured using Phoretix software and the depletion of ADAMTS4-2 from the medium expressed as % of ADAMTS4-2 in the medium at 0 h (start material) against concentration of heparin. **C**, The sample was analysed by Western blotting using anti-TIMP-3 antibody. * denotes glycosylated TIMP-3. **D**, The pixel intensity of each TIMP-3 and TIMP-3* band was measured using Phoretix software and expressed as TIMP-3 accumulation against concentration of heparin.

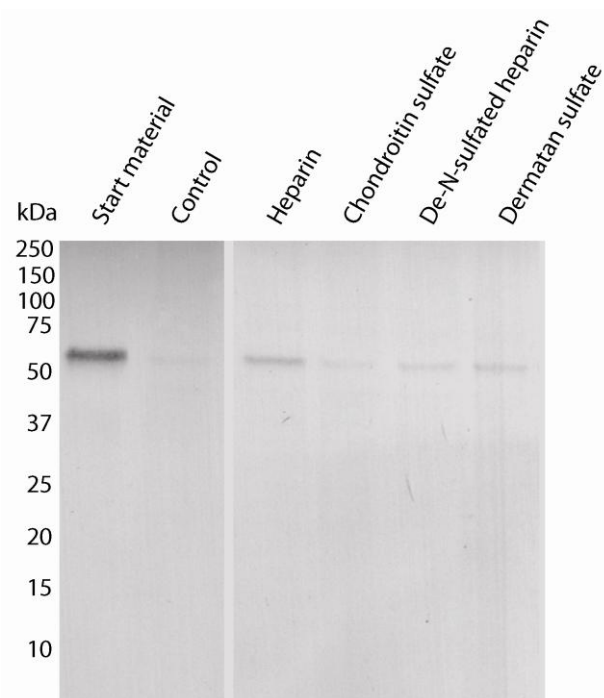


Figure 17: The effect of GAGs on ADAMTS4-2 disappearance from the medium of HTB94 cells.

HTB94 cells were incubated with or without heparin, chondroitin sulfate, de-N-sulfated heparin or dermatan sulfate (100 µg/ml) for 1 h prior to addition of conditioned medium containing ADAMTS4-2 (1.5 nM final). After 24 h, the conditioned medium was harvested and precipitated with TCA. Each sample was analysed by Western blotting using anti-ADAMTS-4 catalytic domain antibody. The start material and control sample was loaded on the same blot as the GAG-treated samples. However, the blot was cropped and is shown as 2 separate images.

3.2.2. Monitoring the cellular uptake of ADAMTS4-2 by confocal microscopy

To support the hypothesis that the disappearance of ADAMTS4-2 from the medium was as a result of endocytosis and intracellular degradation, the cell fraction was analysed to determine whether ADAMTS4-2 could be detected within the cell. However, Western blot analysis of the cell fraction did not detect ADAMTS4-2 within the cell (Figure 8D). An alternative approach to measure internalized ADAMTS4-2 was attempted using fluorescently labeled-ADAMTS4-2. This section describes the purification and *in vitro* labeling of ADAMTS4-2 with Alexa Fluor 488 fluorophore and the detection of intracellular Alexa Fluor 488-ADAMTS4-2 by confocal microscopy.

3.2.2.1. Purification of ADAMTS4-2 by anti-FLAG M2 chromatography and gel filtration

Conditioned medium from ADAMTS4-2-transfected HEK-293/EBNA cells was collected over a 7 day culture and centrifuged to remove cell debris. The conditioned medium was applied to a column of anti-FLAG M2-agarose (1.5 ml bed volume) in 2 batches of 500 ml. The column was washed with 1 M NaCl buffer [50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10 mM CaCl₂, 0.02 % sodium azide and 0.02 % Brij-35], followed by TBS and the bound material was eluted with 200 µg/ml FLAG peptide diluted in TNC-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 0.02 % sodium azide and 0.02 % Brij-35]. Figure 18A shows a silver stain of aliquots of each elution. Elutions 1-3 from each column purification contained ADAMTS4-2. These fractions were pooled (~ 18 ml) and diluted to 25 ml with TNC-Brij. The eluate was then concentrated to 2.5 ml using a Vivaspinn ultrafiltration spin column (MWCO 3000). The concentrated sample was further purified by gel filtration using Sephacryl S-200 equilibrated with TNC-Brij. One ml fractions were collected in 96-well plate format (plate 1 A1-H12, plate 2 A1-E12) and samples from wells spanning this range were analysed by SDS-PAGE and silver staining (Figure 18B). The protein was eluted in fractions H3-H12 (plate 1) and A1-A7 (plate 2). These fractions were pooled and stored in aliquots at -80 °C until further use. The concentration of active ADAMTS4-2 was determined by titration with N-TIMP-3 GST-IGD-FLAG substrate as described in section 3.2.1.2. The concentration of ADAMTS4-2 was 2.4 µM (123 µg/ml). The yield of ADAMTS4-2 was 410 µg/L of conditioned medium, comparable with a yield of 520 µg/L reported by Kashiwagi *et al* (2004).

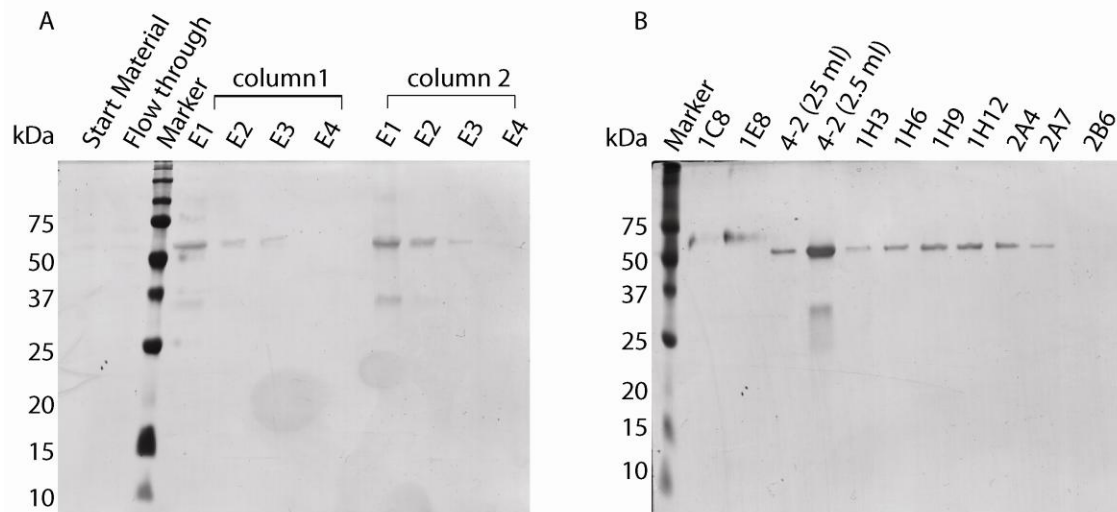


Figure 18: Purification of ADAMTS4-2.

Conditioned medium (1L) from HEK-293/EBNA cells transfected with ADAMTS4-2-FLAG was applied to a column of anti-FLAG M2-agarose (1 ml) in batches of 500 ml (column 1 and column 2). After washing with 1 M NaCl buffer [50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35] (30 ml) and TBS (30 ml), the material bound to the column was eluted (E1-E4) with 3 ml of 200 $\mu\text{g}/\text{ml}$ FLAG peptide in TNV-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35]. **A**, Aliquots (7.5 μl) were analysed by reducing SDS-PAGE and silver staining. Elutions 1-3 from each column (25 ml) were pooled and concentrated 10-fold using a Vivaspinn ultrafiltration spin column (MWCO 3000). The sample was then loaded on to a Sephacryl S-200 column equilibrated with TNC-Brij. One ml fractions were collected in 96-well plate format (plate 1 A1-H12, plate 2 A1-E12). **B**, Aliquots (7.5 μl) were analysed by reducing SDS-PAGE and silver staining.

3.2.2.2. Labeling ADAMTS4-2 with Alexa Fluor 488 fluorophore

After purification, 400 μl of a 2.4 μM solution of ADAMTS4-2 was exchanged into HEPES-Brij buffer [20 mM HEPES (pH 8.2), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35] by dialysis at 4 $^{\circ}\text{C}$ using a Slide-A-Lyzer dialysis cassette (MWCO 3500). Dialysis was necessary to exchange ADAMTS4-2 from TNC-Brij into a buffer free from ammonium ions or primary amines as these groups react with the fluorophore and reduce the efficiency of the labeling reaction. Alexa Fluor 488 was diluted in DMF (1 mg/ml). ADAMTS4-2 was incubated with Alexa Fluor 488 for 1 h at room temperature. The amount of Alexa Fluor 488 in the final reaction was 110 $\mu\text{g}/\text{ml}$. After incubation, glycine was

added to a final concentration of 50 mM to quench the reaction and the solution was passed over a PD10 column equilibrated with TNC-Brij to ensure the removal of unreacted Alexa Fluor 488 fluorophore. As shown in Figure 19, the extent by which ADAMTS4-2 was labeled with the fluorophore was determined by SDS-PAGE and silver staining coupled with fluorescence visualization using a Fujifilm FLA-200 phosphoimager (resolution 50, sensitivity F1000, Fluor 473, filter 7520). An aliquot of Alexa Fluor 488-labeled ADAMTS4-2 was diluted 3-fold with TNC-Brij and the concentration of active enzyme was determined by titration with N-TIMP-3 using the GST-IGD-FLAG substrate. The concentration of Alexa Fluor 488-labeled ADAMTS4-2 was 150 nM (7.7 $\mu\text{g}/\text{ml}$).

The fluorescence intensity of an aliquot of Alexa Fluor 488-ADAMTS4-2 was measured using a SpectraMax Gemini plate reader (ex. 495 nm, em. 519 nm). The enzyme was labeled with ~ 3200 fluorescent units (FU)/ μg ADAMTS4-2.

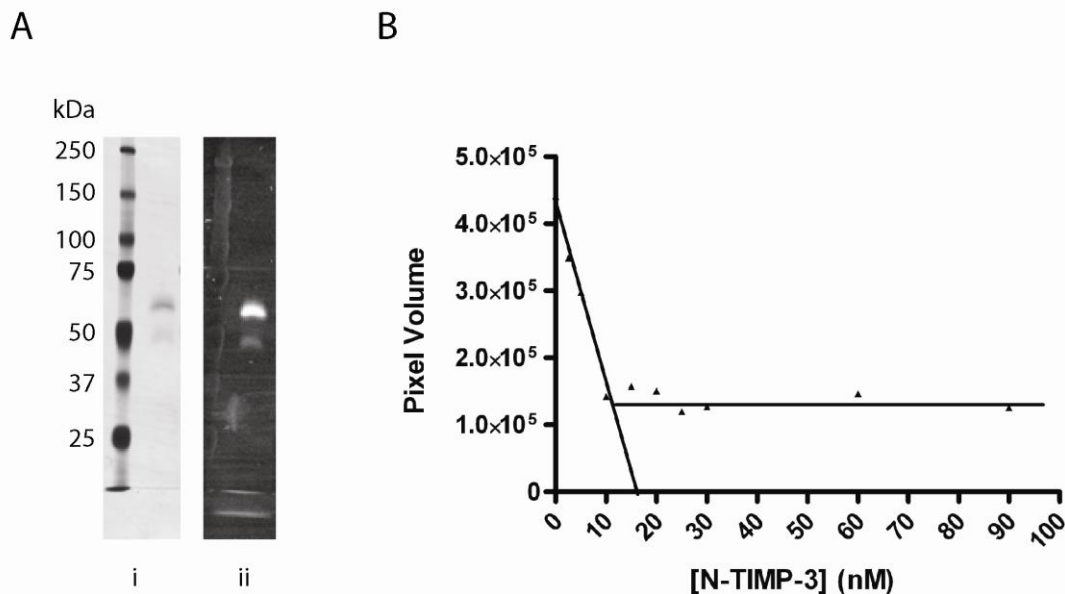


Figure 19: Labeling ADAMTS4-2 with Alexa Fluor 488.

A, Purified ADAMTS4-2 was labeled with Alexa Fluor 488 fluorophore and visualized by reducing SDS-PAGE combined with silver staining (i) and phosphoimaging (ii). B, The active concentration of Alexa Fluor 488-labeled ADAMTS4-2 was determined by titration with N-TIMP-3 using GST-IGD-FLAG substrate, as previously described. [N-TIMP-3] on the graph represents the concentration of N-TIMP-3 in the final reaction volume (1:4 dilution). To determine the concentration of Alexa Fluor488-ADAMTS4-2, the value where the line intercepts the x-axis was multiplied by 4, then by 3 (dilution factor of Alexa Fluor488-ADAMTS4-2).

3.2.2.3. Alexa Fluor 488-ADAMTS4-2 is internalized into HTB94 cells

To determine if ADAMTS4-2 was internalized into cells, HTB94 cells were seeded on glass coverslips (1×10^5 cells in a 12-well plate), grown overnight and washed thrice in phenol red-free, serum-free DMEM. Cells were then incubated at 37 °C in phenol red-free DMEM containing 0.1 % BSA either with or without 250 µg/ml heparin. After 1 h, Alexa Fluor 488-ADAMTS4-2 (15 nM), with a fluorescent reading of ~ 1300 FU was added. As a negative control to demonstrate that unbound fluorophore in the Alexa Fluor 488-ADAMTS4-2 preparation could not be detected, ~ 1300 FU of free Alexa Fluor 488 diluted in TNC-Brij was added to another well.

Cells were incubated overnight. The medium was removed and the cell layer was washed with PBS. The cell layer was then fixed with 4 % PFA (20 min, RT) and the cell surface was stained using Alexa Fluor 568-labeled concanavalin A.

Figure 20 shows 2 representative images taken from each treatment. Sections through the cell (0.2 µm) were scanned and collated in one image using Volocity software. No fluorescence was detected in the negative control where an equivalent amount of free Alexa Fluor 488 fluorescence was added (Figure 20 A, B). Fluorescent-labeled ADAMTS4-2 was detected within the cell (Figure 20 C, D), indicating that Alexa Fluor 488-ADAMTS4-2 could indeed be internalized by HTB94 cells. Heparin reduced the amount of Alexa Fluor 488-ADAMTS4-2 within the cell (Figure 20 E, F), in agreement with its effects on the amount of ADAMTS4-2 remaining in the conditioned medium previously shown in section 3.2.1.6.

Figure 21 shows a representative section through control and heparin-treated cells in higher magnification. In cells incubated with Alexa Fluor 488-ADAMTS4-2, clusters of fluorescence were detected underneath the plasma membrane. In agreement with Figure 20, cells treated with heparin have less fluorescence detectable within the cell.

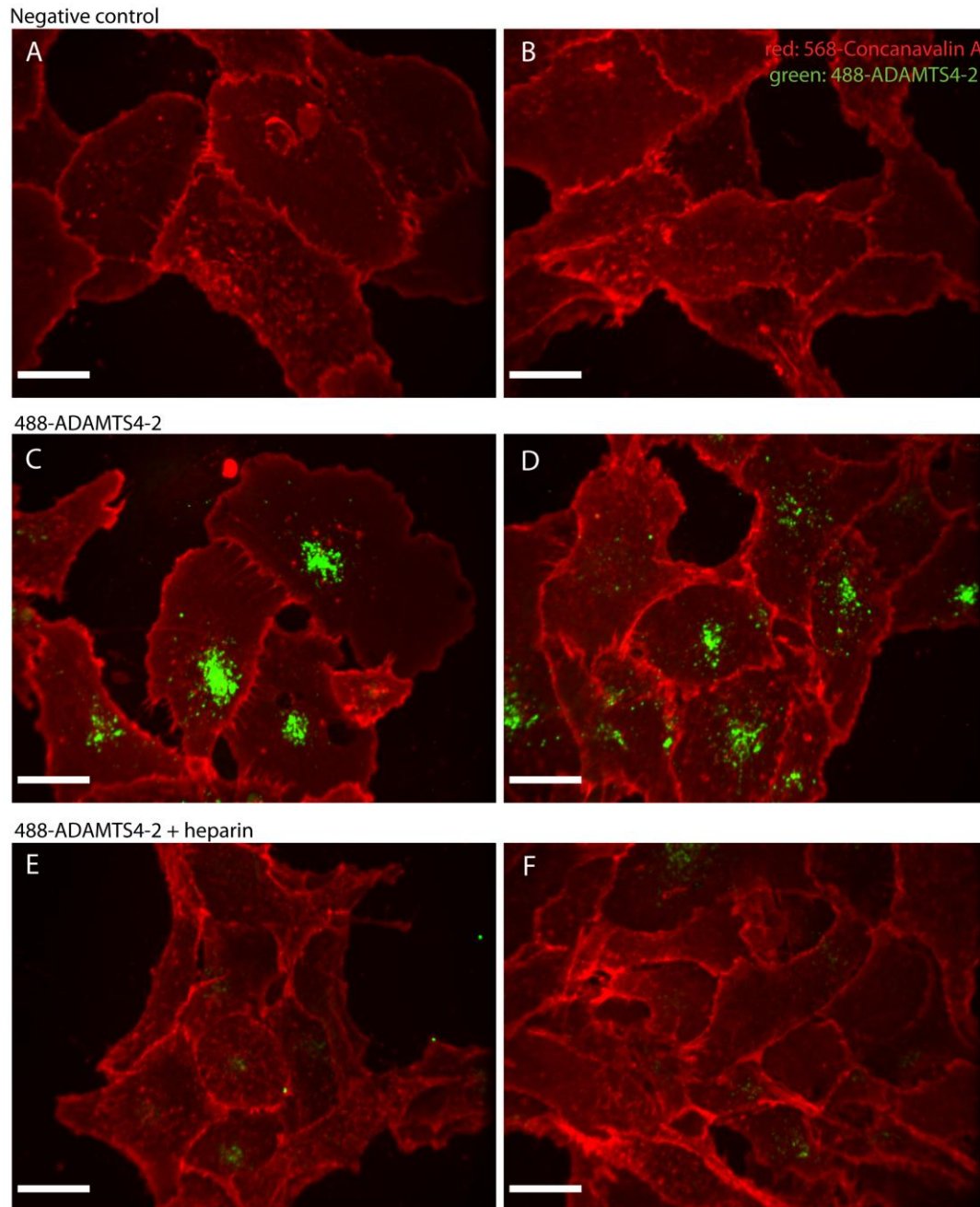


Figure 20: Detection of Alexa Fluor 488-ADAMTS4-2 within the cell.

HTB94 cells were pre-treated for 1 h with phenol red-free DMEM containing 0.1 % BSA with or without 250 µg/ml heparin before addition of Alexa Fluor 488-ADAMTS4-2 (15 nM). After overnight incubation, the medium was removed and the cells were fixed with 4 % PFA. The cell surface was visualized by incubation with Alexa Fluor 568-labeled concanavalin A. Sections through the cell (0.2 µm) were scanned and collated in one image using Volocity software. Shown are 2 representative fields of view per treatment. **A & B**, Negative control containing ~1300 units of Alexa Fluor 488. **C & D**, Alexa Fluor 488-ADAMTS4-2. **E & F**, Alexa Fluor 488-ADAMTS4-2 plus heparin (250 µg/ml). Fluorescent-labeled ADAMTS4-2 (green), fluorescent-labeled concanavalin A (red). Scale bar = 20 µm.

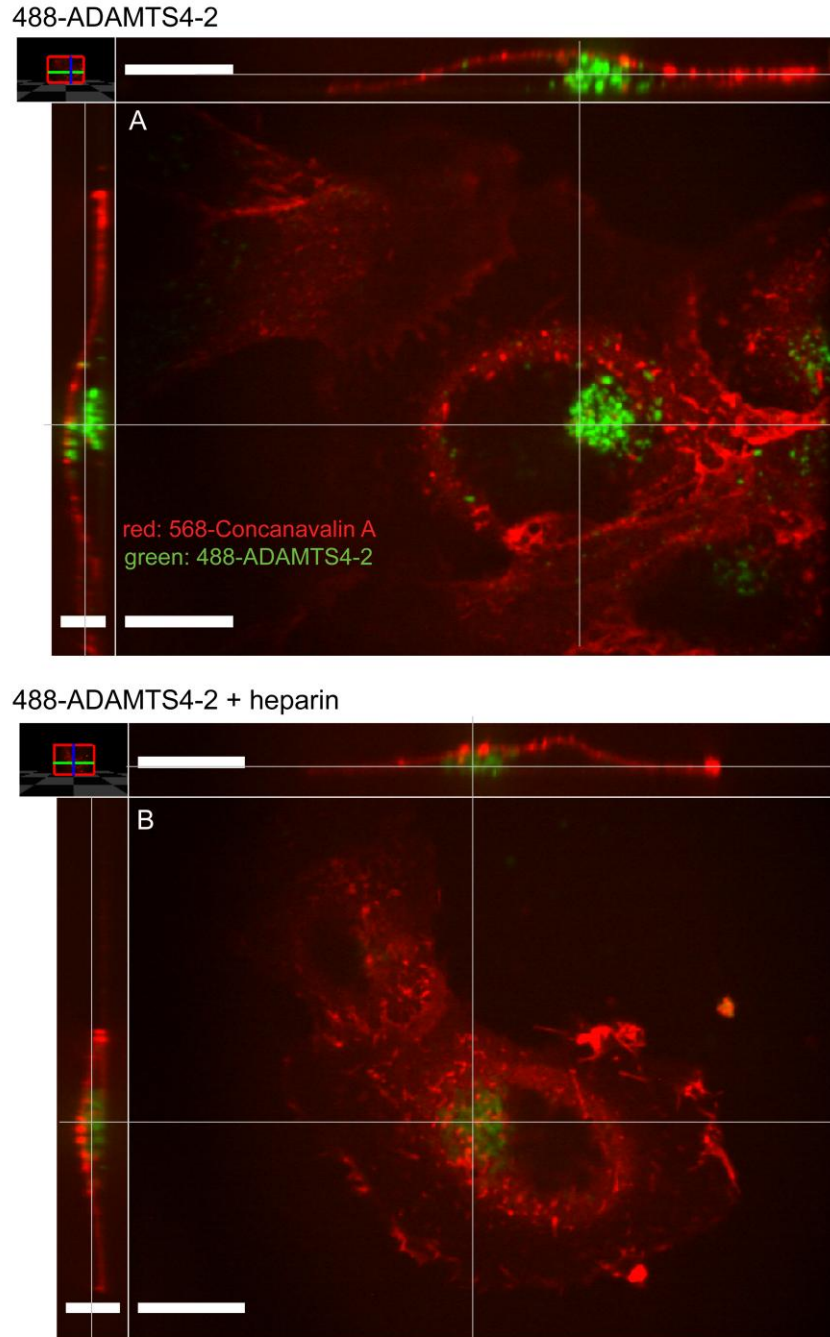


Figure 21: Detection of Alexa Fluor 488-ADAMTS4-2 within the cell.

HTB94 cells were pre-treated for 1 h with phenol red-free DMEM containing 0.1 % BSA with or without 250 µg/ml heparin before addition of Alexa Fluor 488-ADAMTS4-2 (15 nM). After overnight incubation, the medium was removed and the cells were fixed with 4 % PFA. The cell surface was visualized by incubation with Alexa Fluor 568-labeled concanavalin A. Sections of 0.1 µm were imaged and processed in the XYZ plane using Volocity software. **A**, section through a cell incubated with Alexa Fluor 488-ADAMTS4-2. **B**, section through a cell incubated with Alexa Fluor 488-ADAMTS4-2 plus heparin (250 µg/ml). Fluorescent-labeled ADAMTS4-2 (green), fluorescent labeled concanavalin A (red). The white lines indicate the focus point of the image. Scale bar = 20 µm.

3.2.3. Heparin-binding affinity of ADAMTS4-2

Next, the mechanism by which heparin protects against ADAMTS4-2 disappearance was investigated. One possibility is that ADAMTS4-2 binds to HSPGs on the cell surface and that heparin displaces cell surface binding of ADAMTS4-2. Therefore, the heparin-binding affinity of ADAMTS4-2 was analysed by heparin-Sepharose affinity chromatography. ADAMTS4-2 (250 ng) diluted in TNC-Brij (500 μ l) was loaded on a column of heparin-Sepharose under gravity flow. The flow-through was collected and bound protein was eluted with a step-wise gradient of 0.15-1M NaCl in 50 mM Tris-HCl (pH 7.5), 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35. Elutions (500 μ l) were precipitated with TCA (3.3 %) and analysed by reducing SDS-PAGE and Western blotting using the anti-ADAMTS-4-catalytic domain antibody. Figure 22A shows that ADAMTS4-2 did not bind to the heparin-Sepharose column. However, in a similar experiment, full-length ADAMTS4-1 (250 ng) bound to heparin-Sepharose and was eluted with 0.5 -0.75 M NaCl (Figure 22B).

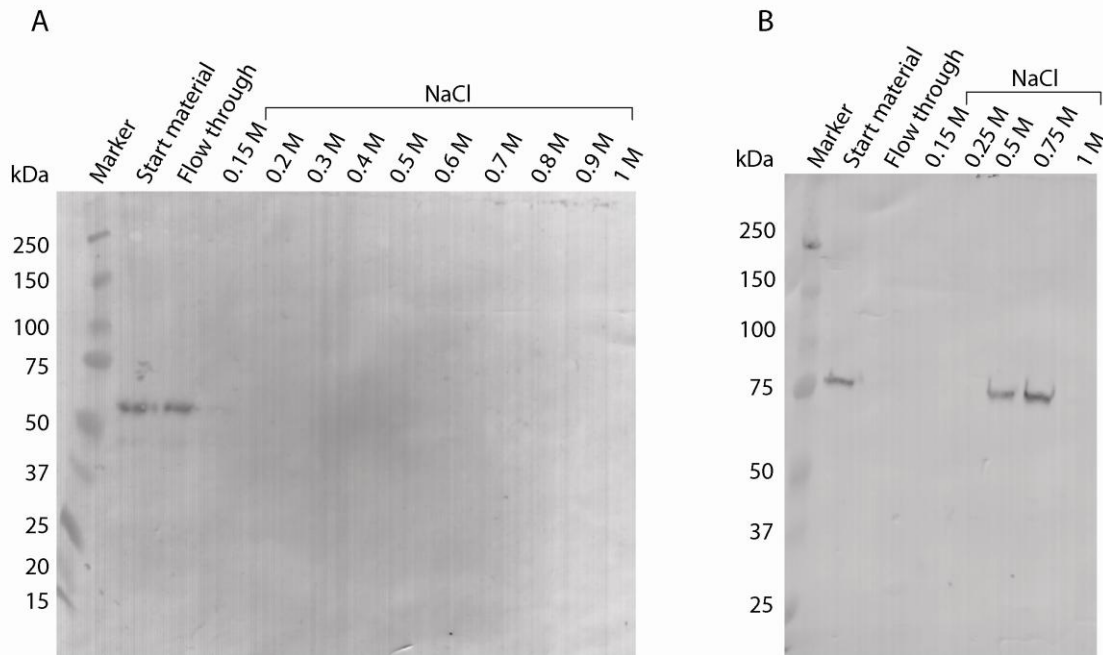


Figure 22: ADAMTS4-2, unlike ADAMTS4-1 does not bind heparin-Sepharose.

A, ADAMTS4-2 or **B**, ADAMTS4-1 (250 ng) diluted in TNC-Brij (500 μ l) were loaded on a column of heparin-Sepharose (100 μ l bed volume) under gravity flow. The flow-through was collected and the column was eluted with a step-wise gradient of 0.15-1 M NaCl in 50 mM Tris-HCl (pH 7.5), 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35. Elutions (500 μ l) were precipitated with TCA (3.3 %) and analysed by reducing SDS-PAGE (7.5 % acrylamide) and Western blotting using the anti-ADAMTS-4-catalytic domain antibody.

3.3. Discussion

In this chapter, the possibility that ADAMTS-4 activity is regulated by endocytosis was examined by monitoring the disappearance of exogenous ADAMTS4-2 from the medium of HTB94 cells by Western blotting and visualizing the appearance of fluorescently labeled ADAMTS4-2 within the cell by confocal microscopy.

HTB94 cells were chosen as a model system to study ADAMTS-4 endocytosis because previous work in our laboratory has shown that TIMP-3, the endogenous inhibitor of ADAMTS-4, accumulates in the medium of HTB94 cells upon treatment with heparin (Troeberg *et al.*, 2008). TIMP-3 also accumulates in these cells in the presence of the LDL-R family antagonist, RAP (Troeberg *et al.*, 2008). These results suggest that endocytic mechanisms exist within this cell

type. Furthermore, HTB94 cells are derived from human chondrocytes and chondrocytes are the only cell-type found in cartilage. Several studies report that IL-1 stimulation can induce the expression of ADAMTS-4 in human, porcine and bovine chondrocytes (Bau *et al.*, 2002; Curtis *et al.*, 2000; Little *et al.*, 2002; Tortorella *et al.*, 2001) and we hypothesised that endocytosis of ADAMTS-4 may be an important regulatory mechanism in these cells.

The Western blotting data demonstrated that when added to HTB94 cells, ADAMTS4-2, a truncated form of full-length ADAMTS-4 lacking the C-terminal spacer domain, disappeared from the medium over time. However, the half-life for disappearance was not reproducible, ranging from 2 – 8h. One reason for this could be that monitoring ADAMTS4-2 disappearance by Western blot analysis relies on developing the blot within its linear range. In addition, internalised ADAMTS4-2 could not be detected by Western blot analysis of the cell lysate. However, if the enzyme was internalized and rapidly degraded by lysosomes, only a very low amount of enzyme or its degradation products would be present within the cell at any one time and the sensitivity limits of Western blotting may be too low for the enzyme to be detected. Therefore, to study the kinetics of ADAMTS4-2 disappearance in further detail and to detect intact ADAMTS4-2 or its degradation products within the cells, it was necessary to develop a more sensitive and quantitative assay. This is the subject of Chapter 4.

The disappearance of ADAMTS4-2 was not due to autodegradation or proteolysis by soluble proteinases. However, the broad-spectrum metalloproteinase inhibitor, GM6001, protected against ADAMTS4-2 disappearance. Cell viability, assessed by the MTT assay was not reduced upon incubation with GM6001. Therefore, the protective effect of GM6001 could indicate that the disappearance of ADAMTS4-2 from the medium was due to metalloproteinase-mediated proteolysis of the enzyme at the cell surface. This will be further investigated in Chapter 4.

The disappearance of ADAMTS4-2 could be inhibited when the cells were pre-treated with heparin but less well when cells were pre-treated with de-N-sulfated heparin or other less sulfated GAGs such as chondroitin sulfate or dermatan sulfate. This suggests that cell surface sulfation is an essential requirement for ADAMTS4-2 disappearance in HTB94 cells and is similar to the effect of each GAG on TIMP-3 accumulation (Troeberg *et al.*, 2008). Cell viability, assessed by the MTT assay was not reduced by treatment with heparin and the protective effect of heparin could indicate that the mechanism by which ADAMTS4-2 disappears is the same as that proposed for TIMP-3 (Troeberg *et al.*, 2008). This study hypothesised that a member of the LDL-R family could be a candidate TIMP-3-endocytic receptor, with HSPGs acting as a co-receptor in this cell type (Troeberg *et al.*, 2008).

To show that the disappearance of ADAMTS4-2 from the medium was a result of endocytosis and intracellular degradation, a fluorescent-based assay was developed. When labeled with Alexa Fluor 488, ADAMTS4-2 retained its activity against GST-IGD-FLAG substrate. Furthermore, when Alexa Fluor 488-ADAMTS4-2 was added to HTB94 cells and incubated at 37 °C overnight, clusters of fluorescence were detected underneath the plasma membrane of the cell. These data indicated that ADAMTS4-2 was taken into the cell. The discrete packages of fluorescence detected within the cell may represent intracellular vesicles. Heparin reduced the fluorescence detectable within the cell, supporting the Western blotting results showing that heparin protects against ADAMTS4-2 disappearance from the medium of HTB94 cells. As a negative control, the same amount of free Alexa Fluor488 was added to other wells and was not detected in the cell. This shows that the uptake of Alexa Fluor 488-ADAMTS4-2 was specific compared to free Alexa Fluor488. Additionally, as the free Alexa Fluor 488 was diluted in TNC-Brij, this shows that the uptake of Alexa Fluor488-ADAMTS4-2 within the cell was not due to permeabilisation of the plasma membrane by the detergent in the buffer. Although detected within the cell, Alexa Fluor 488-ADAMTS4-2 was not detected on the cell surface. The binding of enzyme to the

cell surface could have been displaced by washing with PBS. Alternatively, the enzyme may be bound to the cell surface but the amount of fluorescence emitted may be below the level of detection. Further work would be to analyse surface-bound ADAMTS4-2 by immunostaining with the ADAMTS4-catalytic domain antibody and Alexa Fluor 647-conjugated secondary antibody. It would also be informative to determine the exact location of ADAMTS-4 within the cell by co-staining with an endosomal marker, for example. Co-localisation with lysosomes would provide further evidence of ADAMTS4-2 internalisation and intracellular degradation. Together with the Western blotting results, the confocal data suggest that ADAMTS4-2 is endocytosed by HTB94 cells and that this process can be blocked by heparin.

To investigate the mechanism of heparin protection, the hypothesis that heparin binds to ADAMTS4-2, preventing the binding of the enzyme to HSPGs on the cell surface was considered. Although ADAMTS-4 contains 2 consensus GAG binding sites in the spacer domain ($^{692}\text{F-R-K-F-R-Y}^{697}$) and ($^{829}\text{R-R-R-P-W-A-G-R-K}^{837}$) (Flannery *et al.*, 2002), the spacer domain is completely deleted in the ADAMTS4-2 construct used in this study. However, ADAMTS4-2 retains the cysteine rich domain which has also been reported to contain a GAG binding motif ($^{668}\text{S-K-K-K-F-D-K-C}^{675}$) (Flannery *et al.*, 2002). However, ADAMTS4-2 did not bind to heparin-Sepharose. Additionally, recent work by Dr Linda Troeberg in our laboratory has also shown that ADAMTS4-1 but not ADAMTS4-2 binds to heparin-coated plates (personal communication), suggesting that the major GAG binding sites of ADAMTS-4 are within the spacer domain. These results differ from work by Flannery *et al* (2002) who reported that both the p68 form of ADAMTS-4, containing the spacer domain (similar to ADAMTS4-1) and the p53 form of ADAMTS-4 (similar to ADAMTS4-2) bound heparin-Sepharose and were eluted with 0.8 M NaCl and 0.5 M NaCl, respectively. However, the p53 form retains a small portion of the spacer domain, including part of a consensus GAG binding site ($^{692}\text{F-R-K}^{694}$) (Flannery *et al.*, 2002). This could account for the differences in heparin-binding between the study of Flannery *et al* and the data

presented herein. However, the data presented in this chapter are in agreement with previous work in our laboratory (Kashiwagi *et al.*, 2004). This work showed that in HTB94 cells transfected with ADAMTS4-1, the enzyme was localised to the pericellular space and required heparin for its release into the medium, whilst the enzyme was spontaneously released into the medium when the spacer domain was removed. These results show that the spacer domain plays a major role in localizing ADAMTS-4 to the cell layer. For these reasons, the mechanism of how heparin inhibits the clearance of ADAMTS4-2 from the medium of HTB94 cells is at present unclear. It is speculated that ADAMTS4-2 may bind heparin very weakly. Alternatively, TIMP-3, the endogenous inhibitor of ADAMTS-4, contains heparin-binding domains and binds sulfated GAGs of the ECM (Yu *et al.*, 2000). It is possible that ADAMTS4-2 could be internalized in a complex with TIMP-3 in a similar mechanism as that reported for either MMP-2 and MMP-9 which can be internalised in a complex with TIMP-2 or TIMP-1, respectively (Emonard *et al.*, 2004; Hahn-Dantona *et al.*, 2001). Heparin could inhibit the endocytosis of a TIMP-3-ADAMTS4-2 complex by disrupting the interaction between TIMP-3 and its endocytic receptor. This hypothesis will be considered in future chapters.

The results of this chapter show that ADAMTS-4-2 disappears from the medium of HTB94 cells and can be detected within the cell by fluorescent microscopy. This indicates that ADAMTS4-2 clearance from the medium may be due to endocytosis and intracellular degradation. Both GM6001 and heparin protected against ADAMTS4-2 clearance and, in agreement with this hypothesis, heparin also reduced the amount of Alexa Fluor 488-ADAMTS4-2 detected within the cell. The cellular uptake of ADAMTS-4 by HTB94 cells could have significance in the regulation of ADAMTS-4 activity and localization in normal and/or pathological conditions such as OA. Chapter 4 will describe the development and use of a radioactivity-based assay to study the mechanism of ADAMTS-4 cellular uptake and degradation.

Chapter 4

Development and use of a radioactivity-based assay to study the mechanism of ADAMTS-4 clearance

4.1. Introduction

In Chapter 3, Western blotting was used to demonstrate that ADAMTS4-2 disappeared from the medium of HTB94 cells. In addition, confocal microscopy techniques demonstrated that fluorescent-labeled ADAMTS4-2 could be detected within the cell. The aim of this chapter was to develop a radioactivity-based assay using [^{35}S]-labeled ADAMTS4-2 to investigate the mechanism of ADAMTS4-2 clearance by HTB94 cells.

4.2. Results

4.2.1. Purification of [^{35}S]-labeled ADAMTS-4-2

HEK-293/EBNA cells stably transfected with FLAG-tagged ADAMTS4-2 were grown to confluence. They were metabolically labeled with [^{35}S]Met/[^{35}S]Cys by incubation for 4 days in serum-free DMEM without L-glutamine, cysteine, methionine or cystine, containing 0.2 % lactalbumin enzymatic hydrolysate and [^{35}S]Met/[^{35}S]Cys (25 $\mu\text{Ci/ml}$). [^{35}S]ADAMTS-4-2 was purified from the conditioned medium by anti-FLAG M2 affinity chromatography and FLAG peptide elution. Aliquots of each sample were analysed by SDS-PAGE and silver staining. As the silver stain shows in Figure 23, elution 1 contained a single protein band at a molecular weight of approximately 51 kDa. Similarly, exposure to autoradiographic film indicated that the protein was labeled with [^{35}S] and that there were no other radioactive contaminants in the preparation. The purified protein was verified to be ADAMTS4-2 by Western blot analysis with both anti-FLAG M2- and anti-ADAMTS4-catalytic domain antibodies.

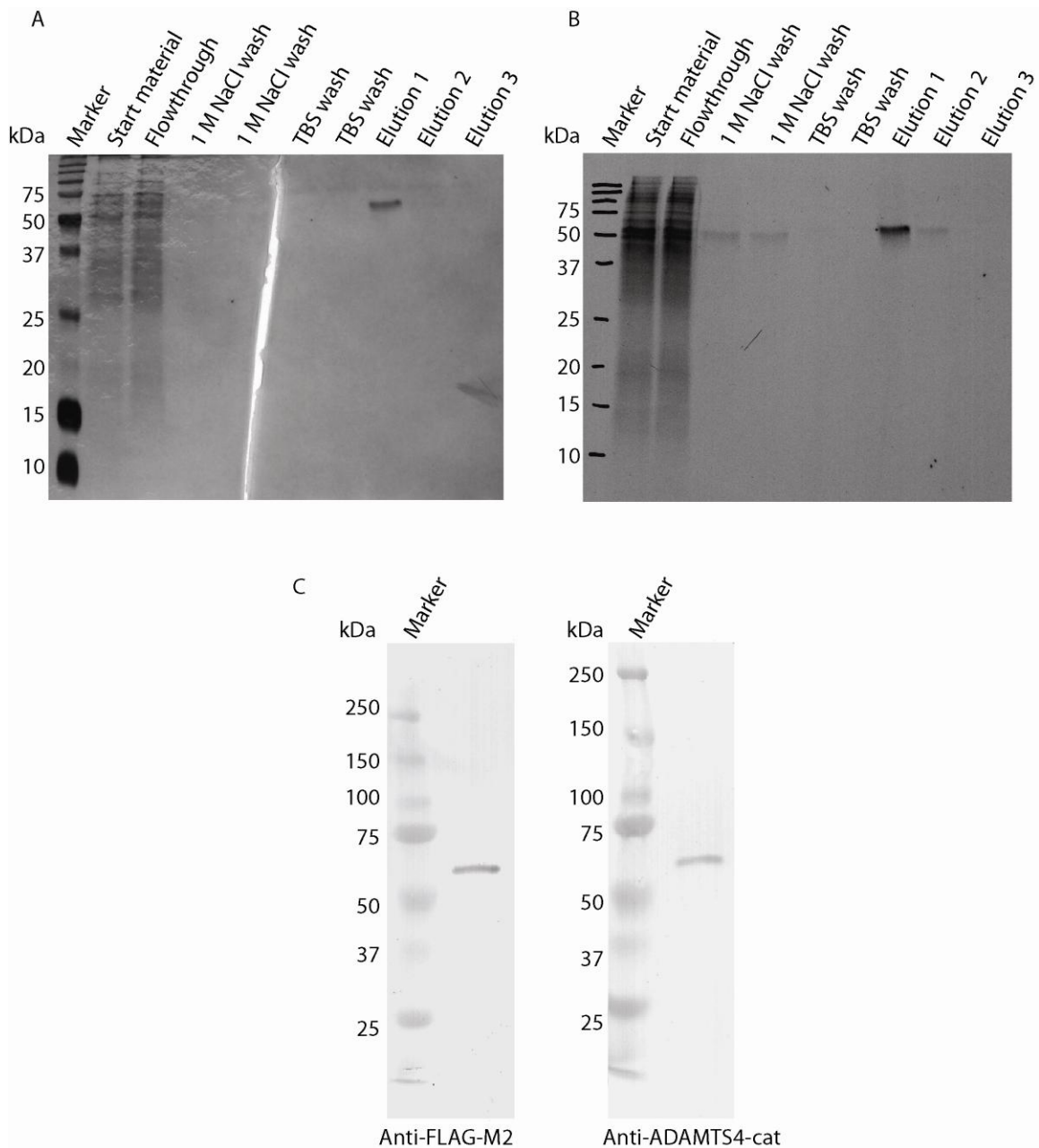


Figure 23: Purification of $[^{35}\text{S}]\text{ADAMTS4-2}$ by anti-FLAG M2 affinity chromatography.

HEK-293/EBNA cells transfected with ADAMTS4-2-FLAG construct were metabolically labeled with $[^{35}\text{S}]\text{Met}/[^{35}\text{S}]\text{Cys}$ for 4 days. The resulting conditioned medium (40 ml) was loaded onto an anti-FLAG M2-agarose column (2 ml). After washing with 1 M NaCl buffer [50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35] (20 ml) and TBS (20 ml), the bound material was eluted with 200 $\mu\text{g}/\text{ml}$ FLAG peptide in TNC-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35] in 3 ml fractions. **A**, Aliquots were analysed by SDS-PAGE and silver staining. **B**, The gel was dried and exposed to autoradiographic film overnight. **C**, Western blot of $[^{35}\text{S}]\text{ADAMTS4-2}$ using anti-FLAG M2-antibody or anti-ADAMTS-4 catalytic domain antibody.

4.2.2. Activity of [^{35} S]ADAMTS4-2 as determined by GST-IGD-FLAG substrate

After purification, an aliquot of [^{35} S]ADAMTS-4-2 elution 1 was diluted with an equal volume of TNC-Brij. The concentration of active [^{35} S]ADAMTS-4-2 was determined by titration with N-TIMP-3, using the GST-IGD-FLAG substrate as described in section 3.2.1.2. Samples were analysed by SDS-PAGE and Coomassie Brilliant Blue R250 staining. The gel was scanned and the pixel volume of the cleaved 21 kDa product was determined using Phoretix software. The concentration of N-TIMP-3 was plotted against the pixel volume of the cleaved product (Figure 24). The concentration of active [^{35} S]ADAMTS-4-2 in elution 1 was 200 nM (10.3 μ g/ml).

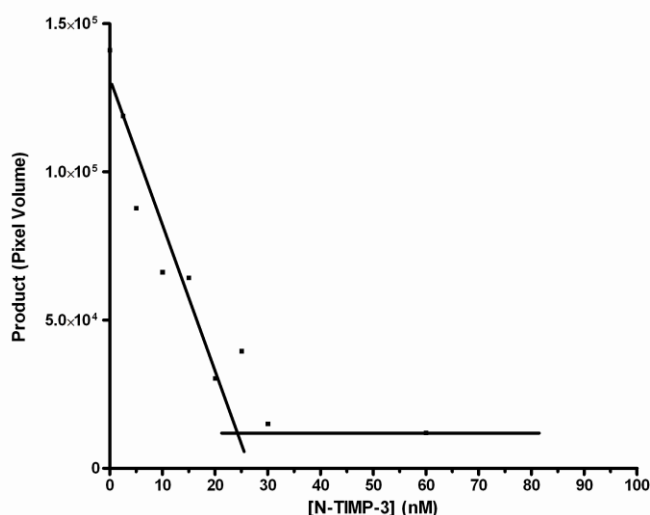


Figure 24: *N-TIMP-3 titration of [^{35} S]ADAMTS4-2.*

The concentration of active [^{35} S]ADAMTS4-2 in elution 1 was determined by titration with known concentrations of N-TIMP-3 using GST-IGD-FLAG substrate. [^{35} S]ADAMTS4-2 elution 1 (2.5 μ l) was diluted with equal volume of TNC-Brij and then incubated with 2.5 μ l of N-TIMP-3 (0-360 nM) for 60 min prior to the addition of GST-IGD-FLAG substrate (5 μ l) for 90 min at 37 °C. Reactions were stopped by addition of 2 x reducing SDS-sample buffer containing 10 mM EDTA (10 μ l). The samples were run on reducing SDS-PAGE and stained with Coomassie Brilliant Blue R250. Pixel volume of the 21 kDa product band was plotted against N-TIMP-3 concentration. [N-TIMP-3] on the graph represents the concentration of N-TIMP-3 in the final reaction volume (1:4 dilution). To determine the concentration of elution 1, the value where the line intercepts the x-axis was multiplied by 4, then by 2 (dilution factor of [^{35} S]ADAMTS4-2).

4.2.3. Detection limits of scintillation counting

In order to use scintillation counting as a read out, it was necessary to determine the linear range of scintillation counting. To achieve this, both [^{35}S]ADAMTS4-2 and [^{35}S]-labeled flow-through material were serially diluted 1:1 with TNC-Brij and the dilutions were counted. The detection limit of [^{35}S]ADAMTS4-2 against background was 50 dpm (Figure 25A). The highest radioactivity counted in the flow-through material was 500,000 dpm and the readings were within linear range until 50 dpm (Figure 25B). All samples were therefore required to be greater than 50 dpm to be detectable against background.

When measured by scintillation counting, the amount of [^{35}S]Met/[^{35}S]Cys incorporation in this batch was 55000 dpm/ μg of [^{35}S]ADAMTS-4-2. Other batches of [^{35}S]ADAMTS-4-2 purified contained comparable [^{35}S]Met/[^{35}S]Cys incorporation.

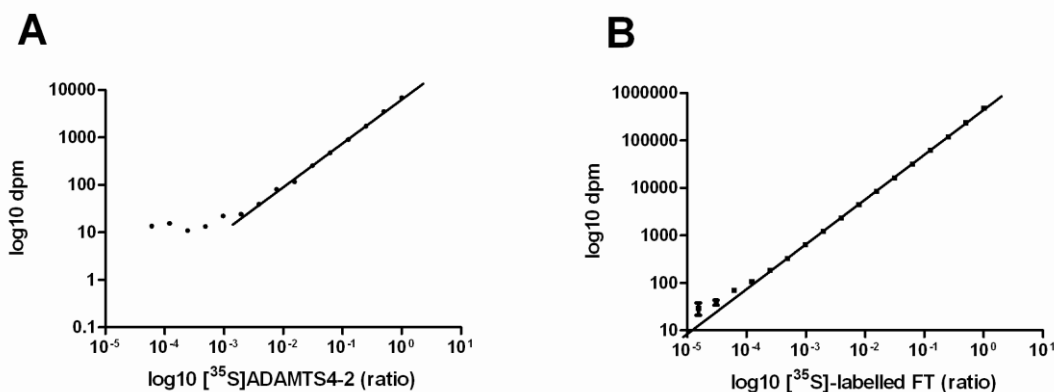


Figure 25: *Linearity of scintillation counting.*

[^{35}S]ADAMTS4-2 or [^{35}S]-labeled flow-through (FT) was serially diluted with TNC Brij and the radioactivity was determined by scintillation counting using a Wallac 1410 Liquid Scintillation Counter (Pharmacia). A, [^{35}S]ADAMTS4-2 (single reading). B, [^{35}S]-labeled flow-through (triplicate readings).

4.2.4. [^{35}S]ADAMTS4-2 clearance by HTB94 cells

To determine whether [^{35}S]ADAMTS4-2 was cleared by HTB94 cells, cells were plated in a 6-well plate at a density of 1×10^6 cells/well and grown overnight before

incubation with DMEM containing 0.1 % BSA. After 1 h, [³⁵S]ADAMTS4-2 (1.5 nM) was added to each well. [³⁵S]ADAMTS4-2 was also added to a 6-well plate in DMEM containing 0.1 % BSA without HTB94 cells. The conditioned medium was removed after 24 h and precipitated with TCA (3.3 %) overnight at 4 °C. After precipitation the samples were centrifuged at 14170 g for 15 min 4 °C to separate TCA-soluble material (degradation products) from TCA-insoluble material (intact enzyme). The TCA-soluble fraction was pipetted directly into a scintillation vial. The TCA-insoluble fraction was dissolved in 1 M NaOH prior to transfer into a scintillation vial. After removal of the conditioned medium, the cells were placed on ice and washed thrice with ice-cold PBS and the wash fractions were transferred to scintillation vials for counting. The cell layer was treated with 0.1 % pronase solution on ice to degrade surface ligands and detach cells from the plate. In wells without cells, the well was treated with pronase to remove radioactivity bound to the plate. After 10 min, the cells readily detached from the plate and were pipetted into an Eppendorf tube. The wells were washed once with ice-cold PBS and this was pooled with the cell/pronase suspension before centrifugation at 755 g for 5 min to separate the surface-bound (pronase-sensitive) fraction from the intracellular (pronase-resistant) fraction. The pronase-sensitive fraction was pipetted directly into a scintillation vial and the pronase-resistant fraction was dissolved in 1 M NaOH prior to scintillation counting.

After 24 h incubation with cells, the amount of [³⁵S]ADAMTS4-2 in the conditioned medium decreased and the TCA-soluble fraction increased compared to incubation in the absence of cells (Figure 26). In addition, radioactivity was detected on the cell surface and within the cell. The radioactivity detected on the surface of the plate incubated without cells reflects the binding of [³⁵S]ADAMTS4-2 to the plasticware and the intracellular fraction is absent because there were no cells to harvest. The wash fractions from wells incubated with [³⁵S]ADAMTS4-2 in the presence or absence of cells were counted and found to be negligible. Additionally, the recovery of the total

number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar (Figure 26E). These results also show that the enzyme was stable upon incubation in the absence of cells and confirms results presented in Chapter 3.

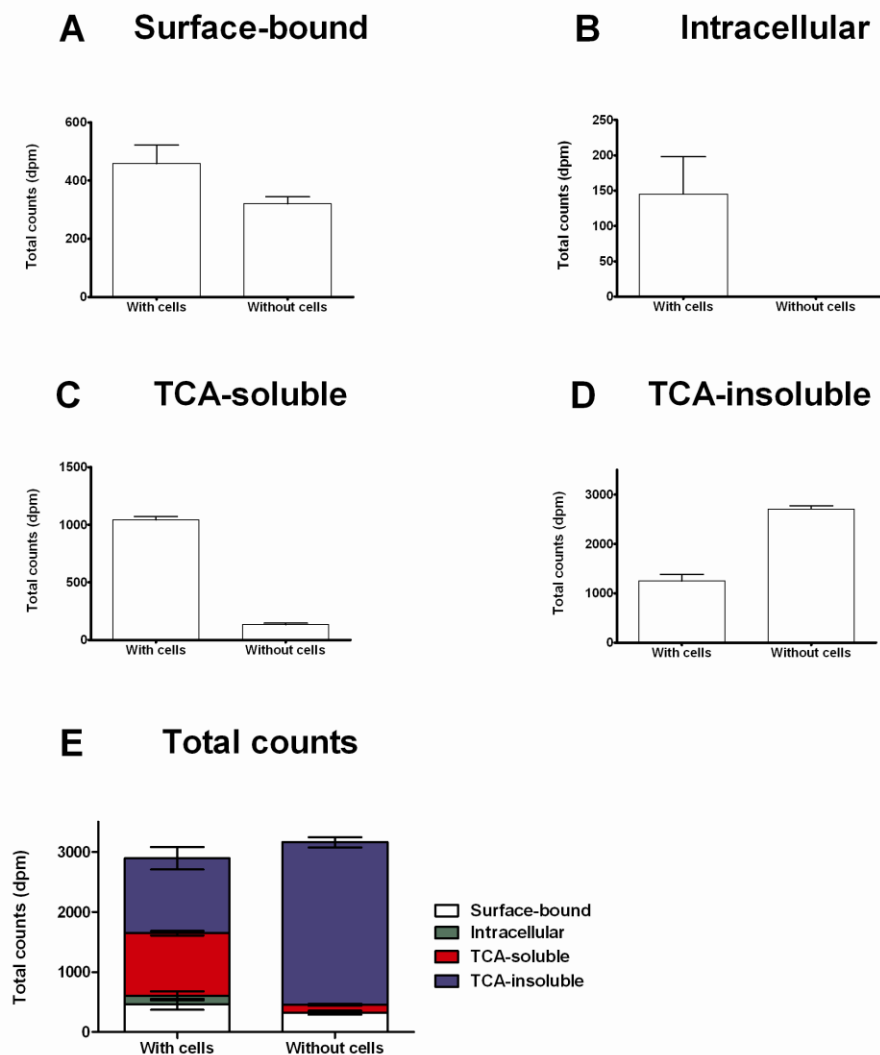


Figure 26: [^{35}S]ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA. After 1 h, [^{35}S]ADAMTS4-2 (1.5 nM) was added. [^{35}S]ADAMTS4-2 was also added to a 6-well plate in DMEM containing 0.1 % BSA without HTB94 cells. After 24 h, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts. **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.2.5. Time course of [³⁵S]ADAMTS4-2 clearance

To investigate the kinetics of ADAMTS-4-2 clearance, a time course experiment was undertaken in order to determine the half-life ($t_{1/2}$) of the disappearance of intact TCA-insoluble [³⁵S]ADAMTS4-2 from the medium of HTB94 cells. The cells were plated at a density of 1×10^6 cells/well and grown overnight before incubation with DMEM containing 0.1 % BSA. After 1 h, [³⁵S]ADAMTS4-2 (1.5 nM) was added to each well. The conditioned medium was removed at 0, 2, 4, 6, 8 and 24 h and precipitated with TCA (3.3 %) overnight at 4 °C. Fractions were harvested as described in section 2.5.2.

Figure 27 shows that the amount of intact TCA-insoluble [³⁵S]ADAMTS4-2 in the conditioned medium decreased over time in accordance with the Western blotting data presented in Chapter 3. The half-life of disappearance was 8 h. Radioactivity was detected within the cell and on the cell surface and the TCA-soluble degraded protein increased in the medium over time. The wash fractions were counted and found to be negligible and the recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar at each time point tested (Figure 27C).

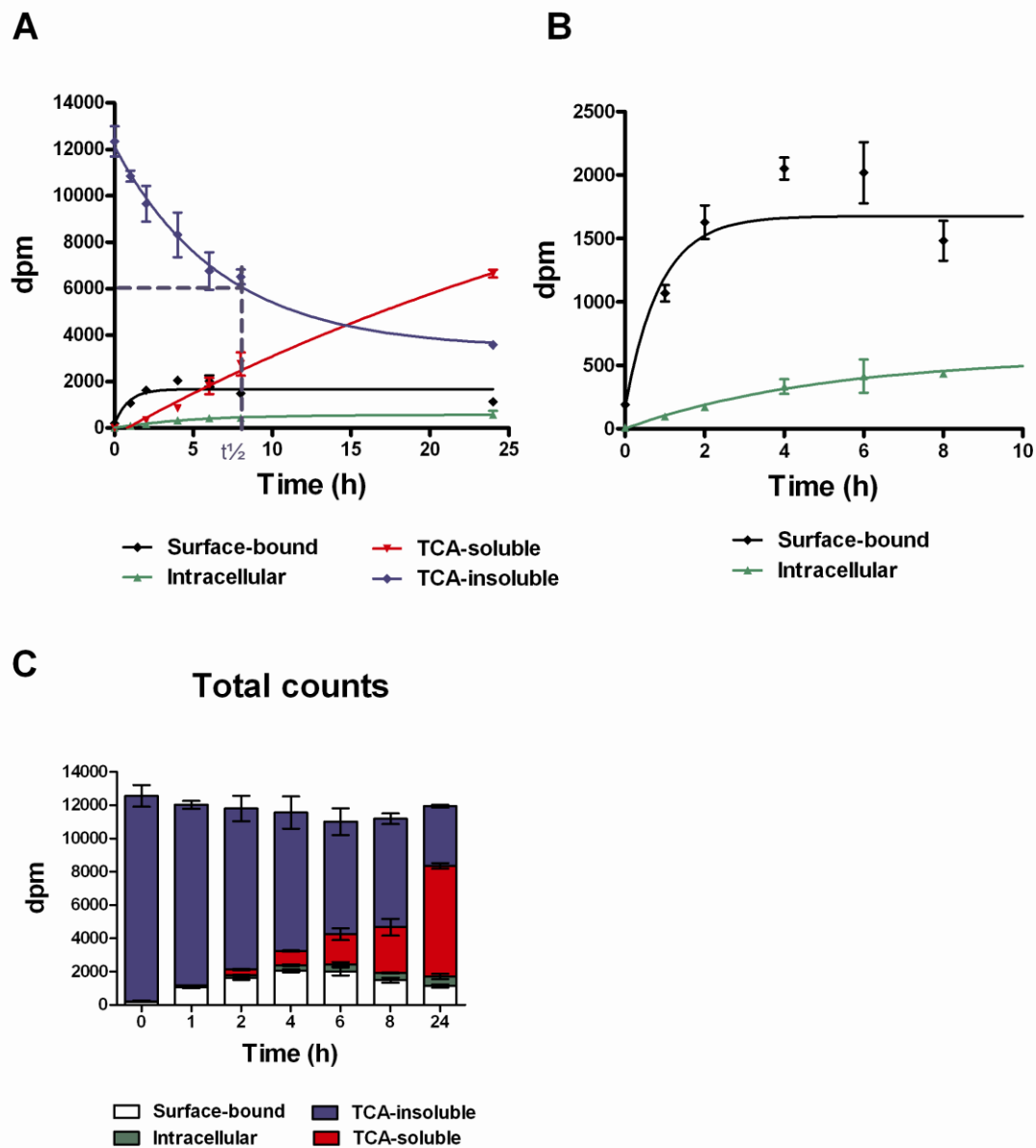


Figure 27: Time course of $[^{35}\text{S}]$ ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA. After 1 h, $[^{35}\text{S}]$ ADAMTS4-2 (1.5 nM) was added. At the time points indicated, the conditioned medium was removed and the surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as detailed in section 2.5.2. Each time point is an average of 3 wells $\pm$ S.D. **A**, Graph of $[^{35}\text{S}]$ ADAMTS4-2 disappearance 0-24 h. **B**, Enlargement of 0-10 h time points, showing the surface-bound and intracellular fractions only. **C**, Total surface-bound, intracellular, TCA-insoluble and TCA-soluble counts. The wash fractions were counted and found to be negligible.

4.2.6. Concentration-dependent clearance of [³⁵S]ADAMTS4-2

To investigate the concentration-dependence of [³⁵S]ADAMTS4-2 clearance, [³⁵S]ADAMTS4-2 was added at a range of concentrations (0.5 - 2 nM) and the medium was harvested at various time points. The TCA-insoluble fraction remaining at each concentration was plotted against time of incubation (Figure 28A). The TCA-insoluble fraction was expressed as % of the TCA-insoluble fraction at 0 h (Figure 28B). At each of the concentrations tested, [³⁵S]ADAMTS4-2 disappeared from the medium at a similar rate. The TCA-soluble fraction also increased in the medium but was only proportional to the concentration of [³⁵S]ADAMTS4-2 added at earlier time points (Figure 28C).

4.2.7. Competition at 4 °C show specificity of binding

The amount of non-specific binding was determined by competition with increasing concentrations of non-radioactive ADAMTS4-2 or BSA. HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 4 °C in DMEM containing 0.1 % BSA for 30 min. HTB94 cells were then incubated with [³⁵S]ADAMTS4-2 (3 nM) at 4 °C in the presence of ADAMTS4-2 (0 - 300 nM) or BSA (0 – 44 μM). After 2 h the medium was removed. The cells were washed thrice with PBS and the cell layer was harvested with 500 μl of 1 M NaOH and the radioactivity bound to the cells was measured by scintillation counting. As Figure 29 shows, the binding of [³⁵S]ADAMTS4-2 was inhibited by addition of increasing concentrations of ADAMTS4-2 but not by BSA.

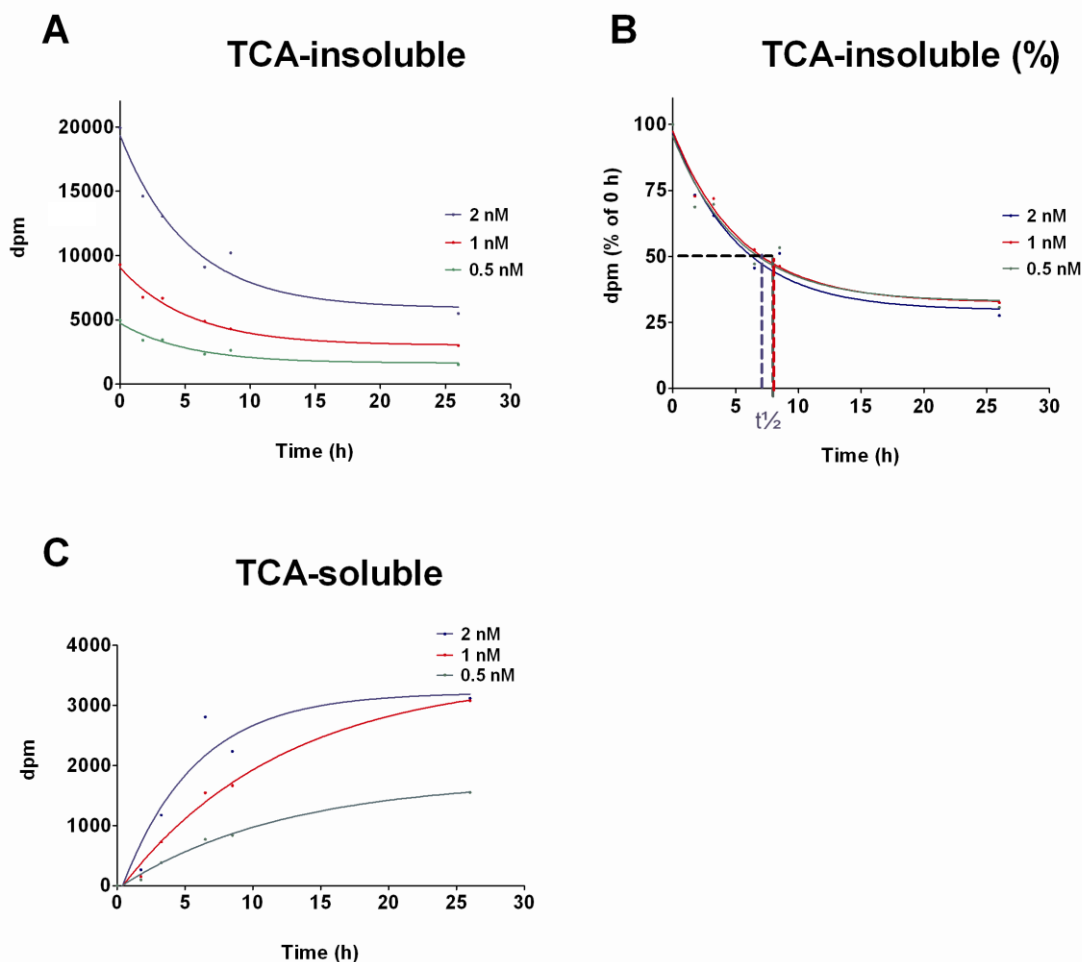


Figure 28: Concentration-dependent $[^{35}\text{S}]\text{ADAMTS4-2}$ clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA. After 1 h, $[^{35}\text{S}]\text{ADAMTS4-2}$ (2 nM, 1 nM or 0.5 nM) was added. At the time points indicated, the conditioned medium was removed and the TCA-soluble and TCA-insoluble fractions were harvested as detailed in section 2.5.2. Each point represents a single reading. **A**, TCA-insoluble fraction. **B**, TCA-insoluble fraction taking dpm at 0 h as 100 %. **C**, TCA-soluble fraction.

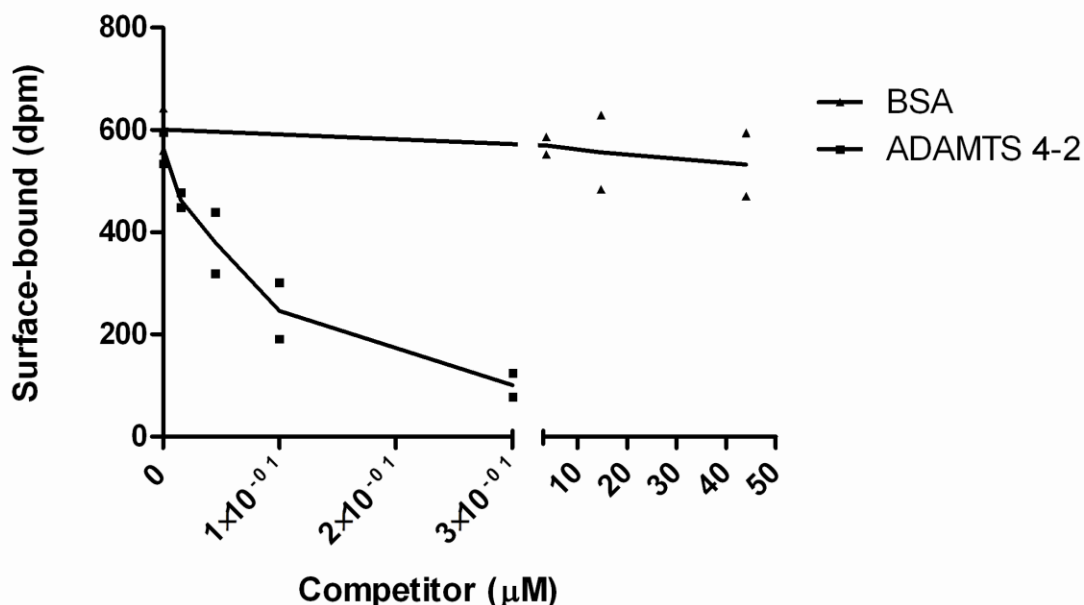


Figure 29: Competition of [³⁵S]ADAMTS4-2 binding to HTB94 cells at 4°C.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 4 °C in DMEM containing 0.1 % BSA for 30 min. HTB94 cells were then incubated with [³⁵S]ADAMTS4-2 (3 nM) at 4 °C for 2 h in the presence of ADAMTS4-2 (0 - 300 nM) or BSA (0 – 44 μM). Surface-bound material was harvested as detailed in section 2.5.2. Data is combined from 2 independent experiments. Each replicate is plotted separately.

4.2.8. The effect of protease inhibitors on [³⁵S]ADAMTS4-2 clearance

To discount the possibility that the clearance of [³⁵S]ADAMTS4-2 from the medium and the appearance of TCA-soluble radioactivity was due to degradation by proteases during culture, HTB94 cells were incubated for 1 h with a variety of protease inhibitors or DMSO carrier control prior to the addition of [³⁵S]ADAMTS4-2 (1.5 nM) for 24 h. The fractions were harvested as described in section 2.5.2. As shown in Figure 30, DMSO did not inhibit [³⁵S]ADAMTS4-2 clearance by HTB94 cells. Of the inhibitors tested, only GM6001 inhibited [³⁵S]ADAMTS4-2 clearance from the medium. GM6001 did not affect the amount of radioactivity bound to the cell surface or the amount detected within the cell. GM6001 reduced the amount of TCA-soluble radioactivity by approximately 50 % compared to untreated cells and the TCA-insoluble fraction

was similarly protected. In this experiment, the recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across each treatment.

This experiment shows that whilst phosphoramidon, E-64 and AEBSF did not affect the clearance of [³⁵S]ADAMTS4-2 from the medium, GM6001 was protective, supporting the Western blot data presented in Chapter 3. In addition, cell viability, as determined by the MTT assay was not adversely affected by GM6001 (Chapter 3). To investigate these results further, a dose experiment was performed with GM6001 and another hydroxamate-based inhibitor, CT1746. Both GM6001 and CT1746 dose-dependently inhibited [³⁵S]ADAMTS4-2 clearance by HTB94 cells but had no dose-dependent effect on the surface-bound fraction, although the intracellular fraction was significantly reduced at higher doses (Figure 31).

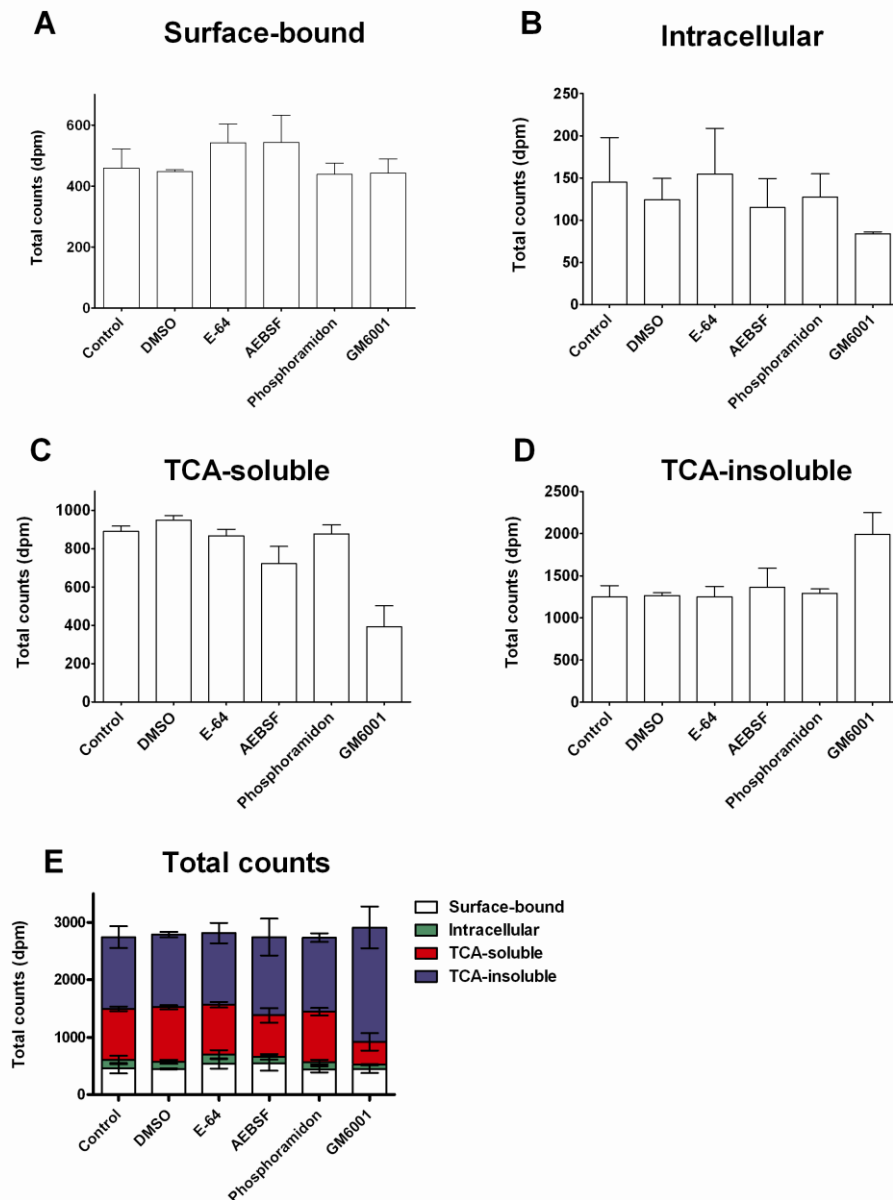


Figure 30: The effect of protease inhibitors on [^{35}S]ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without protease inhibitors (E-64 (10 μM), AEBSF (100 μM), phosphoramidon (10 μM), GM6001 (10 μM) or 1/1000 dilution of DMSO carrier solution. After 1 h, [^{35}S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and the surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as detailed in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

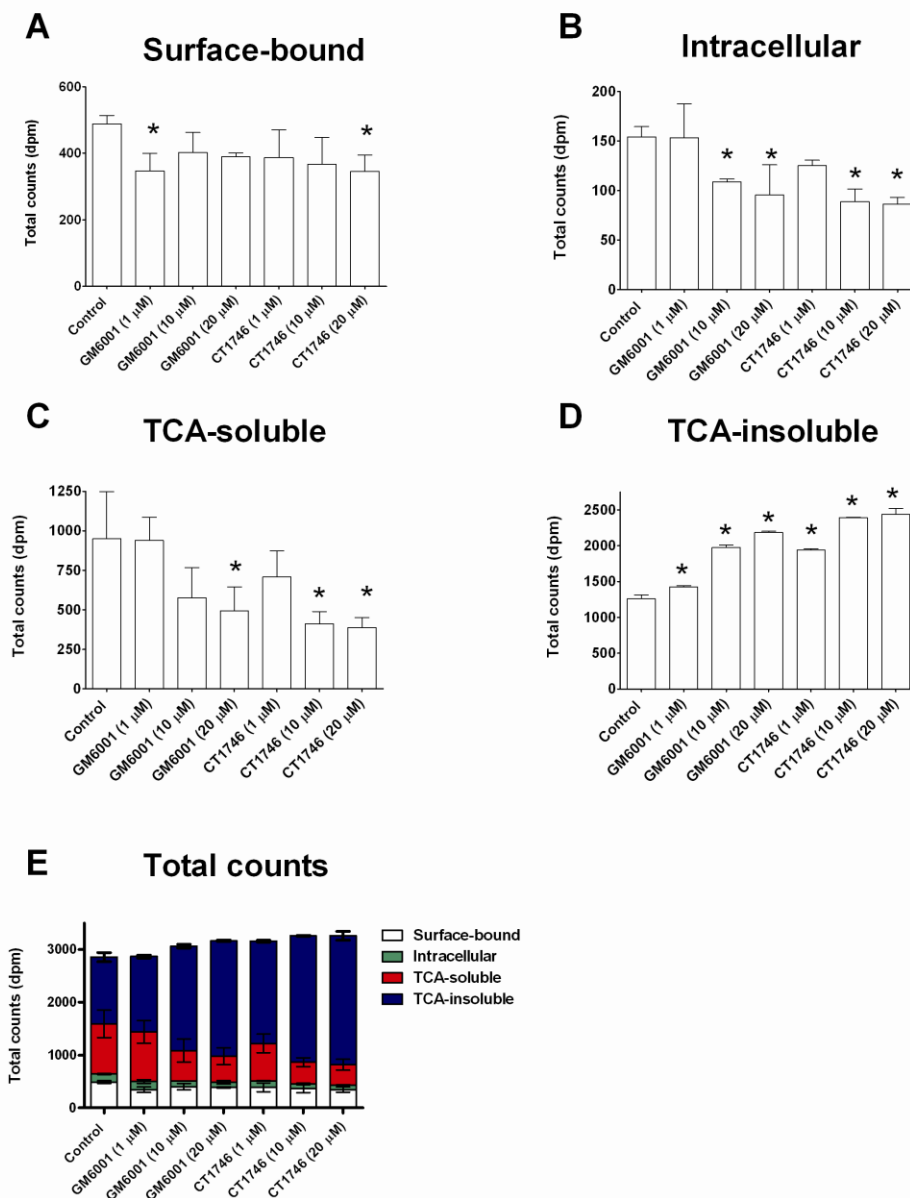


Figure 31: The dose-dependent effect of GM6001 and CT1746 inhibitors on [^{35} S]ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without GM6001 (1-20 μ M) or CT1746 (1-20 μ M). After 1 h, [^{35} S]ADAMTS4-2 (1.5 nM) was added. The control well contained 1/500 dilution of DMSO carrier solution. After 24 h, the conditioned medium was removed and the surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as detailed in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. * = $P \leq 0.05$ as compared to control. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.2.9. The effect of cytochalasin D on [³⁵S]ADAMTS4-2 clearance

4.2.9.1. Assessment of HTB94 cell viability upon cytochalasin D treatment

Cytochalasin D blocks endocytosis by inhibiting actin polymerization (Flanagan and Lin, 1980). However, prolonged incubation with cytochalasin D is toxic. Therefore, in order to determine an appropriate length of incubation of cells with cytochalasin D, the viability of HTB94 cells in the presence of cytochalasin D (10 μ M) was assessed using the MTT assay as previously characterized in section 3.2.1.6. HTB94 cells contracted within 30 min of cytochalasin D treatment. As shown in Figure 32, cell viability was not affected after 6 h incubation with cytochalasin D although a 50 % decrease in cell viability was detected when HTB94 cells were incubated in the presence of cytochalasin D for 24 h. Sodium nitroprusside (SNP) caused a 20 % reduction in cell viability at 6 h and a 90 % reduction in cell viability at 24 h. As a result of this experiment, cells were treated with cytochalasin D for a maximum of 6 h.

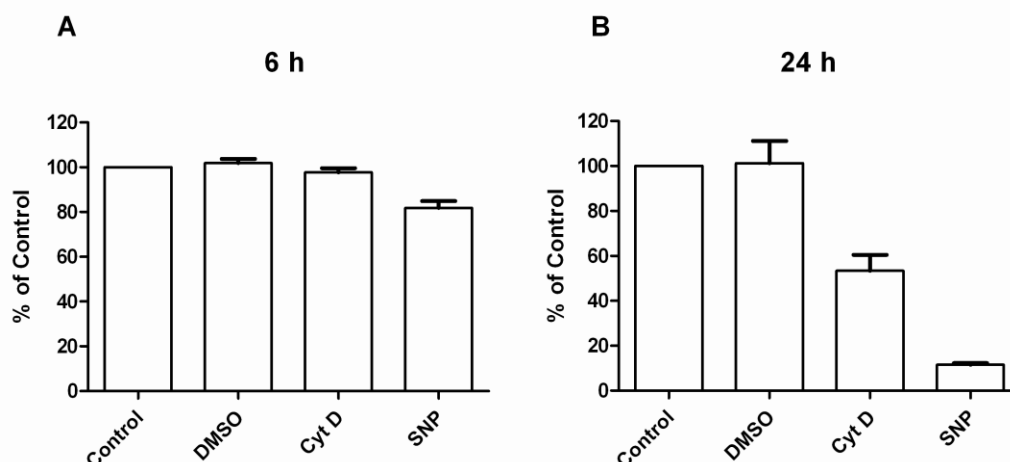


Figure 32: MTT assay of HTB94 cell viability upon incubation with cytochalasin D.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and then treated with cytochalasin D (10 μ M), 1/1000 dilution of DMSO carrier solution or SNP (2 mM) for 6 h (A) or 24 h (B). The medium was removed and the cells were washed once with phenol red-free DMEM before incubation with 975 μ l phenol red-free DMEM and 25 μ l of MTT reagent (5 mg/ml in PBS). After 45 min, 1 ml of 10 % SDS in 10 mM HCl was added to solubilise the formazan crystals. The samples were incubated overnight at 37 $^{\circ}$ C and 200 μ l of each well was transferred to a 96-well plate. The absorbance was read at 574 nm. A, Average of triplicate wells $\pm$ S.D. B, Average of duplicate wells $\pm$ range.

4.2.9.2. Cytochalasin D inhibits the appearance of TCA-soluble fraction

To test the effect of cytochalasin D on [³⁵S]ADAMTS4-2 clearance from the medium of HTB94 cells, cells were treated with 10 µM cytochalasin D or 1/1000 dilution of DMSO carrier solution for 1 h before the addition of [³⁵S]ADAMTS4-2 (1.5 nM). After 5 h, the medium was removed and the TCA-soluble and TCA-insoluble fractions were harvested as previously described. In this experiment, individual surface-bound and intracellular fractions were not harvested, rather the cell layer, after washing with PBS was treated with 1 M NaOH. This gave both surface-bound and intracellular fractions in a single sample (cell-associated fraction). As shown in Figure 33, DMSO did not affect the clearance of [³⁵S]ADAMTS4-2 or the appearance of the TCA-soluble degraded fraction. Although cytochalasin D had no effect on the cell-associated fraction, it reduced the appearance of the TCA-soluble fraction by 50 % and significantly increased the TCA-insoluble fraction compared to control. The recovery of the total number of counts from the cell-associated, TCA-soluble and TCA-insoluble counts was similar across all treatments.

4.2.10. The effect of heparin on [³⁵S]ADAMTS4-2 clearance by HTB94 cells**4.2.10.1. Heparin inhibits [³⁵S]ADAMTS4-2 clearance**

Cells were treated with heparin (250 µg/ml) for 1 h before the addition of [³⁵S]ADAMTS4-2 (1.5 nM). Heparin (250 µg/ml) was sufficient to completely protect against the disappearance of the TCA-insoluble fraction of [³⁵S]ADAMTS4-2 (Figure 34). These data support the Western blotting data presented in Chapter 3 and additionally shows that heparin reduced both the surface-bound and intracellular fractions. Data presented in Chapter 3 show that cell viability was not adversely affected by incubation with heparin for 24 h, even at a concentration of 500 µg/ml (section 3.2.1.9). The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments.

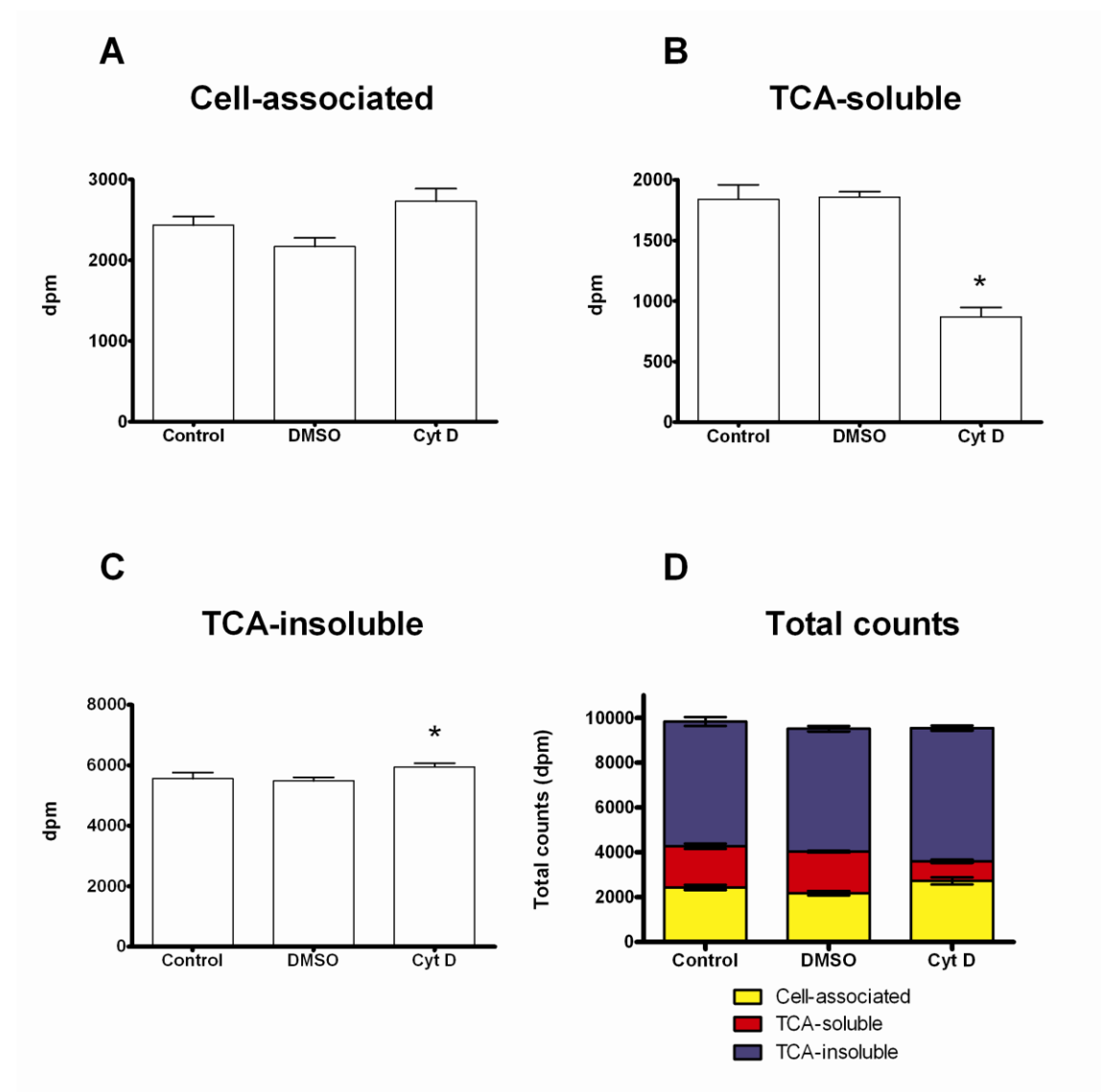


Figure 33: The effect of cytochalasin D on [^{35} S]ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without cytochalasin D (10 μ M) or 1/1000 dilution of DMSO carrier solution. After 1 h, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 5 h, the conditioned medium was removed and the cell-associated, TCA-soluble and TCA-insoluble fractions were harvested as detailed in section 2.5.2. Results are an average of $3 \pm$ S.D. * = $P \leq 0.05$ as compared to control. **A**, Cell-associated counts. **B**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **C**, TCA-insoluble counts. **D**, Total cell-associated, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

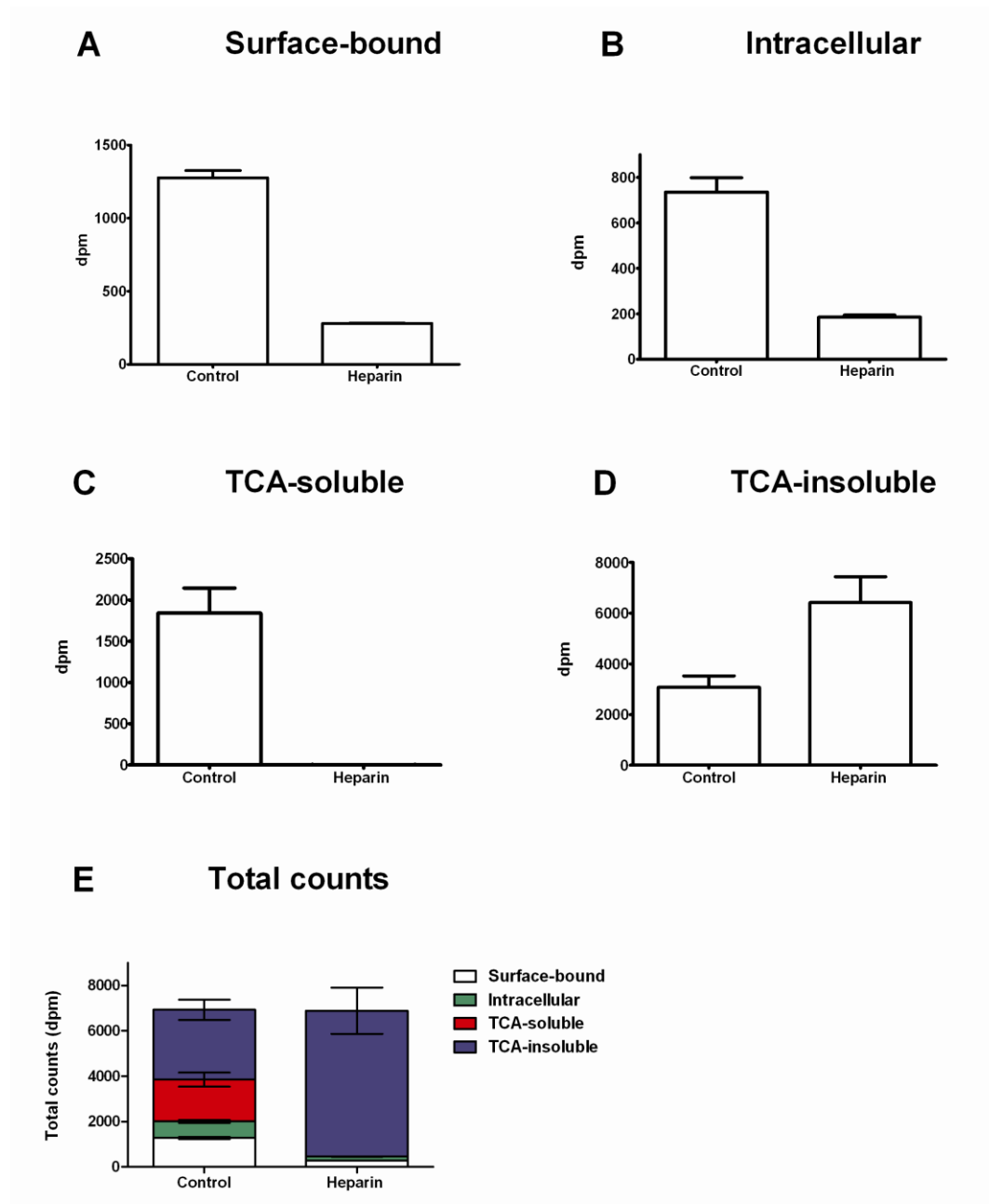


Figure 34: Heparin inhibits [^{35} S]ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml). After 1 h, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.2.10.2. Heparin inhibits surface-binding of [³⁵S]ADAMTS4-2 at 4 °C

To examine the effect of heparin on [³⁵S]ADAMTS4-2 binding at 4 °C, cells were plated as previously described in a 6-well plate and washed thrice with ice-cold PBS prior to incubation at 4 °C for 30 min in 0.1 % BSA with or without heparin (500 µg/ml). After the incubation period, [³⁵S]ADAMTS4-2 (1.5 nM) was added to the cells and incubated at 4 °C. After 2 h incubation, the medium was removed and the cells were washed once with ice-cold PBS. The cells were treated with 0.1 % pronase for 10 min on ice and the surface-bound (pronase-sensitive) fraction was separated from the intracellular (pronase-resistant) fraction, as previously described. The amount of radioactivity in each fraction was determined by scintillation counting. Figure 35A shows that heparin inhibited the binding of [³⁵S]ADAMTS4-2 to the cell surface by 63 %. Figure 35B shows that when incubated at 4 °C, the amount of radioactivity detectable intracellularly was negligible. The recovery of the total number of counts from either control or heparin treatment were similar.

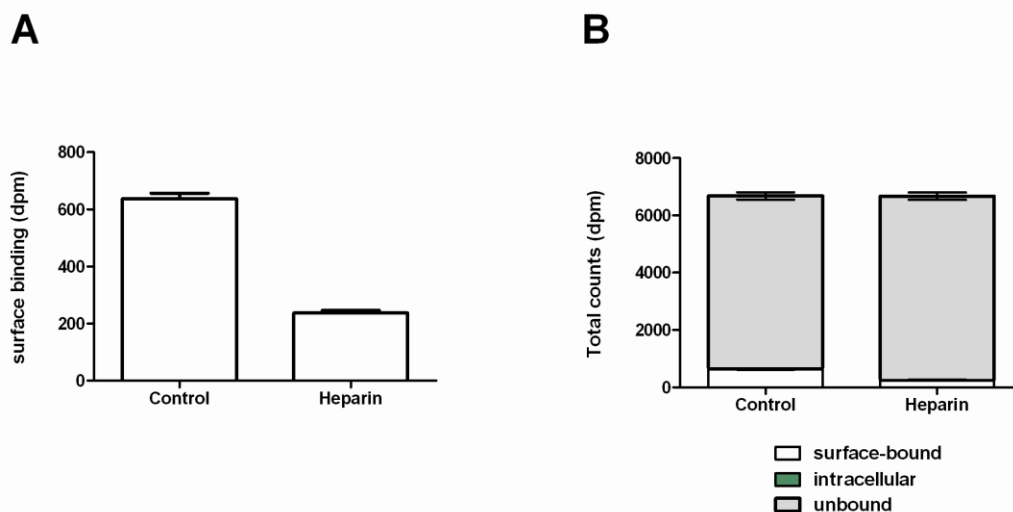


Figure 35: Heparin inhibits surface-binding of [³⁵S]ADAMTS4-2 at 4 °C.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 4 °C in DMEM containing 0.1 % BSA with or without heparin (500 µg/ml). After 30 min, [³⁵S]ADAMTS4-2 (1.5 nM) was added. After incubation for 2 h at 4 °C the medium was removed and the surface-bound, intracellular, wash and unbound fractions were harvested as detailed in section 2.5.2. **A**, surface-bound fraction. **B**, Total surface-bound, intracellular and unbound counts. The wash fractions were counted and found to be negligible. Results are an average of 2 wells $\pm$ range.

4.2.10.3. Heparin dose-dependently inhibits [³⁵S]ADAMTS4-2 clearance

To investigate the effect of heparin on [³⁵S]ADAMTS4-2 clearance in further detail, a dose-response experiment was performed using 0 – 500 µg/ml heparin. Figure 36 shows that heparin resulted in a dose-dependent decrease in both intracellular and surface-bound [³⁵S]ADAMTS4-2. Additionally, treatment of HTB94 cells with heparin dose-dependently inhibited the clearance of [³⁵S]ADAMTS4-2 and the appearance of the TCA-soluble fraction. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments. These data are consistent with the Western blot data presented in Chapter 3.

4.2.10.4. The effect of other GAGs on [³⁵S]ADAMTS4-2 clearance

To determine whether other GAGs aside from heparin were protective against [³⁵S]ADAMTS4-2 clearance, HTB94 cells were treated with a variety of GAGs at a concentration of 250 µg/ml for 1 h prior to the addition of [³⁵S]ADAMTS4-2 in a 24 h assay.

Figure 37 shows that CaPPS, a calcium salt form of chemically sulfated beechwood xylosan, inhibited the appearance of the TCA-soluble fraction as effectively as heparin (>80 %), whereas dermatan sulfate inhibited by 65 % and both chondroitin sulfate and de-N-sulfated heparin inhibited by 30 %. Hyaluronan had no marked effect on the clearance of [³⁵S]ADAMTS4-2 from the medium. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments. These data are consistent with the Western blotting data shown in Chapter 3.

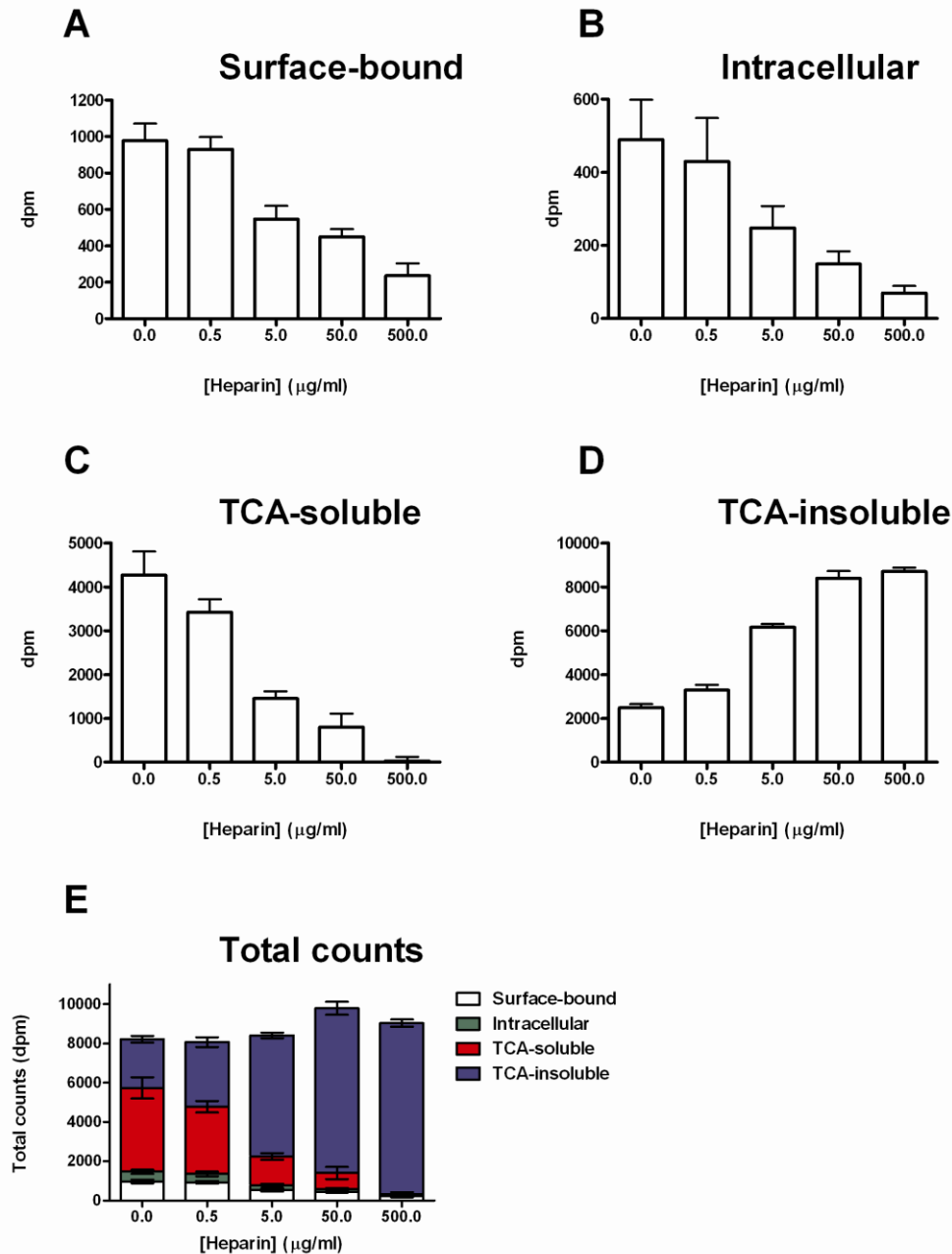


Figure 36: Heparin dose-dependently inhibits [^{35} S]ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (0.5 – 500 μg/ml). After 1 h, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

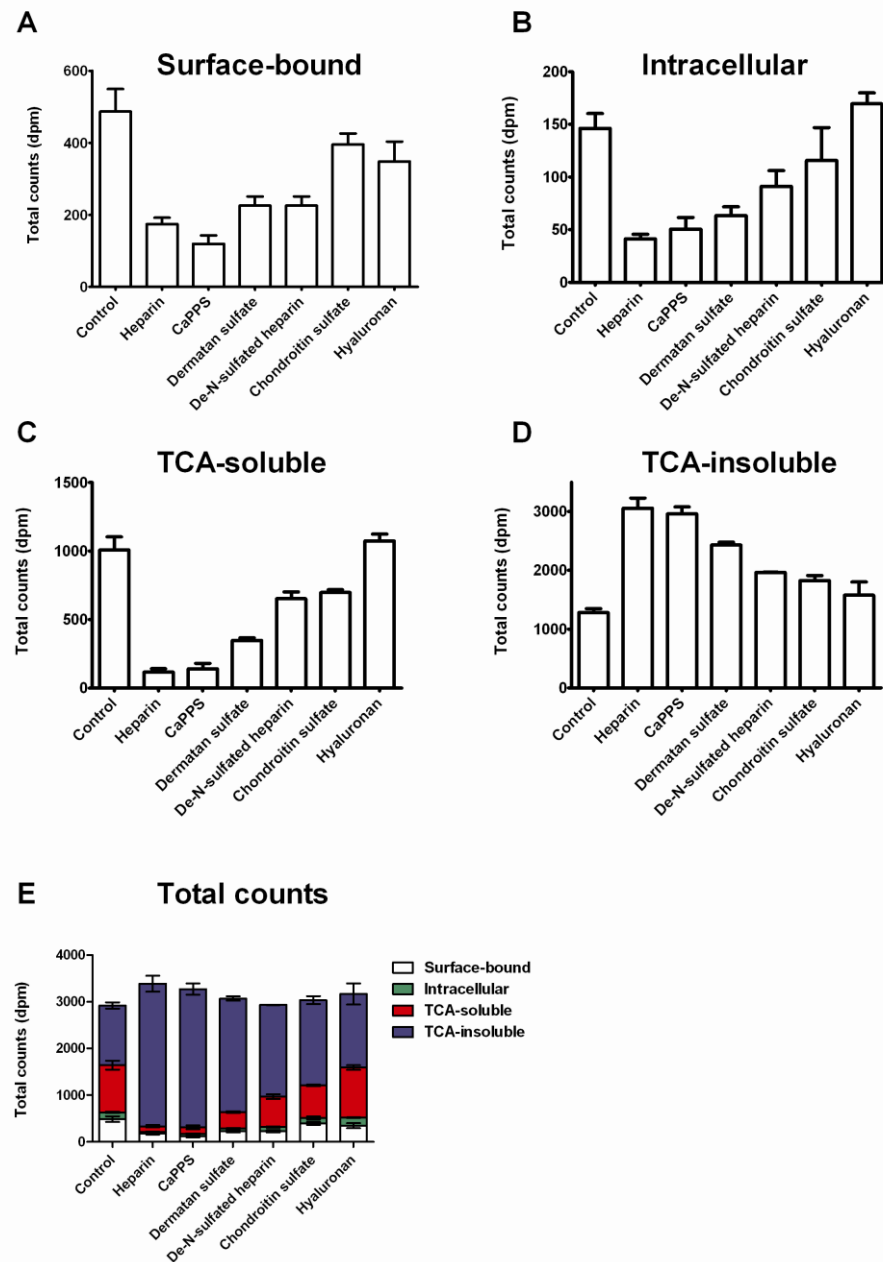


Figure 37: The effect of other GAGs on [^{35} S]ADAMTS4-2 clearance by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without various GAGs (250 μ g/ml). After 1 h, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.2.11. Heparin inhibits [³⁵S]ADAMTS4-2 clearance by porcine and human chondrocytes

The effect of heparin on the clearance of [³⁵S]ADAMTS4-2 by either porcine or human chondrocytes was also investigated. Similar to its effect in HTB94 cells, heparin protected against [³⁵S]ADAMTS4-2 clearance in both porcine and human chondrocytes (Figure 38 and Figure 39, respectively). The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments.

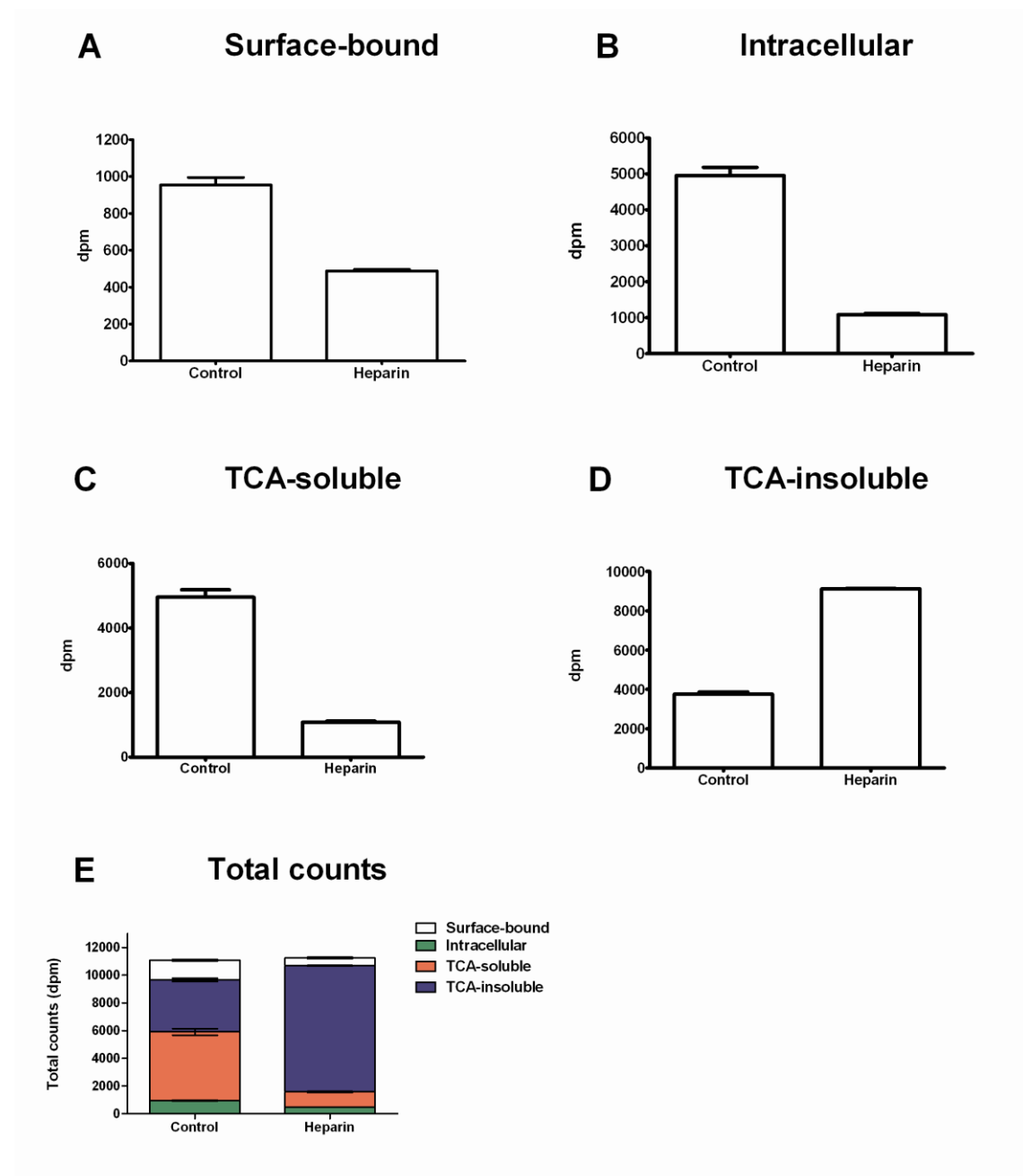


Figure 38: Heparin inhibits [^{35} S]ADAMTS4-2 clearance by porcine chondrocytes. Porcine chondrocytes were seeded in a 12-well plate at a density of 1×10^6 cells, rested for 3 days and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml). After 1 h, [^{35} S]ADAMTS4-2 (0.75 nM) was added. After 24 h, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

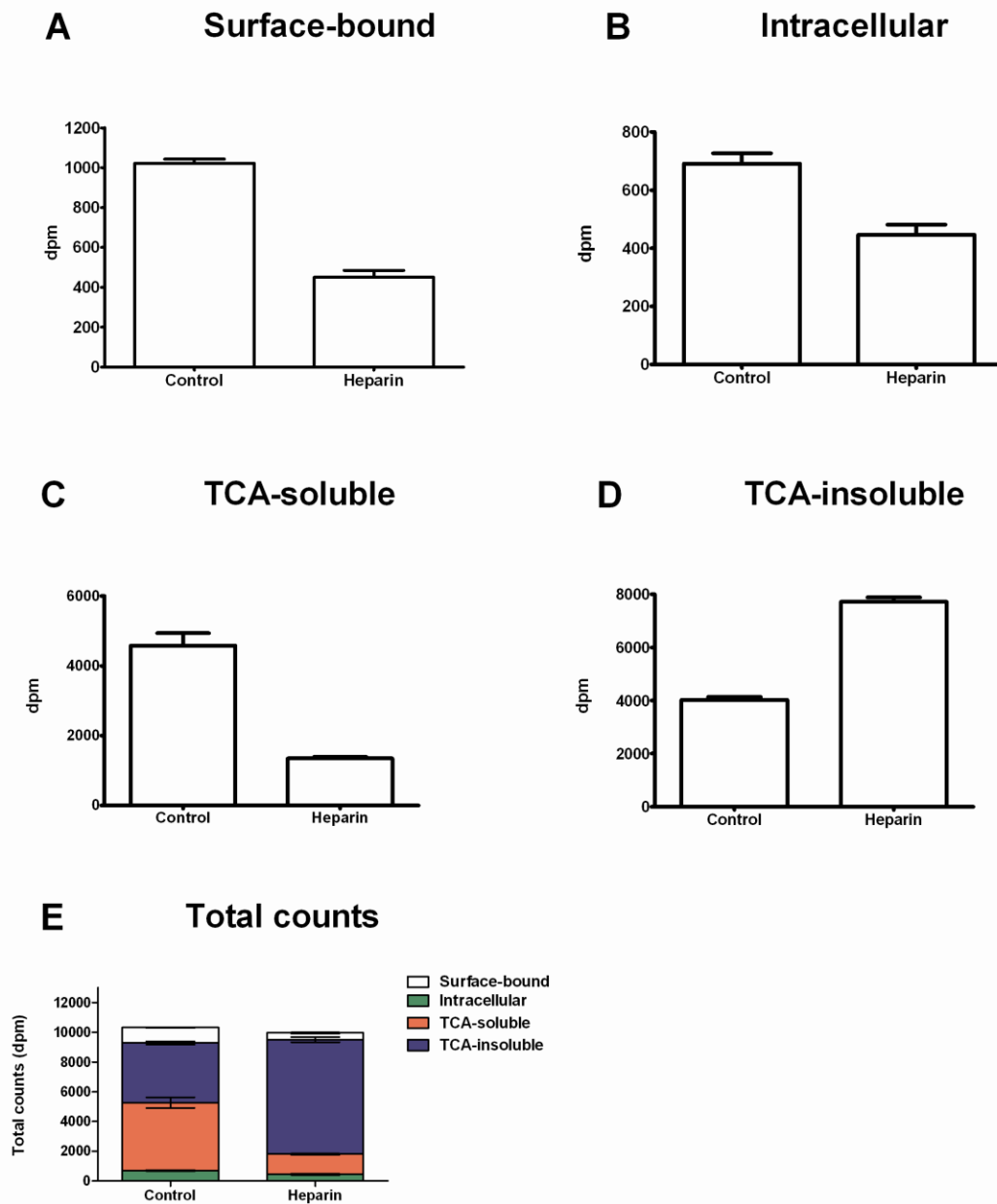


Figure 39: Heparin inhibits [^{35} S]ADAMTS4-2 clearance by human chondrocytes. Human chondrocytes from a healthy 12 year old male were seeded in a 12-well plate at a density of 0.5×10^6 cells, rested for 3 days and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml). After 1 h, [^{35} S]ADAMTS4-2 (0.75 nM) was added. After 24 h, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.2.12. Assessment of [³⁵S]ADAMTS4-2 clearance by CHO-K1 cells and pgsA-745 CHO cells

To investigate whether the cellular uptake of [³⁵S]ADAMTS4-2 required the presence of cell surface GAGs, the clearance of [³⁵S]ADAMTS4-2 from the medium of CHO-K1 cells and pgsA-745 CHO cells was assessed. pgsA-745 CHO cells are deficient in xylosyltransferase, the enzyme which catalyses the attachment of xylose to a serine residue of a core protein. As a result, pgsA-745 CHO cells are deficient in both CS- and HS- proteoglycans (Esko *et al.*, 1985).

4.2.12.1. Surface binding of [³⁵S]ADAMTS4-2 at 4 °C

In a binding experiment similar to that described in section 4.2.10.2., CHO-K1 cells and pgsA-745 CHO cells were incubated for 30 min at 4 °C with or without heparin (250 µg/ml) prior to the addition of [³⁵S]ADAMTS4-2 (1.5 nM) for 2 h at 4 °C. The data in Figure 40 show that under these conditions, CHO-K1 cells bound approximately three times the amount of [³⁵S]ADAMTS4-2 than pgsA-745 CHO cells. Surface binding of [³⁵S]ADAMTS4-2 to CHO-K1 cells but not pgsA-745 CHO cells was sensitive to heparin and incubation with heparin reduced the surface binding in CHO-K1 cells to the background level seen in pgsA-745 CHO cells.

4.2.12.2. [³⁵S]ADAMTS4-2 clearance at 37 °C

To determine the effect of HSPGs on [³⁵S]ADAMTS4-2 cellular uptake and degradation, CHO-K1 and pgsA-745 CHO cells were incubated with [³⁵S]ADAMTS4-2 at 37 °C for 24 h. The surface-bound fraction from both CHO-K1 cells and pgsA-745 cells were similar in the absence of heparin (Figure 41). In the presence of heparin, surface-bound radioactivity was also reduced in both cell types. However, the intracellular fraction of radioactivity in pgsA-745 CHO cells was approximately 80 % less than that of CHO-K1 cells and heparin reduced the intracellular fraction in CHO-K1 cells by approximately 70 % but had no effect on the intracellular fraction of pgsA-745 CHO cells. Heparin reduced

the TCA-soluble fraction in CHO-K1 cells by 50 % compared to control. This resulted in a similar level of counts as was detected from the TCA-soluble fraction harvested from psgA-745 CHO cells. Heparin treatment had no marked effect on the TCA-soluble fraction of psgA-745 CHO cells. In CHO-K1 cells, heparin increased the TCA-insoluble fraction compared to untreated CHO-K1 cells. In addition, the TCA-insoluble fraction was increased in psgA-745 CHO cells compared to CHO-K1 cells. However, the TCA-insoluble fraction harvested from heparin-treated psgA-745 CHO cells was not significantly different to the TCA-insoluble fraction of untreated CHO-K1 cells, although there was a trend towards an increase. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments.

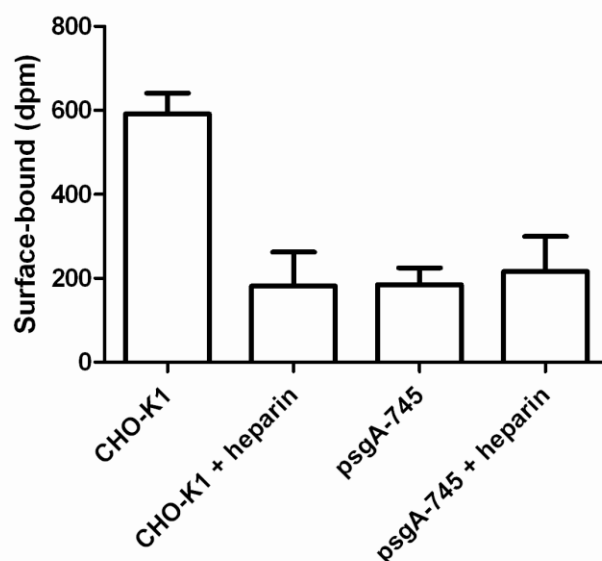


Figure 40: [^{35} S]ADAMTS4-2 binding to the surface of CHO-K1 cells and psgA-745 CHO cells.

Cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free Ham's F12 medium before incubation at 4 °C in Ham's F12 medium containing 0.1 % BSA with or without heparin (250 μ g/ml). After 30 min, [^{35} S]ADAMTS4-2 (1.5 nM) was added. The cells were incubated for 2 h at 4 °C. The medium was removed and the cells were washed with ice-cold PBS. The cell layer was harvested with 1 M NaOH and the radioactivity counted. Results are an average of 3 wells $\pm$ S.D.

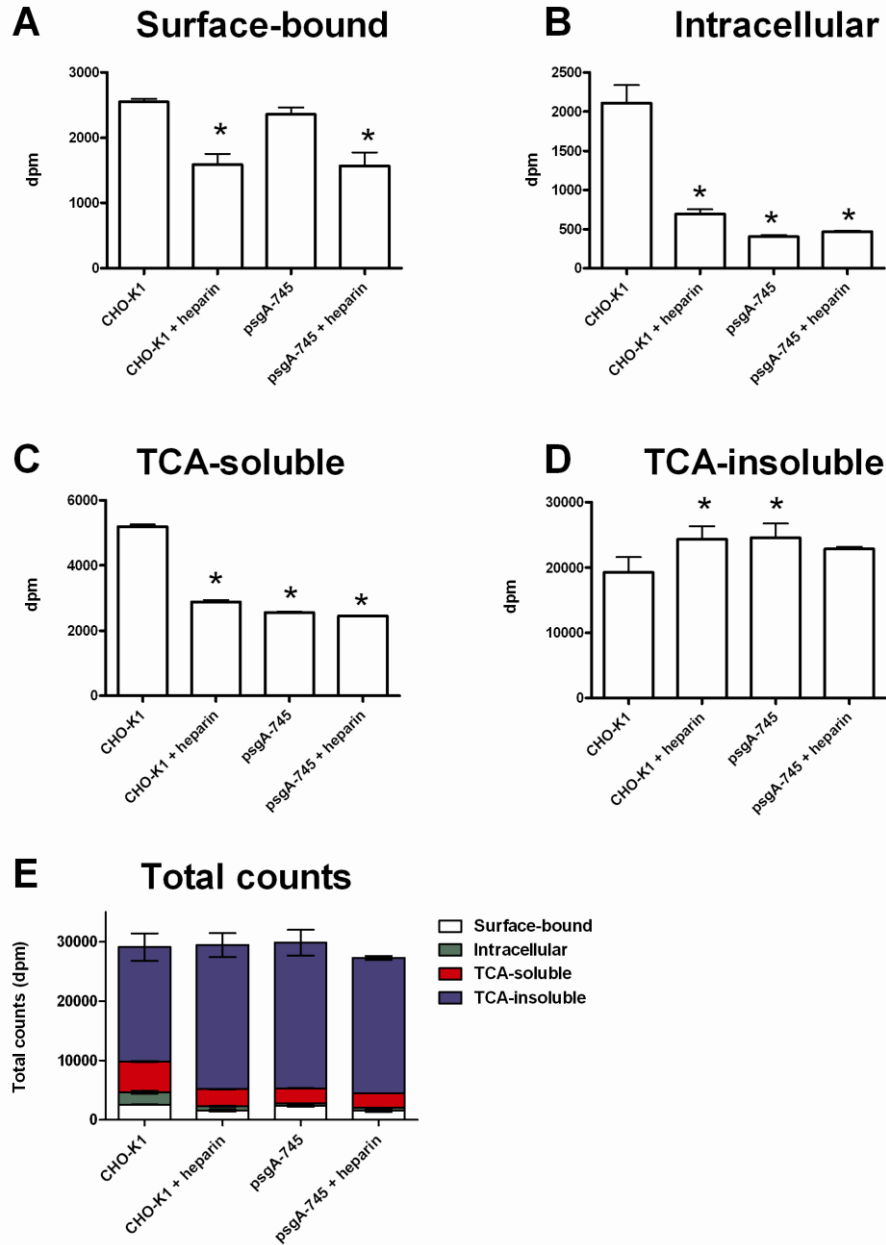


Figure 41: $[^{35}\text{S}]\text{ADAMTS4-2}$ uptake and degradation by CHO-K1 and psgA-745 CHO cells.

Cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free Ham's F12 medium before incubation at 37°C in Ham's F12 medium containing 0.1 % BSA with or without heparin (250 $\mu\text{g}/\text{ml}$). After 1 h, $[^{35}\text{S}]\text{ADAMTS4-2}$ (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. * = $P \leq 0.05$ as compared to CHO-K1 cells. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts. **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.2.13. Uptake and degradation of [³⁵S]ADAMTS4-5 by HTB94 cells

4.2.13.1 Purification of [³⁵S]ADAMTS4-5

To determine whether the ancillary domains of ADAMTS-4 were required for ADAMTS-4 clearance, the catalytic domain of ADAMTS-4 (ADAMTS4-5) was tested for its ability to clear from the medium of HTB94 cells. HEK-293/EBNA cells expressing ADAMTS4-5 were metabolically labeled with [³⁵S]Met/[³⁵S]Cys as described in section 4.2.1. [³⁵S]ADAMTS4-5 was purified from the conditioned medium by anti-FLAG M2 affinity chromatography and FLAG peptide elution. Aliquots of each sample were analysed by SDS-PAGE and silver staining (Figure 42A). The gel was also exposed to autoradiographic film (Figure 42B). [³⁵S]ADAMTS4-5 was collected in elutions 1 & 2, running at a molecular weight slightly higher than the predicted molecular weight of 24 kDa (Figure 42B). This observation is in agreement with Gendron *et al* (2007). In addition, there was a protein band at approximately 50 kDa and at the top of the gel, although the band at the top of the gel appeared to be non-radioactive. ADAMTS4-5 has essentially no aggrecanase activity (Kashiwagi *et al.*, 2004). Therefore, to determine the approximate concentration of [³⁵S]ADAMTS4-5, the amount of protein was determined by standard curve against BSA. Figure 42C shows a silver stain of increasing amounts of BSA (ng) and an aliquot of [³⁵S]ADAMTS4-5 (5 µl). The gel was scanned and the pixel volume of each band was determined using Phoretix software. Figure 42D shows a standard curve of pixel volume of the BSA band against amount of BSA (ng) added. Using the equation $y = mx + c$, the concentration of [³⁵S]ADAMTS4-5 was calculated to be 50 nM (1.2 µg/ml) based on a molecular weight of 24 kDa.

4.2.13.2. [³⁵S]ADAMTS4-5 uptake by HTB94 cells

When added to HTB94 cells, [³⁵S]ADAMTS4-5 (1.5 nM) was cleared from the medium over 24 h (Figure 43). Similar to their effect on [³⁵S]ADAMTS4-2 (1.5 nM), heparin and GM6001 reduced the amount of radioactivity associated with

the surface-bound and intracellular fractions and also inhibited the appearance of the TCA-soluble fraction by >90 % and 50 %, respectively.

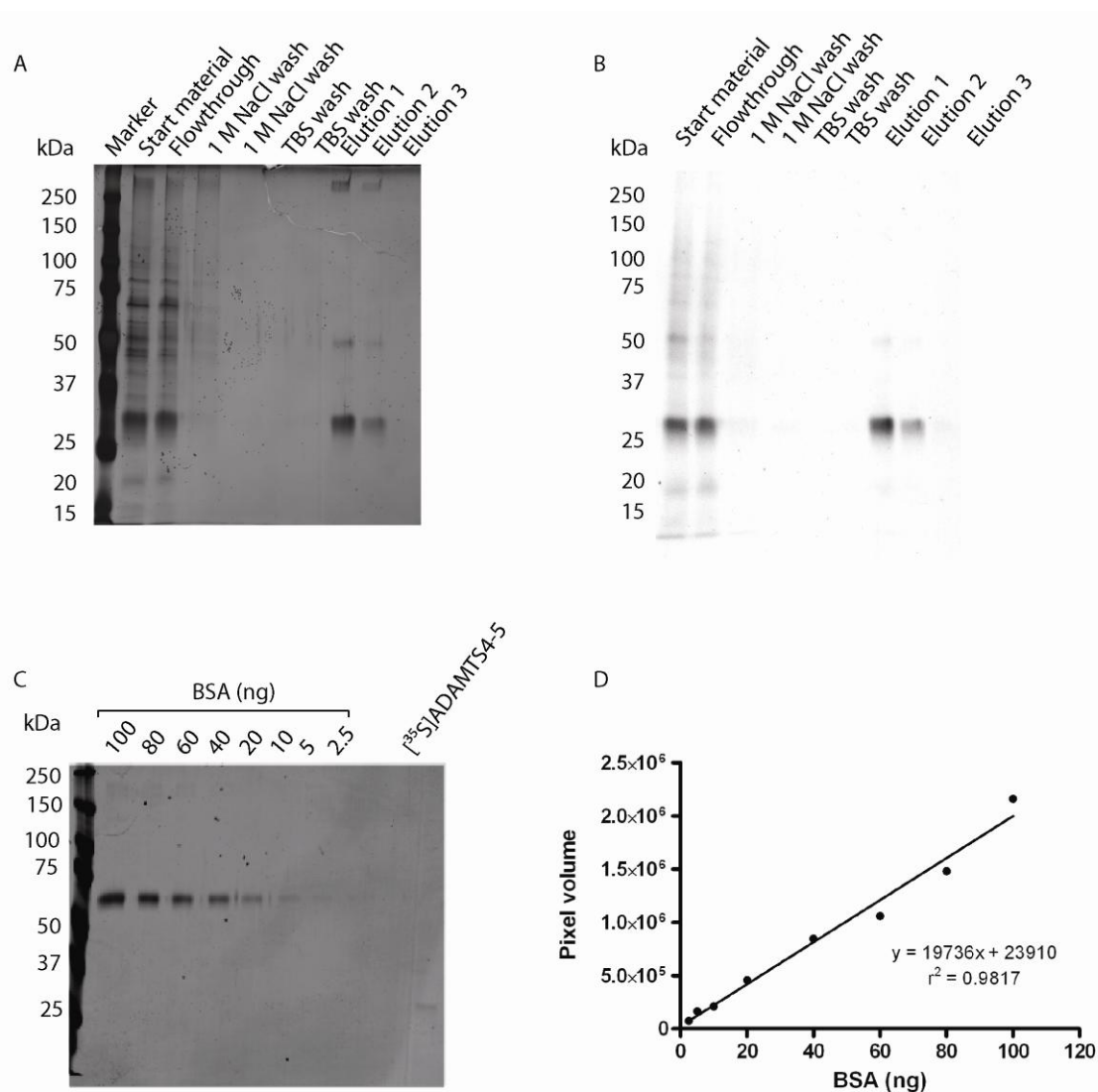


Figure 42: Purification of $[^{35}\text{S}]\text{ADAMTS4-5}$ and determination of concentration using BSA standard curve.

HEK-293/EBNA cells transfected with ADAMTS4-5-FLAG construct were metabolically labeled with $[^{35}\text{S}]\text{Met}/[^{35}\text{S}]\text{Cys}$ for 4 days and purified as described in section 4.2.1. **A**, Aliquots were analysed by reducing SDS-PAGE and silver staining. **B**, The gel was exposed to autoradiographic film overnight. **C**, The amount of $[^{35}\text{S}]\text{ADAMTS4-5}$ in elution 1 was determined by SDS-PAGE analysis of 5 μl of elution 1 and known amounts of BSA (100-2.5 ng). The gel was developed by silver staining and scanned. The pixel volume of each band was determined using Phoretix software. **D**, Standard curve of pixel volume of $[^{35}\text{S}]\text{ADAMTS4-5}$ band against amount of BSA (ng) added.

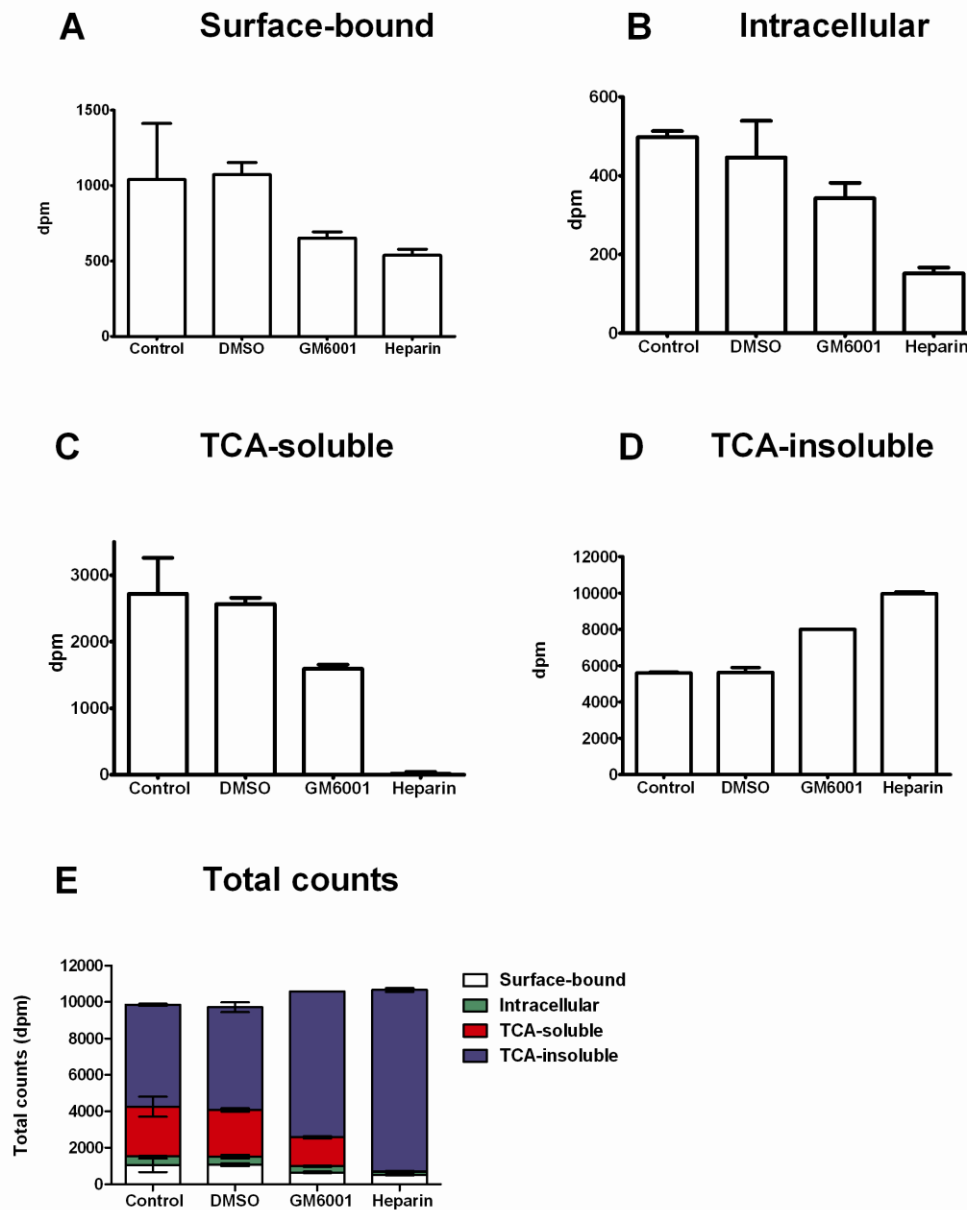


Figure 43: The effect of GM6001 and heparin on the clearance of [^{35} S]ADAMTS4-5 by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, rested overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without either GM6001 (10 μ M), heparin (250 μ g/ml) or 1/1000 dilution of DMSO carrier solution. After 1 h, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and the surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.2.14. [³⁵S]ADAMTS4-2 clearance by wild-type and *Timp-3*-null dermal fibroblasts

In view of the results of Chapter 3, where ADAMTS4-2 did not bind heparin-Sepharose, I considered the hypothesis that ADAMTS4-2 could be internalized in a complex with TIMP-3 and that GM6001 inhibited ADAMTS4-2 clearance by disrupting the interaction between TIMP-3 and ADAMTS4-2. In addition, heparin may inhibit ADAMTS4-2 clearance by blocking the interaction between TIMP-3 and GAGs of the ECM. Wild-type and *Timp-3*-null dermal fibroblasts were incubated for 1 h with GM6001 (50 μ M) or heparin (250 μ g/ml) prior to the addition of [³⁵S]ADAMTS4-2 (1.5 nM) for 24 h. The TCA-insoluble fraction at the start of the experiment (0 h) was 5378 dpm $\pm$ 244. As shown in Figure 44, very little [³⁵S]ADAMTS4-2 was cleared from the medium of either wild-type or *Timp-3*-null mouse dermal fibroblasts over 24 h. Additionally, the amount of TCA-soluble radioactivity detected in the medium after 24 h was comparable between wild-type and *Timp-3*-null fibroblasts. Heparin reduced the TCA-soluble fraction by more than 95 % in either cell type. GM6001 reduced the TCA-soluble fraction by 68 % $\pm$ 7 in wild-type cells and by 40 % $\pm$ 10 in *Timp-3*-null cells. The cell-associated fraction was increased in control wild-type cells compared to control *Timp-3*-null cells. Heparin and GM6001 reduced the amount of cell-associated radioactivity in wild-type fibroblasts but had little effect on the cell-associated fraction from *Timp-3*-null fibroblasts. The recovery of the total number of counts from the cell-associated, TCA-soluble and TCA-insoluble counts was similar across all treatments.

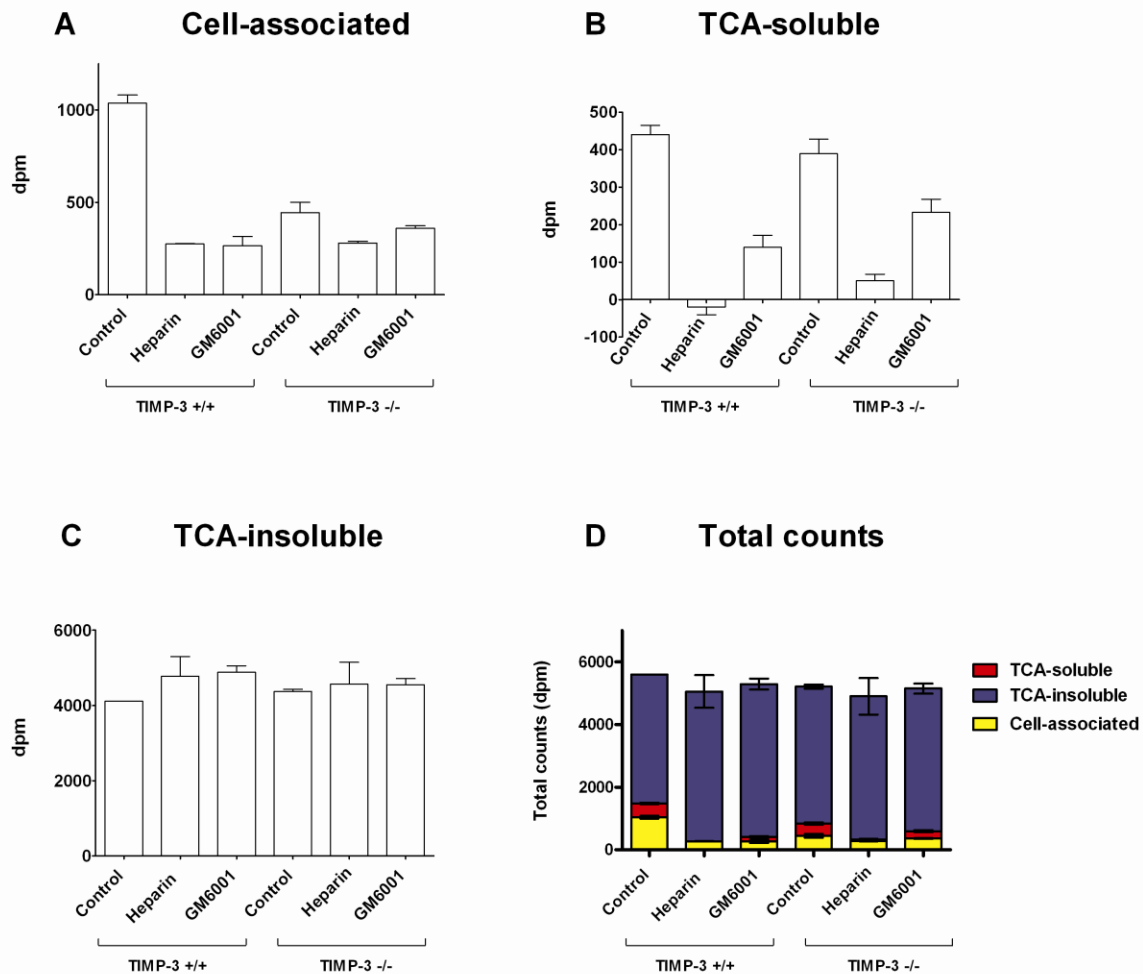


Figure 44: [^{35} S]ADAMTS4-2 clearance by *Timp-3* wild-type (+/+) and null (-/-) mouse dermal fibroblasts.

Cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA and 0.5 % DMSO with or without heparin (250 μ g/ml) or GM6001 (50 μ M). After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and cell-associated, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Cell-associated counts. **B**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **C**, TCA-insoluble counts. **D**, Total cell-associated, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

4.3. Discussion

This chapter describes the development of a radioactive protein-based assay and its use to investigate the clearance of ADAMTS4-2 by HTB94 cells and

chondrocytes. A radioactive protein-based approach offers increased sensitivity over the Western blotting methods described in Chapter 3 and also gives a quantitative read-out.

Time course experiments using [^{35}S]ADAMTS4-2 verified the Western blotting data presented in Chapter 3 because the TCA-insoluble fraction of [^{35}S]ADAMTS4-2 disappeared from the medium upon incubation with HTB94 cells over time. Moreover, radioactivity was detected on the surface and within the cell, supporting the hypothesis that ADAMTS4-2 binds to the cell surface and is endocytosed by HTB94 cells. These data are in contrast with the microscopy data presented in Chapter 3, where although Alexa Fluor 488-ADAMTS4-2 was detected within the cell, it was not detected on the cell surface. The possible reason for not detecting Alexa Fluor 488-ADAMTS4-2 on the cell surface may be due to reduced sensitivity of fluorescence detection as discussed in Chapter 3. However, along with a decrease in the TCA-insoluble fraction over time, there was an increase in the TCA-soluble fraction of the medium upon addition of [^{35}S]ADAMTS4-2. This observation is consistent with the hypothesis that degraded remnants of [^{35}S]ADAMTS4-2 are released into the medium following endocytosis and degradation within the cell.

When a range of [^{35}S]ADAMTS4-2 concentrations were added to HTB94 cells, the TCA-insoluble [^{35}S]ADAMTS4-2 disappeared from the medium at the same rate, regardless of the concentration. This shows that the mechanism by which [^{35}S]ADAMTS4-2 was cleared from the medium was non-saturable under these conditions. This observation is not consistent with a receptor-mediated endocytic process because at high concentrations of ligand, it would be expected that all endocytic receptors become occupied and the clearance of ligand would be dependent on the turnover of the receptor and/or its level of expression on the cell surface. Non-saturable binding of thrombospondin to CHO cells defective in GAG synthesis has been previously described (Murphy-Ullrich *et al.*, 1988). In this study, the authors hypothesised that there may be a second binding phase

that could be saturated when higher concentrations of ligand are added or that binding is non-saturable due to thrombospondin self-aggregation. To investigate the non-saturable binding of [³⁵S]ADAMTS4-2 further, the concentration of [³⁵S]ADAMTS4-2 added to the cells should be increased by at least 100-fold. However, this was not feasible in the present study due to limited reagents. Furthermore, although, there have been no reports that ADAMTS-4 can self-aggregate, ADAMTS4-2 contains a thrombospondin type I-like domain. The thrombospondin type 1-like domain of ADAMTS-4 may mediate adhesion to the cell membrane and/or the ECM, as has been shown for ADAMTS-1 (Kuno *et al.*, 1999).

The binding experiments at 4 °C showed that non-radiolabeled ADAMTS4-2 dose-dependently competed with [³⁵S]ADAMTS4-2 for binding to HTB94 cells. Non-specific binding determined by addition of 100-fold excess of ADAMTS4-2 was less than 20 %. The addition of 10,000-fold excess BSA did not compete with [³⁵S]ADAMTS4-2 binding, showing that the binding to the cell surface was specific.

The Western blotting data presented in Chapter 3 showed that the metalloproteinase inhibitor, GM6001 inhibited ADAMTS4-2 disappearance from the medium of HTB94 cells whilst serine and cysteine protease inhibitors had no effect. In this chapter, the effect of these protease inhibitors was confirmed using [³⁵S]ADAMTS4-2. In addition to GM6001, another metalloproteinase inhibitor CT1746 dose-dependently inhibited the clearance of [³⁵S]ADAMTS4-2, further suggesting that cell surface metalloproteinases may degrade [³⁵S]ADAMTS4-2. However, [³⁵S]ADAMTS4-2 proteolysis would likely fragment the intact protein into smaller TCA-insoluble fragments and no fragments of ADAMTS-4 were detected when the TCA-insoluble fraction of the medium was analysed by Western blotting using the anti-ADAMTS4-catalytic antibody (Chapter 3). Proteolysis within the catalytic domain could destroy the antibody binding epitope, resulting in the failure to detect any proteolysed fragments. To further

investigate the possibility of [³⁵S]ADAMTS4-2 fragmentation, it would be informative to analyse the TCA-insoluble fraction of the medium by autoradiography after SDS-PAGE.

Since the effect of metalloproteinase inhibitors on the clearance of [³⁵S]ADAMTS4-2 by HTB94 cells suggests that some cell surface metalloproteinase-mediated degradation of [³⁵S]ADAMTS4-2 may occur, future work would be to identify which cell surface metalloproteinases are expressed by HTB94 cells. The expression of these metalloproteinases could be knocked down using siRNA and the effect of each metalloprotease on the clearance of [³⁵S]ADAMTS4-2 assessed. In this regard, MMP-17 is reportedly expressed in JJ012 chondrosarcoma cells and has been shown to cleave full-length ADAMTS-4 (68 kDa), generating a protein with a molecular weight of 40 kDa in addition to protein of 53 kDa in size (Gao *et al.*, 2004). This suggests that MMP-17 is a candidate metalloproteinase responsible for cell surface cleavage of ADAMTS4-2 into a 40 kDa protein.

Another approach would be to assess the clearance of [³⁵S]ADAMTS4-2 in the presence of 100-fold excess of either TIMP-1, TIMP-2 or TIMP-3. This would narrow down a candidate metalloproteinase as MT-MMPs are inhibited by TIMP-2, not TIMP-1 and only TIMP-3 inhibits ADAMTSs. However, it may be difficult to interpret the results because HTB94 cells express LRP-1 and both TIMP-1 and TIMP-2 are endocytosed via LRP-1 (Emonard *et al.*, 2004; Hahn-Dantona *et al.*, 2001). Additionally, TIMP-3 also accumulates in the medium of HTB94 cells when treated with heparin or the LDL-R family antagonist RAP (Troeberg *et al.*, 2008). However, in HTB94 cells, heparin and CaPPS did not affect the levels of TIMP-1 or TIMP-2 in the medium (Troeberg *et al.*, 2008) suggesting that at least in HTB94 cells TIMP-1 and TIMP-2 are not endocytosed by a heparin-sensitive mechanism.

The effect of cytochalasin D, which block endocytosis by inhibiting actin polymerization (Flanagan and Lin, 1980) was also examined. This treatment reduced the amount of TCA-soluble radioactivity in the medium, suggesting that ADAMTS4-2 is endocytosed by an actin-dependent mechanism. The endocytosis of many ligands requires an intact actin cytoskeleton. For example, cytochalasin D treatment inhibits CD-14-dependent LPS endocytosis in THP-1 cells (Poussin *et al.*, 1998). Additionally, entry of Kaposi's sarcoma-associated herpesvirus (KSHV) into HUVEC cells was blocked by approximately 40 % upon treatment with cytochalasin D (Raghu *et al.*, 2009). In HTB94 cells, cytochalasin D causes TIMP-3 accumulation (Troeberg *et al.*, 2008) which suggests that it blocks actin-dependent endocytosis of TIMP-3 in these cells.

The production of the TCA-soluble fraction was not completely blocked by treatment with cytochalasin D suggesting that other processes aside from actin-dependent endocytosis may mediate ADAMTS4-2 clearance by HTB94 cells. As discussed above, the TCA-soluble radioactivity produced in the presence of cytochalasin D could be due to metalloprotease-mediated breakdown on the cell surface. Further work would be to investigate the effect of cytochalasin D in combination with GM6001 on the clearance of [³⁵S]ADAMTS4-2 by HTB94 cells. Alternatively, a cytochalasin D-independent mechanism of [³⁵S]ADAMTS4-2 endocytosis may exist. A cytochalasin D-independent but tyrosine kinase-dependent mechanism of endocytosis has been reported for LpL-enriched LDL (Fuki *et al.*, 2000a). To further investigate this, a first step would be to determine the effect of the tyrosine kinase inhibitor genistein on the clearance of [³⁵S]ADAMTS4-2 by HTB94 cells.

Another approach would be to determine the effect of lysosomotropic agents such as monensin or chloroquine on the clearance of [³⁵S]ADAMTS4-2 by HTB94 cells and their effect on the amount of intracellular radioactivity. Monensin and chloroquine neutralize the lysosome and inhibit lysosomal activity causing an accumulation of endocytosed ligand within the cell. The

accumulation of [^{35}S]ADAMTS4-2 within the intracellular fraction would support the hypothesis that [^{35}S]ADAMTS4-2 is endocytosed and degraded by lysosomal hydrolases prior to the release of TCA-soluble amino acids from the cell into the medium.

In this chapter, I showed that treatment of HTB94 cells with heparin prior to the addition of [^{35}S]ADAMTS4-2 reduced the surface-bound, intracellular and TCA-soluble fraction and protected against the clearance of [^{35}S]ADAMTS4-2. In addition, binding studies at 4 °C also demonstrated that heparin reduced the binding of [^{35}S]ADAMTS4-2 to the cell surface. This experiment showed that treatment of the cell layer with pronase for 10 min on ice was sufficient to remove cell surface proteins because pronase was able to effectively separate intracellular from surface-bound [^{35}S]ADAMTS4-2. It also showed that incubation at 4 °C prevents intracellular uptake so in binding studies at 4 °C it was not necessary to discriminate between surface-bound and intracellular fractions meaning that the surface-bound radioactivity could be recovered by harvesting the cell layer with 1 M NaOH. The correlation between the amount of radioactivity within the cell and the amount of TCA-soluble radioactivity in the medium in experiments conducted at 37 °C suggests that [^{35}S]ADAMTS4-2 is endocytosed and degraded within the cell.

The investigation of the effect of different GAGs on the clearance of [^{35}S]ADAMTS4-2 by HTB94 cells yielded similar results to that reported by Western blot analysis in Chapter 3. GAGs with low levels of sulfation (chondroitin or dermatan sulfate) or no sulfation (hyaluronan) were not as effective as heparin or CaPPS, both of which are highly sulfated. Furthermore, N-desulfated heparin was less protective than heparin. This indicates that the clearance of [^{35}S]ADAMTS4-2 by HTB94 cells depends on the level of sulfation.

[^{35}S]ADAMTS4-5 was also cleared by HTB94 cells over 24 h. This indicates that the cysteine-rich, thrombospondin type-1 and disintegrin domains of ADAMTS-4

are not essential for ADAMTS-4 clearance by HTB94 cells. In addition to heparin, the metalloprotease inhibitor GM6001 also inhibited the clearance of [³⁵S]ADAMTS4-5. The inhibitory effect of heparin was greater than that of GM6001 for both [³⁵S]ADAMTS4-2 and [³⁵S]ADAMTS4-5, suggesting that the heparin-sensitive mechanism of clearance by HTB94 cells is more dominant than the GM6001-sensitive mechanism. Similar to ADAMTS4-2, ADAMTS4-5 does not bind heparin-coated plates (Dr Linda Troeberg, personal communication). Together, these data indicate that the mechanisms of both [³⁵S]ADAMTS4-2 and [³⁵S]ADAMTS4-5 clearance are the same. However, the rate of clearance may differ between these proteins and should be the subject of future study.

It would also be informative to determine whether full-length ADAMTS-4 is cleared by HTB94 cells. During the course of this study, the purification of [³⁵S]ADAMTS4-1 in a manner similar to that described for [³⁵S]ADAMTS4-2 was attempted several times. However, ADAMTS4-1 loses about 80 % of its FLAG-tag during culture (Kashiwagi *et al.*, 2004) and sufficient quantities of [³⁵S]ADAMTS4-1 were unattainable for use in this study. As discussed in Chapter 3, ADAMTS4-1 binds to the ECM and cell surface via interaction of the spacer domain (Kashiwagi *et al.*, 2004). Therefore, ADAMTS4-1 would likely bind with greater affinity to the ECM and cell surface of HTB94 cells. It would be informative to see whether ADAMTS4-1 clearance by HTB94 cells proceeded at the same rate as ADAMTS4-2. One approach to the successful purification of ADAMTS4-1 may be to purify the protein and then radiolabel the purified protein with ¹²⁵Iodine. Alternatively, a new construct with the FLAG-tag at the N-terminus could be created to determine whether this improves the yield of intact ADAMTS4-1.

In addition, heparin prevented the clearance of [³⁵S]ADAMTS4-2 by both porcine and human chondrocytes, showing that the cellular uptake of [³⁵S]ADAMTS4-2 also occurs in primary cells. It would be of further interest to investigate the rate

of [³⁵S]ADAMTS4-2 clearance by normal chondrocytes compared to OA chondrocytes to determine if the mechanism is altered in disease.

The experiments using CHO-K1 and pgsA-745 CHO cells demonstrated that cell surface HSPGs are required for the internalisation of [³⁵S]ADAMTS4-2 although similar amounts of radioactivity were detected on the surface of both CHO-K1 cells and psgA-745 CHO cells. This could be due to non-specific binding of [³⁵S]ADAMTS4-2 at the cell surface during the 24 h culture. However, the amount of enzyme detected within the cell correlated with the amount of TCA-soluble radioactivity detected in the medium suggesting that HSPGs mediate the binding, internalisation and lysosomal degradation of ADAMTS4-2. These observations support the hypothesis that ADAMTS-4 clearance is dependent on the cellular level of sulfation. Heparin inhibited the appearance of the TCA-soluble fraction of CHO-K1 cells compared to psgA-745 CHO cells. However, heparin treatment did not completely block the appearance of the TCA-soluble fraction in either CHO-K1 cells and psgA-745 CHO cells, suggesting that other mechanisms of degradation may exist. To eliminate the possibility of metalloproteinase-mediated degradation at the cell surface, future work would be to repeat these experiments in the presence of GM6001.

Similar studies have shown that HSPGs mediate the catabolism of thrombospondin by CHO-K1 cells (Murphy-Ullrich *et al.*, 1988). Comparable to the results presented for [³⁵S]ADAMTS4-2, cell surface binding and degradation of thrombospondin by CHO-K1 cells was inhibited by heparin, whereas heparin had little effect in pgsA-745 CHO cells (Murphy-Ullrich *et al.*, 1988). In addition, Yang *et al* showed that LRP-1-mediated endocytosis of MMP2-thrombospondin-2 complex was inhibited by heparin (Yang *et al.*, 2001), suggesting that a HSPG co-receptor is involved. However, it has been suggested that HSPG binding is not necessary for thrombospondin-1 to bind LRP-1 at the cell surface but that subsequent endocytosis requires high affinity heparin binding (Wang *et al.*, 2004b).

To further characterize the dependence of cell surface HSPGs on the mechanism of [³⁵S]ADAMTS4-2 clearance, future studies would also include using other strains of CHO cells defective in GAG synthesis: CHO-761 cells are deficient in galactosyltransferase, CHO-803 cells are defective in heparan sulfate but have normal levels of chondroitin sulfate and CHO-677 cells are defective in heparan sulfate but have elevated levels of chondroitin sulfate (Murphy-Ullrich *et al.*, 1988). Additionally, heparitinase and/or chondroitinase treatment of wild-type CHO-K1 cells prior to the addition of [³⁵S]ADAMTS4-2 would be informative. To further characterize the effect of HSPGs on the clearance of ADAMTS4-2 by HTB94 cells, future work would be to identify which HSPGs are expressed by HTB94 cells. The expression of these HSPGs could be knocked-down using siRNA and the effect of each HSPG on the clearance of [³⁵S]ADAMTS4-2 assessed.

In view of the results of Chapter 3, where ADAMTS4-2 did not bind heparin-Sepharose, I considered the hypothesis that ADAMTS4-2 could be internalized in a complex with TIMP-3. This hypothesis was considered because of reports that the LRP-1 ligands MMP-2 and -9 can be internalized alone or in complex with TIMP-2 or TIMP-1, respectively (Emonard *et al.*, 2004; Hahn-Dantona *et al.*, 2001). I also hypothesised that GM6001 may inhibit endocytosis by disrupting the interaction between TIMP-3 and [³⁵S]ADAMTS4-2. In addition, since TIMP-3 contains heparin-binding domains and binds sulfated GAGs of the ECM (Yu *et al.*, 2000), heparin could inhibit the internalization of [³⁵S]ADAMTS4-2 from the medium by blocking the interaction between TIMP-3 and cell surface HSPGs, with HSPGs acting as co-receptors for TIMP-3 endocytosis. Potential HSPG co-receptor candidates include perlecan, syndecans and glypicans. Perlecan may be the HSPG involved in TIMP-3 binding to the matrix since, in a review article, Knox and Whitelock quote an unpublished study where TIMP-3 reportedly binds perlecan and is localized pericellularly (Knox and Whitelock, 2006). However, preliminary experiments showed that very little [³⁵S]ADAMTS4-2 was cleared by either wild-type or *Timp-3*-null mouse dermal fibroblasts after 24 h. Additionally,

the amount of TCA-soluble radioactivity detected in the medium after 24 h was comparable between wild-type and *Timp-3*-null fibroblasts. Although heparin had a greater inhibitory effect than GM6001, reducing the TCA-soluble fraction by more than 95 %, GM6001 reduced the TCA-soluble fraction by 68 % in wild-type cells and by 40 % in *Timp-3*-null cells. The differential effect of GM6001 between wild-type and *Timp-3*-null cells suggests that a mechanism whereby ADAMTS-4 is endocytosed as a complex with TIMP-3 may exist. The cell-associated fraction was increased in control wild-type cells compared to control *Timp-3*-null cells. Heparin and GM6001 reduced the amount of cell-associated radioactivity in wild-type fibroblasts but had little effect on the cell-associated fraction from *Timp-3*-null fibroblasts. This suggests that [³⁵S]ADAMTS4-2 may interact with cell surface TIMP-3. However, the cells used in this experiment were thawed from liquid nitrogen stocks and were originally isolated from mice which were culled due to infection. The infection may be the reason why very little ADAMTS4-2 was cleared by either wild-type or *Timp-3*-null fibroblasts. To confirm that the results are reproducible, the above experiment needs to be repeated using freshly isolated fibroblasts from healthy mice or in HTB94 cells where TIMP-3 expression is silenced by siRNA.

In summary, this chapter describes the development and use of a radioactivity-based assay to monitor the clearance of ADAMTS-4 by HTB94 cells and chondrocytes. The results indicate that both ADAMTS-4-2 and ADAMTS4-5 are cleared by HTB94 cells, showing that this process is not dependent on sites within the ancillary domains of ADAMTS-4. Degradation at the cell surface may account for some of the observations. However, preliminary studies using *Timp-3*-null fibroblasts have indicated that ADAMTS-4 may be endocytosed in a complex with TIMP-3. It is possible that GM6001 may disrupt this interaction. The clearance of ADAMTS-4 is also heparin-sensitive, indicating that the mechanism requires cell surface HSPGs. In the case of ADAMTS4-2, this requirement has been confirmed because cells deficient in HSPGs accumulated

50 % less TCA-soluble degradation products of [³⁵S]ADAMTS4-2 in the medium than wild-type cells and less radioactivity was detected within the cell.

Numerous studies report the requirement of HSPGs for endocytosis of some LRP-1 ligands. With this in mind, the aim of Chapter 5 is to investigate whether LRP-1 plays a role in the clearance of ADAMTS4-2 by HTB94 cells.

Chapter 5

Investigating the role of LDL-R family in the clearance of ADAMTS4-2

5.1. Introduction

In the previous chapter, a radioactive-based assay was developed and used to characterize the clearance of ADAMTS-4 by HTB94 cells. It was shown that ADAMTS-4 clearance may be due in part to proteolysis at the cell surface. However, the protein was detectable within the cell, suggesting endocytosis may also occur. The cellular uptake and clearance of ADAMTS-4 was also shown to be dependent on cell surface HSPGs and preliminary work indicated that ADAMTS-4 may be endocytosed in a complex with TIMP-3. In view of previous work in our laboratory showing that TIMP-3 accumulates in the conditioned medium of HTB94 cells upon treatment with either heparin or RAP, a ligand antagonist of the LDL-R family (Troeberg *et al.*, 2008), I tested whether ADAMTS-4 clearance was also RAP-sensitive. Therefore, the aims of this chapter were to: i) determine whether RAP inhibits ADAMTS-4 clearance and ii) compare the clearance of ADAMTS-4 to that of methylamine-activated α_2M (α_2M^*), the endocytosis of which is characteristic of LRP-1 (Strickland *et al.*, 1990).

5.2. Results

5.2.1. Assessing the effect of RAP on [³⁵S]ADAMTS4-2 clearance

5.2.1.1. SDS-PAGE analysis of recombinant RAP

Recombinant RAP protein was a kind gift from Dr Hervé Emonard (Christian de Duve Institute of Cellular Pathology and Université Catholique de Louvain, Brussels, Belgium). Before using RAP in cell experiments, the purity of the protein was confirmed by SDS-PAGE and staining with Coomassie Brilliant Blue R-250. As shown in Figure 45, the protein ran at a molecular weight of approximately 39 kDa with minor fragments of a lower molecular weight.

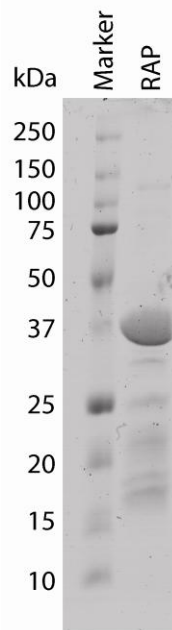


Figure 45: *Purified recombinant RAP.*

Recombinant RAP was a kind gift from Dr Hervé Emonard (Christian de Duve Institute of Cellular Pathology and Université Catholique de Louvain, Brussels, Belgium). RAP (20 μ g) was run on SDS-PAGE (10 % acrylamide) and the protein was stained using Coomassie Brilliant Blue R-250.

5.2.1.2. RAP inhibits [^{35} S]ADAMTS4-2 clearance by HTB94 cells

HTB94 cells were incubated with RAP (500 nM) or heparin (250 μ g/ml) for 1 h before addition of [^{35} S]ADAMTS4-2 (1.5 nM). After 24 h, the fractions were harvested as previously described. Figure 46 shows that incubation with RAP did not significantly affect the amount of radioactivity bound to the cell surface or within the cell. However, the TCA-soluble fraction was reduced by approximately 50 % by RAP. Although the TCA-insoluble fraction showed a slight increase compared to control, this was not statistically significant. In addition, heparin was more protective than RAP. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments.

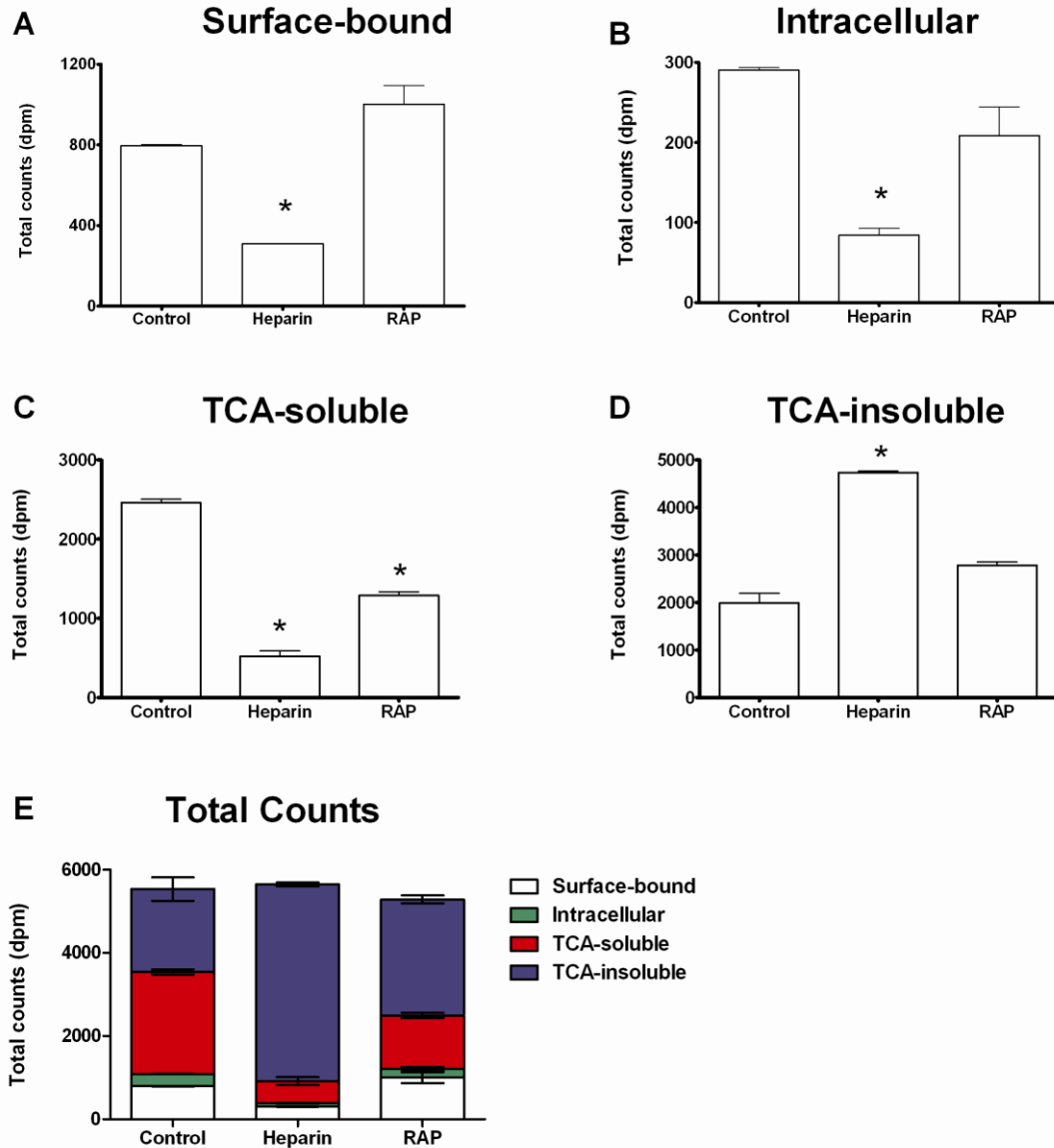


Figure 46: RAP inhibits clearance of [^{35} S]ADAMTS4-2 by HTB94 cells.

Cells were seeded in a 6-well plate at a density of 1×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml) or RAP (500 nM). After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. * = $P \leq 0.05$ as compared to control. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts. **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

5.2.1.3. RAP inhibits clearance of [³⁵S]ADAMTS4-5 by HTB94 cells

In order to determine whether the clearance of [³⁵S]ADAMTS4-5 (catalytic domain) was RAP-sensitive, HTB94 cells were incubated with RAP (500 nM) or heparin (250 µg/ml) for 1 h before addition of [³⁵S]ADAMTS4-5 (1.5 nM) in a 24 h assay. As shown in Figure 47, RAP had no effect on the surface-bound fraction but reduced the intracellular fraction by approximately 40 %. RAP also significantly protected against the clearance of the TCA-insoluble fraction and protected against the appearance of the TCA-soluble fraction by 80 % compared to control. However, the protective effect of RAP was not as pronounced as heparin. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments. These data indicate that the appearance of the TCA-soluble fraction upon incubation of HTB94 cells with either ADAMTS4-2 or ADAMTS4-5 was inhibited by RAP. However, the amount by which RAP protected against the appearance of the TCA-soluble fraction differed between experiments.

5.2.1.4. RAP does not inhibit surface binding of [³⁵S]ADAMTS4-2 at 4 °C

To determine whether RAP affected the binding of [³⁵S]ADAMTS4-2 to the surface of HTB94 cells, cells were equilibrated at 4 °C for 30 min with either heparin (500 µg/ml) or RAP (500 nM) before addition of [³⁵S]ADAMTS4-2 (1.5 nM) for 2 h. The surface bound fraction was determined as described in section 2.5.2. Figure 48 shows that whilst incubation with heparin reduced the binding of [³⁵S]ADAMTS4-2 to the cell surface by 60 %, RAP had no effect.

5.2.1.5. Viability of HTB94 cells in the presence of RAP

Figure 49 shows that treatment of HTB94 cells with RAP (500 nM) for 24 h did not affect cell viability as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. As a positive control for cell death, treatment with sodium nitroprusside (2mM) inhibited cell viability by 93 %.

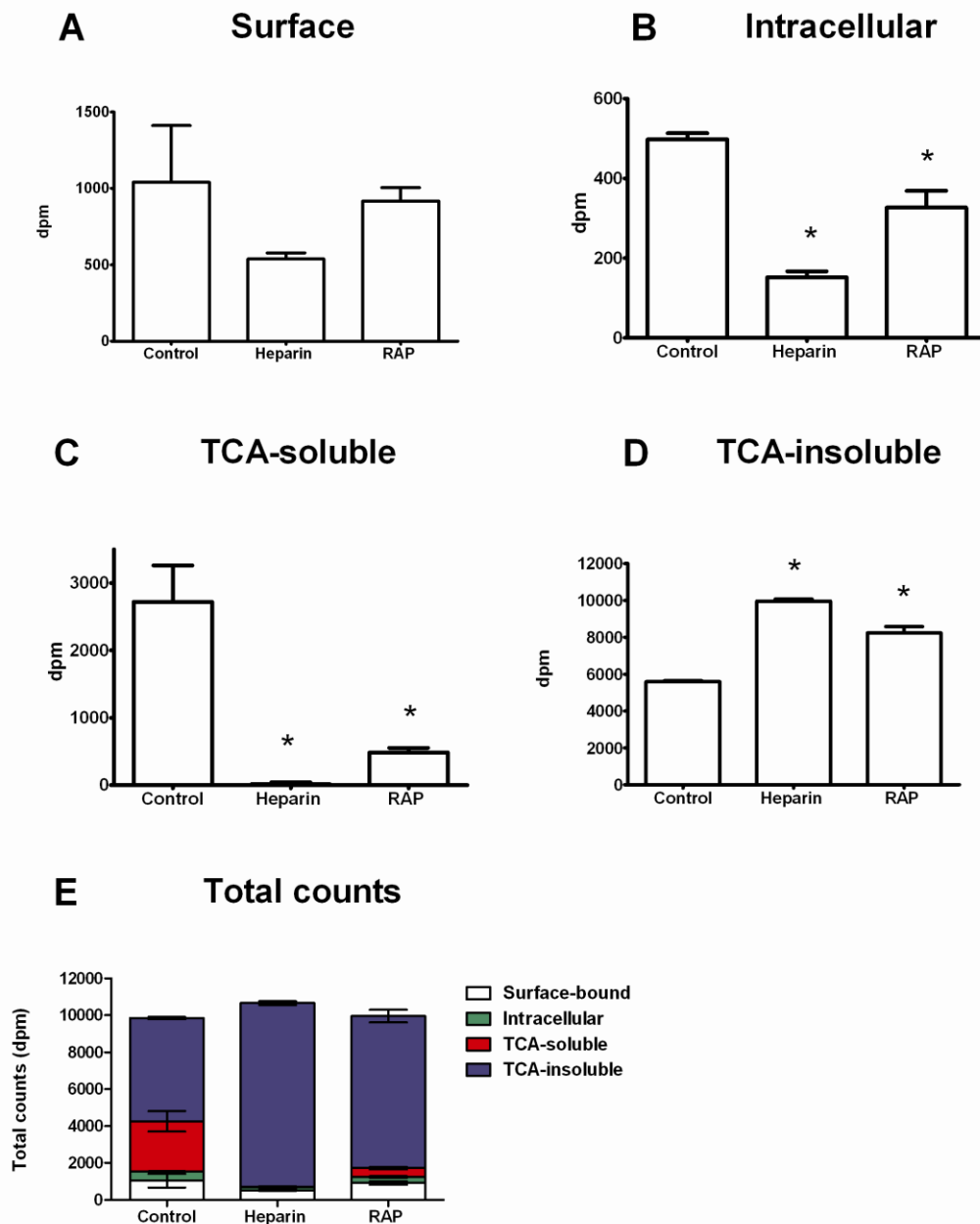


Figure 47: RAP inhibits clearance of [^{35} S]ADAMTS4-5 by HTB94 cells.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml) or RAP (500 nM). After 1 hour, [^{35} S]ADAMTS4-5 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. * = $P \leq 0.05$ as compared to control. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, wash, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

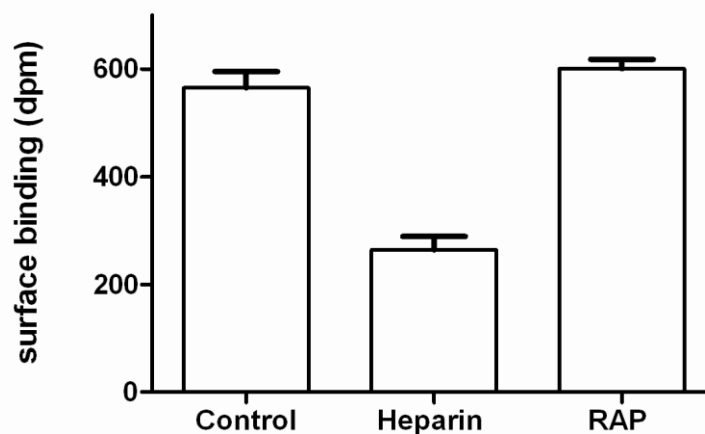


Figure 48: RAP does not inhibit surface binding of [^{35} S]ADAMTS4-2 at 4 °C.

HTB94 cells were seeded in a 6-well plate at a density of 1×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 4 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml) or RAP (500 nM). After 30 min, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After incubation for 2 h at 4 °C the medium was removed and the surface bound fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range.

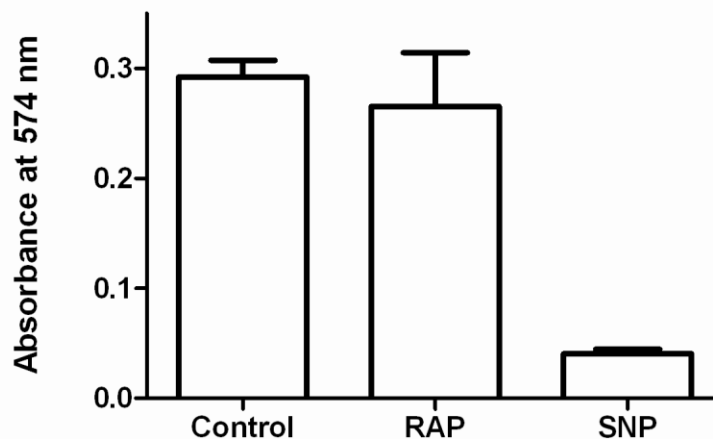


Figure 49: MTT assay of HTB94 cell viability upon incubation with RAP.

HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and then treated with RAP (500 nM) or sodium nitroprusside (SNP) (2 mM) for 24 h. The medium was removed and the cells were washed once with phenol red-free DMEM before incubation with 975 μ l phenol red-free DMEM and 25 μ l of MTT reagent (5 mg/ml in PBS). After 45 min, 1 ml of 10 % SDS in 10 mM HCl was added to solubilise the formazan crystals. The samples were incubated overnight at 37 °C and 200 μ l of each well was transferred to a 96-well plate. The absorbance was read at 574 nm. Results are an average of triplicate wells $\pm$ S.D.

5.2.1.6. RAP inhibits clearance of [³⁵S]ADAMTS4-2 by porcine and human chondrocytes

Whilst heparin reduced the surface-bound and intracellular fractions isolated from porcine chondrocytes, in this experiment, RAP significantly increased both the surface bound fraction and intracellular fraction (Figure 50). However, RAP protected against the clearance of the TCA-insoluble fraction of [³⁵S]ADAMTS4-2 and the appearance of the TCA-soluble fraction was reduced by 60 % compared to control. Similar to its effect on porcine chondrocytes, RAP inhibited the clearance of the TCA-insoluble fraction of [³⁵S]ADAMTS4-2 from the medium of chondrocytes isolated from a young normal human donor (male, 12-year old) (Figure 51). RAP also inhibited the appearance of the TCA-soluble fraction by 70 %. In this experiment, RAP reduced the TCA-soluble fraction by as much as heparin treatment. Figure 52 shows that RAP had no effect on the surface-bound fraction but decreased the intracellular fraction isolated from another young normal human donor (male, 17-year old). The TCA-soluble fraction was reduced by 75 % compared to control and again RAP was as protective as heparin. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments within each experiment. Although RAP consistently reduced the TCA-soluble fraction, the amount of protection was variable and requires further investigation. However, these data show RAP-dependent mechanisms of ADAMTS4-2 clearance by both primary porcine and human chondrocytes.

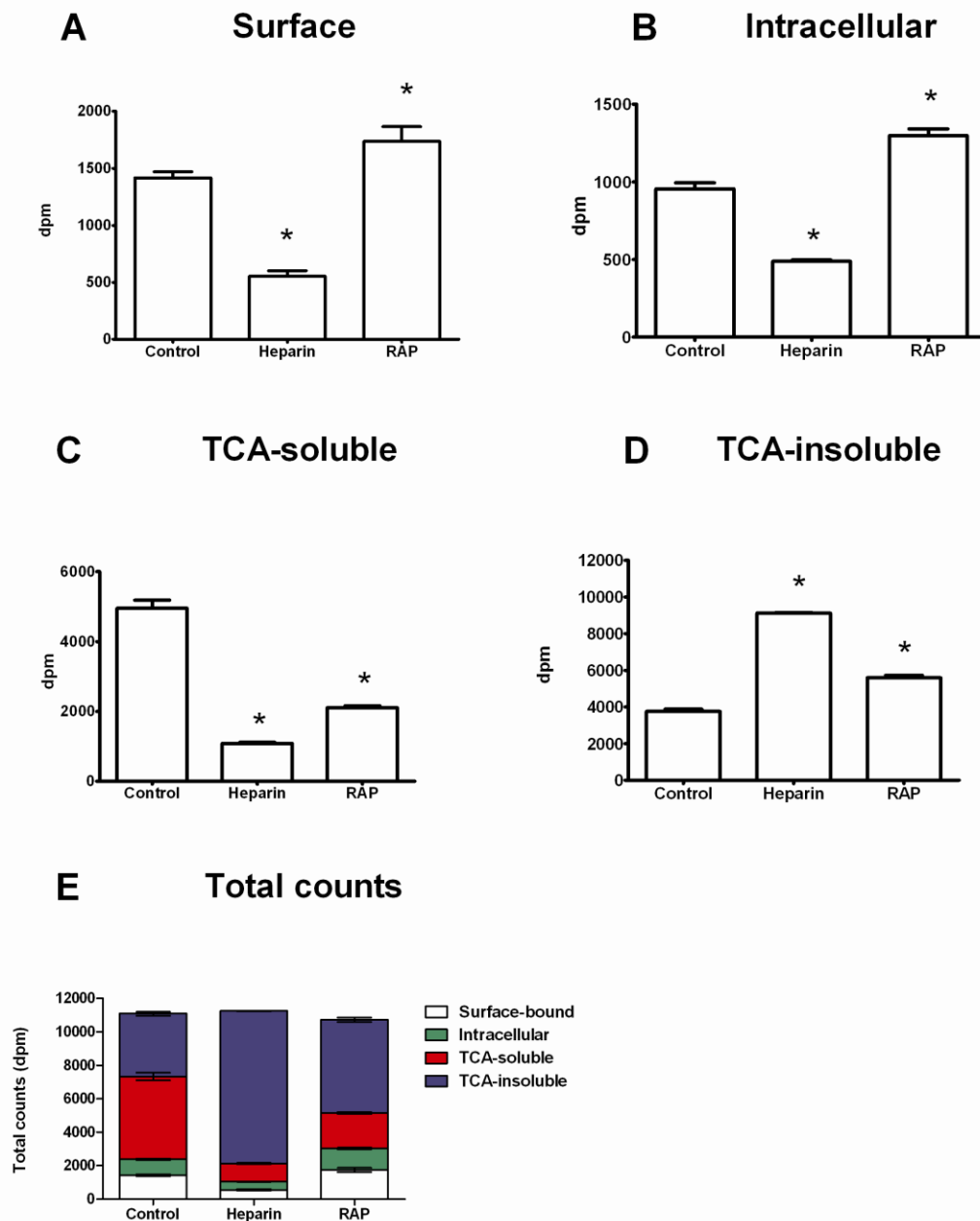


Figure 50: RAP inhibits clearance of [^{35} S]ADAMTS4-2 by porcine chondrocytes. Porcine chondrocytes were seeded in a 12-well plate at a density of 1×10^6 cells, grown for 3 days and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml) or RAP (500 nM). After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. * = $P \leq 0.05$ as compared to control. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, wash, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

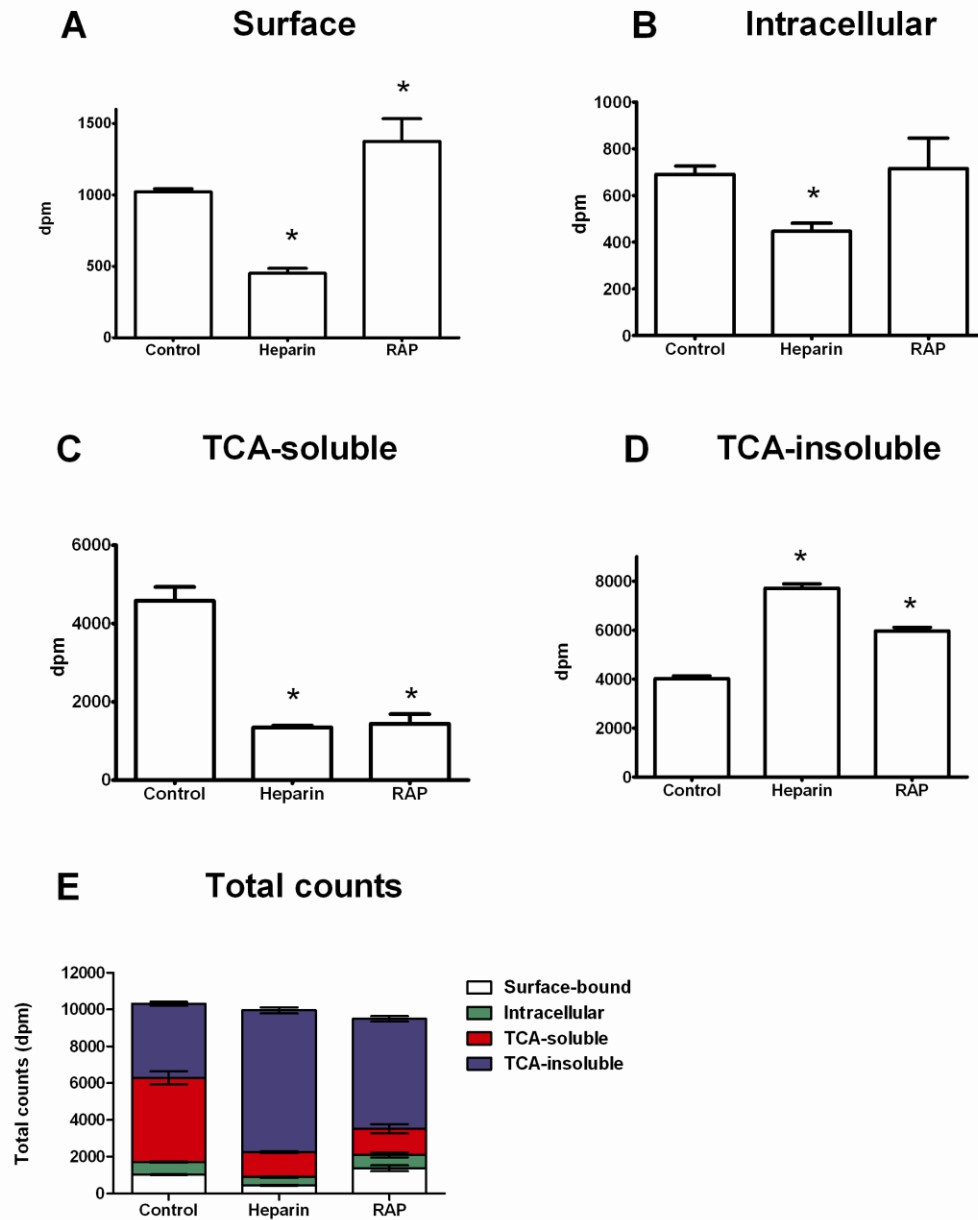


Figure 51: RAP inhibits clearance of [^{35} S]ADAMTS4-2 by human chondrocytes.

Human chondrocytes from a healthy 12 year old male were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown for 3 days and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml) or RAP (500 nM). After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. * = $P \leq 0.05$ as compared to control. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

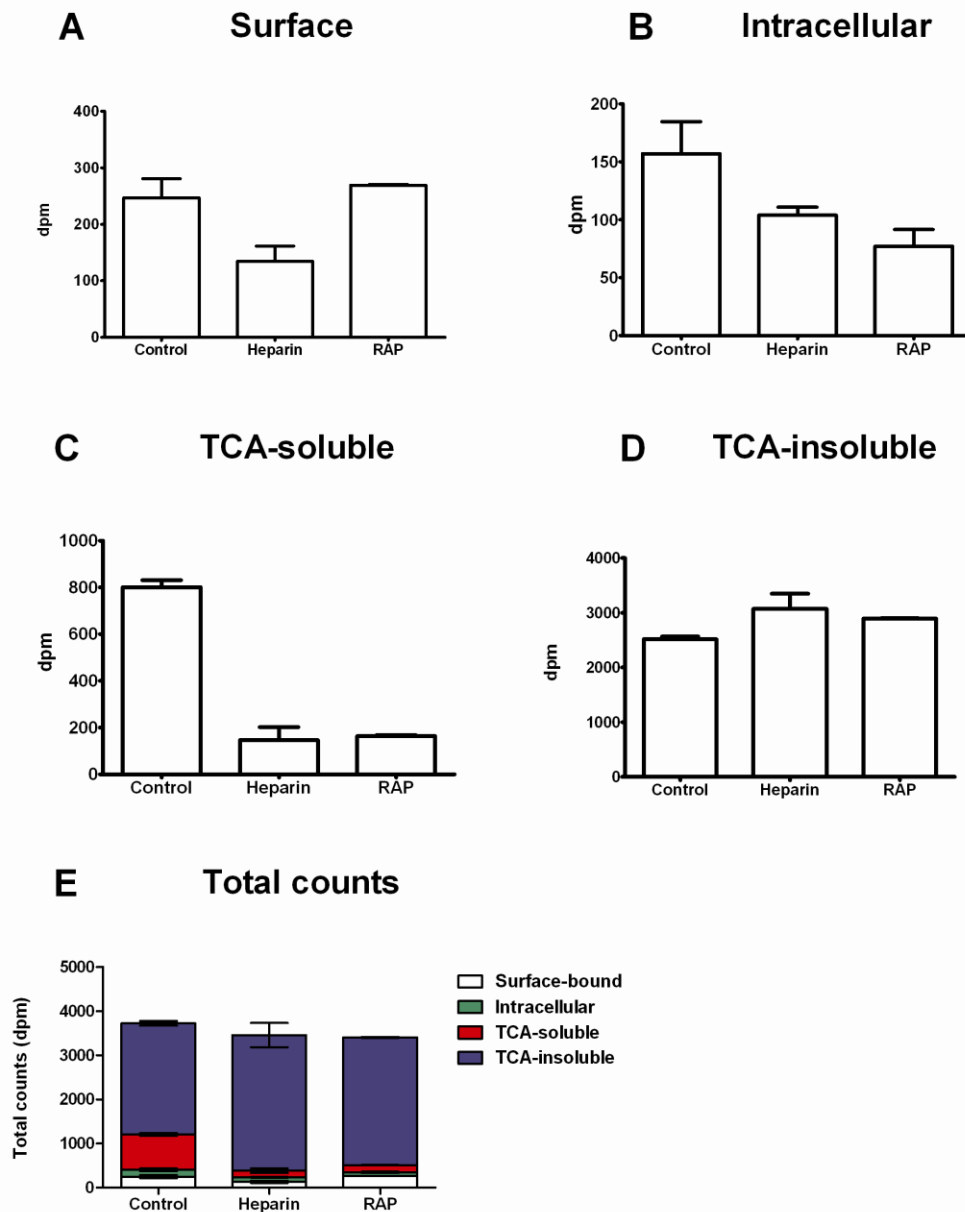


Figure 52: RAP inhibits clearance of [^{35} S]ADAMTS4-2 by human chondrocytes.

Human chondrocytes from a healthy 17 year old male were seeded in a 6-well plate at a density of 1×10^6 cells, grown for 3 days and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml) or RAP (500 nM). After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

5.2.2. Investigating the role of LRP-1 in the clearance of [³⁵S]ADAMTS4-2

5.2.2.1. siRNA knockdown of LRP-1 in HTB94 cells

The reduction in the TCA-soluble fraction upon incubation of HTB94 cells and primary chondrocytes with RAP suggested a RAP-sensitive mechanism of [³⁵S]ADAMTS4-2 clearance by these cell types. The most studied member of the LDL-R family with respect to endocytosis of proteolytic enzymes and their inhibition is LRP-1. Therefore, the effect of knockdown of LRP-1 by siRNA on [³⁵S]ADAMTS4-2 clearance was investigated, using previously validated LRP-1 primers (Dedieu *et al.*, 2008).

Firstly, to establish the conditions for LRP-1 knockdown, HTB94 cells were transfected overnight in a 12-well plate with 0-300 pmol of control siRNA or LRP-1 siRNA using Lipofectamine RNAiMAX reagents. After transfection, the medium was replaced with DMEM containing 10 % FCS. After 3 days in culture, whole cell lysates were harvested in CHAPS buffer [10 mM CHAPS, 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl₂], supplemented with protease inhibitor cocktail (1:40) and samples were analysed by non-reducing SDS-PAGE (5 % Tris glycine) and Western blotting with anti-LRP-1 (8G1) antibody. As a loading control, equal volumes of each sample were analysed by non-reducing SDS-PAGE (7.5 % acrylamide) and Western blotting with an anti- α -tubulin antibody.

Figure 53 shows that LRP-1 ran with a molecular weight of approximately 515 kDa whilst the α -tubulin ran with a molecular weight of approximately 45 kDa as expected. The blots were scanned and the pixel volume of the LRP-1 specific band (~515 kDa) and the α -tubulin band (~45 kDa) were determined using Phoretix software. Under the transfection conditions used, 50 pmol of LRP-1 siRNA was sufficient to attenuate LRP-1 expression in HTB94 cells by 73 %. Transfection with control siRNA had no effect on the expression of LRP-1 compared to untransfected cells (control) or transfection with Lipofectamine RNAimax reagent alone. Transfection also had no effect on the expression of α -

tubulin. These results validate the specific siRNA knockdown of LRP-1 in HTB94 cells.

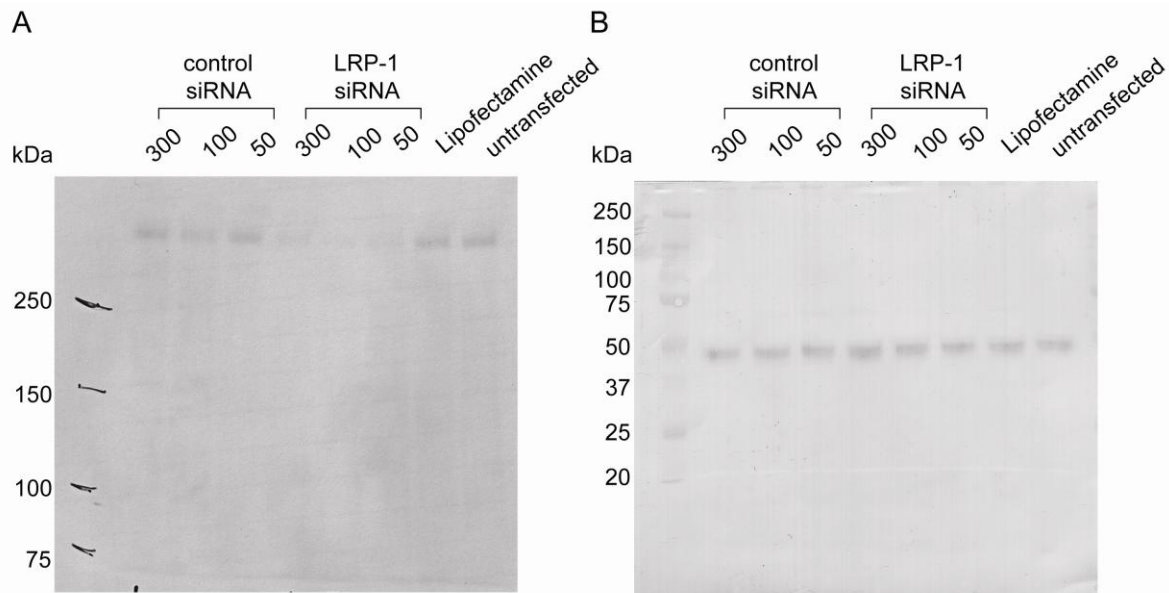


Figure 53: *siRNA knockdown of LRP-1 in HTB94 cells.*

HTB94 cells were seeded in a 12-well plate at a density of 1.5×10^4 cells and grown overnight. Cells were left untransfected (control) or transfected with either Lipofectamine RNAiMax reagent alone, or Lipofectamine RNAiMax containing control siRNA or LRP-1 siRNA (300 pmol, 100 pmol or 50 pmol) for 24 h. Cells were then grown for 3 days in DMEM containing 10 % FCS. Cells were then lysed with CHAPS buffer (10 mM CHAPS, 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl_2), supplemented with protease inhibitor cocktail (1:40). Samples were then diluted 1:1 with non-reducing SDS-loading buffer and equal volumes of each sample were analysed by **A**, non-reducing SDS-PAGE (5 % Tris glycine) and Western blotting with anti-LRP-1 (8G1) antibody. **B**, As a loading control, equal volumes of each sample were analysed by non-reducing SDS-PAGE (7.5 % acrylamide) and Western blotting with an anti- α -tubulin antibody.

5.2.2.2. The effect of attenuated LRP-1 expression on clearance of [^{35}S]ADAMTS4-2

The effect of LRP-1 knockdown on the clearance of [^{35}S]ADAMTS4-2 was investigated in duplicate so that 1 set of cells were used for Western blot analysis to confirm the knockdown of LRP-1 expression whilst [^{35}S]ADAMTS4-2 was added to the other set of cells to determine LRP-1-dependent [^{35}S]ADAMTS4-2 clearance. HTB94 cells were transfected with 50 pmol of either control siRNA or LRP-1 siRNA overnight and allowed to grow in DMEM containing 10 % FCS for 3 days. After 3 days in culture, cell lysates were either harvested with

CHAPS buffer and frozen at -20 °C (0 h) or incubated at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 µg/ml). After 24 h, the cell lysates were harvested as previously described. Equal volumes of each sample were analysed by non-reducing SDS-PAGE (5 % Tris glycine) and Western blotting with anti-LRP-1 (2629) antibody. As a loading control, equal volumes of each sample were analysed by non-reducing SDS-PAGE (7.5 % acrylamide) and Western blotting with an anti- α -tubulin antibody. The blots were scanned and the pixel volume of the LRP-1 specific band (~515 kDa) and the α -tubulin band (~45 kDa) were determined using Phoretix software. The other set of transfected cells was incubated at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 µg/ml) for 1 h prior to the addition of [³⁵S]ADAMTS4-2 (1.5 nM). After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as previously described.

Compared to untransfected cells, the cells transfected with either control siRNA or LRP-1 siRNA did not grow as well because the expression of α -tubulin is greater in untransfected cells than the cells transfected with either LRP-1 or control siRNA (Figure 54). Transfection with LRP-1 siRNA knocked down the expression of LRP-1 at 0 h by 81 %. However, the expression of LRP-1 in the LRP-1 siRNA treated cells was reduced by only 56 % compared to control siRNA treated cells at the 24 h time point. The specificity of the LRP-1 antibody (2629) was confirmed by Western blotting using the secondary antibody alone (Figure 54B). Expression of α -tubulin in control and LRP-1 siRNA transfected cells was comparable throughout the experiment. Similarly, treatment with heparin did not affect the expression of either LRP-1 or α -tubulin in control and LRP-1 siRNA transfected cells.

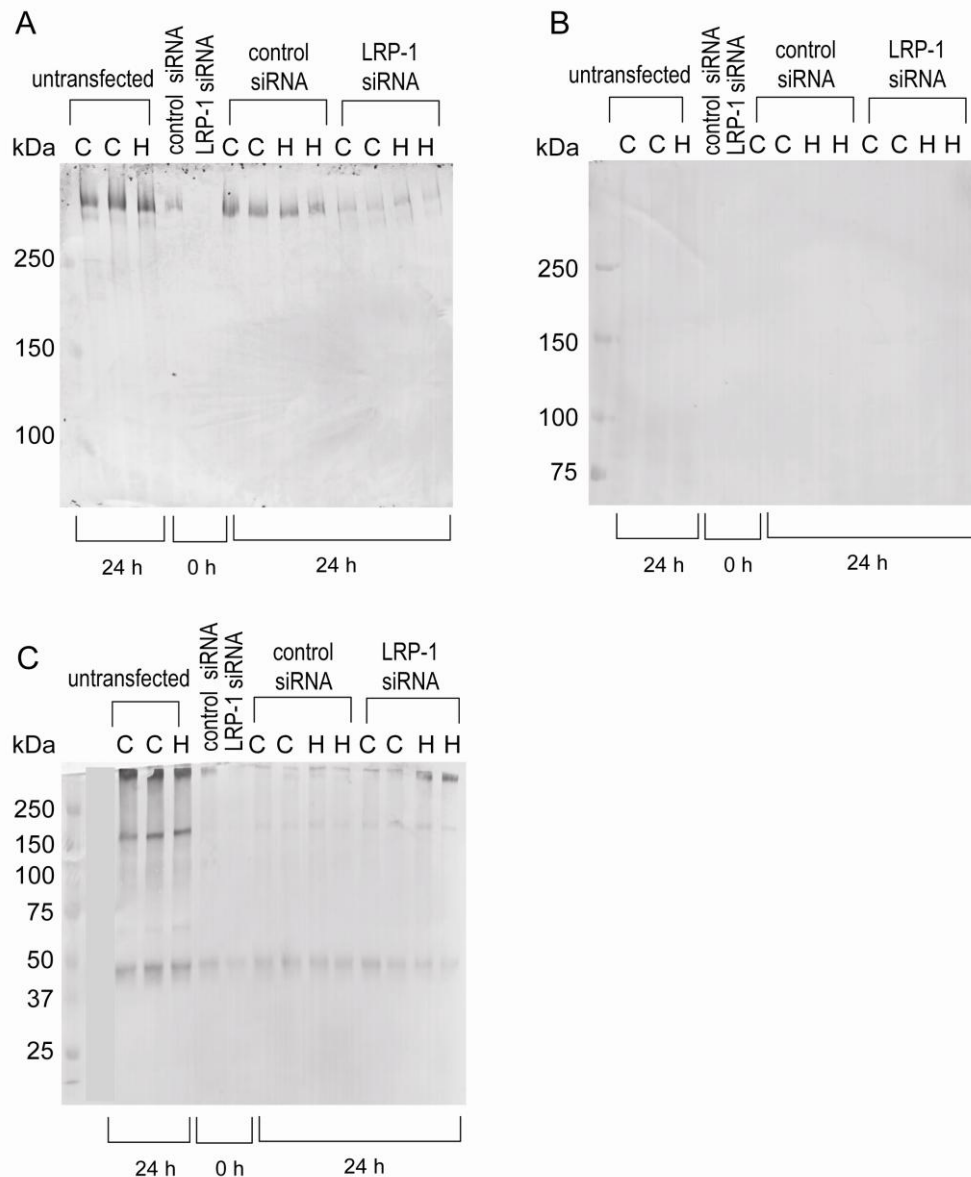


Figure 54: siRNA knockdown of LRP-1 in HTB94 cells.

HTB94 cells were seeded in a 12-well plate at a density of 2.5×10^4 cells and grown overnight. Cells were transfected with control siRNA or LRP-1 siRNA (50 pmol) using Lipofectamine RNAi max reagent for 24 h. Cells were then grown for 3 days in DMEM containing 10 % FCS. Cells were either lysed immediately (0 h) with CHAPS buffer (10 mM CHAPS, 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl_2), supplemented with protease inhibitor cocktail (1:40) or incubated in DMEM containing 0.1 % BSA for 24 h with or without heparin (250 $\mu\text{g}/\text{ml}$) before lysis. Samples were then diluted 1:1 with non-reducing SDS-loading buffer and equal volumes of each sample analysed by **A**, non-reducing SDS-PAGE (5 % Tris glycine) and Western blotting with anti-LRP-1 (2629) antibody. **B**, Non-reducing SDS-PAGE (5 % Tris glycine) and Western blotting with secondary antibody and no primary antibody. **C**, As a loading control, equal volumes of each sample were analysed by non-reducing SDS-PAGE (7.5 % acrylamide) and Western blotting with an anti- α -tubulin antibody. C = control, H = heparin treated (250 $\mu\text{g}/\text{ml}$). Each treatment was performed in duplicate.

Figure 55 shows the effect of LRP-1 attenuation on the clearance of [³⁵S]ADAMTS4-2 by HTB94 cells. In accordance with the Western blotting data, untransfected cells produced more TCA-soluble [³⁵S] degradation products than transfected cells because there was greater cell growth in untransfected cells than cells transfected with either control siRNA or LRP-1 siRNA. The amount of radioactivity in surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were comparable between control siRNA-transfected and LRP-1 siRNA-transfected cells. Additionally, heparin protected against the clearance of [³⁵S]ADAMTS4-2 by control siRNA- or LRP-1 siRNA-transfected cells to the same extent. Although the expression of LRP-1 was increased at 24 h compared to 0 h, these data indicate that under the conditions of the experiment, LRP-1 knockdown of at least 50 % compared to control transfection had no effect on the uptake of [³⁵S]ADAMTS4-2. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments.

5.2.2.3. [³⁵S]ADAMTS4-2 clearance by wild-type and LRP-1-null mouse embryonic fibroblasts

To further investigate whether LRP-1 plays a role in ADAMTS4-2 clearance, the uptake of [³⁵S]ADAMTS4-2 (1.5 nM) from the medium by wild-type mouse embryonic fibroblasts (MEF-1) and LRP-1-deficient mouse embryonic fibroblasts (PEA-13) were compared. As shown in Figure 56, the disappearance of [³⁵S]ADAMTS4-2 and the appearance of the TCA-soluble fraction were similar in both cell types. The surface-bound fraction was less in the MEF-1 cells compared to PEA-13 cells although the intracellular fraction was similar in both cell types.

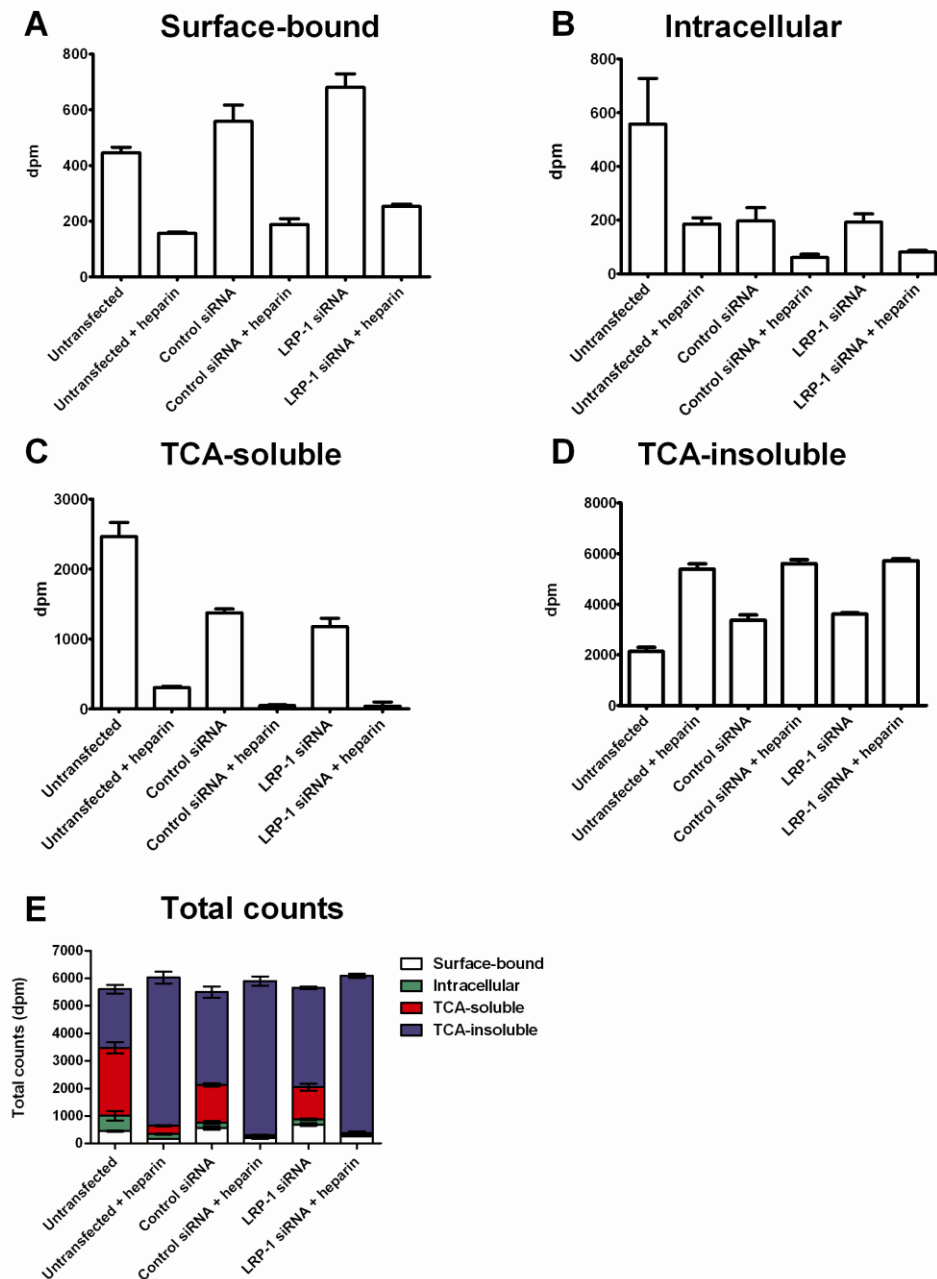


Figure 55: The effect of attenuated LRP-1 expression on clearance of [^{35} S]ADAMTS4-2 by HTB94 cells.

HTB94 cells were transfected as shown in Figure 5.9 and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 µg/ml). After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

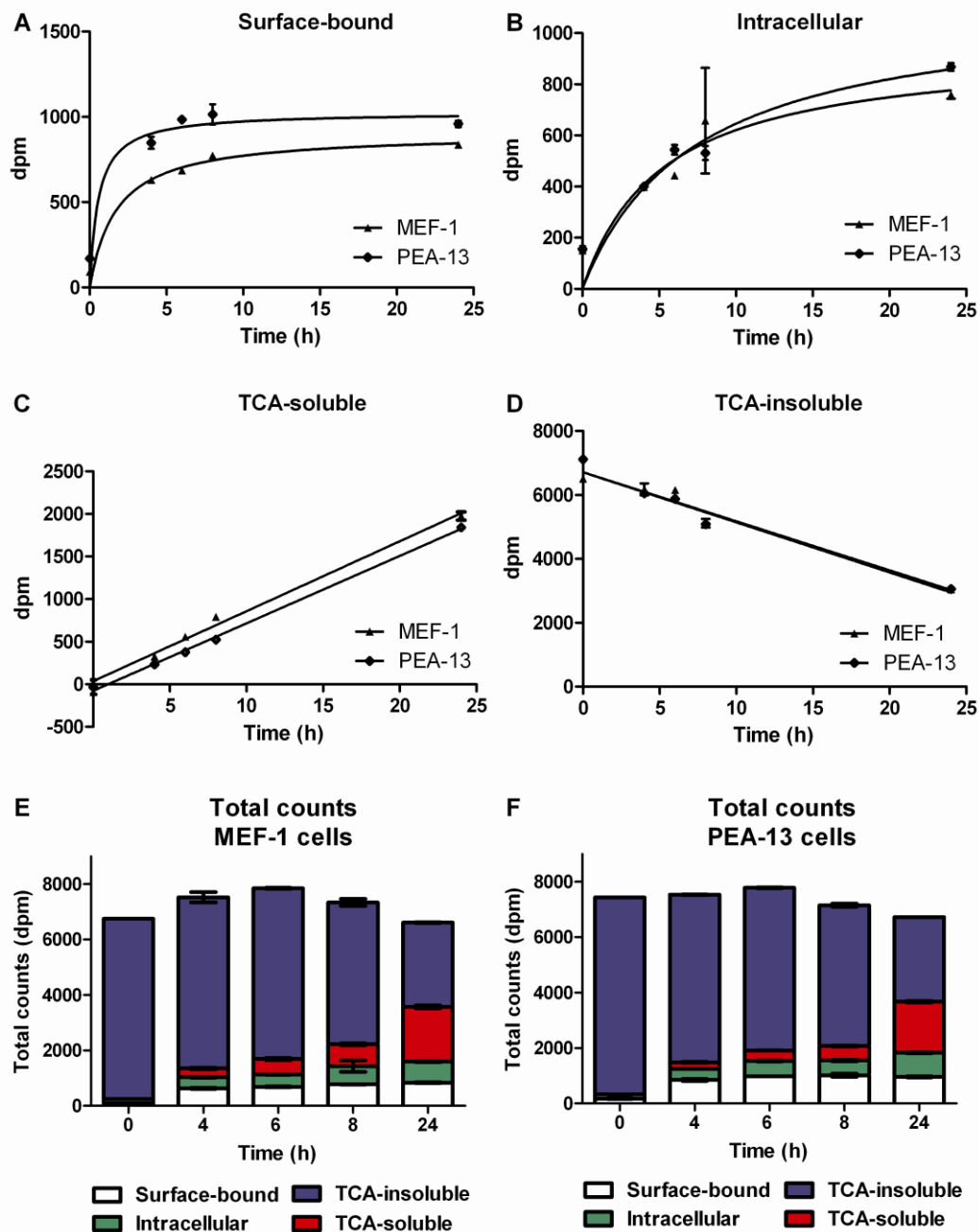


Figure 56: Clearance of [^{35} S]ADAMTS4-2 by MEF-1 and PEA-13 cells.

MEF-1 and PEA-13 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA. After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. At the time points indicated, the conditioned medium was removed and surface-bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Surface-bound counts, **B**, Intracellular counts, **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E & F**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts from MEF-1 and PEA-13 cells, respectively. The wash fractions were counted and found to be negligible.

5.2.2.4. Heparin more effectively inhibits the clearance of [³⁵S]ADAMTS4-2 from the medium of HTB94 cells than of MEF-1 cells

Next, the clearance of [³⁵S]ADAMTS4-2 by MEF-1 cells and HTB94 cells was compared. MEF-1 and HTB94 cells were plated at a density of 0.5×10^6 cells/well, grown overnight and incubated with heparin (250 µg/ml) for 1 h before addition of [³⁵S]ADAMTS4-2 (1.5 nM). The surface-bound and intracellular fractions isolated from HTB94 cells were more radioactive than those isolated from MEF-1 cells, although both surface-bound and intracellular fractions upon heparin treatment were similar in either cell type (Figure 57 A, B). Although the TCA-soluble fraction was significantly different between MEF-1 and HTB94 cells, the TCA-insoluble fraction was comparable in either cell type. However, heparin was more protective against the appearance of the TCA-soluble fraction in HTB94 cells: heparin reduced the TCA-soluble fraction by 83 % in HTB94 cells compared to a 28 % reduction in MEF-1 cells. The recovery of the total number of counts from the surface-bound, intracellular, TCA-soluble and TCA-insoluble counts was similar across all treatments.

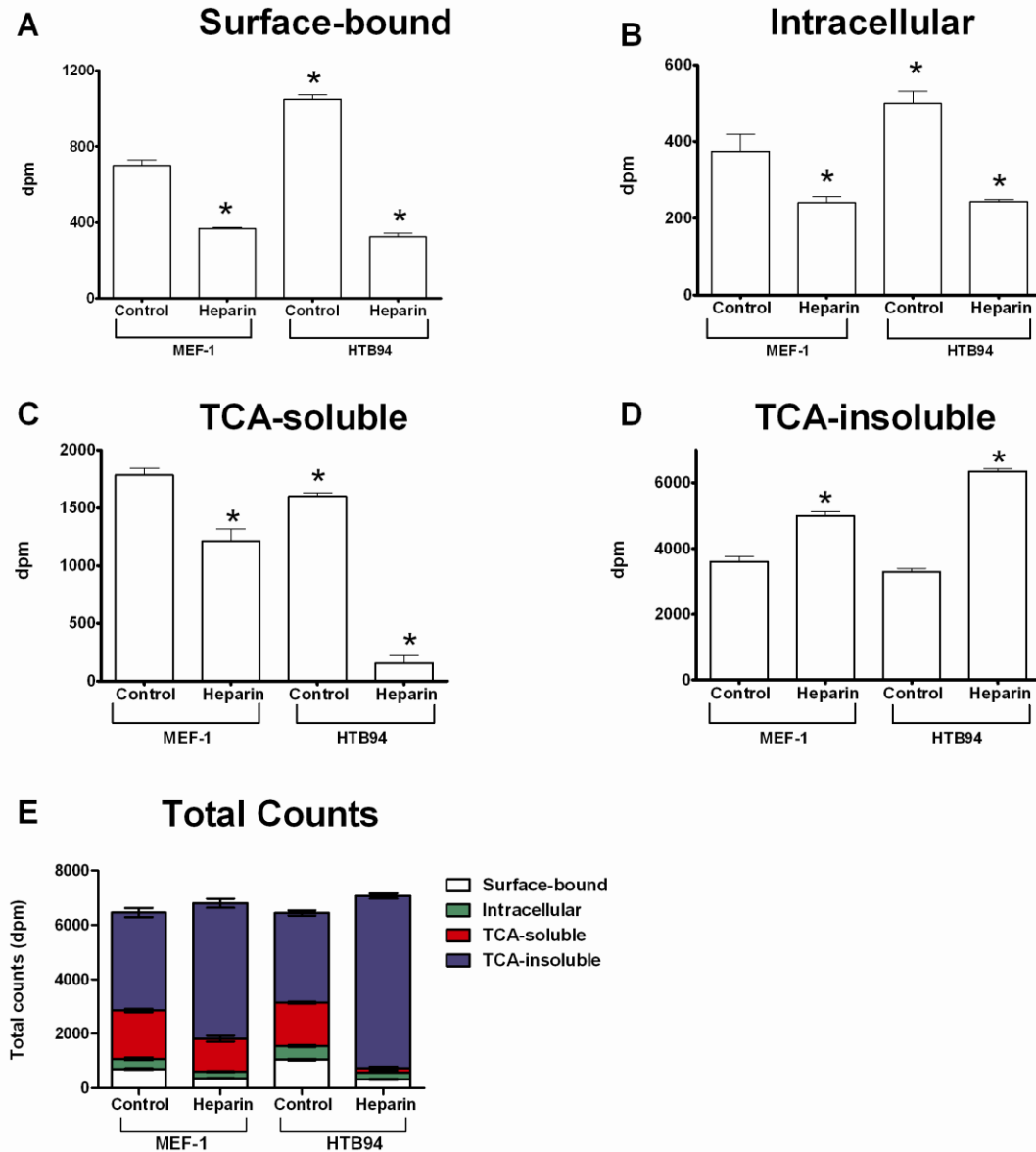


Figure 57: Heparin more effectively inhibits the clearance of [^{35} S]ADAMTS4-2 by HTB94 cells than by MEF-1 cells.

MEF-1 and HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without heparin (250 μ g/ml). After 1 hour, [^{35} S]ADAMTS4-2 (1.5 nM) was added. After 24 h, the conditioned medium was removed and surface bound, intracellular, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 3 wells $\pm$ S.D. * = $P \leq 0.05$ as compared to control. **A**, Surface-bound counts. **B**, Intracellular counts. **C**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **D**, TCA-insoluble counts. **E**, Total surface-bound, intracellular, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

5.2.3. Endocytosis of methylamine-treated $\alpha_2\text{M}$ by MEF-1, PEA-13 and HTB94 cells

The aim of the following section was to compare the clearance of ADAMTS4-2 by HTB94 cells to the endocytosis of methylamine-treated $\alpha_2\text{M}$ ($\alpha_2\text{M}^*$), a well-studied ligand of LRP-1. In order to study $\alpha_2\text{M}^*$ endocytosis, $\alpha_2\text{M}^*$ was prepared by reacting $\alpha_2\text{M}$ with 264 mM methylamine-HCl in 100 mM Tris-HCl (pH 8.0) for 1 h at room temperature. Tyrosine residues of $\alpha_2\text{M}^*$ were then labeled with ^{125}I using Iodo-Beads.

5.2.3.1. Use of Azocoll substrate to confirm conversion of $\alpha_2\text{M}$ to $\alpha_2\text{M}^*$

Azocoll is an insoluble protein-dye conjugate which can be hydrolysed by a variety of proteinases, giving a soluble coloured product which is proportional to enzyme activity (Chavira *et al.*, 1984). $\alpha_2\text{M}$ inhibits most proteinases irreversibly to form an $\alpha_2\text{M}$ -proteinase complex. Any proteinase within an $\alpha_2\text{M}$ -proteinase complex is able to cleave low molecular mass substrates but can not cleave large substrates such as Azocoll. Therefore, the addition of $\alpha_2\text{M}$ to the reaction inhibits proteinase-mediated Azocoll cleavage. However, $\alpha_2\text{M}^*$ can no longer bind to proteinases and so addition of $\alpha_2\text{M}^*$ to the reaction does not inhibit proteinase-mediated Azocoll cleavage. To determine whether $\alpha_2\text{M}$ was converted to $\alpha_2\text{M}^*$ by methylamine-HCl treatment, trypsin-mediated cleavage of Azocoll and the inhibition of cleavage by $\alpha_2\text{M}$ but not $\alpha_2\text{M}^*$ was tested.

5.2.3.1.1. Linearity of Azocoll assay

Before using the Azocoll assay to measure trypsin activity in the presence of either $\alpha_2\text{M}$ or $\alpha_2\text{M}^*$, the linearity of the assay was determined. Azocoll substrate (0.15 g) was washed with 50 ml of buffer (50 mM Tris-HCl, 1 mM CaCl_2 , pH 7.8) with rotation for 4 h at room temperature. The suspension was then centrifuged and the pellet was resuspended in 30 ml of buffer to give a 5 mg/ml suspension. Azocoll (490 μl) was then incubated with either 10 μl of 50 mM Tris-HCl, 1 mM CaCl_2 (pH 7.8) or trypsin (4 μM in 50 mM Tris-HCl, 1 mM CaCl_2 , pH 7.8). At the

time intervals indicated, the reaction was stopped by centrifugation and the supernatant was transferred to a 96-well plate. The absorbance of the soluble coloured product was read at 550 nm. The absorbance reading was plotted against the time incubated with 50 mM Tris-HCl, 1 mM CaCl₂ (pH 7.8), either with or without trypsin. Figure 58A shows that in the absence of trypsin, Azocoll was not cleaved but when trypsin was added to the buffer, the substrate was cleaved and the cleavage was linear up to 25 min (enlarged view in Figure 58B). To allow for a greater incubation period before stopping the reaction, the concentration of trypsin in the reaction was diluted 10-fold with 50 mM Tris-HCl, 1 mM CaCl₂ (pH 7.8). Figure 58C shows that under these conditions, the assay was linear up to 3.5 h (enlarged view in Figure 58D). As a consequence of these results, future experiments were executed using a stock solution of 400 nM trypsin. The reaction was stopped between 90 min – 2 h after addition of Azocoll substrate.

5.2.3.1.2. AEBSF and α_2 M inhibit trypsin-mediated Azocoll cleavage

The ability of trypsin to cleave Azocoll substrate could be inhibited by incubation of the serine protease inhibitor AEBSF (50 mM) with trypsin for 15 min prior to the addition of Azocoll substrate (Figure 59A). In addition, the proteolytic activity of trypsin could also be dose-dependently inhibited by α_2 M (Figure 59B), reaching a maximum level of inhibition at an approximate ratio of 1:1 (α_2 M:trypsin).

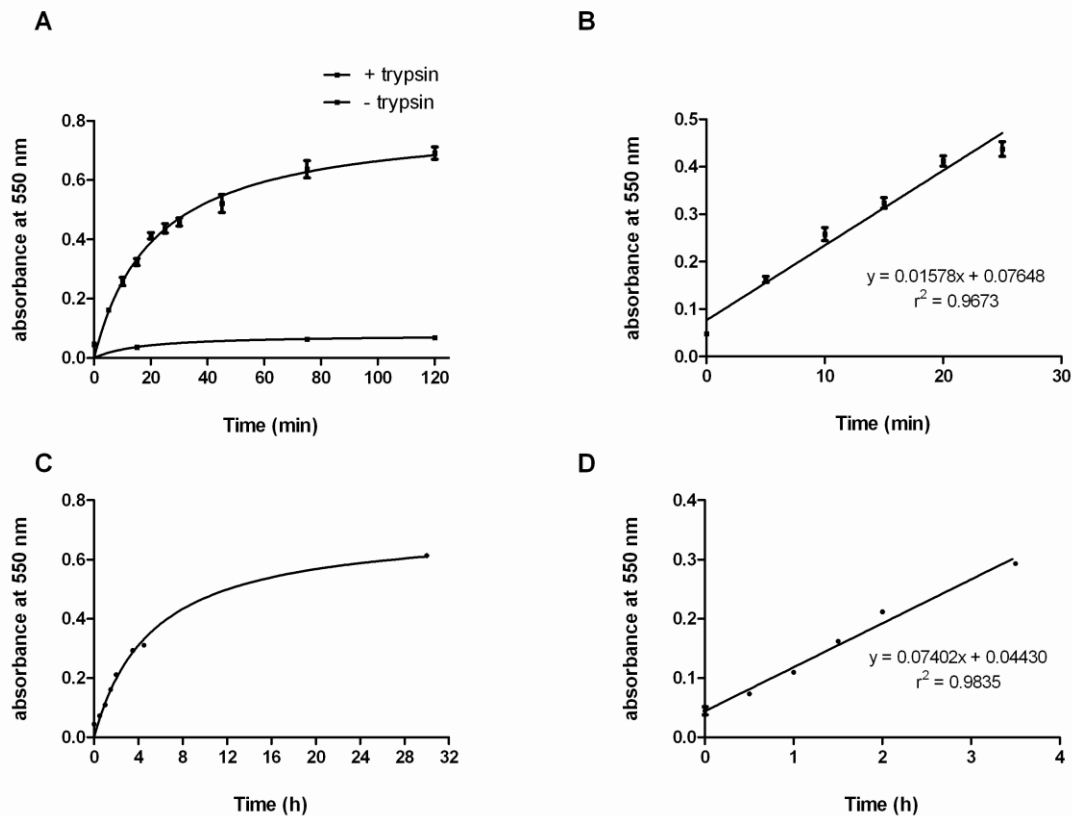


Figure 58: Linearity of the Azocoll assay.

Azocoll was suspended in 0.05 M Tris-HCl, 1 mM CaCl_2 (pH 7.8) to give a 5 mg/ml slurry whereas trypsin was diluted to either 4 μM or 400 nM in 50 mM Tris-HCl, 1 mM CaCl_2 (pH 7.8). **A**, Azocoll (490 μl) was incubated at room temperature with 10 μl of trypsin (4 μM) or buffer alone (10 μl). Results are an average of 3 $\pm$ S.D. **B**, Enlargement of 0 - 25 min time points. **C**, Azocoll (490 μl) was incubated at room temperature with 10 μl of trypsin (400 nM). **D**, Enlargement of 0 - 3.5 h time points. At the time intervals indicated, the reaction was stopped by centrifugation at 14170 g for 5 min at 4 $^\circ\text{C}$ and transfer of the supernatant (150 μl aliquot) to a 96-well plate. The absorbance was read at 550 nm using a FLUOstar Omega (BMG Labtech) plate reader.

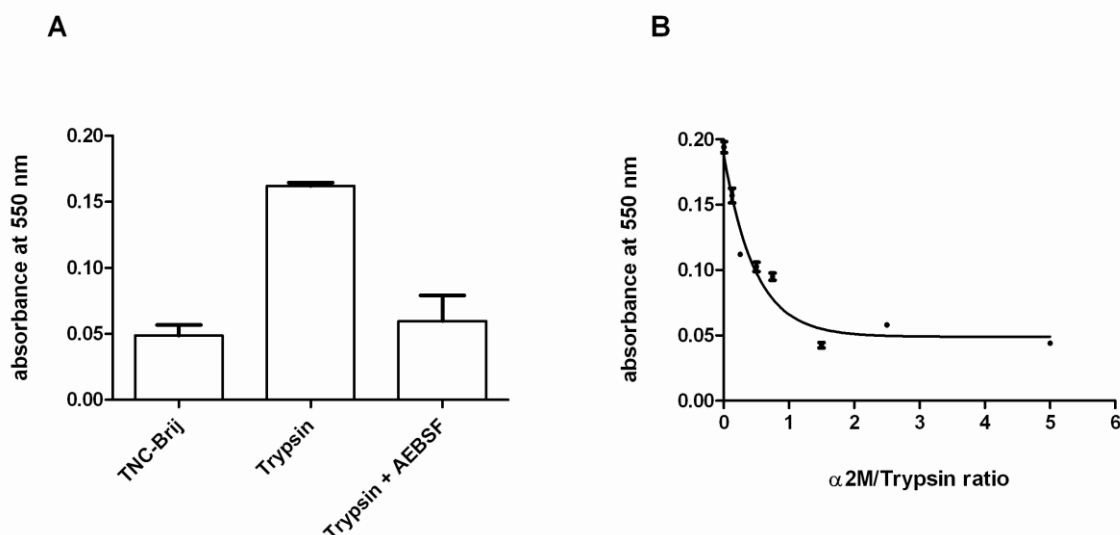


Figure 59: AEBSF and α_2 M inhibit trypsin-mediated cleavage of Azocoll.

Azocoll was suspended in 0.05 M Tris-HCl, 1 mM CaCl_2 (pH 7.8) to give a 5 mg/ml slurry and trypsin was diluted to 400 nM in 50 M Tris-HCl, 1 mM CaCl_2 (pH 7.8). **A**, Trypsin (10 μ l) was incubated with 10 μ l of TNC-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35] or AEBSF (10 μ l of 100 mM stock) for 15 min at room temperature prior to the addition of Azocoll (490 μ l). TNC-Brij (20 μ l) was the negative control. The reaction was incubated at room temperature with rotation for 90 min before centrifugation at 14170 g for 5 min at 4 °C and transfer of the supernatant (150 μ l aliquot) to a 96-well plate. Results are an average of 3 $\pm$ S.D. **B**, Trypsin (10 μ l) was incubated with 10 μ l of TNC-Brij or α_2 M (50 nM, 100 nM, 200 nM, 300 nM, 600 nM, 1 μ M or 2 μ M, diluted in TNC-Brij) for 15 min at room temperature prior to the addition of Azocoll (490 μ l). The reaction was incubated at room temperature with rotation for 2 h before centrifugation at 14170 g for 5 min at 4 °C and transfer of the supernatant (150 μ l aliquot) to a 96-well plate. The absorbance was read at 550 nm using a FLUOstar Omega (BMG Labtech) plate reader. The absorbance was plotted against the ratio of α_2 M:trypsin in the assay. Results are an average of 2 readings $\pm$ range.

5.2.3.1.3. α_2 M* does not inhibit trypsin-mediated cleavage of Azocoll

To test that the buffers used to react with α_2 M did not cause Azocoll hydrolysis, Azocoll substrate was incubated with 50 mM TNC-Brij (pH 7.8) or 100 mM Tris-HCl (pH 8.0) either with or without 264 mM methylamine-HCl. Azocoll substrate was not hydrolysed by any of the buffers tested. In addition, trypsin cleavage of Azocoll was comparable in 100 mM Tris-HCl (pH 8.0) either with or without the addition of 264 mM methylamine-HCl (Figure 60A).

$\alpha_2\text{M}$ was incubated with 100 mM Tris-HCl (pH 8) either with or without the addition of 264 mM methylamine-HCl. The final concentration of $\alpha_2\text{M}$ was 6 μM . After 1 h incubation at room temperature, the samples were exchanged into TNC buffer with 20 % glycerol [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 20 % glycerol] using G-50 Sepharose. Aliquots of $\alpha_2\text{M}$ and $\alpha_2\text{M}$ treated with methylamine-HCl were then incubated with trypsin for 15 min at room temperature prior to the addition of Azocoll substrate. As shown in Figure 60B, the addition of $\alpha_2\text{M}$ inhibited Azocoll cleavage whereas $\alpha_2\text{M}$ treated with methylamine-HCl did not inhibit Azocoll cleavage. These results show that $\alpha_2\text{M}$ was converted to $\alpha_2\text{M}^*$ by treatment with methylamine-HCl.

5.2.3.1.4. Heat-induced fragmentation of $\alpha_2\text{M}$ but not $\alpha_2\text{M}^*$

Heating $\alpha_2\text{M}$ at pH 9.5 with SDS (2 %) and DTT (28 mM) for 30 min at 100 °C generated bands at 125 kDa and 62 kDa (Figure 61). In contrast, $\alpha_2\text{M}^*$ did not generate either the 125 kDa or the 62 kDa band. This is in accordance with published work (Harpel *et al.*, 1979) and further demonstrated $\alpha_2\text{M}$ was converted to $\alpha_2\text{M}^*$ by reaction with methylamine-HCl.

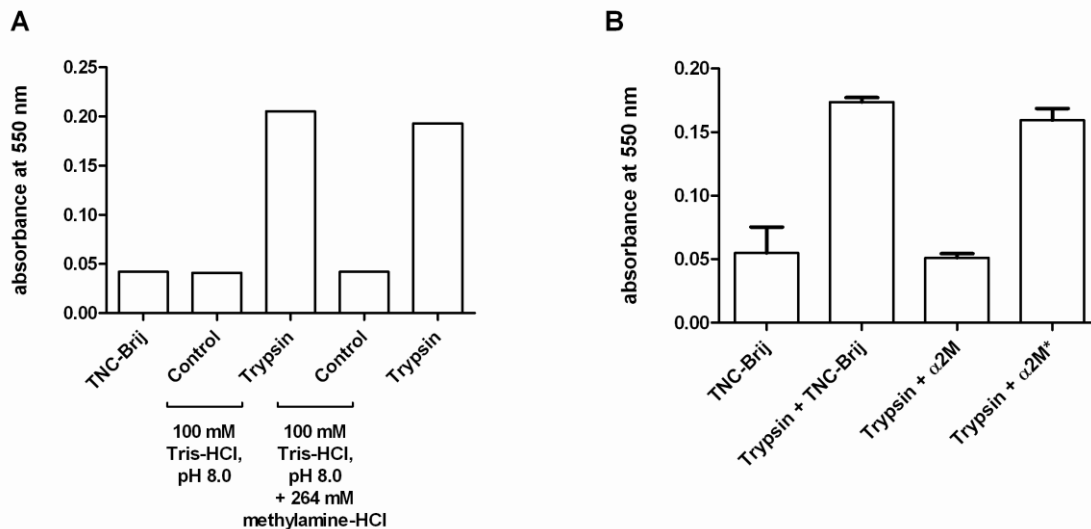


Figure 60: α_2M^* does not inhibit trypsin-mediated cleavage of Azocoll.

Azocoll was suspended in 0.05 M Tris-HCl, 1 mM $CaCl_2$ (pH 7.8) to give a 5 mg/ml slurry and trypsin was diluted to 400 nM in 0.05 M Tris-HCl, 1 mM $CaCl_2$ (pH 7.8). **A**, Trypsin (8 μ l) was incubated with 2 μ l of 100 mM Tris-HCl (pH 8) either with or without 264 mM methylamine-HCl for 15 min at room temperature prior to the addition of Azocoll substrate (490 μ l). Azocoll was not hydrolyzed by TNC-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.02 % sodium azide, 0.02 % Brij-35] (10 μ l) or 100 mM Tris-HCl (pH 8.0) either with or without 264 mM methylamine HCl (10 μ l). The reaction was incubated at room temperature with rotation for 2 h before centrifugation at 14170 g for 5 min at 4 °C and transfer of the supernatant (150 μ l aliquot) to a 96-well plate. The absorbance was read at 550 nm using a FLUOstar Omega (BMG Labtech) plate reader. **B**, α_2M was incubated with 100 mM Tris-HCl (pH 8) either with or without 264 mM methylamine-HCl (6 μ M α_2M final concentration) at room temperature. The samples were exchanged into TNC-20 % glycerol buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.02 % sodium azide, 20 % glycerol] using G-50 Sepharose and activity against trypsin assayed as follows: Two μ l of α_2M or α_2M^* were incubated with 8 μ l of trypsin for 15 min prior to the addition of Azocoll substrate (490 μ l). Trypsin (8 μ l) with TNC-Brij (2 μ l) was the positive control. TNC-Brij (10 μ l) was the negative control. The reaction was incubated at room temperature with rotation for 2 h before centrifugation at 14170 g for 5 min at 4 °C and transfer of the supernatant (150 μ l aliquot) to a 96-well plate. The absorbance was read at 550 nm using a FLUOstar Omega (BMG Labtech) plate reader. Results are an average of 3 readings $\pm$ S.D.

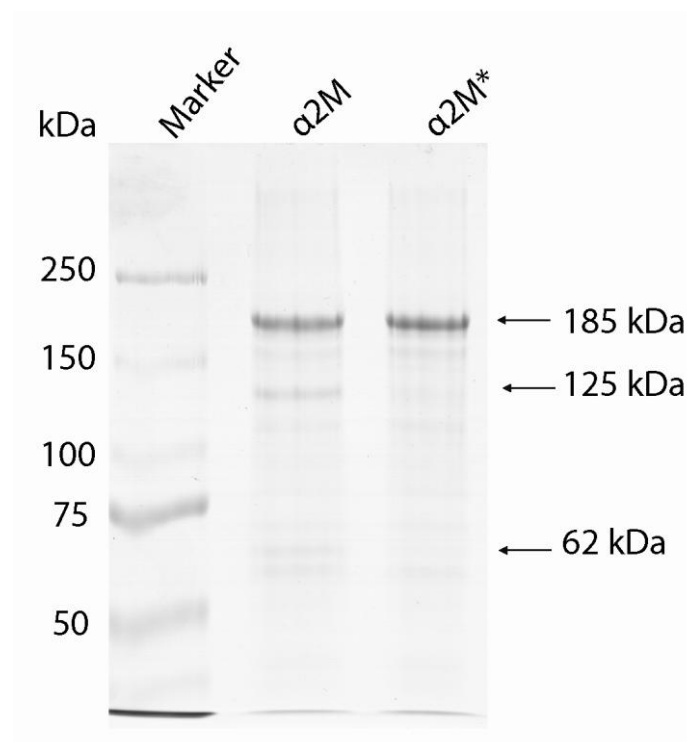


Figure 61: Heat-induced fragmentation of α_2M .

α_2M or α_2M^* (2.2 mg/ml) was incubated with 35 μ l of 0.05 M Tris-HCl (pH 9.5), 2 % SDS, 28 mM DTT and heated at 100 $^{\circ}$ C for 30 min. The samples were analysed by SDS-PAGE (6 % acrylamide) and stained with Coomassie Brilliant Blue R-250.

5.2.3.2. Iodination of α_2M^*

α_2M^* (100 μ g) was iodinated with ^{125}I using Iodo-Beads. Excess $Na^{125}I$ or unincorporated ^{125}I was separated from iodinated α_2M^* by gel filtration in TNC-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.02 % sodium azide, 0.02 % Brij-35] using SephadexG-50 (5 ml column). Elutions (200 μ l) were collected and 5 μ l aliquots of each elution was analysed by reducing SDS-PAGE (6 % acrylamide) and silver staining.

$[^{125}I]-\alpha_2M^*$, with a molecular weight of approximately 185 kDa, eluted in fractions 5 to 7 (Figure 62). However, there were minor bands at a higher molecular weight which became more apparent when the gel was exposed to a phosphoimaging plate. This indicates that the sample was not completely reduced when analysed by reducing SDS-PAGE as the samples were not boiled

for 5 min prior to SDS-PAGE because of the risk of creating volatile ^{125}I under these conditions. Elution 6 was selected for use in subsequent experiments.

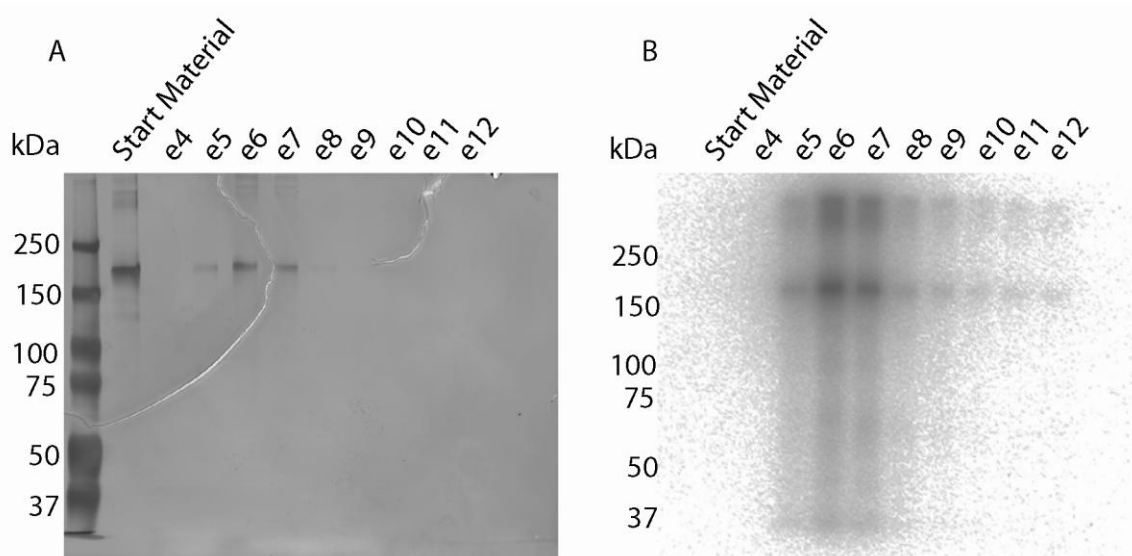


Figure 62: ^{125}I -labeling of $\alpha_2\text{M}^*$ using Iodo-Beads.

Two iodination beads were washed with 1000 μl PBS to remove any loose particles and dried on filter paper. The beads were then added to carrier-free Na^{125}I (1 mCi/37 MBq) in PBS (100 μl). The beads and carrier-free Na^{125}I were incubated at room temperature for 5 min before addition of $\alpha_2\text{M}^*$ (100 μg) to a final volume of 200 μl . The reaction was incubated for 15 min. Excess Na^{125}I or unincorporated ^{125}I was separated from iodinated $\alpha_2\text{M}^*$ by gel filtration into TNC-Brij [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl_2 , 0.02 % sodium azide, 0.02 % Brij-35] using G-50 Sepharose (5 ml column). Two hundred μl elutions were collected and aliquots (5 μl) were analysed by **A**, reducing SDS-PAGE (6 % acrylamide) and silver staining. **B**, the gel was exposed to a phosphorimaging plate for 30 min. e = elution.

5.2.3.2.1. Silver stain of [^{125}I]- $\alpha_2\text{M}^*$ to determine concentration

To determine the approximate concentration of [^{125}I]- $\alpha_2\text{M}^*$ in elution 6, the amount of protein was estimated using a standard curve constructed relative to known amounts of $\alpha_2\text{M}$. Figure 63 shows a silver stain of increasing amounts of $\alpha_2\text{M}$ (ng) and aliquots of [^{125}I]- $\alpha_2\text{M}$ (5 μl and 10 μl). The gel was scanned and the pixel volume of each band was determined using Phoretix software. Figure 63B shows a standard curve of pixel volume of $\alpha_2\text{M}$ band against amount of $\alpha_2\text{M}$ (ng)

added. Using the equation $y = mx + c$, the concentration of [^{125} I]- α_2 M* was calculated to be 50 nM based on the molecular weight of the tetramer (740 kDa).

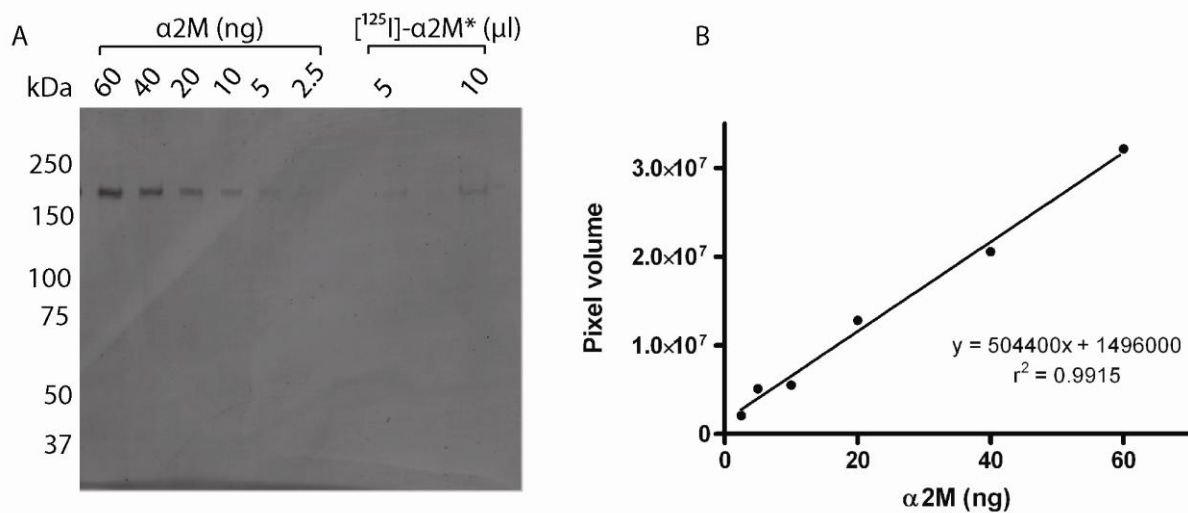


Figure 63: Determination of approximate [^{125} I]- α_2 M* concentration using α_2 M standard curve.

The amount of [^{125} I]- α_2 M* was determined using a standard curve of known amounts of α_2 M. **A**, α_2 M (60-2.5 ng) was analysed by reducing SDS-PAGE (6 % acrylamide) together with 5 μ l and 10 μ l aliquots of [^{125} I]- α_2 M* from elution 6. The gel was developed by silver staining and scanned. The pixel volume of each band was determined using Phoretix software. **B**, Standard curve of pixel volume of α_2 M band against amount of α_2 M (ng) added.

5.2.3.2.2. Detection limits of [^{125} I]- α_2 M* counting

^{125} I emits gamma radiation which can be measured in the absence of scintillation fluid using a gamma counter. In a similar manner to that described in Chapter 3, the linear range of [^{125} I]- α_2 M* detection was determined. To achieve this, [^{125} I]- α_2 M* was serially diluted 1:1 with TNC-Brij and the dilutions were counted. The highest radioactivity counted was 700,000 dpm and the readings were linear down to 50 dpm (Figure 64). The amount of ^{125} I incorporation was 10811 dpm/ μ g of [^{125} I]- α_2 M*.

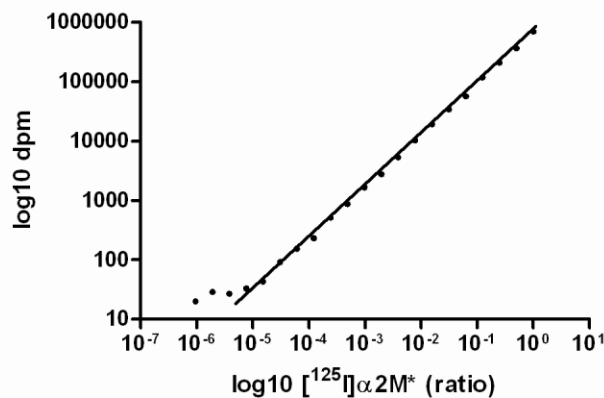


Figure 64: Linearity of $[^{125}\text{I}]\text{-}\alpha_2\text{M}^*$ counting.

$[^{125}\text{I}]\text{-}\alpha_2\text{M}^*$ was serially diluted with TNC Brij and the radioactivity was measured using a 1282 COMPUGAMMA Universal Gamma Counter (Wallac, Pharmacia).

5.2.2.3. $[^{125}\text{I}]\text{-}\alpha_2\text{M}^*$ endocytosis by wild-type and LRP-1-deficient embryonic fibroblasts

To confirm that $[^{125}\text{I}]\text{-}\alpha_2\text{M}^*$ and RAP were behaving as expected, the endocytosis of $[^{125}\text{I}]\text{-}\alpha_2\text{M}^*$ by either MEF-1 (wild-type) or PEA-13 (homozygous LRP-1-deficient) cells was assessed.

MEF-1 or PEA-13 cells were incubated with or without RAP (500 nM) for 1 h before the addition of $[^{125}\text{I}]\text{-}\alpha_2\text{M}^*$ (0.05 nM). After 6 h, the medium was harvested and TCA precipitated (3.3 %). The cell layer was washed thrice with PBS and solubilised with 1 M NaOH. Radioactivity in each fraction was determined by gamma counting. Figure 65 shows that $[^{125}\text{I}]\text{-}\alpha_2\text{M}^*$ was endocytosed by MEF-1 but not PEA-13 fibroblasts since MEF-1 cells showed increased radioactivity in both the cell-associated and TCA-soluble fractions as well as decreased radioactivity in the TCA-insoluble fraction compared to PEA-13 cells. In addition, when MEF-1 cells were treated with RAP, both the cell-associated and TCA-soluble fractions harvested from MEF-1 cells were reduced by 60 % and 80 %, respectively. RAP reduced both the cell-associated and TCA-soluble fractions to levels similar to those of PEA-13 cells. Furthermore, the amount of TCA-

insoluble radioactivity harvested from RAP-treated MEF-1 cells was similar to the TCA-insoluble fraction from PEA-13 cells. In contrast, treatment with RAP had no effect on any of the fractions harvested from PEA-13 cells. The recovery of the total number of counts from the cell-associated, TCA-soluble and TCA-insoluble counts was similar across all treatments.

5.2.2.4. [^{125} I]- α_2 M* endocytosis by HTB94 cells and the effect of RAP

HTB94 cells were incubated with or without RAP (500 nM) for 1 h before the addition of [^{125} I]- α_2 M* (0.025 nM). After 6 h or 24 h, the medium was harvested and TCA precipitated (3.3 %). The cell layer was washed thrice with PBS and solubilised with 1 M NaOH. Radioactivity in each fraction was determined by gamma counting. Figure 66 shows that upon addition of [^{125} I]- α_2 M* to HTB94 cells, the TCA-soluble fraction increased and the TCA-insoluble fraction decreased with time. RAP reduced the amount of cell-associated radioactivity at both 6 h and 24 h. Additionally, RAP reduced the TCA-soluble fraction by 50 % at both 6 h and 24 h. There was no significant change in the TCA-insoluble fraction by RAP at 6 h but RAP partially blocked the clearance of TCA-insoluble [^{125} I]- α_2 M* at 24 h. In addition, RAP did not completely protect against the appearance of the TCA-soluble fraction at either 6 h or 24 h. The recovery of the total number of counts from the cell-associated, TCA-soluble and TCA-insoluble counts was similar across all treatments.

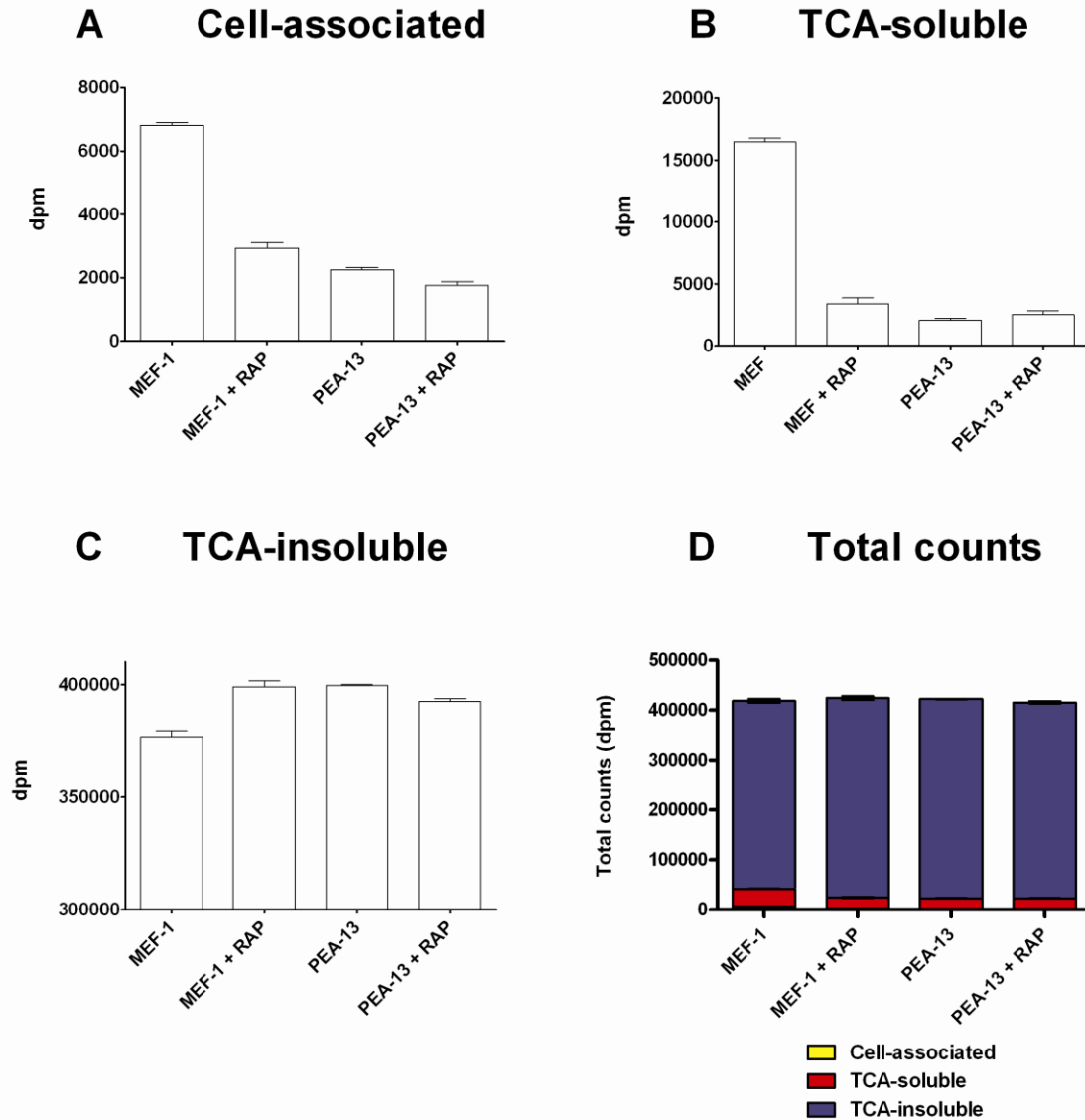


Figure 65: [^{125} I]- α_2 M* endocytosis by MEF-1 and PEA-13 cells.

MEF-1 and PEA-13 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without RAP (500 nM). After 1 hour, [^{125} I]- α_2 M* (0.05 nM) was added. After 6 h, the conditioned medium was removed and cell-associated, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Cell-associated counts. **B**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **C**, TCA-insoluble counts. **D**, Total cell-associated, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

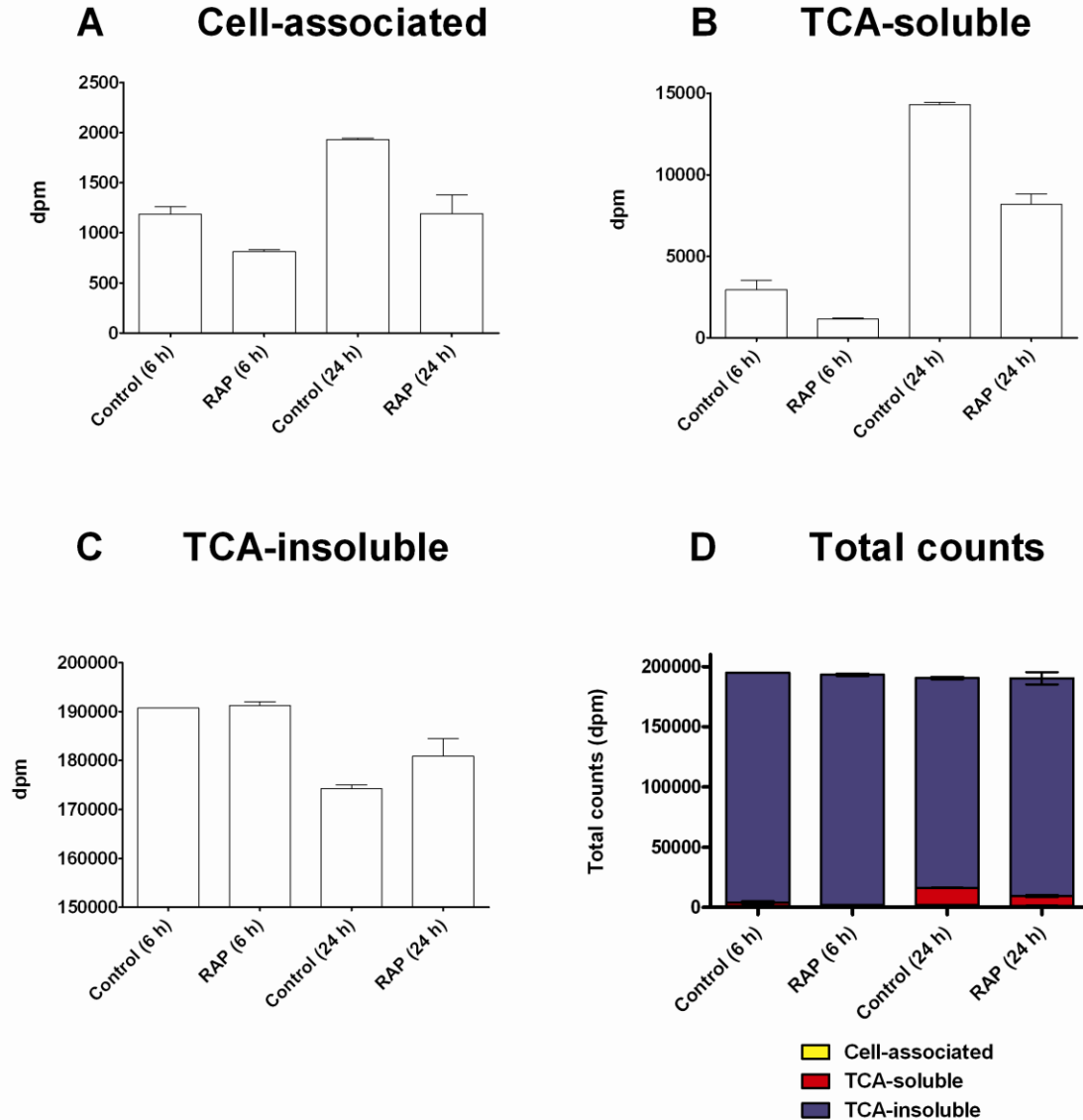


Figure 66: [^{125} I]- α_2 M* endocytosis by HTB94 cells.

HTB94 cells were seeded in a 12-well plate at a density of 0.5×10^6 cells, grown overnight and washed thrice in serum-free DMEM before incubation at 37 °C in DMEM containing 0.1 % BSA with or without RAP (500 nM). After 1 hour, [^{125} I]- α_2 M* (0.025 nM) was added. After 6 h or 24 h, the conditioned medium was removed and cell-associated, TCA-soluble and TCA-insoluble fractions were harvested as described in section 2.5.2. Results are an average of 2 wells $\pm$ range. **A**, Cell-associated counts. **B**, TCA-soluble counts (corrected for TCA-soluble counts in start material). **C**, TCA-insoluble counts. **D**, Total cell-associated, TCA-soluble and TCA-insoluble counts. The wash fractions were counted and found to be negligible.

5.3. Discussion

In this chapter, the role of the LDL-R family in the clearance of ADAMTS-4 by HTB94 cells was investigated. RAP blocked the clearance of [^{35} S]ADAMTS4-2 from the medium exposed to HTB94 cells and also from the medium exposed to both porcine and human chondrocytes. The clearance of [^{35} S]ADAMTS4-5 by HTB94 cells was also inhibited by RAP. This indicates that neither the cysteine-rich, thrombospondin type-1 or disintegrin domain of ADAMTS-4 are essential for RAP-sensitive ADAMTS-4 clearance by HTB94 cells. However, RAP showed greater protection against the appearance of the TCA-soluble fraction with [^{35} S]ADAMTS4-5 than [^{35} S]ADAMTS4-2. However, the level of protection by RAP also varied between HTB94 cells and chondrocytes. Since there is variability in these results, it will be important to investigate the protective effect of RAP further by experimental repetition. Experiments at 4 °C indicated that RAP did not affect surface binding of [^{35} S]ADAMTS4-2. In this regard, RAP has been shown to have no effect on the binding of [^{125} I]-pro-MMP-2:TIMP-2 to HT1080 cells. However, RAP reportedly inhibits endocytosis of the complex suggesting that an unknown RAP-insensitive co-receptor may be involved in the initial binding to the cells surface, followed by RAP-sensitive endocytosis mediated by LRP-1 (Emonard *et al.*, 2004). Additionally, although internalisation of MMP-13 is via the LRP-1 receptor, MMP-13 binds to wild-type (MEF-1) and LRP-1-null (MEF-2) cells at equal levels, suggesting that the LRP-1 receptor is not involved in the primary binding of MMP-13 to the cell surface (Barmina *et al.*, 1999). Ca^{2+} is necessary for MMP-13 binding to an unidentified 170 kDa receptor before transfer to the LRP-1 receptor (Barmina *et al.*, 1999). Future work would be to conduct a binding assay in the presence and absence of Ca^{2+} to investigate whether binding of [^{35} S]ADAMTS4-2 to the surface of HTB94 cells is dependent on this ion.

The possibility that RAP inhibited ADAMTS-4 clearance by compromising cell viability was ruled out because incubation of HTB94 cells with RAP for 24 h had no effect on cell viability assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT) assay. Future work would be to measure the effect of RAP and other agent tested in this study on cell apoptosis by staining cells with FITC conjugated-annexin V and propidium iodide or by TUNEL staining. Additionally, to confirm that ADAMTS-4 clearance is inhibited by a mechanism that is absolutely due to the action of RAP, one could test whether the His-tag alone has any effect on ADAMTS-4 clearance. In view of the data presented in Chapter 4, where GM6001 was found to block ADAMTS4-2 clearance, the effect of RAP in combination with GM6001 on ADAMTS4-2 clearance by HTB94 cells should also be assessed.

The role of LRP-1 in the clearance of ADAMTS4-2 was examined because RAP blocks the endocytosis of all known LRP-1 ligands (Herz *et al.*, 1991) although it also competes for the binding of ligands to several receptors of the LDL-R family aside from LRP-1 (Bu, 1998; Herz *et al.*, 1991). The first approach was to knockdown the expression of LRP-1 in HTB94 cells using siRNA. In a 24 h experiment, knockdown of LRP-1 expression by more than 50 % had no effect on the clearance of [³⁵S]ADAMTS4-2 compared to cells transfected with control siRNA. These data indicate that LRP-1 does not mediate the cellular uptake and clearance of ADAMTS4-2 by HTB94 cells. Future work would be to harvest the medium at earlier time points because during the course of the experiment (24 h), the expression of LRP-1 in cells treated with LRP-1 siRNA increased compared to the expression of LRP-1 at the start of the experiment. Alternatively, the efficiency of LRP-1 knockdown may be improved using vector-based short hairpin RNA (shRNA) system. Dedieu *et al* (Dedieu *et al.*, 2008) reported the efficiency of shRNA knockdown of LRP-1 in FTC133 cells (human follicular thyroid carcinoma cells) to be greater than 90 % compared to a 70 % efficiency using siRNA. Nonetheless, it would also be informative to assess the effect of RAP on the clearance of ADAMTS4-2 by HTB94 cells where LRP-1 expression was attenuated by either approach as this may reveal whether an LRP-1-independent but RAP-dependent mechanism of ADAMTS4-2 clearance exists in HTB94 cells.

To further investigate whether LRP-1 mediates ADAMTS4-2 endocytosis, the clearance of ADAMTS4-2 by wild-type mouse embryonic fibroblasts (MEF-1) and LRP-1-deficient mouse embryonic fibroblasts (PEA-13) was compared. The uptake of ADAMTS4-2 from the medium was found to be similar in both cell types. In contrast, RAP, uPA/PAI-1 complexes or α_2M^* are not endocytosed by PEA-13 cells although they are endocytosed by MEF-1 cells (Willnow and Herz, 1994). The results reported herein suggest that ADAMTS4-2 is not a ligand for LRP-1. The effect of RAP on the clearance of ADAMTS4-2 by either MEF-1 cells or PEA-13 cells would not yield any additional information since ligand blotting experiments have shown that RAP does not bind to any other membrane protein in PEA-13 fibroblasts (Willnow and Herz, 1994). This suggests that LRP-1 is the only RAP-binding LDL-R family member expressed in embryonic fibroblasts. However, this experiment should be conducted in order to confirm this.

Although a similar amount of intact [^{35}S]ADAMTS4-2 disappeared from the medium of MEF-1 cells compared to HTB94, the protective effect of heparin was more pronounced in HTB94 cells. This suggests that the mechanism of [^{35}S]ADAMTS4-2 clearance may differ between these cell types and that HTB94 cells may be more dependent on HSPGs than MEF cells, for example. It would be informative to compare the expression of HSPGs such as perlecan, syndecans and glypicans between these cell types.

To confirm that the MEF-1 and PEA-13 cells were behaving as expected and to ensure that the preparation of RAP inhibited LRP-1-mediated endocytosis, the endocytosis of [^{125}I]- α_2M^* was assessed. More [^{125}I]- α_2M^* was endocytosed by MEF-1 cells compared to PEA-13 cells and RAP inhibited the uptake of [^{125}I]- α_2M^* in MEF-1 cells but had no effect in PEA-13 cells. This shows that the cells were behaving as expected and that the preparation of RAP inhibited LRP-1 mediated endocytosis of [^{125}I]- α_2M^* . However, the TCA-soluble fraction of MEF-1 cells treated with RAP or of PEA-13 cells either with or without RAP increased during the course of the experiment compared to the amount of TCA-soluble

radioactivity at the start of the experiment. This suggests that an LRP-1 independent mechanism of [^{125} I]- α_2 M* degradation exists. The results reported here are contrary to a study demonstrating that LRP-1 is the only receptor mediating the uptake of [^{125} I]- α_2 M* in mouse embryonic fibroblasts (Willnow and Herz, 1994).

[^{125} I]- α_2 M* endocytosis by HTB94 cells was also assessed after 6 h and 24 h incubation. TCA-soluble radioactivity increased in the medium of HTB94 cells with incubation and was in part inhibited by RAP. The effect of RAP on the TCA-insoluble fraction was not significant at 6 h but RAP inhibited the TCA-insoluble fraction at 24 h. However, RAP was not fully protective against [^{125} I]- α_2 M* disappearance, supporting the hypothesis that an LRP-1-independent mechanism of [^{125} I]- α_2 M* endocytosis and intracellular degradation may exist in HTB94 cells. This is also contrary to reports that endocytosis of [^{125} I]- α_2 M* is unique to LRP-1 (Willnow and Herz, 1994).

Future work to yield information about the mechanism of ADAMTS4-2 clearance would be to compare the rate of ADAMTS4-2 clearance by HTB94 cells to that of α_2 M*. Since α_2 M* was labeled with ^{125} I, preparing [^{125} I]-ADAMTS4-2 would ensure that the sensitivity of detection could be comparable between proteins. As a first step to this goal, ADAMTS4-2 was subject to iodination following the same method used to prepare [^{125} I]- α_2 M*. However, iodination of ADAMTS4-2 yielded an inactive enzyme, possibly due to the harsh oxidation conditions of the reaction. Further attempts to maintain enzyme activity upon iodination, by reducing the incubation time of the iodination reaction for example could be attempted.

The data presented in this chapter support a RAP-sensitive mechanism of ADAMTS-4 clearance by HTB94 cells. However, RAP only inhibits partially, so RAP-insensitive mechanisms also exist. As previously mentioned, pressing work will be to test the effect of RAP in combination with GM6001 to determine

whether RAP-insensitive, GM6001-insensitive mechanisms of ADAMTS-4 clearance exist before conducting further studies. In addition to binding to LRP-1, RAP binds to other members of the LDL-R family. For example, RAP also binds to megalin (LRP-2) (Kounnas *et al.*, 1992b) and VLDL-R (Battey *et al.*, 1994) with high affinity although it binds the LDL-R weakly (Medh *et al.*, 1995). Given the data suggesting that LRP-1 knockdown in HTB94 cells or genetic deficiency of LRP-1 in embryonic fibroblasts does not prevent ADAMTS-4 clearance, it is hypothesised that RAP may inhibit ADAMTS-4 cellular uptake mediated by another member of the LDL-R family. Additionally, RAP has been reported to bind to other receptors, including cubilin (Birn *et al.*, 1997), sortilin (Petersen *et al.*, 1997; Tauris *et al.*, 1998) and lipolysis-stimulated receptor (Troussard *et al.*, 1995). Furthermore, the C-terminal domain of RAP also binds to heparin (Orlando and Farquhar, 1993, 1994), suggesting that RAP could inhibit the clearance of ADAMTS4-2 by HTB94 cells and chondrocytes by binding to HSPGs at the cell surface.

To further investigate this hypothesis, the identification of proteins in cell membrane extracts capable of binding to ADAMTS-4 would be informative. Cell membrane extracts from HTB94 cells could be separated under conditions of non-reducing SDS-PAGE followed by transfer to PVDF membrane. The membrane could then be subjected to ligand binding using [¹²⁵I]-ADAMTS4-2, which would offer increased sensitivity over [³⁵S]ADAMTS4-2. Potential cell surface ADAMTS-4 binding proteins could also be identified by immunoprecipitation using an ADAMTS-4 antibody or ADAMTS-4 could be immobilized onto a resin and the membrane extract passed over the resin. Any bound proteins could be identified by SDS-PAGE followed by mass spectrometry. Alternatively, cell membrane extracts could be used in ligand binding experiments with RAP to determine RAP-binding proteins. This may reveal which proteins RAP interacts with to inhibit [³⁵S]ADAMTS4-2 cellular uptake and clearance by HTB94 cells. Another strategy would be to identify which members of the LDL-R family HTB94 cells express and then perform

knockdown studies to determine the effect of each receptor on the ADAMTS4-2 clearance.

In summary, the results reported herein have eliminated the role of LRP-1 in the clearance of ADAMTS-4 by HTB94 cells and suggest that other RAP-binding proteins such as another member of the LDL-R family may be responsible.

Chapter 6

General discussion and concluding remarks

As discussed in Chapter 1, ADAMTS-4 is one of several members of the ADAMTS family with the ability to cleave the IGD domain of aggrecan at the Glu³⁷³-Ala³⁷⁴ bond. Although both ADAMTS-4 and ADAMTS-5 were initially purified from IL-1 α -stimulated cartilage explants, suggesting that they both may be involved in cartilage matrix degradation, studies in knockout mice have indicated that ADAMTS-5 may be a more important aggrecanase in the progression of osteoarthritis, at least in the mouse (Glasson *et al.*, 2005; Stanton *et al.*, 2005). However, siRNA knockdown of either ADAMTS-4 or ADAMTS-5 reduced aggrecan degradation in both OA and normal human cartilage stimulated with TNF α or oncostatin M (Song *et al.*, 2007). In addition, a recent study using immunohistochemistry techniques showed that ADAMTS-4 was detected in human OA chondrocytes and not control cartilage and that the expression of ADAMTS-4 was increased with disease severity (Cheung *et al.*, 2009). Furthermore, the expression of ADAMTS-4 correlated with demethylation of CpG sites within the ADAMTS-4 promoter region, suggesting that ADAMTS-4 is epigenetically regulated. Together, these studies suggest that degradation of aggrecan by ADAMTS-4 could contribute to human OA.

Whilst the activity of ADAMTS-4 can be regulated in a number of ways including gene expression, prodomain removal, C-terminal processing and inhibition by TIMP-3, the focus of this thesis was to assess whether endocytosis of ADAMTS-4 from the extracellular milieu could be an additional mechanism regulating ADAMTS-4 activity in the ECM. Further research into the mechanisms by which ADAMTS-4 activity may be regulated is essential in order to understand the role of ADAMTS-4 in the progression of osteoarthritis. In addition, such work may ultimately assist with the development of agents to prevent and/or delay the onset of arthritic disease.

To determine whether ADAMTS-4 could be endocytosed, I first assessed whether exogenously added ADAMTS4-2, a truncated form of full-length ADAMTS-4 lacking the C-terminal spacer domain, could disappear from the

medium of HTB94 chondrosarcoma cells (Chapter 3). I discovered that ADAMTS4-2 disappeared from the medium of HTB94 cells over time and fluorescent-labeled ADAMTS4-2 could be detected within the cell by confocal microscopy. Further study using a radioactive-based assay (Chapter 4 & 5) showed that the clearance of ADAMTS4-2 by HTB94 cells was reduced by hydroxamate-based metalloproteinase inhibitors (e.g. GM6001), cytochalasin D and sulfated GAGs such as heparin and calcium pentosan polysulfate. In addition, I identified that the clearance of ADAMTS4-2 was partially dependent on cell surface HSPGs as well as the LDL receptor family antagonist RAP. Although the majority of this work was conducted using ADAMTS4-2, a limited number of experiments using the catalytic domain of ADAMTS-4 alone (ADAMTS4-5) showed that the clearance of ADAMTS-4 by HTB94 cells is not dependent on sites within the ancillary domain. The uptake of the catalytic domain of ADAMTS-4 was also partially inhibited by heparin, GM6001 or RAP. As previously discussed in Chapter 4, future work will be to determine whether full-length ADAMTS4 (ADAMTS4-1) is endocytosed by HTB94 cells, particularly given that ADAMTS4-1 has high affinity for the ECM and cell surface (Kashiwagi *et al.*, 2004).

RAP binds to LRP-1, one of several members of the LDL receptor family with high affinity, inhibiting the uptake of several LRP-1 ligands (Bu *et al.*, 1992; Herz *et al.*, 1992; Nykjaer *et al.*, 1992). However, the work presented in this thesis showed that ADAMTS4-2 clearance was not blocked by LRP-1 deficient cells compared to wild-type cells nor by siRNA knockdown of LRP-1 expression in HTB94 cells. This suggests that another member of the LDL receptor family aside from LRP-1 may be responsible for mediating the clearance of ADAMTS4-2 by HTB94 cells. This is supported by studies in which RAP has been shown to bind to other members of the LDL receptor family with high affinity including megalin (Kounnas *et al.*, 1992a), VLDL-R (Battey *et al.*, 1994), apoER2 (Stockinger *et al.*, 1998) and sorLA/LR11 (Jacobsen *et al.*, 1996). Future work would be to determine which members of the LDL receptor family aside from

LRP-1 are expressed by HTB94 cells and perform knockdown experiments to determine whether any of them mediate ADAMTS4-2 cellular uptake and degradation. RAP, in addition to binding to members of the LDL receptor family also binds directly to heparin via residues within the C-terminal domain (Orlando and Farquhar, 1993, 1994), suggesting that RAP could inhibit ADAMTS4-2 cellular uptake by HTB94 cells and chondrocytes by binding to an unidentified HSPG receptor at the cell surface, displacing bound ADAMTS4-2. However, in our experiments, RAP did not affect the binding of ADAMTS4-2 to the cell surface at 4 °C suggesting that a RAP-insensitive co-receptor is involved in the initial binding of ADAMTS4-2 to the cell surface, followed by RAP-sensitive endocytosis, as has been described for MMP-2 (Emonard *et al.*, 2004) and MMP-13 (Barmina *et al.*, 1999). Alternatively, the role of other receptors in the cellular uptake of ADAMTS-4 should also be considered since RAP also binds to several other receptors including lipolysis-stimulated receptor (Troussard *et al.*, 1995), cubilin (Birn *et al.*, 1997) and sortilin (Petersen *et al.*, 1997; Tauris *et al.*, 1998), for example.

The data presented in this thesis indicate that metalloproteinase-mediated degradation of ADAMTS-4 at the cell surface may account for as much as 50 % of ADAMTS-4 clearance by HTB94 cells. Pressing work will be to determine the effect of GM6001 on the cellular uptake and degradation of ADAMTS4-2 by HSPGs-deficient CHO745 cells compared to wild-type cells, in both the presence and absence of RAP. This experiment may enable the relative contribution of each pathway to be assessed and conclusively determine whether the clearance of ADAMTS4-2 from the medium of HTB94 cells in the presence of RAP is in fact due to metalloproteinase-mediated degradation at the cell surface.

In view of the observation that the clearance of ADAMTS-4 from the medium of HTB94 cells can be inhibited by GM6001, published studies investigating LRP-1 mediated endocytosis have reported that the activity of the LRP-1 receptor can be regulated by MT1-MMP in malignant cells (Rozanov *et al.*, 2004). MT1-MMP

proteolysis of LRP-1 was reported to inactivate the ligand binding domain of LRP-1 and inhibit the internalisation of LRP-1 ligands whilst GM6001 blocked MT1-MMP-mediated proteolysis of LRP-1. Although the effect of GM6001 on ligand endocytosis was not directly assessed in the published study, I hypothesize that GM6001 would enhance the endocytosis of LRP-1 ligands by inhibiting LRP-1 shedding from the cell surface. This is in contrast with the effect of GM6001 on the clearance of ADAMTS-4 by HTB94 cells reported in this thesis and further suggests that LRP-1 is not involved in the mechanism of ADAMTS-4 clearance reported herein.

A RAP-dependent mechanism of MMP-13 endocytosis exists in human chondrocytes although the receptor was identified to be LRP-1 since the internalisation of MMP-13 from the medium was reduced by LRP-1-deficient cells compared to wild type cells (Barmina *et al.*, 1999). A subsequent study demonstrated that MMP-13 internalisation from the medium was reduced by OA chondrocytes suggesting that impaired MMP-13 internalisation could contribute to cartilage matrix degradation in OA (Walling *et al.*, 2003). Since the data presented herein suggest that ADAMTS-4 is regulated by endocytosis, further study would be to determine whether ADAMTS-4 endocytosis is different between healthy and OA chondrocytes, as is the case for MMP-13. If so, it would be of interest to determine the relative importance of impaired ADAMTS-4 internalisation compared to that of MMP-13 internalisation in the development and progression of OA.

The HSPG-dependent mechanism of ADAMTS-4 endocytosis will also be the subject of future study. Whilst HSPGs have been suggested to be co-receptors for the endocytosis of some LRP-1 ligands, they have also been identified as independent receptors mediating the endocytosis of bound ligands as part of the normal HSPG turnover on the cell surface. For example, the HSPG perlecan has been shown to mediate the uptake of HIV-1 Tat, a transcriptional transactivator of the HIV-1 virus (Argyris *et al.*, 2004). However, only a small amount of HIV-1 Tat

protein was degraded, which suggests that perlecan-mediated internalisation of HIV-1 Tat does not deliver HIV-1 Tat to the lysosomes. Fuki *et al* reported that the syndecan family of proteoglycans internalise lipoprotein lipase (LpL)-enriched low density lipoprotein (LDL) via a mechanism independent to the LRP-1 pathway (Fuki *et al.*, 1997) but dependent on cholesterol-rich membrane rafts (Fuki *et al.*, 2000b). In addition, perlecan has been reported to mediate the endocytosis of LpL-enriched LDL independent to either syndecan or LRP-1, via a mechanism sensitive to the tyrosine kinase inhibitor genistein (Fuki *et al.*, 2000a). Since the chondrocyte pericellular matrix is rich in perlecan (Melrose *et al.*, 2006), experiments to determine whether perlecan mediates the clearance of ADAMTS-4 from the medium of HTB94 cells and chondrocytes will be an interesting line of future study.

Our group has recently reported that endogenous TIMP-3 accumulated in HTB94 cells upon treatment with heparin (Troeberg *et al.*, 2008) and unpublished work within our laboratory has also shown that exogenously added TIMP-3 clears from the medium of HTB94 cells in a manner similar to that observed with exogenously added ADAMTS4-2, with both heparin and RAP also inhibiting TIMP-3 clearance. These findings suggest that ADAMTS4-2 and TIMP-3 may be regulated in the same way. TIMP-3 contains heparin-binding domains and binds sulfated GAGs of the ECM (Yu *et al.*, 2000) and also binds to ADAMTS-4 with high affinity (Kashiwagi *et al.*, 2001). As depicted in Figure 67, it is possible that ADAMTS4-2 could be internalized in a complex with TIMP-3. This hypothesis is based on the observations that ADAMTS4-2 does not bind heparin-Sepharose and the major GAG binding sites of ADAMTS-4 have been mapped to the spacer domain, which is absent from ADAMTS4-2. Without considering the possibility that ADAMTS-4 is internalised in a complex with TIMP-3, it is difficult to explain why heparin inhibits the clearance of ADAMTS-4 by HTB94 cells. If internalised as a complex, this observation could be explained by the possibility that heparin blocks the association between TIMP-3 and cell surface HSPGs. I also considered this hypothesis because the LRP-1 ligands MMP-2 and -9 can be

internalized alone or in complex with TIMP-2 or TIMP-1, respectively (Emonard *et al.*, 2004; Hahn-Dantona *et al.*, 2001). Thus, I further hypothesize that whilst GM6001 may inhibit metalloproteinase-mediated proteolysis of ADAMTS-4 at the cell surface, GM6001 may also inhibit endocytosis by disrupting the interaction between the ADAMTS-4 and TIMP-3 complex. RAP may inhibit the clearance of ADAMTS-4 by HTB94 cells and chondrocytes by disrupting the interaction of the ADAMTS-4-TIMP-3-HSPG complex with a RAP-sensitive, as yet unidentified endocytic receptor. Although I have eliminated LRP-1 as the RAP-sensitive receptor in this mechanism, further work to validate the ADAMTS-4-TIMP-3 complex hypothesis would be to first determine whether exogenous TIMP-3 can be endocytosed by LRP-1 using LRP-1 wild-type and knockout cells. In this study, I conducted preliminary experiments to determine whether ADAMTS-4 could be internalised in the absence of TIMP-3 by investigating the clearance of ADAMTS4-2 by wild-type and *Timp-3*-null mouse dermal fibroblasts. However, these experiments were preliminary and very little intact ADAMTS4-2 disappeared from the medium during culture. Heparin had a greater inhibitory effect than GM6001, reducing the TCA-soluble fraction by more than 95 %, GM6001 reduced the TCA-soluble fraction by 68 % in wild-type cells and by 40 % in *Timp-3*-null cells. However, the differential effect of GM6001 between wild-type and *Timp-3*-null cells suggests that a mechanism whereby ADAMTS-4 is endocytosed as a complex with TIMP-3 may exist. The cell-associated fraction was increased in control wild-type cells compared to control *Timp-3*-null cells and heparin and GM6001 reduced the amount of cell-associated radioactivity in wild-type fibroblasts but had no effect on the cell-associated fraction from *Timp-3*-null fibroblasts. This suggests that [³⁵S]ADAMTS4-2 may interact with cell surface TIMP-3. However, it is clear that more work will be required in order to determine whether ADAMTS-4 is actually endocytosed in a complex with TIMP-3. An alternative approach other than using *Timp-3*-null fibroblasts may be to knockdown the expression of TIMP-3 in HTB94 cells by siRNA.

Figure 67 summarizes the possible mechanisms by which ADAMTS-4 clearance by HTB94 cells and chondrocytes may be inhibited by GM6001, heparin and RAP. However, it is likely that more than one mechanism of ADAMTS-4 clearance exists and that cell surface metalloproteinase-mediated degradation of ADAMTS-4 in addition to RAP-sensitive and/or HSPG-sensitive mechanisms each contribute to the cellular uptake and clearance of ADAMTS-4 by HTB94 cells and chondrocytes reported in this study.

Concluding remarks

The work described in this thesis has identified a novel mechanism of ADAMTS-4 regulation by HTB94 cells and chondrocytes. These results indicate that ADAMTS4-2 is cleared from the medium by HTB94 cells and chondrocytes via a mechanism that is sensitive to hydroxamate-based metalloproteinase inhibitors, HSPGs and the LDL receptor family antagonist RAP. It will be important to investigate whether the endocytosis of ADAMTS-4 changes with age and/or with the development of OA as well as extending the study to other aggrecanases, notably ADAMTS-5. Additionally, endocytosis may be a general mechanism regulating ADAMTS activity in tissues other than cartilage. For example, as the expression of both ADAMTS-4 and -5 have been reported to be increased in human glioblastomas (Held-Feindt *et al.*, 2006; Matthews *et al.*, 2000; Nakada *et al.*, 2005), it is tempting to suggest that impaired endocytosis of either/both enzymes may contribute to their invasive potential in the brain. Whilst the results reported within this thesis provide the first steps to understanding the regulation of ADAMTS-4 activity by endocytosis, studies are required to further understand the role of HSPGs in this process. Further work to characterize the RAP-sensitive mechanism of ADAMTS4-2 clearance in addition to addressing whether impaired endocytosis of ADAMTS-4 contributes significantly to the development of osteoarthritis are also important questions worthy of further investigation.

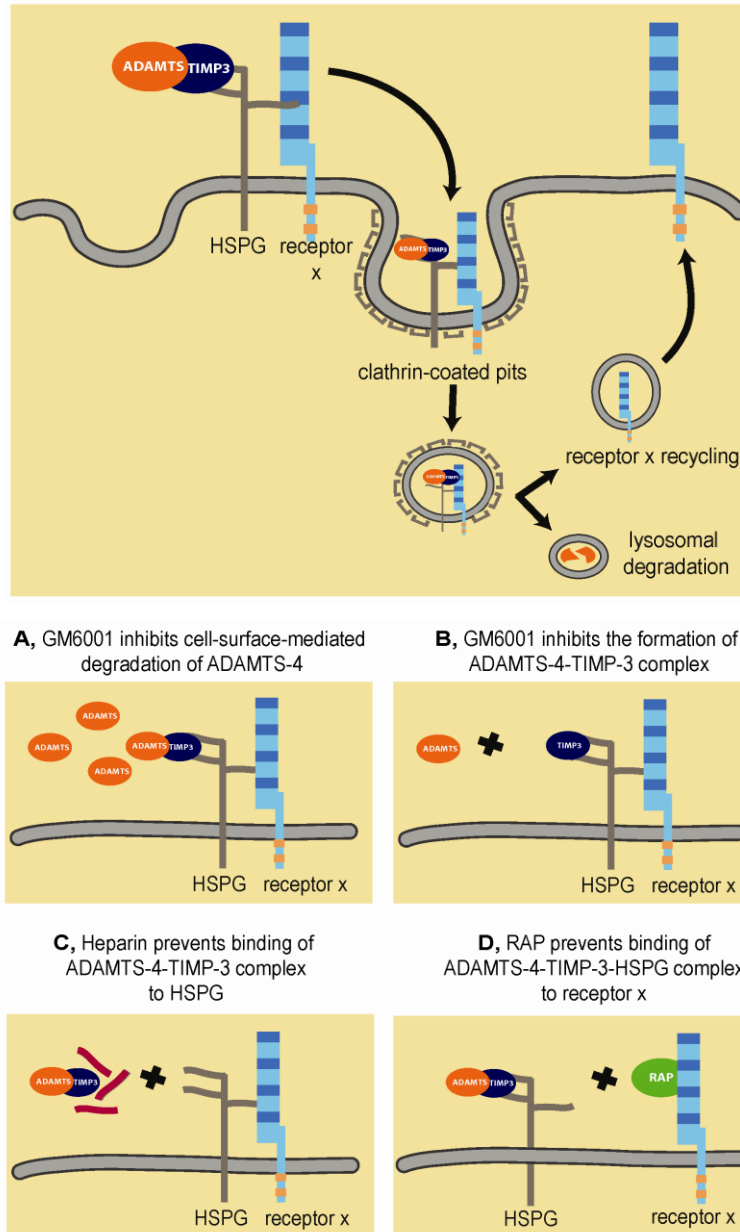


Figure 67: Hypothetical mechanism of ADAMTS-4 clearance by HTB94 cells and chondrocytes.

I propose that ADAMTS-4 may be endocytosed in a complex with TIMP-3. The complex first binds to cell surface HSPGs via interaction with TIMP-3 and is then transferred to a second receptor which mediates internalisation. **A**, GM6001 may inhibit ADAMTS-4 proteolysis at the cell surface and/or **B**, disrupt the formation of the ADAMTS-4-TIMP-3 complex. This reduces TIMP-3 uptake from the medium and intracellular degradation. **C**, I propose that heparin inhibits internalisation of the complex by preventing the binding of TIMP-3 to HSPGs whilst **D**, RAP inhibits the internalisation of the ADAMTS-4-TIMP-3-HSPG complex by preventing the interaction between the complex and an unidentified RAP-sensitive endocytic receptor. As depicted here, HSPGs may be endocytosed in a complex with receptor x. Alternatively, they may mediate transfer to receptor x and remain on the cell surface.

Chapter 7

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