METALLOPROTEINASE CLEAVAGE OF VERSICAN AT THE FIBROBLAST CELL SURFACE

by

Sean Bertram Maurice

B.Kin., University of Calgary, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Dental Science)

THE UNIVERSITY OF BRITISH COLUMBIA

October, 2009

© Sean Bertram Maurice, 2009

ABSTRACT

Versican is a large aggregating proteoglycan expressed in the pericellular matrix of fibroblast cells. It is highly expressed during development and remodeling. The regulated synthesis and degradation of versican are associated with physiological remodeling. Versican is expressed in fibroproliferative lesions of human pulmonary fibrosis and atherosclerosis. Stromal expression of versican is associated with many forms of cancer and may be predictive of poor prognosis. Abnormal persistence of the versican-rich matrix may contribute to fibroproliferative and oncogenic processes. The process of versican degradation is not understood, but as versican is a pericellular molecule, physiological degradation likely involves cell surface-associated proteolysis. As such, the overarching hypothesis for this work is that **regulated versican turnover involves the cell surface-associated metalloproteinases ADAMTS-2, MMP-2 and MT1-MMP, that are expressed in versican-rich remodeling lesions.**

ADAMTS-2 is a procollagen N-propeptidase involved in collagen fibrillogenesis. As procollagen is synthesized in a versican-rich matrix, it was hypothesized that ADAMTS-2 might bind and process versican. MMP-2 and MT1-MMP in complex with TIMP-2, are activated at the cell surface during wound healing, pulmonary fibrosis and cancer.

Versican was purified from human fetal lung fibroblast cultures for *in vitro* proteolysis experiments. The purified versican preparation was characterized by electrophoresis, chromatography, spectrophotometry and mass spectrometry. ADAMTS-2 and versican localization in normal and fibrotic human lungs were investigated. ADAMTS-2 was shown to co-purify with versican from human fetal lung fibroblasts. Bovine ADAMTS-2 was purified from fetal calf skin and shown to cleave purified human versican.

The plant lectin concanavalin-A (ConA) induces a matrix degradative phenotype and is used here to investigate the process of versican degradation relative to apoptotic

ii

events in human fetal lung fibroblast cultures. Con-A induced increased expression of MMP-2 and MT1-MMP in human fetal lung fibroblasts and a concomitant loss of versican from the matrix. Microarray analysis was used to investigate expression of possible versican-degrading enzymes and their inhibitors, expressed in response to ConA. Recombinant MMP-2 and MT1-MMP were shown to process purified versican *in vitro*.

This work expands upon the body of knowledge of versican turnover and should help in the search for therapeutic avenues to treat fibroproliferative and oncogenic processes.

TABLE OF CONTENTS

| Abstract | ii |
|-------------------------|------|
| Table of Contents | iv |
| List of Tables | ix |
| List of Figures | x |
| Abbreviations | xiii |
| Dedication | xiv |
| Acknowledgements | xv |
| Co-authorship Statement | xvi |

CHAPTER 1 – BACKGROUND

| 1.1. Vers | sican in normal physiology | . 1 |
|-----------|----------------------------------|-----|
| 1.1.1. | Development | . 2 |
| 1.1.2. | Reproduction | . 4 |
| 1.2. Vers | sican in wound healing disorders | . 5 |
| 1.2.1. | Wound healing and fibrosis | . 5 |
| 1.2.2. | Pulmonary fibrosis | 10 |
| 1.2.3. | Atherosclerosis | 13 |
| 1.2.4. | Cancer stroma | 15 |
| 1.2.5. | Arthritis | 19 |
| 1.2.6. | Tendinopathies | 19 |
| 1.3. Vers | sican structure | 20 |
| 1.3.1. | Hyalectans | 21 |
| 1.3.2. | Gene organization and regulation | 21 |
| 1.3.3. | Splice variants | 24 |

| 1.3.4. Glycosaminoglycans | 27 |
|---|----|
| 1.3.5. N-terminus/G1 | 30 |
| 1.3.6. C-terminus/G3 | 30 |
| 1.4. Versican turnover in tissue remodeling | 31 |
| 1.4.1. Matrix metalloproteinases | 32 |
| 1.4.2. ADAMTS | 37 |
| 1.4.3. Known and unknown versican proteolytic events | 40 |
| 1.4.4. Versican proteolysis | 41 |
| 1.4.5. Glial hyaluronate binding protein | 41 |
| 1.4.6. Hyaluronectin | 43 |
| 1.4.7. Matrikines | 44 |
| 1.4.8. Potential mechanisms of altered proteolysis in aberrant tissue | |
| remodeling | 45 |
| 1.5. Rationale | 45 |
| 1.5.1. Overarching hypothesis | 48 |
| 1.5.2. Specific aims | 48 |
| 1.6. References | 50 |
| | |
| CHAPTER 2 – PURIFICATION AND CHARACTERIZATION OF VERSICAN | |
| 2.1. Summary | 78 |
| 2.2. Introduction | 78 |
| | |

| | 2.3.4. Electrophoretic techniques | 82 |
|-----|--|-------|
| | 2.3.5. Western blotting | 83 |
| | 2.3.6. Quantification of chondroitin sulfate concentration with the DMMB | |
| | assay | 84 |
| | 2.3.7. Gel filtration chromatography | 84 |
| | 2.3.8. Characterization of proteolytic fragments | 84 |
| | 2.3.9. Enzyme incubation | 85 |
| | 2.3.10. Proteomic identification | 85 |
| 2.4 | I. Results | 86 |
| | 2.4.1. Versican purification from human fetal lung fibroblast cells | 86 |
| | 2.4.2. Optimization of versican purification | 89 |
| | 2.4.3. Gel filtration analysis of versican | 91 |
| | 2.4.4. Characterization of versican degradation | 91 |
| | 2.4.5. Mass spectrometric characterization of versican | 93 |
| | 2.4.6. Versican glycosylation and characterization | 95 |
| 2. | 5. Discussion | 97 |
| 2.6 | 6. References | . 100 |

CHAPTER 3 – VERSICAN-ADAMTS-2 INTERACTIONS IN HUMAN

PULMONARY FIBROSIS

| 3.1. | Summary | 106 |
|------|-------------------------|-----|
| 3.2. | Introduction | 107 |
| 3.3. | Experimental procedures | 109 |
| 3. | 3.1. Patient samples | 109 |
| 3. | 3.2. Histology | 110 |

| | 3.3.3. Immunohistochemistry | 110 |
|-----|---|-----|
| | 3.3.4. Release of ADAMTS-2 from normal lung tissues | 111 |
| | 3.3.5. Cell culture | 111 |
| | 3.3.6. Isolation of versican | 112 |
| | 3.3.7. Co-purification of versican and ADAMTS-2 | 113 |
| | 3.3.8. Separate elution of ADAMTS-2 and versican | 113 |
| | 3.3.9. Electrophoretic techniques | 113 |
| | 3.3.10. Purification of fetal bovine skin ADAMTS-2 | 115 |
| | 3.3.11. Versican digestion and ADAMTS-2 incubations | 115 |
| 3.4 | . Results | 116 |
| | 3.4.1. Versican and ADAMTS-2 co-localize in normal human lungs | 116 |
| | 3.4.2. Versican and ADAMTS-2 localization in BOOP | 116 |
| | 3.4.3. Versican and ADAMTS-2 are localized to remodeling areas in UIP | 118 |
| | 3.4.4. Release of ADAMTS-2 from normal human lung tissue | 118 |
| | 3.4.5. ADAMTS-2 co-purifies with versican at physiological pH | 120 |
| | 3.4.6. ADAMTS-2 co-purification with versican is pH dependent | 123 |
| | 3.4.7. Purification of bovine ADAMTS-2 | 125 |
| | 3.4.8. ADAMTS-2 degrades versican | 127 |
| | 3.4.9. Versican inhibits auto-degradation of ADAMTS-2 | 129 |
| 3.5 | Discussion | 131 |
| 3.6 | . References | 135 |

CHAPTER 4 – VERSICAN DEGRADATION AT THE CELL SURFACE BY

MMP-2 AND MT1-MMP

| 4.1. | Summary | , | 14 | 1 | J |
|------|---------|---|----|---|---|
|------|---------|---|----|---|---|

| 4.2. Introduction | 142 |
|--|-----|
| 4.3. Experimental procedures | 144 |
| 4.3.1. Tissue culture | 144 |
| 4.3.2. Immunofluorescence staining and microscopy | 145 |
| 4.3.3. SDS-PAGE | 146 |
| 4.3.4. Gelatin zymography | 147 |
| 4.3.5. RNA preparation | 147 |
| 4.3.6. Microarrays | 148 |
| 4.3.7. Enzyme assays | 149 |
| 4.4. Results | 150 |
| 4.4.1. ConA induces degradation of versican | 150 |
| 4.4.2. ConA induces changes in MMP-2 and MT1-MMP localization | 150 |
| 4.4.3. Microarrays | 153 |
| 4.4.4. Caspase and MMP inhibition alters the apoptotic response of | |
| fibroblast cells to ConA | 155 |
| 4.4.5. MMP-2 and MT1-MMP cleave versican in vitro. | 157 |
| 4.4.6. MT1-MMP cleaves and disaggregates recombinant versican G3 | 159 |
| 4.5. Discussion | 159 |
| 4.6. References | 165 |

| CHAPTER 5 – CONCLUDING REMARKS AND FUTURE DIRECTIONS | 172 |
|---|-----|
| 5.1. References | 179 |
| Appendix 1 – Complete data set of CLIP-CHIP proteinase, inhibitor and | |
| control spot fold changes and <i>p</i> -values | 183 |

LIST OF TABLES

| Table 1.1 | Versican expres | sion in cancer | | . 16 | 3 |
|-----------|-----------------|----------------|--|------|---|
|-----------|-----------------|----------------|--|------|---|

LIST OF FIGURES

| Figure 1.1 Structures of interstitial proteoglycans revealed by electron |
|--|
| microscopy 3 |
| Figure 1.2 Granulation tissue in a cutaneous wound at five days post injury7 |
| Figure 1.3 Myofibroblasts in normal and pathological wound healing |
| Figure 1.4 Versican expression associated with proliferating fibroblasts in |
| idiopathic pulmonary fibrosis 12 |
| Figure 1.5 Versican localization in atherosclerosis |
| Figure 1.6 Hyalectan family members in the central nervous system |
| Figure 1.7 Versican splice variants showing domain composition and potential |
| glycosylation sites |
| Figure 1.8 Versican V0 primary sequence and potential glycosylation attachment |
| sites |
| Figure 1.9 Glycosaminoglycan monomer constituents |
| Figure 1.10 MMP family domain organization, active site consensus sequence |
| and propeptide 'cysteine switch' |
| Figure 1.11 Common and divergent pathways of regenerative versus pathological |
| wound healing |
| Figure 1.12 ADAMTS family domain organization and evolutionary |
| relationships |
| Figure 1.13 Detection of versican proteolytic fragments |
| Figure 1.14 Normal and aberrant proteolysis of versican at the cell surface 46 |
| Figure 2.1 Versican at the cell surface of human fetal lung fibroblasts |
| Figure 2.2 Purification of versican from human fetal lung fibroblast cells at |
| рН 7.5 |

| Figure 2.3 | Chondroitin sulfate concentration measured by the DMMB assay 90 |
|---------------|--|
| Figure 2.4 | Analysis of versican separated by gel filtration |
| Figure 2.5 | N-terminal sequencing of versican degradation product |
| Figure 2.6 | Tryptic versican peptides detected in MS/MS |
| Figure 2.7 | Versican cleavage sites and peptides detected in proteomics |
| experiment | s compared with potential glycosylation sites |
| Figure 3.1 \ | Versican and ADAMTS-2 localization in normal lungs and in |
| bronchiolitis | s obliterans organizing pneumonia (BOOP)117 |
| Figure 3.2 \ | Versican and ADAMTS-2 localization in usual interstitial pneumonia |
| (UIP) | |
| Figure 3.3 | Release of ADAMTS-2 from normal human lung tissue |
| Figure 3.4 | ADAMTS-2 and versican co-purify 122 |
| Figure 3.5 | ADAMTS-2 co-purification with versican is pH dependent 124 |
| Figure 3.6 | Purification of bovine ADAMTS-2 from fetal calf skin |
| Figure 3.7 | ADAMTS-2 degrades versican in vitro128 |
| Figure 3.8 | Versican inhibits ADAMTS-2 auto-degradation |
| Figure 4.1 | Concanavalin A induces degradation of versican concomitant with |
| fibroblast ce | ell apoptosis 151 |
| Figure 4.2 | Concanavalin A induced changes in metalloproteinase expression |
| and localiza | ation |
| Figure 4.3 | Microarray analysis of differentially expressed fibroblast proteases |
| and inhibito | ors in response to Concanavalin-A154 |
| Figure 4.4 | Caspase and MMP inhibition alters the apoptotic response of |
| fibroblast ce | ells to Concanavalin-A 156 |
| Figure 4.5 | MMP-2 and MT1-MMP cleave versican <i>in vitro</i> |

Figure 4.6 MT1-MMP cleaves and disaggregates recombinant versican G3..... 160

ABBREVIATIONS

- ADAMTS-2 a disintegrin and metalloproteinase with thrombospondin motifs-2;
- MMP-2 matrix metalloproteinase-2;
- MT1-MMP membrane-type matrix metalloproteinase-1;
- IPF idiopathic pulmonary fibrosis;
- CSPG chondroitin sulfate proteoglycan;
- BOOP bronchiolitis obliterans organizing pneumonia;
- HUVEC human umbilical vein endothelial cell;
- BSA bovine serum albumin;
- HFL-1 human fetal lung fibroblast;
- ECM extracellular matrix;
- PBS phosphate-buffered saline;
- TBS tris-buffered saline;
- UIP usual interstitial pneumonia;
- DMEM Dulbecco's modified Eagle medium;
- CM conditioned medium;
- DMMB 1,9-dimethylmethylene blue chloride.
- ConA concanavalin A;
- Z-FA-FMK carboxybenzyl-phenylalanine, alanine, fluoromethylketone;
- Z-VAD-FMK carboxybenzyl-valine, alanine, aspartate, O-methylated fluoromethylketone;
- SAM significance analysis of microarrays.

DEDICATION

I am deeply indebted to many people who have helped, encouraged and supported me over the past 6 years. Above all, I would like to dedicate this work to my family:

To my wonderful, long-suffering wife Andrea, who fulfills my heart and soul, and has enabled me to live up to my fullest potential.

To my children, Zachary, Mary-Grace and Susan, for giving me a break from my work, for keeping my worries in perspective, for making me smile, laugh and play, when I might otherwise forget to.

To my parents who worked hard to provide a decent upbringing for me and my sisters, who challenged me to be my best and at the same time allowed me to learn from my own mistakes.

To the Lord God, without whom nothing would be possible and by who's grace I live each day.

ACKNOWLEDGEMENTS

I am forever indebted to Dr. Clive Roberts for many years of dedicated supervision. Over the last six years Clive has always been generous with his time, offering extensive guidance and thought provoking suggestions. This work would not have been possible without the kind supervision, trust and congeniality that was offered to me in the lab. It has been a privilege having Clive as a mentor.

I am indebted to members of the Roberts lab, Saloumeh Pourmalek and Rendi Yan for friendship and technical assistance in the lab. I also thank prior lab member Dennis Lee who performed significant groundwork on versican purification that benefited my studies enormously.

I am extremely grateful to Dr. Chris Overall for his assistance, advice and encouragement throughout my thesis work. I am also grateful to Dr. Overall and Dr. Roberts for the collaborative atmosphere that I was able to train in. I thank my committee members Dr. Ed Putnins and Dr. Doug Waterfield for taking time to meet one on one, for offering thorough and thoughtful feedback on my work and for their encouragement. I would like to thank the many members of the Overall lab for providing advice, mentorship and friendship, and allowing me to feel like a member of their lab, more than just a collaborator.

I am also grateful to Dr. Ross MacGillivray and the Centre for Blood Research that is an outstanding interdisciplinary facility and a privilege to work in. My future as a scientist is brighter due to the diverse scientists and techniques that I was exposed to within this centre.

CO-AUTHORSHIP STATEMENT

Chapter 2 - I was involved in all aspects of the experimental design, data analysis and writing of the manuscript, with assistance from Dr. Clive Roberts, Dr. Chris Overall and Dr. Alain Doucet. I performed all the experiments described with the exception of the purification of versican at pH 6.0 that was performed by Dennis Lee and which was used for most of the experiments described. I thank Dr. Richard Dean and Dr. Oded Kleifeld for intellectual discussion surrounding the proteomics work. I thank Suzanne Perry for expert assistance with Edman Degradation chemical N-terminal sequencing.

Chapter 3 - I was involved in all aspects of the experimental design, data analysis and writing of the manuscript with assistance from Dr. Clive Roberts. I performed all experiments described with the exception of the immunohistochemical staining and lung tissues extractions which were performed by Rendi Yan.

Chapter 4 - I was involved in all aspects of the experimental design, data analysis and writing of the manuscript with assistance from Dr. Clive Roberts, Dr. Chris Overall, Dr. Reinhild Kapplehoff and Dr. Alain Doucet. I performed all experiments described with assistance from Reinhild Kapplehoff in performing and analyzing the microarray experiments; and assistance from Alain Doucet in performing and analyzing the proteomics experiments. Yili Wang expressed and purified recombinant MMP-2 and soluble recombinant MT1-MMP. Recombinant versican His-G3 and His-LC constructs were cloned, expressed, purified and refolded by Clive Roberts, Heidi Kai and Saloumeh Pourmalek. Anti-His-LC antibody LC2 was generated by Clive Roberts and Saloumeh Pourmalek.

xvi

This work was supported by grants from the Canadian Institutes for Health Research (MT 15171) and the British Columbia Lung Association.

CHAPTER 1 – BACKGROUND

1.1. Versican in normal physiology

Versican is expressed in many tissues during development and also in adult tissues. First named because it appeared to be a 'versatile proteoglycan' (Zimmermann & Ruoslahti, 1989), versican is indeed associated with many different tissues and functions. Versican is expressed in the brain, vasculature, intervertebral discs, liver, myometrium and prostate (Dours-Zimmermann & Zimmermann, 1994). It is associated with elastic microfibrils in the skin and loose connective tissue of many organs (Bode-Lesniewska et al, 1996; Zimmermann et al, 1994). Versican contributes to fibrous networks in the pancreas and biliary tracts (Fukata et al, 1989).

Versican performs several mechanical and biochemical functions. Its glycosaminoglycan side chains are involved in creation of a hydrated space that allows the resistance to stretch and compression through the reversible redistribution of water, a property termed visco-elasticity (Kinsella et al, 2004). The physical size of the molecule allows it to alter accessibility of the cell surface and thereby indirectly alter cell surface binding and signaling (Kinsella et al, 2004; Roberts, 2003). Creation of an expanded and hydrated matrix is also associated with facilitation of cell migration and proliferation prior to collagen deposition and tissue remodeling.

Versican appears to aid in the ordered deposition of collagen during normal wound healing. However versican is also associated with contributing to disorganization of collagen fibrils during cervical dilation (Uldbjerg & Malmstrom, 1991), suggesting that the concentration and localization of the molecule is critical to determining its effect on collagen organization. Versican binds numerous ligands through its protein domains

and its glycosaminoglycan side chains. Variable ligand binding may allow versican to alter matrix permissiveness and glycosaminoglycan binding of cytokines contributes to ECM cell signaling and chemotactic gradients. Versican has been referred to as an anti-adhesive molecule and it is specifically excluded from sites of focal adhesion (Yamagata & Kimata, 1994; Yamagata et al, 1993). Because of its association with cell migration and proliferation, versican is proposed to be a haptotactic factor, promoting cell movement through creation of a gradient of cell-extracellular matrix adhesions (Cattaruzza & Perris, 2005).

Versican's multiple functions are accomplished through its composite structure that contains globular and linear protein domains with a substantial mass of glycosaminoglycans. Compared to the related proteoglycan aggrecan, versican is less glycosylated, but it is still a very large hydrodynamic component of the ECM. As visualized by electron microscopy, versican's core protein is extended through the glycosaminoglycan attachment domains, the glycosaminoglycans are fairly linear and the globular terminal domains are small compared to the size of the whole protein (Fig. 1.1).

1.1.1. Development

Versican was first identified because of its association with skeletal development in the embryo (Kimata et al, 1986). A transgene insertional mutation into mouse chromosome 13 identified a locus critical to endocardial cushion formation in the heart (Yamamura et al, 1997). The homozygous mice are embryonic lethal by day 10.5 and the gene responsible was identified as versican (Mjaatvedt et al, 1998). Hyaluronan synthase-2 knockout mice develop a very similar phenotype of cardiac malformation suggesting a crucial interaction (Camenisch et al, 2000). Versican proteolysis is also



Figure 1.1. Structures of interstitial proteoglycans revealed by electron microscopy. Bovine interstitial proteoglycans analyzed by glycerol spraying/rotary shadowing electron microscopy. A. Cartilage proteoglycan. E. Aorta proteoglycan (versican). Adapted from Morgelin, M. et. al. Shared and distinct features of interstitial proteoglycans from different bovine tissues revealed by electron microscopy. © *J. Biol. Chem.* 1989; 264: 12080-12090. Copied under licence from Access Copywright. Further reproduction prohibited.

critical in heart development where cleavage products alter cell behaviour differently from the intact molecule (Kern et al, 2007; Kern et al, 2006).

Versican appears to guide neural crest cell (NCC) migration and axon outgrowth through inhibitory interactions (Landolt et al, 1995; Schmalfeldt et al, 2000). Overexpression the transcription factor Pax3 leads to overexpression of versican and defective NCC migration in mice (Henderson et al, 1997). Purified versican inhibits NCC attachment and migration in vitro. (Perris et al, 1996; Schmalfeldt et al, 2000). However, versican coated membranes attract migrating NCC and co-polymerization of versican with collagen increases NCC migration (Perissinotto et al, 2000). Versican may thus direct NCC migration through a combination of permissive and inhibitory interactions construed by different parts of the molecule. Specific proteolytic events may be necessary to end versican's role in facilitating NCC migration and proliferation (Dutt et al, 2006).

1.1.2. Reproduction

In most normal adult tissues little is known about versican turnover. However significant production and loss of versican is associated with numerous aspects of reproduction. Versican is associated with loose connective tissue and blood vessel walls of the normal ovary (Voutilainen et al, 2003). In both the pre-ovulatory follicle and in the cytoplasm of granulosa cells after ovulation versican is a stromal component adjacent to the basement membrane (Irving-Rodgers et al, 2006a). Stromal versican is widely dispersed in the bovine corpus luteum (Irving-Rodgers et al, 2006b). Versican expression and cleavage are associated with maturation of the cumulus-oocyte complex *in vivo* (Dunning et al, 2007).

A versican-like proteoglycan assists human sperm cell migration in vitro (Eriksen et al, 1994), and versican has since been shown to be a significant component of human follicular fluid (Eriksen et al, 1999). High levels of stromal versican are observed in development of the prostate, but expression is decreased greater than 10 fold during puberty (Sakko et al, 2007).

A large hyaluronan binding chondroitin sulfate proteoglycan (CSPG) similar to versican has been suggested to contribute to the disorganization of collagen fibrils during cervical dilation (Uldbjerg & Malmstrom, 1991). Indeed versican expression increases in association with matrix remodeling and collagen degradation in the cervical connective tissue just before birth (Westergren-Thorsson et al, 1998).

1.2. Versican in wound healing disorders

1.2.1. Wound healing and fibrosis

Wound healing processes can result in a spectrum of end results under different circumstances. In oral wound healing or dermal wound healing in the fetus, tissue regeneration is complete. In normal dermal wound healing, limited scar formation occurs and in dermal scarring disorders such as hypertrophic scarring and keloids, scarring is sustained and proliferative. These are just two of a large group of disorders that involve prolonged scarring and proliferation of connective tissue. Fibroproliferative diseases include pulmonary fibrosis, systemic sclerosis, hepatic fibrosis, renal fibrosis, atherosclerosis and some aspects of cancer metastasis (Wynn, 2007). In each, normal organ tissue is progressively replaced by functionally abnormal tissue. Despite common mechanisms underlying many of these diseases, there are no currently approved therapies that directly target mechanisms of fibrosis (Wynn, 2007).

There are three overlapping phases of wound healing: inflammation, tissue formation and tissue remodeling (Singer & Clark, 1999). In response to a wounding event, injured epithelial and endothelial cells become activated and trigger coagulation and inflammation. Activated cells secrete growth factors, cytokines, chemokines and proteinases that participate in the chemical and physical recruitment of inflammatory cells. This includes the regulated proteolysis of ECM proteins of the basement membrane and fibrin clot.

Fibroblast cells become activated to myofibroblasts that migrate and proliferate, expressing α -smooth muscle actin (α -SMA). In the tissue formation phase, myofibroblasts secrete ECM components that form a temporary scaffold to assist inflammatory cell recruitment and neovascularization. This temporary extracellular matrix is rich in fibrin, fibronectin, hyaluronan and versican. It is designated the provisional matrix to indicate its normal expression is both essential and transient (Clark et al, 1982). The new tissue rich in myofibroblast cells and the provisional matrix they synthesize is termed the granulation tissue (Fig. 1.2). The granulation tissue allows neovascularization, wound closure and tissue remodeling by proteases including the MMP's.

During tissue remodeling, the provisional matrix is gradually replaced by a collagenous matrix. Collagen deposition is necessary for the structural arrangement of the remodeling tissue and for a return to normal tissue function, however excess deposition of collagen leads to fibrosis. A balance between synthesis and catabolism of collagen is necessary for appropriate resolution of wounding. Catabolism is controlled by different groups of metalloproteinases during the different phases of wound healing (Madlener et al, 1998).



Figure 1.2. Granulation tissue in a cutaneous wound at five days post injury. Activated myofibroblasts synthesize a provisional matrix and together form the granulation tissue in which reepithelization, neovascularization and tissue remodeling occur. Adapted from Singer, A.D. & Clark, R.A.F. Cutaneous Wound Healing. © *N. Engl. J. Med.* 1999; 341: 738-746. Copied under licence from Access Copywright. Further reproduction prohibited. As α -SMA expressing myofibroblasts shorten collagen networks to effect wound closure, proteolytic removal of excess ECM molecules and cells is necessary (Tomasek et al, 2002). While it is the activated myofibroblasts that are principally responsible for resolution of the granulation tissue provisional matrix, changes in extracellular matrix molecules can influence the ability of myofibroblasts to remodel the extracellular matrix (Clark et al, 1995; Xu & Clark, 1996). Therefore ECM remodeling in the resolution of wound healing both regulates and is regulated by activated myofibroblasts. Myofibroblasts eventually cease α -SMA expression and contractility, and disappear by apoptosis (Desmouliere et al, 1995). Myofibroblast contractility is important for physiological tissue remodeling but excessive contractility can also lead to tissue deformations as seen in fibrosis (Hinz, 2007). The timely loss of excess ECM molecules and myofibroblasts allows a return to normal architecture, whereas, persistence of the myofibroblasts and the provisional matrix may contribute to fibrosis (Fig. 1.3).

Compared to fetal wound healing, myofibroblasts involved in adult wound healing show an increased expression of α -SMA and increased contractile capacity (Estes et al, 1994; Moulin et al, 2001). Exogenous TGF- β induces scar formation in healing fetal wounds suggesting a role for this growth factor in fibrotic processes (Lin et al, 1995; Sullivan et al, 1995). Rapid re-epithelialization appears to be critical to scarless healing of fetal wounds. (Martin, 1997). In fetal wound healing, high expression of chondroitin sulfate is associated with scarless healing and may alter collagen fibril formation (Whitby & Ferguson, 1991). However, high levels of versican and reduced fibroblast apoptosis are associated with hypertrophic scarring (Armour et al, 2007; Scott et al, 1996). These observations suggests that versican expression and degradation are



Figure 1.3. Myofibroblasts in normal and pathological wound healing.

Adapted from Tomasek, J.T. et. al. Myofibroblasts and Mechanoregulation of Connective Tissue Remodelling. © *Nat. Rev. Mol. Cell Biol.* 2002; 5:349-363. Copied under licence from Access Copywright. Further reproduction prohibited.

essential components of normal wound healing and that persistence of versican may lead to aberrant healing.

Fibrosis is invariably preceded by inflammation, however fibrogenic processes are largely independent of inflammatory processes (Wynn, 2004). Therefore improved therapies will need to be directed at fibrogenic pathways rather than at the primary causes of the fibrosis (Friedman, 2007; Wynn, 2007).

1.2.2. Pulmonary fibrosis

Pulmonary fibrosis is a scarring disorder of the lungs involving progressive replacement of lung parenchyma by non-functional collagen-rich scar tissue. Idiopathic pumonary fibrosis (IPF) is a specific form of pulmonary fibrosis differing from the other idiopathic interstitial pneumonias (Travis et al, 2002). It is identified by the histopathological pattern of usual interstitial pneumonia and exhibits characteristic fibroblastic foci (King et al, 2000). IPF is driven largely if not entirely by noninflammatory mechanisms (Selman et al, 2001; Travis et al, 2002). Median survival of patients with idiopathic pulmonary fibrosis is 3 to 5 years and there is no currently effective therapy (Khalil & O'Connor, 2004). The current 'standard of care' therapy of prednisone and azathioprine is in fact potentially detrimental to patients as these cytotoxic agents have not been proven to be effective for IPF (Hunninghake, 2005). In addition to chronic injury, genetic factors contribute to the susceptibility to pulmonary fibrosis (Chung et al, 2003). One such genetic susceptibility factor in humans is mutations in telomerase genes (Armanios et al, 2007).

In pulmonary fibrosis, myofibroblasts are primarily responsible for the excess deposition of collagen (Zhang et al, 1994). In fact myofibroblasts are the effector cells

that are primarily responsible for all synthesis of ECM proteins and immune mediators that perpetuate pulmonary fibrosis (Thannickal et al, 2004).

In the developing lung, versican is the predominant chondroitin sulfate bearing proteoglycan and its expression is associated with alveolar tissue volume changes (Faggian et al, 2007). Versican is expressed in the most prevalent forms of human lung fibrosis, including those associated with non-granulomatous inflammation: organizing diffuse alveolar damage in patients with adult respiratory distress syndrome; usual interstitial pneumonia in patients with IPF and idiopathic BOOP (Bensadoun et al, 1996). Versican is also expressed in association with myofibroblasts in granulomatous forms of lung fibrosis including the lesions of tuberculosis, sarcoidosis and extrinsic allergic alveolitis. In all of these forms of human lung fibrosis, versican is found in association with migratory, proliferating alpha actin-positive myofibroblasts (Fig. 1.4) (Bensadoun et al, 1997). Myofibroblasts synthesize type 1 procollagen within the versican-rich matrix (Bensadoun et al, 1996; Bensadoun et al, 1997).

Versican is a significant component of the expanded interstitial tissue in the interstitial lung disease lymphangioleiomyomatosis (Merrilees et al, 2004). Interestingly, it has been suggested that asthma bears certain similarities to fibrosis (Roberts, 1995) and increased versican expression contributes to an expanded bronchial ECM that is detrimental in asthma (Potter-Perigo et al, 2004; Roberts & Burke, 1998). In patients with mild asthma, activated fibroblasts producing versican are present in bronchoalveolar lavage fluid and appear to contribute to peribronchial fibrosis (Larsen et al, 2004).

Pulmonary fibrosis seems to be perpetuated by a proteolytic imbalance. The tissue inhibitor of metalloproteinases (TIMPs) are all highly expressed in IPF compared to normal and they may therefore inhibit normal proteolytic activity required for normal



Figure 1.4. Versican expression associated with proliferating fibroblasts in idiopathic pulmonary fibrosis. Versican stains strongly in association with proliferating myofibroblasts in the thickened interstitium and in a subepithelial fibroblast rich focus which is in the process of colonizing an alveolus. *Scale bar* = 100 μ m. Image courtesy of Dr. Clive R. Roberts.

remodeling (Ramos et al, 2001; Selman et al, 2000). Yet there are also many MMP and ADAMTS proteases that are up-regulated in IPF (Pardo et al, 2008). It remains to be clarified which enzymes and inhibitors are critical for normal resolution. It is also unclear which enzymes and inhibitors function in a predominantly pro-fibrotic versus anti-fibrotic manner. While the up-regulation of these proteases as a whole can be assumed to indicate their role in facilitating the progression of IPF, it is also possible that in response to the events of fibrosis the body is attempting to restore a proteolytic balance and encourage remodeling through increasing protease expression. The reality is quite likely a complex interplay of these two opposing processes.

While inflammation is often believed to precede and lead to pathways of fibrosis, it is possible that in IPF inflammation occurs subsequent to the formation of fibroblastic foci and that the pathology is driven by chronic epithelial injury leading to aberrant fibroblastic wound healing (Selman & Pardo, 2002). Numerous new therapies to combat IPF by inhibition of fibrotic events independent of inflammation are now in development and clinical trials (Rogliani et al, 2008).

1.2.3. Atherosclerosis

Versican is a major component of smooth muscle pericellular matrix in the normal aorta (Yao et al, 1994). Yet versican expression is also abundant in atherosclerotic lesions (Evanko et al, 1998; Halpert et al, 1996), transplant arteriopathy (Lin et al, 1996a; Lin et al, 1996b) and in restenotic lesions after angioplasty (Wight et al, 1997). Dermatan sulfate glycosaminoglycans on versican bind platelets and may contribute to platelet accumulation at ruptured atherosclerotic plaques (Mazzucato et al, 2002). Under normal conditions, versican is part of a hydrated viscoelastic matrix that assists in normal vessel functioning, yet its pathological expression appears to be at the centre



Figure 1.5. Versican localization in atherosclerosis.

A. Movats staining of human artery with early intimal thickening shows proteoglycans in light blue. B. Versican immunostaining shows reactivity in same thickened intima. C. Human coronary atherosclerotic plaque staining shows proteoglycans adjacent to a thrombus. D. Versican staining is abundant in the same layer. Adapted from Wight, T.N. and Merilees, M.J. Proteoglycans in atherosclerosis and restenosis, key roles for versican. © *Circ. Res.* 2004; 94:1158-1167. Copied under licence from Access Copywright. Further reproduction prohibited.

of many of the events of atheroslerosis and restenosis (Wight, 2008; Wight & Merrilees, 2004). High concentrations of versican are transiently associated with myofibroblasts in the adventitia and neointima during coronary artery repair (Shi et al, 2000). In atherosclerosis, versican staining is abundant in the thickened intima (Fig. 1.5) (Wight & Merrilees, 2004).

Vascular smooth muscle cells require a versican-rich matrix for migration and proliferation (Evanko et al, 1999). Likewise, antisense inhibition of versican synthesis reduces cell proliferation in injured rat carotid arteries (Huang et al, 2006). Versican binding to fibulin-2 appears to contribute to this promotion of growth and migration (Olin et al, 2001; Strom et al, 2006).

Versican proteolysis is associated with neointimal regression (Kenagy et al, 2005). Versican turnover in the neointima appears to involve multiple proteasese acting in sequential steps (Kenagy et al, 2005; Kenagy et al, 2006). Fragments of versican are detected in normal and diseased blood vessels (Formato et al, 2004; Sandy et al, 2001; Theocharis et al, 2003a) indicating that versican turnover occurs in both normal and pathological vessel remodeling.

1.2.4. Cancer stroma

Versican was first identified as a cancer-related gene in 1990. It was observed to be hypomethylated in colorectal cancer, potentially allowing its excessive expression to contribute to malignancy (Adany & lozzo, 1990). Since then there have been many more reports of versican as a major component of the stroma surrounding tumors in most if not all the bodies organ systems. A number of reports have found that versican expression is correlated with poor prognosis and reduced incidences of relapse-free survival (Table 1.1). In fact expression of versican has been found to be predictive of

| | Present in tumour stroma | Associated with growth & metastasis | Associated wtih invasive potential | Associated with angiogenesis | Related to disease free survival | Potential indicator Of poor prognosis | |
|------------------------------|-----------------------------|---|---------------------------------------|---------------------------------|-------------------------------------|--|---|
| Brain | $\overline{}$ | | | | | | (Paulus et al, 1996) |
| Oral squamous cell carcinoma | | \checkmark | | | \checkmark | | (Pukkila et al, 2007) |
| Oral malignant melanoma | \checkmark | \checkmark | \checkmark | | | | (Banerjee et al, 2005; Docampo et al, 2007) |
| Salivary gland | \checkmark | | | | | | (Nara et al, 1991) |
| Odontogenic | \checkmark | | | | | | (Ito et al, 2002; Zhao et al, 1999) |
| Laryngeal squamous cell | V | \checkmark | \checkmark | | | | (Skandalis et al, 2006b; Skandalis et al, 2004; Stylianou et al, 2008; Vynios et al, 2008) |
| Pharygeal squamous cell | \checkmark | \checkmark | | | | | (Pukkila et al, 2004) |
| Esophageal | \checkmark | \checkmark | | | | | (Hao et al, 2006) |
| Non small cell lung | \checkmark | \checkmark | | | | | (Pirinen et al, 2005) |
| Gastric | \checkmark | | | | | | (Theocharis et al, 2003) |
| Pancreatic | \checkmark | \checkmark | | | | | (Fukata et al, 1989; Koninger et al, 2004; Mauri et al, 2005; Skandalis et al, 2006a) |
| Liver | \checkmark | \checkmark | | | | | (Lin et al, 2007) |
| Breast | \checkmark | \checkmark | \checkmark | \checkmark | | \checkmark | (Castronovo et al, 2007) |
| Prostate | \checkmark | | | | \checkmark | \checkmark | (Ricciardelli et al, 1998; Ricciardelli et al, 2007; Sakko et al, 2003) |
| Testicular | \checkmark | \checkmark | \checkmark | \checkmark | | | (Labropoulou et al, 2006) |
| Cervical | \checkmark | \checkmark | | | \checkmark | \checkmark | (Kodama et al, 2007a) |
| Endometrial | \checkmark | \checkmark | | | | | (Kodama et al, 2007b) |
| Ovarian | \checkmark | \checkmark | | | \checkmark | | (Voutilainen et al, 2003) |
| Uterine | \checkmark | | | | | | (Catherino et al, 2004; Malik & Catherino, 2007) |
| Colorectal | \checkmark | \checkmark | \checkmark | | | | (Adany & lozzo, 1990; Lin et al, 2007; Mukaratirwa et al, 2004; Theocharis, 2002; Tsara et al, 2002) |
| Basal cell carcinoma | \checkmark | | | | | | (Karvinen et al, 2003) |
| Cutaneous malignant melanoma | \checkmark | \checkmark | \checkmark | | | \checkmark | (Docampo et al, 2007; Touab et al, 2003; Touab et al, 2002) |
| Leukemic monocytes | | \checkmark | | | | | (Makatsori et al, 2003) |
| Chondromyxoid fibroma | \checkmark | | | | | | (Romeo et al, 2007) |
| Yolk sac tumour | \checkmark | | | | | | (Isogai et al, 1996; Nakashima et al, 1990; Sobue et al, 1989) |

 Table 1.1. Versican expression in cancer

poor prognosis in oral (Pukkila et al, 2007), breast (Ricciardelli et al, 2002; Suwiwat et al, 2004), prostate (Ricciardelli et al, 1998), cervical (Kodama et al, 2007a), endometrial (Kodama et al, 2007b) and cutaneous (Touab et al, 2003; Touab et al, 2002) cancers. Versican expression may contribute to anchorage independence that precedes neoplastic conversion (Oba-Shinjo et al, 2006).

Modifications of versican are associated with aggressiveness and metastatic potential in laryngeal, gastric, pancreatic, colon and rectal tumor stroma (Skandalis et al, 2006a; Skandalis et al, 2006b; Theocharis, 2002; Theocharis et al, 2003b; Tsara et al, 2002). Modifications observed include a lower hydrodynamic size with a higher percentage of chodroitin sulfate chains, higher percentage of 6 sulfated dissacharides and reduced average molecular weight of both chondroitin and dermatan sulfate side chains.

It has long been known that growing tumors induce the formation of extracellular matrix surrounding them in a process known as the stromal reaction (loachim, 1976). Within an appropriately permissive ECM a tumor can grow, recruit vasculature, evade host immune response and eventually metastasize. Tumor derived factors have been shown to induce versican expression in cultured benign prostatic and pancreatic stromal cells (Koninger et al, 2004; Sakko et al, 2001). Creation of a versican-rich matrix may also facilitate neo-angiogenesis that is required for continued tumor growth (Brown et al, 1999; Koyama et al, 2007), suggesting that versican is permissive for tumor growth. Host fibroblasts are likely responsible for most versican synthesis in response to tumor signaling. However, there is evidence of cancer cells secreting versican into the stroma (Bouterfa et al, 1999; Dobra et al, 2000; Mauri et al, 2005; Touab et al, 2002).

Tumor stroma and the granulation tissue of healing wounds share a number of properties. Through the release of vascular permeability factor, tumor cells induce fibrinogen extravasation and formation of a fibrin-fibronectin gel (Dvorak, 1986). Proliferation of fibroblasts, recruitment of inflammatory cells and angiogenesis gradually transform the fibrin-fibronectin gel into a vascular tumor stroma that is much like wound granulation tissue (Dvorak, 1986). The forming granulation tissue exhibits strong expression of CSPGs (Yeo et al, 1991). Since tumor growth is dependent on a suitable stroma, the altered tumor stromal itself can be considered carcinogenic and it is likely that successful cancer therapies of the future will need to address normalization of the tumor microenvironment (Bissell & Radisky, 2001).

Myofibroblasts play a central role in cancer invasiveness (De Wever et al, 2008). The induction of activated fibroblasts by dysregulation of proteases is proposed to be similar in fibrosis and tumor microenvironments (Radisky et al, 2007). Type 1 collagen is a metastasis-associated gene that is suggested to be a potential anti-metastasis target (Fingleton, 2007; Ramaswamy et al, 2003). As in fibrosis the excess deposition of type 1 collagen occurs in a versican-rich matrix.

While host induction of a permissive stroma seems to be a consistent requirement of cancer progression and this stroma is clearly versican-rich under most circumstances, it is not necessarily the case that versican expression promotes growth and metastasis in all cases. In advanced laryngeal cancer, increased expression of versican is seen concomitant with a decrease in high molecular weigh versican extractable from the tissue and an increase in versican fragments (Stylianou et al, 2008). Thus it is possible that versican expression is part of a host encapsulation reaction that the tumor must circumvent through proteolytic means in order to continue growth and progression. As the tumor stroma is complex and is stimulated by various host-tumor interactions that

are not fully understood, we need to be cautious in assigning functions to highly expressed molecules (lozzo, 1995). Thus, elucidating versican's role and turnover in the tumor stroma is crucial to the design of future therapies that would target the aberrant tumor stroma.

1.2.5. Arthritis

The prototypical cartilage proteoglycan is aggrecan, however versican is also involved in cartilage growth and turnover. Versican was first identified due to its association with limb chondrogenesis (Kimata et al, 1986). It is highly expressed in the perichondrium and presumptive joint interzone (Matsumoto et al, 2006; Shepard et al, 2007). Versican expression is up-regulated by chondrogenic stimuli and knock-down results in compromised mesenchymal chondensations (Kamiya et al, 2006; Shepard et al, 2008). Human mesenchymal stem cells undergoing osteoblast differentiation express versican (Foster et al, 2005).

Increased versican to aggrecan ratio is associated with osteoarthritis and dedifferentiation of chondrocytes (Martin et al, 2001). Collagen-induced arthritis in rats results in increased versican production by peripheral blood mononuclear cells (Shou et al, 2006). It may be that increased expression of versican occurs with proliferative elements of arthritic processes.

1.2.6. Tendinopathies

Versican is a component of normal achilles tendon (Corps et al, 2006) and the scapholunate interosseous ligament (Milz et al, 2006). Versican expression is associated with small arteries and in the neointima of severe carpal tunnel syndrome
(Tsujii M et al, 2006) and with proliferating fibroblasts and microvessels in patellar tendonosis (Scott et al, 2008).

Several catabolic products of versican have been detected and characterized in bovine tendon (Samiric et al, 2004) and many more have been detected in human patellar tendons of patients with tendinosis (Scott et al, 2008). The role of versican degradation relative to tendinosis disease progression is not clear.

1.3. Versican structure

The spatial orientation of versican's larger isoforms was demonstrated in a seminal paper two decades ago that analyzed large interstitial proteoglycans including aorta proteoglycans by electron microscopy (Fig. 1.1) (Morgelin et al, 1989). The aorta proteoglycans appears to be versican V0 and V1 as the size and domain compositions are appropriate and we know that these isoforms are highly expressed in the aorta. These were later refered as 'versican type' proteoglycans (Morgelin et al, 1994) and based on our current knowlege, there is no other logical candidate proteoglycan. This early work demonstrated that versican has more diversity in the length of its glycosaminoglycans and an average glycosaminoglycan length about twice that of aggrecan's (Morgelin et al, 1989). Due to its fewer glycosaminoglycan side chains that create less electrostatic repulsion, versican's core protein was less extended than aggrecan's and contracted less upon glycosaminoglycan digestion.

Based on its composite structure, versican may span between two extracellular binding interactions as 'molecular bridge' (Miosge et al, 1998; Ruoslahti, 1996) with variable size and degrees of glycosylation determined by expression of different splice variants.

1.3.1. Hyalectans

In addition to versican, there are several proteoglycans that share a homologous Cterminal domain with a selectin-like domain composition. These include versican, aggrecan, neurocan and brevican; and they have been referred to as 'lecticans' to indicate proteoglycans with lectin-like domains (Ruoslahti, 1996). The term 'hyalectans' has since been dubbed to designate proteoglycans with lectin-like domains and hyaluronan binding N-terminal domains (lozzo, 1998). This hyalectan family appears to offer a repertoire of proteoglycans that may bind identical substrates. Through the altered expression of versican splice variants and hyalectan family members, cells could regulated expansion or contraction of the extracellular matrix and alter its chemotactic properties (Fig. 1.6).

1.3.2. Gene organization and regulation

The human versican gene lies on chromosome 5 and is composed of 15 exons (lozzo et al, 1992; lto et al, 1995; Naso et al, 1994; Zako et al, 1995). All the versican isoforms contain N and C terminal globular domains termed G1 (comprising exons 1-6) and G3 (comprising exons 9-15) respectively. Exon 7 encodes the smaller of the GAG attachment domains termed GAG- α and exon 8 encodes the larger one termed GAG- β (Dours-Zimmermann & Zimmermann, 1994; Zimmermann & Ruoslahti, 1989). The isoform containing both GAG- α and GAG- β is called V0, containing only GAG- β is V1, only GAG- α is V2, and V3 is the smallest (Figs. 1.6, 1.7)

Reduced activity of the tumor suppressor gene *TP53* results in decreased versican expression (Yoon et al, 2002). Dihydrotestosterone increases versican expression



Figure 1.6. Hyalectan family members in the central nervous system.

Versican splice variants compared with other hyalectan family members showing putative glycosaminoglycans only. Adapted from Bandtlow, C.E. and Zimmermann, D.R. Proteoglycans in the Developing Brain: New Conceptual Insights for Old Proteins. © *Physiol. Rev.* 2000; 80:1267-1290. Copied under licence from Access Copywright. Further reproduction prohibited.

| | ↓ ↓ | 4 | ↓↓ | Į, | <mark>∦↓</mark> ↓ | <u>t</u> 11 | | ∎ 1 1 | # # 11 | t t | # # † | ↓↓ ↓ | ₩. | 111 | ł. | ttt 🖬 | † † | ↓ ↓ | Hut I | . ↓↓ , | 1 | ł | ↓ ↓ ↓ | #Ŧ | 1 | 1 1 | . † | ŧ | |
|----|----------------|----------------|-------|------|-------------------|-------------|--------------------|-------------|---------------------|----------------------|--------------|--------------|------------|--------------|----|-------|------------|------------|---------------------------|----------------|----------|---|-------|----|---|-----|------------|------|-----|
| V0 | lg | Link Link | | | | GA | G-α | | | | | | | | | | | GAC | G-β | | | | | | | | ΕE | Lect | CCP |
| V1 | ↓ Ig | Link Link | | 111 | t fff | | <u>k</u> 14 | udd 4 | GAG- | Hai li i β | | n i i | i 1 | ↓ ↓ ↓ | #1 | ↓ s↓ | ↓ ↓ | ↓ EEL | ↓ .ect <mark>CC</mark> | # P | | | | | | | | | |
| V2 | ↓ Ig | ↓ Link Link | ↓↓, | ţ. | i₩ı ↓ | GA(| μι τ G-α | t † † | ha di sa | ∔ ↓ ↓ E | E Lect | | 1 | | | | | | | | | | | | | | | | |
| V3 | ↓ Ig | Link Link E | E Leo | t CC | P | | | | | | | | | | | | | | | | | | | | | | | | |

Figure 1.7. Versican splice variants showing domain composition and potential glycosylation sites. Domain composition: Ig = Ig-like V-type; Link = link domain; GAG- α = glycosaminoglycan attachment domain alpha; GAG- β = glycosaminoglycan attachment domain beta; E = Calcium-binding EGF-like domain; Lect = C-type lectin-like domain; CCP = complement control protein, short consensus repeat or sushi domain. Arrows illustrate potential attachment sites for glycosaminoglycans (black arrows), O-linked glycosylations (red arrows) and N-linked glycosylations (blue arrows). Based on Zimmermann & Ruoslahti, 1989; Dours-Zimmermann & Zimmermann, 1994. during puberty (Sakko et al, 2007). Versican expression in smooth muscle cells is regulated by a beta-catenin-T-cell factor complex (Rahmani et al, 2005).

Versican is upregulated by TGF- β in a variety of cells (Arslan et al, 2007; Kahari et al, 1991; Robbins et al, 1997; Schonherr et al, 1991; Venkatesan et al, 2002; Wolf et al, 1994; Zhao & Russell, 2005). Though TGF- β is sometimes considered a pro-fibrotic cytokine, it is also essential in tissue remodeling wherein excess or inadequate TGF- β are linked to numerous disease states (Blobe et al, 2000). TGF- β null mice exhibit multiple defects of development and the inflammatory system (Kaartinen et al, 1995; Proetzel et al, 1995; Sanford et al, 1997; Shull et al, 1992). It is possible that excess TGF- β contributes to fibrosis through stimulating excess versican synthesis, but the data is not yet conclusive.

1.3.3. Splice variants

Alternative splicing of versican mRNA results in four different gene products with identical N- and C-terminal globular domains but different sized central glycosaminoglycan-attachment domains due to the inclusion of one or both or neither of the GAG α and GAG β domains (Ito et al, 1995; Naso et al, 1994; Zako et al, 1995). The first cloning of the versican gene involved a partial V1 transcript (Krusius & Ruoslahti, 1986) that was later fully cloned (Zimmermann & Ruoslahti, 1989). The alternatively spliced GAG-beta domain was later discovered and cloned, uncovering both the V0 and V2 isoforms (Dours-Zimmermann & Zimmermann, 1994).

There are as many as 41 potential glycosaminoglycan attachment sites in versican V0 though it is predicted that *in vivo* V0 has between 17-23 glycosaminoglycans attached, V1 has 12-15, V2 has 5-8 and V3 has none (Fig. 1.7 & 1.8) (Dours-





Versican V0 primary sequence with colours representing different protein domains. The N-terminal globular domain is composed of the signal peptide (black), Ig-like domain (blue), Link 1 (light green) and Link 2 (dark green). GAG- α is yellow and GAG- β is light orange. Note *in vivo* these two glycosaminoglycan attachment domains are considerably extended due to glycosaminoglycan hydrophilic interactions and repulsion. The C-terminal globular domain is composed of two EGF domains (brown and orange), the C-type lectin domain (red) and the complement regulatory protein-like domain (purple). Potential attachment sites are marked for glycosaminoglycans (hexagons) and N- and 0-linked glycosylations (circles). Based on Zimmermann & Ruoslahti, 1989 and Dours-Zimmermann & Zimmermann, 1994.

Zimmermann & Zimmermann, 1994; Zimmermann & Ruoslahti, 1989). Versican V1 and V3 may also occasionally retain and express intron 14 in the carboxy terminal, presumably altering its binding properties (Lemire et al, 1999). While V3 may be completely void of glycosylations, it does contain potential glycosylation and glycosaminoglycan attachment sites (Fig. 1.7 & 1.8). Whether V3 might be glycosylated *in vivo* has yet to be determined.

Alternative splicing is common in ECM proteins (Boyd et al, 1993), yet the majority of known alternative splicing events add or subtract protein-protein interaction domains resulting in modest changes in molecular size (Resch et al, 2004). Alternative splicing of versican's large and heavily glycosylated central domains is thus a unique case of alternative splicing that has dramatic effects on the hydration and viscoelastic properties of the ECM with a 20 fold size difference between the largest and smallest isoform (Figs. 1.6 - 1.8).

Numerous studies have documented RNA levels of versican transcripts in different tissues and at different stages, though there is some conflict between reports. Generally, V0 and V1 seem to be associated with cell proliferation and migration where they are expressed at high levels, followed by a drastic reduction that is necessary for tissue maturation. V0 and V1 are associated with guiding neural crest cell migration (Dutt et al, 2006). V0 and V1 are expressed in melanoma cell lines and V0 predominates with the less differentiated cells (Touab et al, 2002). The larger isoforms are still detectable at lower levels in mature blood vessels (Bode-Lesniewska et al, 1996; Yao et al, 1994) and skin (Zimmermann et al, 1994). V2 is a major component of the brain ECM and the predominant versican isoform present in the brain (Paulus et al, 1996; Schmalfeldt et al, 2000; Schmalfeldt et al, 1998). The V3 transcript can be expressed by cytokine stimulated endothelial cells (Cattaruzza et al, 2002). V3

overexpressing cells grow more slowly, are less extended and adhere more firmly (Lemire et al, 2002; Serra et al, 2005). Overexpression of V3 perturbs vascular elastic fibre assembly (Merrilees et al, 2002). In the developing Cornea, V0 is the most expressed isoform during the early postnatal period, V1 and V2 are expressed moderately and V3 is expressed more abundantly in later periods (Koga et al, 2005).

Versican V3 may have an opposite effect on cell proliferation and migration from the larger isoforms. Since V3 would shorten the putative 'molecular bridge' between the two terminal binding domains it would vastly alter the hydrodynamic properties of the pericellular matrix compared with a proliferative matrix rich in versican V0 and V1.

Wagner disease is a rare, autosomal dominant, vitreoretinopathy. Recent work has identified mutations in versican's exons 7 and 8, and intron 7, in several families with this disease (Kloeckener-Gruissem et al, 2006; Miyamoto et al, 2005; Mukhopadhyay et al, 2006). These mutations appear to result in a splice variant imbalance, potentially the inability to synthesize V0, that disrupts the ultrastructure of the vitreous gel and leads to the characteristic liquefaction.

1.3.4. Glycosaminoglycans

Glycosaminoglycans are specialized glycans that differ from N- and O-linked glycosylations by being consistently linear, repeating dimers usually containing one uronic acid (glucuronic acid or iduronic acid) and a hexosamine residue (Fig. 1.9). Proteoglycans like versican have glycosaminoglycans bound to the core protein through a four residue linker attached to a serine residue (Sugahara et al, 2003). Glycosaminoglycans are increasingly being recognized to bind protein substrates in the ECM. Post-translational structural fine tuning through epimerization of uronic acids and variable sulfation patterning provides an enormous variability and alters substrate



Figure 1.9. Glycosaminoglycan monomer constituents.

Disaccharide monomers of the four classes of glycosaminoglycans showing their variable epimerization of glucuronic acid to iduronic acid and positions of variable sulfation. Adapted from Bandtlow, C.E. and Zimmermann, D.R. Proteoglycans in the Developing Brain: New Conceptual Insights for Old Proteins. © *Physiol. Rev.* 2000; 80:1267-1290. Copied under licence from Access Copywright. Further reproduction prohibited.

binding ability (Raman et al, 2005). As these side chains are also capable of being modified in a number of ways, they have been referred to as the most informationdense biological molecules (Turnbull et al, 2001) and it has been proposed that their modifications may form a code necessary for proper development (Bulow & Hobert, 2006).

Mechanical properties of glycosaminoglycans include binding water to create a hydrated matrix. Just as versican deficiency abolishes the capacity to form endocardial cushions in heart morphogenesis (Mjaatvedt et al, 1998), chondroitin synthesis is necessary to create osmotic swelling pressure in *caenorhabditis elegans* tissue morphogenesis (Hwang et al, 2003). This suggests that versican's glycosaminoglycans are directly responsible for the its space filling and viscoelastic properties (Kinsella et al, 2004).

In addition to binding water, versican's glycosaminoglycans bind numerous substrates and further affect the physical and chemical composition of the pericellular matrix. Versican's glycosaminoglycans bind various chemokines and growth factors (Hirose et al, 2001). Dermatan sulfate glycosaminoglycans on versican bind platelets and may contribute to platelet accumulation at ruptured atherosclerotic plaques (Mazzucato et al, 2002). Versican may aid in lymphocyte homing through glycosaminoglycan binding to L- and P-selectin (Kawashima et al, 2000; Kawashima et al, 1999) with binding involving over-sulfated portions of the glycosaminoglycan chain (Kawashima et al, 2002). The 70kDa heavy-chain component of inter- α -trypsin inhibitor (ITI) binds to follicular fluid versican (Eriksen et al, 1999).

Changes in size, sulfation and epimerization of glycosaminoglycans are all mechanisms that fine tune versican's functions in the ECM. In advanced laryngeal squamous cell carcinoma there is a reduction in 6-sulfated disaccharides and an

increase in 4-sulfated disaccharides (Skandalis et al, 2004). Versican's glycosaminoglycans have been measured at different masses and with different sulfation patterns (Skandalis et al, 2006a; Skandalis et al, 2006b; Theocharis et al, 2003b). Versican's hydrodynamic size changes on stimulation with platelet-derived growth factor or TGF- β in a manner that does not alter the core protein size (Schonherr et al, 1991).

1.3.5. N-terminus/G1

Versican's N-terminus contains an immunoglobulin-like domain followed by two link domains. This is domain organization is consistent throughout the hyalectan family. This domain binds hyaluronan (HA) with high affinity through a ternary complex with link protein (LeBaron et al, 1992; Matsumoto et al, 2003; Shi et al, 2004). We now know there to be four different link protein family members (Spicer et al, 2003). Interestingly these four link proteins are located on chromosomes next to the four hyalectan family members versican, brevican, aggrecan and neurocan respectively, suggesting several early gene duplications. However, it does not appear that the link protein adjacent to a given hyalectan is the physiological link used. Link protein-3 (HAPLN-3) colocalizes and is coordinately upregulated with versican in arterial smooth muscle cells (Ogawa et al, 2004), despite HAPLN-1 and versican being paralogous genes (Spicer et al, 2003).

1.3.6. C-terminus/G3

Understanding the binding activities of the G3 domain of versican is of significance importance since it could form the second end of the putative 'molecular bridge' with

hyaluronan. Additionally, the selectin-like domain composition of the G3 domain suggests a possible cell surface binding activity that has yet to be described.

A number of different ECM binding partners have been documented and it seems plausible that binding to different ECM proteins or cell surface ligands is regulated and plays an important role in modulating the hydration and visco-elastic properties of the ECM. Versican has been shown to bind fibrillin-1 (Aspberg et al, 1999; Isogai et al, 2002) and HA-versican-fibrillin-1 complexes seem to be critical to maintaining physiological properties of the ciliary and vitreous bodies in the eye (Ohno-Jinno et al, 2008). Versican has also been shown to bind fibulin-2 (Olin et al, 2001), supporting previous observations that versican colocalizes with fibulin-1 and -2 during heart development and appears to make a switch in molecular associations as development proceeds (Miosge et al, 1998). Versican forms a complex with HA and tenascin-R in the brain (Aspberg et al, 1997; Ruoslahti, 1996). In addition to mediating binding with matrix or cell surface ligands, the G3 domain has been reported to self associate in a calcium dependent manner (Ney et al, 2006).

1.4. Versican turnover in tissue remodeling

Versican is clearly a ubiquitious proteoglycan that is critically involved in development, remodeling and homeostasis of numerous tissues. As versican is heavily expressed in remodeling tissues and present at more modest levels under homeostatic conditions, its regulated turnover is necessary for formation of normal tissue architecture. Versican expression and turnover are involved in both normal and pathological remodeling. Therefore elucidating the proteolytic pathways of normal versican turnover is essential to determining the dysregulated events that contribute to aberrant remodeling.

1.4.1. Matrix metalloproteinases

Tissue remodeling involves not only the synthesis of new matrix but also the regulated proteolysis of matrix proteins and bioactive molecules (Page-McCaw et al, 2007). Proteases perform irreversible, post-translational modifications to proteins and thereby govern many aspects of normal and aberrant cell physiology. Proteolysis is necessary for infiltration of inflammatory cells, migration and proliferation of the granulation tissue, as well as resolution of the provisional matrix. There are over 560 proteases and homologues in the human body including 194 metalloproteinases to date (Overall & Blobel, 2007; Puente et al, 2003). The metalloproteinases or metzincins are characterized by a HExxHxxGxxH catalytic zinc binding motif and conserved methionine turn (Stocker et al, 1995). This family includes the astacins, adamalysins, matrix metalloproteinases (MMPs) and the serralysins.

The first MMP was discovered in 1962, detected during tadpole morphogenesis for an ability to cleave fibrillar collagen, which is otherwise largely resistant to proteolysis (Gross & Lapiere, 1962). MMPs are synthesized as inactive zymogens that contain a propeptide "cysteine switch" complexed to the active site zinc atom (Figure 1.10) (Wart & Birkedal-Hansen, 1990). As the known spectrum of MMP substrates grew for several decades, our understanding of MMP functions was largely limited to their roles in degrading ECM structural proteins. In the last decade we have seen numerous examples of MMPs participating in cell signalling through the precise modification of bioactive molecules. The first example of an MMP processing a bioactive molecule was MMP-2 processing of monocyte chemoattractant protein-3 that converted a proinflammatory chemokine into a chemokine receptor antagonist through the removal of four N-terminal amino acids (McQuibban et al, 2000). This conversion had kinetic



Figure 1.10. MMP family domain organization, active site consensus sequence and propeptide 'cysteine switch.' Adapted from Cauwe, B. et. al. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. © *Crit. Rev. Biochem. Mol. Biol.* 2007; 42: 113-185. Copied under licence from Access Copywright. Further reproduction prohibited.

parameters that were greater than for MMP-2 cleavage of gelatin, the substrate for which MMP-2 is commonly entitled (gelatinase-A). Since then there have been numerous reports of various MMPs cleaving and altering bioactivity of substrates through cleavages of similarly small numbers of amino acids (Wolf et al, 2008). A recent report demonstrates not only conversion of a chemokine from a receptor agonist into an antagonist, but also loss of glycosaminoglycan binding upon a second Cterminal cleavage (Cox et al, 2008).

Dysregulation of MMPs is correlated with many pathologies, especially cancer where MMPs contribute to all stages of cancer growth, including but not limited to invasion and metastasis (McCawley & Matrisian, 2000). Based on the high levels of MMP expression and correlations with progression in many cancers, clincal inhibition of MMPs was a rational and promising approach to treating cancer (Stetler-Stevenson et al, 1996). Numerous MMP inhibitor clinical trials were attempted for cancer treatment but all were unsuccessful for a variety of reasons, not the least of which is that MMPs are involved in a much greater diversity of processes than merely degrading ECM to facilitate metastasis (Coussens et al, 2002; Egeblad & Werb, 2002). Likewise in rheumatoid arthritis and osteoarthritis, current MMP inhibitors have been unaffective due our lack of a precise and complete understanding of MMP activities in these diseases (Murphy & Nagase, 2008). MMPs and inhibitors are necessary for normal wound healing but an imbalance of MMPs to TIMP inhibitors is a associated with pathological scarring (Fig. 1.11).

Despite the dissapointing results of many clinical trials for MMP inhibition in cancer, improving our understanding of the precise roles of individual proteases and their substrates may lead to the development of more specific inhibitors that will provide effective therapies. As MMPs can have 'dual personalities' in inflammatory processes it



Figure 1.11. Common and divergent pathways of regenerative versus pathological wound healing. Adapted from Wynn, T.A. Common and Unique Mechanisms Regulate Fibrosis in Various Fibroproliferative Diseases. © *J. Clin. Invest.* 2007; 117:524-529. Copied under licence from Access Copywright. Further reproduction prohibited.

is clearly important to understand which MMPs are beneficial and which are detrimental in a particular disease state before improved therapies will be possible (Le et al, 2007; Lopez-Otin & Matrisian, 2007; Overall & Kleifeld, 2006). For example, MT1-MMP is highly expressed and plays a major role in cancer (Sabeh et al, 2004; Sato et al, 2005). Based on its critical importance in tumor progression, it has been suggested that MT1-MMP is a promising target for future attempts at more specific inhibition in cancer (Lah et al, 2006). Yet on the other hand, it would be wise to consider MT1-MMP a potential cancer anti-target until its role in adults is more thoroughly elucidated (Overall & Kleifeld, 2006), since it plays a critical role in development and cell signalling pathways (Holmbeck et al, 1999; Tam et al, 2004). Thus much work is needed to understand the spectrum of roles performed by individual proteases before we can design more rational targeted therapies.

In addition to realizing that MMPs exert their effects on diverses classes of extracellular substrates, we are also in the process of elucidating how MMPs act more in precisely regulated cell surface-associated microenvironments than as soluble enzymes (Cauwe et al, 2007). Thus understanding the constituents of cell surface associated microenvironments may be more relevant to physiological proteolysis than merely knowning if a given protease is capable of processing a particular substrate.

Regulation of MMP activation is extremely complex, involving regulation at multiple levels. MMPs do not act in isolation but act in webs and cascades, necessitating an understanding of the interplay between proteases (Keller et al, 2007; Overall & Blobel, 2007; Overall & Kleifeld, 2006). MT1-MMP activity and localization are altered through a variety of post-translational mechanisms. MT1-MMP is internalized through both caveolae and dynamin dependent endocytosis, is recycled to the cell surface or degraded, is involved in activating other proteases, cleaving non-protease substrates

itself, autocatalytically cleaving itself to generate non-catalytic soluble forms and being cleaved by other proteases to relase catalytic soluble forms (Cauwe et al, 2007; Itoh & Seiki, 2004).

An additional level of complexity in metalloproteinase activation is conferred by the tissue inhibitors of metalloproteinases (TIMPs). These physiological inhibitors of metalloproteinases are also sometimes necessary for MMP activation. TIMP-2 for example forms a membrane activation complex with MT1-MMP in order to activate MMP-2 and either inadequate or excessive TIMP-2 impairs MMP-2 activation (Will et al, 1996). TIMPs are multifunctional and in addition to direct interaction with proteases, can regulate signaling events through direct binding of cell surface receptors and initiating signaling cascades (Chirco et al, 2006; Stetler-Stevenson, 2008).

TIMP-3 is unique in its ability to bind to ECM and appears to be the primary TIMP involved in regulating ADAMTS activities (Kashiwagi et al, 2001). TIMP-3 is the only TIMP to inhibit ADAMTS-2 and this inhibition is improved by glycosaminoglycan binding (Wang et al, 2006). Increased expression of TIMP-3 may contribute to the accumulation of versican in prostate cancer stroma by inhibiting versican-degrading ADAMTS enzymes (Cross et al, 2005).

1.4.2. ADAMTS

The 'a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motifs' (ADAMTS) family of enzymes are members of the adamalysin family and differ from the related membrane bound ADAM enzymes in their ancillary domains that contain glycosaminoglycan binding thrombospondin domains (Apte, 2004). These enzymes have only fairly recently been described, with ADAMTS-1 being identified in 1997 (Kuno et al, 1997). ADAMTS enzymes have been found to perform many of the

roles previously thought to be exclusively performed by MMPs. The search for the putative aggrecan degrading MMP responsible for arthritis resulted in the eventual discovery of ADAMTS-5 as an essential enzyme for mouse arthritis (Glasson et al, 2005; Stanton et al, 2005). Both MMP and ADAMTS enzymes seem to be involved in different aspects of normal and pathological aggrecan turnover though all the details are still not worked out (Fosang et al, 2008; Sandy, 2006).

As with the MMPs, regulation of ADAMTS activation appears to be complex, involving processing by other metalloproteinases or autolysis (Colige et al, 2005; Flannery et al, 2002; Gao et al, 2004; Tortorella et al, 2005). ADAMTS-2 processing by by proprotein convertases and C-terminal processing results in 7 different forms of the enzyme (Colige et al, 2005). In breast and lung cancer, ADAMTS expression is dysregulated (Porter et al, 2004; Rocks et al, 2006). As with the MMPs, it is unclear whether these enzymes promote or inhibit cancer growth, but several ADAMTSs have been found to suppress tumour growth (Lopez-Otin & Matrisian, 2007).

ADAMTS-1, -4, & -9 have been shown to cleave versican (Jonsson-Rylander et al, 2005; Sandy et al, 2001; Somerville et al, 2003; Westling et al, 2004). They are considered to form a proteoglycan processing super-clade that process hyalectans and are evolutionarily distinct from the other ADAMTS enzymes (Fig. 1.12) (Apte, 2004). The remaining 'super-clade' contains the procollagen processing enzymes ADAMTS-2, -3 and -14, along with 8 other enzymes: ADAMTS-6, 7, 10, 12, 16-19; with few or no known substrates. Based on homologies to other proteins that are critical in development and cell signaling, it has been suggested that ADAMTS-2 likely plays important roles in development and cell signaling independent of its role in procollagen processing (Prockop et al, 1998). Corresponding with this observation, ADAMTS-2 expression appears to be in excess of that required for its role in procollagen



Figure 1.12. ADAMTS family domain organization and evolutionary relationships.

A. ADAMTS domain organization in the minimal structure of ADAMTS-4 with only one thrombospondin motif. All other ADAMTS family members contain additional thrombospondin motifs C-terminal to the spacer domain. B. Domain structure in the procollagen N-propeptidases. C. Evolutionary relationships of human ADAMTS enzymes - ADAMTS-1, 4, 5, 8, 9, 15 & 20 are thought to form a "super-clade" of proteoglycan processing enzymes. ADAMTS 2, 3 & 14 are procollagen N-propeptidases with no other known substrates to date. ADAMTS-13 is the von Willebrand factor cleaving protease. The other proteases (ADAMTS-6, 7, 10, 12, 16-19) have few if any known substrates. Adapted from Apte S.S. A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motifs: the ADAMTS family. © *Int. J. Biochem. Cell Biol.* 2004; 36:981-985. Copied under licence from Access Copywright. Further reproduction prohibited. processing in several tissues (Colige et al, 1997) and it can exist in multiple activation states, again suggesting the possibility of other roles than processing procollagen (Colige et al, 2005).

1.4.3. Known and unknown versican proteolytic events

Several MMPs have a versican-degrading capacity but few have been well characterized. MMP-1, -2, -3, -7 and -9 have all been shown to process versican preparations *in vitro* (Halpert et al, 1996; Passi et al, 1999; Perides et al, 1995). However, no characterization of proteolytic products has yet been done. Just as aggrecan cleavage at specific sites is performed by numerous MMPs (Fosang et al, 1992), it is probable that numerous MMPs are capable of cleaving versican *in vitro*, but the physiological relevance remains to be worked out.

Several members of the ADAMTS family cleave versican. There are four currently known and characterized versican cleavage sites produced by ADAMTS metalloproteinases (Jonsson-Rylander et al, 2005; Sandy et al, 2001; Westling et al, 2004). These cleavage sites were discovered using synthetic peptides representing a portion of versican that was predicted to be cleaved and neo-epitope antibodies to the predicted cleavage sites. These cleavage sites have been confirmed *in vivo* and in the case of DPEAAE there is much evidence for the cleaved product to exist in different remodeling processes. Versican cleavage occurs at several stages of cardiac development and produces the DPEAAE neo-epitope in an asymmetric pattern, suggesting a possible function for the cleavage product (Kern et al, 2007; Kern et al, 2006). Proteolysis may affect cell migration wherein an anti-DPEAAE antibody inhibits TGF- β 2 induced cell migration (Arslan et al, 2007).

1.4.4. Versican proteolysis

In addition to the well known cleavage sites, it is likely that numerous as yet uncharacterized cleavages are relevant to versican's normal and aberrant turnover (Kenagy et al, 2006). We and others have observed multiple fragments of versican detected in cell culture media of when extracted from tissues (Fig. 1.13). Multiple versican degradation proucts are detected in the stroma of pancreatic carcinoma (Skandalis et al, 2006a). N- and C-terminal cleavage products of versican have recently been documented in the vitreous body of the eye (Ohno-Jinno et al, 2008). An increase in versican expression and versican fragments corresponds with increasing stages of laryngeal cancer (Stylianou et al, 2008; Vynios et al, 2008). Cytokine stimulation of human umbilical vein endothelial cells (HUVEC) results in different Nand C-terminal proteolytic fragments of versican depending on the cytokine used (Cattaruzza et al, 2002). Numerous proteolytic fragments of versican can be immunoprecipitated from human brain astrocytoma with a C-terminal antibody (Zheng et al, 2004). Degradation products of versican are detected in the vitreous body of the eye (Ohno-Jinno et al, 2008).

1.4.5. Glial hyaluronic acid binding protein

Glial hyaluronic acid binding protein (GHAP) was first identified as a 60 kDa glycoprotein that could be reduced to 47kDa with enzymatic removal of N- and Olinked glycosylations (Perides et al, 1989). Versican was found to colocalize with GHAP in the brain (Perides et al, 1992). It was later suggested to potentially be a metalloproteinase induced versican fragment (Perides et al, 1995) and eventually identified as a versican V2 cleavage product with cleavage at the Glu405-Gln-406 bond (Westling et al, 2004). Production of GHAP corresponds with a reduction in the



Figure 1.13. Detection of versican proteolytic fragments.

Versican purified by ion exchange at physiological pH exhibits numerous fragments detected by electrophoresis and Western blotting. Large arrows indicate aggregate and monomer versican, small arrows indicate proteolytic fragments. A. Proteoglycan staining with Alcian Blue. B. Versican immunostaining with 2B1 antibody. C. Versican immunostaining with LC2 antibody. D. Versican purified by ion exchange from bovine and mouse aorta, digested with chondroitinase ABC and detected by Western blotting shows intact versican V0 and V1 along with numerous proteolytic fragments. Part D adapted from Kenagy, R.D. et. al. Versican degradation and vascular disease. © *Trends Cardiovasc. Med.* 2006; 16:209-215. Copied under licence from Access Copywright. Further reproduction prohibited.

extracellular space, suggesting that versican proteolysis is required for normal brain development (Bignami et al, 1993).

1.4.6. Hyaluronectin

Hyaluronectin is a hyaluronan binding glycoprotein that has been found in the central nervous system (Delpech et al, 1989) and is expressed by fibroblasts and smooth muscle cells (Ponting & Kumar, 1995). It exists as a range of different protein sizes, with the major protein being approximately 60 kDa (Delpech et al, 1989; Ponting & Kumar, 1995). Hyaluronectin is strongly expressed in the intima and surrounding deposits in human atherosclerotic lesions (Levesque et al, 1994). It is produced and lost from the neointima to a greater extent in young rats than old in response to aortic injury (Chajara et al, 1998). Hyaluronectin is associated with benign and malignant mesenchymal carcinomas (Girard et al, 1988); is in gliomal and menigiomal stroma (Delpech et al, 1993); and is elevated in invasive areas of breast carcinoma compared to non-invasive areas (Bertrand et al, 1992). N-terminal sequencing of brain derived hyaluronectin revealed numerous sequences of versican's N-terminal domain (Delpech et al, 1997). Consistent with hyaluronectin being a fragment of versican, immunoprecipitation of fibroblast culture media with an anti-hyaluronectin polyclonal antibody detects a large protein product greater than 200 kDa (Ponting & Kumar, 1995). Higher levels of hyaluronectin appear to be beneficial in cancer (Delpech et al, 1997; Delpech et al, 1993), suggesting that versican proteolysis might be a host response that limits tumor growth. Whereas, mice injected with cells expressing high levels of hyaluronectin grew larger tumours and had more metastases than control mice (Paris et al, 2006). Interestingly, cells expressing low levels of hyaluronectin had fewer metastases than control mice, but the significance of this finding is not

understood (Paris et al, 2006). Together, these studies indicate that versican proteolysis is related to cancer progression and that multiple proteolytic fragments of versican are detectable in tumor stroma.

1.4.7. Matrikines

The discoveries that GHAP and hyaluronectin are versican degradation products serves to underscore the extent and significance of versican proteolytic events that are yet to be described and understood. Thus, elucidating the inventory of versicandegrading proteases and their cleavage events is crucial to future improvements in diagnosis and therapy of many fibroproliferative disorders.

Extracellular matrix proteins were historically considered as structural entities with few biologically interesting functions. However, we now know that ECM proteins participate in signaling cascades through binding to cell surface receptors; can alter the location and availability of bioactive cytokines, chemokines and growth factors; and can in fact contain cryptic bioactive components that may be released upon proteolysis. The term matrikines was coined to denote bioactive ECM products produced by regulated proteolysis (Maquart et al, 2004).

Well known matrikines include the angiogenesis inhibitors angiostatin and endostatin that are proteolytically derived from plasminogen and type XVIII collagen respectively (O'Reilly et al, 1997; O'Reilly et al, 1994). Endorepellin is an angiogenesis inhibitor derived from the heparan sulfate proteoglycan perlecan through BMP-1/Tolloid-like proteinase cleavage (Gonzalez et al, 2005; Mongiat et al, 2003). Just as we know that MMPs themselves can have pleiotropic roles (Overall & Dean, 2006), single ECM proteins and proteoglycans can perform opposite functions under different stimuli (Bix & lozzo, 2005).

1.4.8. Potential mechanisms of altered proteolysis in aberrant tissue remodeling

Based on the detection of numerous proteolytic fragments of versican, it is predictable that versican produces cryptic matrixines that have yet to be described. Versican directs cell migration through haptotaxis (Cattaruzza & Perris, 2005) and such guidance could be specifically altered or even reversed through proteolytic events. Thus, there are multiple different potential pathways for versican turnover wherein its properly regulated turnover allows a return of normal tissue architecture and its aberrant turnover appears to contribute to the excess deposition of collagen and fibrosis (Figure 1.14). Normal and pathological turnover may vary in the activity and activation state of the proteases involved. Differences could be merely in the level of protease expression or the ratio of proteases to inhibitors present. Differences could also involve different proteolytic pathways stimulated in the different remodeling conditions, just as aggrecan turnover in disease and normal remodeling appears to involve different groups of metalloproteinases (Sandy, 2006). Clearly elucidating the relevant pathways and alterations is critical to understanding aberrant versican turnover in disease.

1.5. Rationale

Versican is a large chondroitin sulfate proteoglycan associated with cell migration and proliferation in development. Its expression allows creation of a hydrated and expanded extracellular space that is critical for endocardial cushion formation in the heart (Mjaatvedt et al, 1998). Likewise, versican-rich barrier tissues are involved in guidance of neural crest cell migration (Dutt et al, 2006; Landolt et al, 1995). Proteolysis of versican appears to regulate these developmental processes both through the loss of the expanded extracellular space and through the creation of



Figure 1.14. Normal and aberrant proteolysis of versican at the cell surface.

Schematic representation of versican at the cell surface of fibroblast cells where it may bind ADAMTS-2 and contribute to collagen fibrillogenesis and deposition. Excess and persistent versican expression may contribute to excessive collagen deposition as seen in fibrosis. Alternatively, appropriate versican proteolysis may be critical to physiological resolution of remodeling and a return to normal tissue architecture. Metalloproteinases MT1-MMP and MMP-2 form an activation complex at the cell surface and ADAMTS-2 binds to sulfated glycosaminoglycans. All three enzymes are well localized to contribute to versican degradation at the cell surface. Proteolysis may release large or small portions of the C-terminal domain, releasing fibroblast cells from the matrix which versican is bound to and altering its hydrodynamic and chemotactic properties. Proteolysis may also result in partial or complete loss of versican from the matrix and further degradation intracellularly.

cleavage products which may themselves be functional (Dutt et al, 2006; Kern et al, 2007; Kern et al, 2006).

Versican expression is associated with fibroproliferative remodeling in many forms of human pulmonary fibrosis (Bensadoun et al, 1996; Bensadoun et al, 1997; Roberts, 2003). It is also abundant in atherosclerotic and restenotic lesions (Evanko et al, 1998; Halpert et al, 1996; Wight et al, 1997). In numerous cancers, stromal versican expression is predictive of poor prognosis (Kodama et al, 2007a; Kodama et al, 2007b; Pukkila et al, 2007; Ricciardelli et al, 2002; Ricciardelli et al, 1998; Suwiwat et al, 2004; Touab et al, 2003; Touab et al, 2002).

Whereas versican turnover appears to be an essential part of physiological tissue remodeling, dysregulation of versican turnover may contribute to pathological remodeling. Proteolytic pathways of versican turnover are to date poorly understood. Improving our understanding of proteolytic events involved in versican turnover is essential to understanding how versican turnover might be pathologically dysregulated. As versican is a pericellular molecule, regulated turnover of versican likely involves proteolysis in proximity to the cell surface.

In human pulmonary fibrosis, procollagen is synthesized within a versican-rich matrix (Bensadoun et al, 1996). Thus it was hypothesized that the procollagen Npropeptidase ADAMTS-2 might bind and process versican. MMP-2 and MT1-MMP are activated at the cell surface during wound healing (Okada et al, 1997; Overall et al, 2000), pulmonary fibrosis (Garcia-Alvarez et al, 2006) and many cancers (Sato et al, 2005). These enzymes were likewise hypothesized to play a role in versican proteolysis. Concanavalin-A (ConA) is a lectin that is known to bind fibroblast cell surface receptors and induce a matrix degrading phenotype that includes up-regulation of MMP-2 and MT1-MMP (Overall & Sodek, 1990; Yu et al, 1995).

1.5.1. Overarching hypothesis

Regulated versican turnover involves the cell-surface associated metalloproteinases ADAMTS-2, MMP-2 and MT1-MMP, that are expressed in versican-rich remodeling lesions.

1.5.2. Specific aims

- 1. To purify versican from human fetal lung fibroblast cultures
- 2. To investigate the potential of the procollagen N-propeptidase ADAMTS-2 to cleave versican
- 3. To investigate mechanisms of versican degradation in the fibroblast degradative phenotype induced by concanavalin-A
- 4. To assess the capacity of the cell-surface associated metalloproteinases MMP-2 and MT1-MMP to degrade versican *in vitro*
- 5. To characterize proteolytic fragments of versican

In order to investigate versican proteolysis *in vitro*, versican was purified from human fetal lung fibroblast cultures. Versican was found to be labile and contain degradation products at physiological pH, allowing insight into contaminating proteases and leading to improved purification conditions. Following previous work performed in the lab, conditions for optimized purification of versican were characterized and described. Attempts were made to use mass spectrometry to detect versican fragments.

To investigate ADAMTS-2 as a potential versican processing enzyme, the immunohistochemical localization of both was determined in lung tissues of patients with idiopathic pulmonary fibrosis and in normal lung tissues. The co-purification of versican and ADAMTS-2 from human fetal lung fibroblast cultures was investigated and a pH dependence of co-purification was shown. ADAMTS-2 was purified from fetal calf skin and used to investigate its capacity to process purified human versican *in vitro*.

ConA stimulation of human fetal lung fibroblasts was employed as a possible tissue culture model of versican degradation. Loss of versican from the culture media was assessed concurrent with the up-regulation and activation of MMP-2 and MT1-MMP. Microarray analysis was used to investigate expression of possible versican-degrading enzymes and their inhibitors, expressed in response to ConA.

Using recombinant MMP-2 and MT1-MMP, versican proteolysis by these enzymes was investigated. Proteolytic production of different degradation products was investigated with two different antibodies and with purified versican and a recombinant versican C-terminal construct.

Before findings of versican degradation can be translated from the laboratory into diagnostic or therapeutically valuable information, detailed characterization of proteolytic events including cleavage sites is required. As such, traditional N-terminal sequencing was attempted and in light of the challenges of using this technique with a large proteoglycan, new mass spectrometry based techniques were investigated.

It is hoped that improving our knowledge of versican degrading proteases and characterizing degradation processes will lead to improved diagnosis and therapy for many fibroproliferative disorders.

1.6. References

Adany R, lozzo RV (1990) Altered methylation of versican proteoglycan gene in human colon carcinoma. *Biochem Biophys Res Commun* **171**(3): 1402-1413

Apte SS (2004) A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motifs: the ADAMTS family. *Int J Biochem Cell Biol* **36**(6): 981-985

Armanios MY, Chen JJL, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips JA, III, Lansdorp PM, Greider CW, Loyd JE (2007) Telomerase Mutations in Families with Idiopathic Pulmonary Fibrosis. *N Engl J Med* **356**(13): 1317-1326

Armour A, Scott PG, Tredget EE (2007) Cellular and molecular pathology of HTS: basis for treatment. *Wound Repair Regen* **15**: S6-S17

Arslan F, Bosserhoff AK, Nickl-Jockschat T, Doerfelt A, Bogdahn U, Hau P (2007) The role of versican isoforms V0/V1 in glioma migration mediated by transforming growth factor-beta2. *Brit J Cancer* **96**: 1560-1568

Aspberg A, Adam S, Kostka G, Timpl R, Heinegard D (1999) Fibulin-1 is a ligand for the C-type lectin domains of aggrecan and versican. *J Biol Chem* **274**(29): 20444-20449

Aspberg A, Miura R, Bourdoulous S, Shimonaka M, Heinegard D, Schachner M, Ruoslahti E, Yamaguchi Y (1997) The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. *Proc Natl Acad Sci U S A* **94**(19): 10116-10121

Banerjee AG, Bhattacharyya I, Vishwanatha JK (2005) Identification of genes and molecular pathways involved in the progression of premalignant oral epithelia. *Mol Cancer Ther* **4**(6): 865-875

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1996) Proteoglycan deposition in pulmonary fibrosis. *Am J Respir Crit Care Med* **154**(6 Pt 1): 1819-1828

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1997) Proteoglycans in granulomatous lung diseases. *Eur Respir J* **10**(12): 2731-2737

Bertrand P, Girard N, Delpech B, Cuval C, d'Anjou J, Dauce JP (1992) Hyaluronan (hyaluronic acid) and hyaluronectin in the extracellular matrix of human breast carcinomas: comparison between invasive and non-invasive areas. *Int J Cancer* **52**(1): 1-6

Bignami A, Hosley M, Dahl D (1993) Hyaluronic acid and hyaluronic acid-binding proteins in brain extracellular matrix. *Anat Embryol (Berl)* **188**(5): 419-433

Bissell MJ, Radisky D (2001) Putting tumours in context. Nat Rev Cancer 1(1): 46-54

Bix G, Iozzo RV (2005) Matrix revolutions: 'tails' of basement-membrane components with angiostatic functions. *Trends Cell Biol* **15**(1): 52-60

Blobe GC, Schiemann WP, Lodish HF (2000) Role of Transforming Growth Factor *beta* in Human Disease. *N Engl J Med* **342**(18): 1350-1358

Bode-Lesniewska B, Dours-Zimmermann MT, Odermatt BF, Briner J, Heitz PU, Zimmermann DR (1996) Distribution of the large aggregating proteoglycan versican in adult human tissues. *J Histochem Cytochem* **44**(4): 303-312

Bouterfa H, Darlapp AR, Klein E, Pietsch T, Roosen K, Tonn JC (1999) Expression of different extracellular matrix components in human brain tumor and melanoma cells in respect to variant culture conditions. *J Neurooncol* **44**(1): 23-33

Boyd CD, Pierce RA, Schwarzbauer JE, Doege K, Sandell LJ (1993) Alternate exon usage is a commonly used mechanism for increasing coding diversity within genes coding for extracellular matrix proteins. *Matrix* **13**(6): 457-469

Brown LF, Guidi AJ, Schnitt SJ, Van De Water L, Iruela-Arispe ML, Yeo TK, Tognazzi K, Dvorak HF (1999) Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin Cancer Res* **5**(5): 1041-1056

Bulow HE, Hobert O (2006) The Molecular Diversity of Glycosaminoglycans Shapes Animal Development. *Annual Review of Cell and Developmental Biology* **22**(1): 375-407

Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro AJ, Kubalak S, Klewer SE, McDonald JA (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* **106**(3): 349-360

Castronovo V, Kischel P, Guillonneau F, Leval L, Defechereux T, Pauw E, Neri D, Waltregny D (2007) Identification of specific reachable molecular targets in human breast cancer using a versatile ex vivo proteomic method. *Proteomics* **7**(8): 1188-1196 Catherino WH, Leppert PC, Stenmark MH, Payson M, Potlog-Nahari C, Nieman LK, Segars JH (2004) Reduced dermatopontin expression is a molecular link between uterine leiomyomas and keloids. *Genes, Chromosomes and Cancer* **40**(3): 204-217

Cattaruzza S, Perris R (2005) Proteoglycan control of cell movement during wound healing and cancer spreading. *Matrix Biol* **24**(6): 400-417

Cattaruzza S, Schiappacassi M, Ljungberg-Rose A, Spessotto P, Perissinotto D, Morgelin M, Mucignat MT, Colombatti A, Perris R (2002) Distribution of PG-M/versican variants in human tissues and de novo expression of isoform V3 upon endothelial cell activation, migration, and neoangiogenesis in vitro. *J Biol Chem* **277**(49): 47626-47635

Cauwe B, Steen PEVd, Opdenakker G (2007) The Biochemical, Biological, and Pathological Kaleidoscope of Cell Surface Substrates Processed by Matrix Metalloproteinases. *Crit Rev Biochem Mol Biol* **42**(3): 113 - 185

Chajara A, Delpech B, Courel M-N, Marcelle L, Basuyau J-P, Levesque H (1998) Effect of aging on neointima formation and hyaluronan, hyaluronidase and hyaluronectin production in injured rat aorta. *Atherosclerosis* **138**(1): 53-64

Chirco R, Liu X-W, Jung K-K, Kim H-R (2006) Novel functions of TIMPs in cell signaling. *Cancer Metastasis Rev* **25**(1): 99-113

Chung MP, Monick MM, Hamzeh NY, Butler NS, Powers LS, Hunninghake GW (2003) Role of Repeated Lung Injury and Genetic Background in Bleomycin-Induced Fibrosis. *Am J Respir Cell Mol Biol* **29**(3): 375-380

Clark RA, Nielsen LD, Welch MP, McPherson JM (1995) Collagen matrices attenuate the collagensynthetic response of cultured fibroblasts to TGF-beta. *J Cell Sci* **108**(3): 1251-1261

Clark RAF, Lanigan JM, DellaPelle P, Manseau E, Dvorak HF, Colvin RB (1982) Fibronectin and Fibrin Provide a Provisional Matrix for Epidermal Cell Migration During Wound Reepithelialization. *J Investig Dermatol* **79**(5): 264-269

Colige A, Li S-W, Sieron AL, Nusgens BV, Prockop DJ, Lapiere CM (1997) cDNA cloning and expression of bovine procollagen I N-proteinase: A new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. *Proc Natl Acad Sci U S A* **94**(6): 2374-2379

Colige A, Ruggiero F, Vandenberghe I, Dubail J, Kesteloot F, Van Beeumen J, Beschin A, Brys L, Lapiere CM, Nusgens B (2005) Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. *J Biol Chem* **280**(41): 34397-34408

Corps AN, Robinson AH, Movin T, Costa ML, Hazleman BL, Riley GP (2006) Increased expression of aggrecan and biglycan mRNA in Achilles tendinopathy. *Rheumatology (Oxford)* **45**(3): 291-294

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix Metalloproteinase Inhibitors and Cancer--Trials and Tribulations. *Science* **295**(5564): 2387-2392

Cox JH, Dean RA, Roberts CR, Overall CM (2008) Matrix Metalloproteinase Processing of CXCL11/I-TAC Results in Loss of Chemoattractant Activity and Altered Glycosaminoglycan Binding. *J Biol Chem* **283**(28): 19389-19399

Cross NA, Chandrasekharan S, Jokonya N, Fowles A, Hamdy FC, Buttle DJ, Eaton CL (2005) The expression and regulation of ADAMTS-1, -4, -5, -9, and -15, and TIMP-3 by TGFbeta1 in prostate cells: relevance to the accumulation of versican. *Prostate* **63**(3): 269-275

De Wever O, Demetter P, Mareel M, Bracke M (2008) Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* **123**(10): 2229-2238

Delpech B, Delpech A, Bruckner G, Girard N, Maingonnat C (1989) Hyaluronan and hyaluronectin in the nervous system. *Ciba Found Symp* **143:** 208-220

Delpech B, Girard N, Olivier A, Maingonnat C, van Driessche G, van Beeumen J, Bertrand P, Duval C, Delpech A, Bourguignon J (1997) The origin of hyaluronectin in human tumors. *Int J Cancer* **72**(6): 942-948

Delpech B, Maingonnat C, Girard N, Chauzy C, Maunoury R, Olivier A, Tayot J, Creissard P (1993) Hyaluronan and hyaluronectin in the extracellular matrix of human brain tumour stroma. *Eur J Cancer* **29A**(7): 1012-1017

Desmouliere A, Redard M, Darby I, Gabbiani G (1995) Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* **146**(1): 56-66

Dobra K, Andang M, Syrokou A, Karamanos NK, Hjerpe A (2000) Differentiation of mesothelioma cells is influenced by the expression of proteoglycans. *Exp Cell Res* **258**(1): 12-22

Docampo MJ, Rabanal RM, Miquel L, Hernandez D, Domenzain C, Bassols A (2007) Altered expression of versican and hyaluronan in melanocytic tumors of dogs. *Am J Vet Res* **68**(12): 1376-1385

Dours-Zimmermann MT, Zimmermann DR (1994) A novel glycosaminoglycan attachment domain identified in two alternative splice variants of human versican. *J Biol Chem* **269**(52): 32992-32998

Dunning KR, Lane M, Brown HM, Yeo C, Robker RL, Russell DL (2007) Altered composition of the cumulus-oocyte complex matrix during in vitro maturation of oocytes. *Hum Reprod* **22**(11): 2842-2850

Dutt S, Kleber M, Matasci M, Sommer L, Zimmermann DR (2006) Versican V0 and V1 guide migratory neural crest cells. *J Biol Chem* **281**(17): 12123-12131

Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**(26): 1650-1659

Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* **2**(3): 161-174

Erdelyi I, Nieskens DH, Van Dijk JE, Vass L, Nederbragt H (2003) Immunohistochemical evaluation of versican, in relation to chondroitin sulphate, in canine mammary tumours. *Histol Histopathol* **18**(4): 1067-1080

Erdelyi I, van Asten AJ, van Dijk JE, Nederbragt H (2005) Expression of versican in relation to chondrogenesis-related extracellular matrix components in canine mammary tumors. *Histochem Cell Biol*: 1-11

Eriksen GV, Carlstedt I, Morgelin M, Uldbjerg N, Malmstrom A (1999) Isolation and characterization of proteoglycans from human follicular fluid. *Biochem J* **340** (**Pt 3**): 613-620

Eriksen GV, Malmstrom A, Uldbjerg N, Huszar G (1994) A follicular fluid chondroitin sulfate proteoglycan improves the retention of motility and velocity of human spermatozoa. *Fertil Steril* **62:** 618-623

Estes JM, Berg JSV, Adzick NS, MacGillivray TE, Desmouliere A, Gabbiani G (1994) Phenotypic and functional features of myofibroblasts in sheep fetal wounds. *Differentiation* **56**(3): 173-181

Evanko SP, Angello JC, Wight TN (1999) Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **19**(4): 1004-1013

Evanko SP, Raines EW, Ross R, Gold LI, Wight TN (1998) Proteoglycan distribution in lesions of atherosclerosis depends on lesion severity, structural characteristics, and the proximity of plateletderived growth factor and transforming growth factor-beta. *Am J Pathol* **152**(2): 533-546

Faggian J, Fosang AJ, Zieba M, Wallace MJ, Hooper SB (2007) Changes in versican and chondroitin sulfate proteoglycans during structural development of the lung. *Am J Physiol Regul Integr Comp Physiol* **293**(2): R784-792

Fingleton B (2007) Molecular targets in metastasis: lessons from genomic approaches. *Cancer Genomics Proteomics* **4**(3): 211-221

Flannery CR, Zeng W, Corcoran C, Collins-Racie LA, Chockalingam PS, Hebert T, Mackie SA, McDonagh T, Crawford TK, Tomkinson KN, LaVallie ER, Morris EA (2002) Autocatalytic cleavage of ADAMTS-4 (Aggrecanase-1) reveals multiple glycosaminoglycan-binding sites. *J Biol Chem* **277**(45): 42775-42780

Formato M, Farina M, Spirito R, Maggioni M, Guarino A, Cherchi GM, Biglioli P, Edelstein C, Scanu AM (2004) Evidence for a proinflammatory and proteolytic environment in plaques from endarterectomy segments of human carotid arteries. *Arterioscler Thromb Vasc Biol* **24**(1): 129-135

Fosang AJ, Neame PJ, Last K, Hardingham TE, Murphy G, Hamilton JA (1992) The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases, and cathepsin B. *J Biol Chem* **267**(27): 19470-19474

Fosang AJ, Rogerson FM, East CJ, Stanton H (2008) ADAMTS-5: the story so far. *Eur Cell Mater* **5**(15): 11-26

Foster LJ, Zeemann PA, Li C, Mann M, Jensen ON, Kassem M (2005) Differential Expression Profiling of Membrane Proteins by Quantitative Proteomics in a Human Mesenchymal Stem Cell Line Undergoing Osteoblast Differentiation. *Stem Cells* **23**(9): 1367-1377

Friedman S (2007) Reversibility of hepatic fibrosis and cirrhosis—is it all hype? *Nat Clin Pract Gastroenterol Hepatol* **4**: 236-237

Fukata S, Fukatsu T, Nagasaka T, Ohiwa N, Nara Y, Nakashima N, Sobue M, Takeuchi J (1989) Immunohistochemical localization of proteoglycans in interstitial elements of human pancreas and biliary system. *The Histochemical Journal* **21**(12): 707-714
Gao G, Plaas A, Thompson VP, Jin S, Zuo F, Sandy JD (2004) ADAMTS4 (aggrecanase-1) activation on the cell surface involves C-terminal cleavage by glycosylphosphatidyl inositol-anchored membrane type 4-matrix metalloproteinase and binding of the activated proteinase to chondroitin sulfate and heparan sulfate on syndecan-1. *J Biol Chem* **279**(11): 10042-10051

Garcia-Alvarez J, Ramirez A, Sampieri CL, Nuttall RK, Edwards DR, Selman M, Pardo A (2006) Membrane type-matrix metalloproteinases in idiopathic pulmonary fibrosis. *Sarcoidosis Vasc Diffuse Lung Dis* **23**: 13-21

Girard N, Courel MN, Maingonnat C, Delpech B (1988) Hyaluronectin: detection with monoclonal antibodies in human tumors. *Hybridoma* **7**(4): 333-340

Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, Flannery CR, Peluso D, Kanki K, Yang Z, Majumdar MK, Morris EA (2005) Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* **434**(7033): 644-648

Gonzalez EM, Reed CC, Bix G, Fu J, Zhang Y, Gopalakrishnan B, Greenspan DS, Iozzo RV (2005) BMP-1/Tolloid-like Metalloproteases Process Endorepellin, the Angiostatic C-terminal Fragment of Perlecan. *J Biol Chem* **280**(8): 7080-7087

Gross J, Lapiere CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci U S A* **48**(6): 1014-1022

Halpert I, Sires UI, Roby JD, Potter-Perigo S, Wight TN, Shapiro SD, Welgus HG, Wickline SA, Parks WC (1996) Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. *Proc Natl Acad Sci U S A* **93**(18): 9748-9753

Hao Y, Triadafilopoulos G, Sahbaie P, Young HS, Omary MB, Lowe AW (2006) Gene Expression Profiling Reveals Stromal Genes Expressed in Common Between Barrett's Esophagus and Adenocarcinoma. *Gastroenterology* **131**(3): 925-933

Henderson DJ, Ybot-Gonzalez P, Copp AJ (1997) Over-expression of the chondroitin sulphate proteoglycan versican is associated with defective neural crest migration in the Pax3 mutant mouse (splotch). *Mech Dev* **69**(1-2): 39-51

Hinz B (2007) Formation and Function of the Myofibroblast during Tissue Repair. *J Invest Dermatol* **127**(3): 526-537

Hirose J, Kawashima H, Yoshie O, Tashiro K, Miyasaka M (2001) Versican interacts with chemokines and modulates cellular responses. *J Biol Chem* **276**(7): 5228-5234

Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Gehron Robey P, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H (1999) MT1-MMP-Deficient Mice Develop Dwarfism, Osteopenia, Arthritis, and Connective Tissue Disease due to Inadequate Collagen Turnover. *Cell* **99**(1): 81-92

Huang R, Merrilees MJ, Braun K, Beaumont B, Lemire J, Clowes AW, Hinek A, Wight TN (2006) Inhibition of versican synthesis by antisense alters smooth muscle cell phenotype and induces elastic fiber formation in vitro and in neointima after vessel injury. *Circ Res* **98**(3): 370-377

Hunninghake GW (2005) Antioxidant Therapy for Idiopathic Pulmonary Fibrosis. *N Engl J Med* **353**(21): 2285-2287

Hwang H-Y, Olson SK, Esko JD, Robert Horvitz H (2003) Caenorhabditis elegans early embryogenesis and vulval morphogenesis require chondroitin biosynthesis. *Nature* **423**(6938): 439-443

loachim HL (1976) The stromal reaction of tumors: an expression of immune surveillance. *J Natl Cancer Inst* **57**(3): 465-475

lozzo RV (1995) Tumor stroma as a regulator of neoplastic behavior. Agonistic and antagonistic elements embedded in the same connective tissue. *Lab Invest* **73**(2): 157-160

Iozzo RV (1998) Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* **67**: 609-652

Iozzo RV, Naso MF, Cannizzaro LA, Wasmuth JJ, McPherson JD (1992) Mapping of the versican proteoglycan gene (CSPG2) to the long arm of human chromosome 5 (5q12-5q14). *Genomics* **14**(4): 845-851

Irving-Rodgers HF, Catanzariti KD, Aspden WJ, D'Occhio MJ, Rodgers RJ (2006a) Remodeling of extracellular matrix at ovulation of the bovine ovarian follicle. *Mol Reprod and Dev* **73**(10): 1292-1302

Irving-Rodgers HF, Roger J, Luck MR, Rodgers RJ (2006b) Extracellular matrix of the corpus luteum. *Semin Reprod Med* **24**(4): 242-250

Isogai Z, Aspberg A, Keene DR, Ono RN, Reinhardt DP, Sakai LY (2002) Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. *J Biol Chem* **277**(6): 4565-4572 Isogai Z, Shinomura T, Yamakawa N, Takeuchi J, Tsuji T, Heinegard D, Kimata K (1996) 2B1 antigen characteristically expressed on extracellular matrices of human malignant tumors is a large chondroitin sulfate proteoglycan, PG-M/versican. *Cancer Res* **56**(17): 3902-3908

Ito K, Shinomura T, Zako M, Ujita M, Kimata K (1995) Multiple forms of mouse PG-M, a large chondroitin sulfate proteoglycan generated by alternative splicing. *J Biol Chem* **270**(2): 958-965

Ito Y, Abiko Y, Tanaka Y, Rahemtulla F, Kaku T (2002) Immunohistochemical localization of large chondroitin sulfate proteoglycan in odontogenic tumor. *Med Electron Microsc* **35**(3): 173-177

Itoh Y, Seiki M (2004) MT1-MMP: an enzyme with multidimensional regulation. *Trends Biochem Sci* **29**(6): 285-289

Jonsson-Rylander AC, Nilsson T, Fritsche-Danielson R, Hammarstrom A, Behrendt M, Andersson JO, Lindgren K, Andersson AK, Wallbrandt P, Rosengren B, Brodin P, Thelin A, Westin A, Hurt-Camejo E, Lee-Sogaard CH (2005) Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican. *Arterioscler Thromb Vasc Biol* **25**(1): 180-185

Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J (1995) Abnormal lung development and cleft palate in mice lacking TGF-[beta]3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* **11**(4): 415-421

Kahari VM, Larjava H, Uitto J (1991) Differential regulation of extracellular matrix proteoglycan (PG) gene expression. Transforming growth factor-beta 1 up-regulates biglycan (PGI), and versican (large fibroblast PG) but down-regulates decorin (PGII) mRNA levels in human fibroblasts in culture. *J Biol Chem* **266**(16): 10608-10615

Kamiya N, Watanabe H, Habuchi H, Takagi H, Shinomura T, Shimizu K, Kimata K (2006) Versican/PG-M Regulates Chondrogenesis as an Extracellular Matrix Molecule Crucial for Mesenchymal Condensation. *J Biol Chem* **281**(4): 2390-2400

Karvinen S, Kosma VM, Tammi MI, Tammi R (2003) Hyaluronan, CD44 and versican in epidermal keratinocyte tumours. *Br J Dermatol* **148**(1): 86-94

Kashiwagi M, Tortorella M, Nagase H, Brew K (2001) TIMP-3 Is a Potent Inhibitor of Aggrecanase 1 (ADAM-TS4) and Aggrecanase 2 (ADAM-TS5). *J Biol Chem* **276**(16): 12501-12504

Kawashima H, Atarashi K, Hirose M, Hirose J, Yamada S, Sugahara K, Miyasaka M (2002) Oversulfated chondroitin/dermatan sulfates containing GlcAbeta1/IdoAalpha1-3GalNAc(4,6-O-disulfate) interact with L- and P-selectin and chemokines. *J Biol Chem* **277**(15): 12921-12930

Kawashima H, Hirose M, Hirose J, Nagakubo D, Plaas AH, Miyasaka M (2000) Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. *J Biol Chem* **275**(45): 35448-35456

Kawashima H, Li YF, Watanabe N, Hirose J, Hirose M, Miyasaka M (1999) Identification and characterization of ligands for L-selectin in the kidney. I. Versican, a large chondroitin sulfate proteoglycan, is a ligand for L-selectin. *Int Immunol* **11**(3): 393-405

Keller UAD, Doucet A, Overall CM (2007) Protease research in the era of systems biology. *Biol Chem* **388**(11): 1159-1162

Kenagy RD, Fischer JW, Lara S, Sandy JD, Clowes AW, Wight TN (2005) Accumulation and loss of extracellular matrix during shear stress-mediated intimal growth and regression in baboon vascular grafts. *J Histochem Cytochem* **53**(1): 131-140

Kenagy RD, Plaas AH, Wight TN (2006) Versican Degradation and Vascular Disease. *Trends in Cardiovascular Medicine* **16**(6): 209-215

Kern CB, Norris RA, Thompson RP, Argraves WS, Fairey SE, Reyes L, Hoffman S, Markwald RR, Mjaatvedt CH (2007) Versican proteolysis mediates myocardial regression during outflow tract development. *Developmental Dynamics* **236**(3): 671-683

Kern CB, Twal WO, Mjaatvedt CH, Fairey SE, Toole BP, Iruela-Arispe ML, Argraves WS (2006) Proteolytic cleavage of versican during cardiac cushion morphogenesis. *Dev Dyn*

Khalil N, O'Connor R (2004) Idiopathic pulmonary fibrosis: current understanding of the pathogenesis and the status of treatment. *CMAJ* **171**(2): 153-160

Kimata K, Oike Y, Tani K, Shinomura T, Yamagata M, Uritani M, Suzuki S (1986) A large chondroitin sulfate proteoglycan (PG-M) synthesized before chondrogenesis in the limb bud of chick embryo. *J Biol Chem* **261**(29): 13517-13525

King TE, Costabel U, Cordier J-F, DoPico GA, du Bois RM, Lynch III JP, Myers J, Panos R, Raghu G, Schwartz D, Smith CM (2000) Idiopathic Pulmonary Fibrosis: Diagnosis and Treatment . International Consensus Statement. Americal Thoracic Society. In *Am J Respir Crit Care Med* Vol. 161, pp 646-664.

Kinsella MG, Bressler SL, Wight TN (2004) The regulated synthesis of versican, decorin, and biglycan: extracellular matrix proteoglycans that influence cellular phenotype. *Crit Rev Eukaryot Gene Expr* **14**(3): 203-234

Kloeckener-Gruissem B, Bartholdi D, Abdou MT, Zimmermann DR, Berger W (2006) Identification of the genetic defect in the original Wagner syndrome family. *Mol Vis* **12**: 350-355

Kodama J, Hasengaowa, Kusumoto T, Seki N, Matsuo T, Nakamura K, Hongo A, Hiramatsu Y (2007a) Versican expression in human cervical cancer. *Eur J Cancer* **43**(9): 1460-1466

Kodama J, Hasengaowa, Kusumoto T, Seki N, Matsuo T, Ojima Y, Nakamura K, Hongo A, Hiramatsu Y (2007b) Prognostic significance of stromal versican expression in human endometrial cancer. *Ann Oncol* **18**(2): 269-274

Koga T, Inatani M, Hirata A, Inomata Y, Zako M, Kimata K, Oohira A, Gotoh T, Mori M, Tanihara H (2005) Expression of a chondroitin sulfate proteoglycan, versican (PG-M), during development of rat cornea. *Curr Eye Res* **30**(6): 455-463

Koninger J, Giese T, di Mola FF, Wente MN, Esposito I, Bachem MG, Giese NA, Buchler MW, Friess H (2004) Pancreatic tumor cells influence the composition of the extracellular matrix. *Biochem Biophys Res Commun* **322**(3): 943-949

Koyama H, Hibi T, Isogai Z, Yoneda M, Fujimori M, Amano J, Kawakubo M, Kannagi R, Kimata K, Taniguchi Si, Itano N (2007) Hyperproduction of Hyaluronan in Neu-Induced Mammary Tumor Accelerates Angiogenesis through Stromal Cell Recruitment: Possible Involvement of Versican/PG-M. *Am J Pathol* **170**(3): 1086-1099

Krusius T, Ruoslahti E (1986) Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. *Proceedings of the National Academy of Sciences* **83**(20): 7683-7687

Kuno K, Kanada N, Nakashima E, Fujiki F, Ichimura F, Matsushima K (1997) Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene. *J Biol Chem* **272**(1): 556-562

Labropoulou VT, Theocharis AD, Ravazoula P, Perimenis P, Hjerpe A, Karamanos NK, Kalofonos HP (2006) Versican but not decorin accumulation is related to metastatic potential and neovascularization in testicular germ cell tumours. *Histopathology* **49**(6): 582-593

Lah TT, Alonso MBD, Van Noorden CJF (2006) Antiprotease therapy in cancer: hot or not? *Expert Opin Biol Ther* **6**(3): 257-279

Landolt RM, Vaughan L, Winterhalter KH, Zimmermann DR (1995) Versican is selectively expressed in embryonic tissues that act as barriers to neural crest cell migration and axon outgrowth. *Development* **121**(8): 2303-2312

Larsen K, Tufvesson E, Malmstrom J, Morgelin M, Wildt M, Andersson A, Lindstrom A, Malmstrom A, Lofdahl CG, Marko-Varga G, Bjermer L, Westergren-Thorsson G (2004) Presence of activated mobile fibroblasts in bronchoalveolar lavage from patients with mild asthma. *Am J Respir Crit Care Med* **170**(10): 1049-1056

Le NT, Xue M, Castelnoble LA, Jackson CJ (2007) The dual personalities of matrix metalloproteinases in inflammation. *Front Biosci* **12**: 1475-1487

LeBaron RG, Zimmermann DR, Ruoslahti E (1992) Hyaluronate binding properties of versican. *J Biol Chem* **267**(14): 10003-10010

Lemire JM, Braun KR, Maurel P, Kaplan ED, Schwartz SM, Wight TN (1999) Versican/PG-M isoforms in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **19**(7): 1630-1639

Lemire JM, Merrilees MJ, Braun KR, Wight TN (2002) Overexpression of the V3 variant of versican alters arterial smooth muscle cell adhesion, migration, and proliferation in vitro. *J Cell Physiol* **190**(1): 38-45

Levesque H, Girard N, Maingonnat C, Delpech A, Chauzy C, Tayot J, Courtois H, Delpech B (1994) Localization and solubilization of hyaluronan and of the hyaluronan-binding protein hyaluronectin in human normal and arteriosclerotic arterial walls. *Atherosclerosis* **105**(1): 51-62

Lin H, Ignatescu M, Wilson JE, Roberts CR, Horley KJ, Winters GL, Costanzo MR, McManus BM (1996a) Prominence of apolipoproteins B, (a), and E in the intimae of coronary arteries in transplanted human hearts: geographic relationship to vessel wall proteoglycans. *J Heart Lung Transplant* **15**(12): 1223-1232

Lin H, Wilson JE, Roberts CR, Horley KJ, Winters GL, Costanzo MR, McManus BM (1996b) Biglycan, decorin, and versican protein expression patterns in coronary arteriopathy of human cardiac allograft: distinctness as compared to native atherosclerosis. *J Heart Lung Transplant* **15**(12): 1233-1247

Lin HM, Chatterjee A, Lin YH, Anjomhsoaa A, Fukuzawa R, McCall JL, Reeve AE (2007) Genome wide expression profiling identifies genes associated with colorectal liver metastasis. *Oncol Rep* **17**(6): 1541-1549

Lin RY, Sullivan KM, Argenta PA, Meuli M, Lorenz HP, Adzick NS (1995) Exogenous transforming growth factor-beta amplifies its own expression and induces scar formation in a model of human fetal skin repair. *Ann Surg* **222**(2): 146-154

Lopez-Otin C, Matrisian LM (2007) Emerging roles of proteases in tumour suppression. *Nat Rev Cancer* **7**(10): 800-808

Madlener M, Parks WC, Werner S (1998) Matrix Metalloproteinases (MMPs) and Their Physiological Inhibitors (TIMPs) Are Differentially Expressed during Excisional Skin Wound Repair. *Exp Cell Res* **242**(1): 201-210

Makatsori E, Lamari FN, Theocharis AD, Anagnostides S, Hjerpe A, Tsegenidis T, Karamanos NK (2003) Large matrix proteoglycans, versican and perlecan, are expressed and secreted by human leukemic monocytes. *Anticancer Res* **23**(4): 3303-3309

Malik M, Catherino WH (2007) Novel method to characterize primary cultures of leiomyoma and myometrium with the use of confirmatory biomarker gene arrays. *Fertil Steril* **87**(5): 1166-1172

Maquart F-X, Pasco S, Ramont L, Hornebeck W, Monboisse J-C (2004) An introduction to matrikines: extracellular matrix-derived peptides which regulate cell activity: Implication in tumor invasion. *Critical Reviews in Oncology/Hematology* **49**(3): 199-202

Martin I, Jakob M, Schafer D, Dick W, Spagnoli G, Heberer M (2001) Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. *Osteoarthritis Cartilage* **9**(2): 112-118

Martin P (1997) Wound Healing--Aiming for Perfect Skin Regeneration. Science 276(5309): 75-81

Matsumoto K, Kamiya N, Suwan K, Atsumi F, Shimizu K, Shinomura T, Yamada Y, Kimata K, Watanabe H (2006) Identification and Characterization of Versican/PG-M Aggregates in Cartilage. *J Biol Chem* **281**(26): 18257-18263

Matsumoto K, Shionyu M, Go M, Shimizu K, Shinomura T, Kimata K, Watanabe H (2003) Distinct interaction of versican/PG-M with hyaluronan and link protein. *J Biol Chem* **278**(42): 41205-41212

Mauri P, Scarpa A, Nascimbeni AC, Benazzi L, Parmagnani E, Mafficini A, Della Peruta M, Bassi C, Miyazaki K, Sorio C (2005) Identification of proteins released by pancreatic cancer cells by multidimensional protein identification technology: a strategy for identification of novel cancer markers. *Faseb J* **19**(9): 1125-1127

Mazzucato M, Cozzi MR, Pradella P, Perissinotto D, Malmstrom A, Morgelin M, Spessotto P, Colombatti A, De Marco L, Perris R (2002) Vascular PG-M/versican variants promote platelet adhesion at low shear rates and cooperate with collagens to induce aggregation. *Faseb J* **16**(14): 1903-1916

McCawley LJ, Matrisian LM (2000) Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today* **6**(4): 149-156

McQuibban GA, Gong J-H, Tam EM, McCulloch CAG, Clark-Lewis I, Overall CM (2000) Inflammation Dampened by Gelatinase A Cleavage of Monocyte Chemoattractant Protein-3. *Science* **289**(5482): 1202-1206

Merrilees MJ, Hankin EJ, Black JL, Beaumont B (2004) Matrix proteoglycans and remodelling of interstitial lung tissue in lymphangioleiomyomatosis. *J Pathol* **203**(2): 653-660

Merrilees MJ, Lemire JM, Fischer JW, Kinsella MG, Braun KR, Clowes AW, Wight TN (2002) Retrovirally mediated overexpression of versican v3 by arterial smooth muscle cells induces tropoelastin synthesis and elastic fiber formation in vitro and in neointima after vascular injury. *Circ Res* **90**(4): 481-487

Milz S, Aktas T, Putz R, Benjamin M (2006) Expression of extracellular matrix molecules typical of articular cartilage in the human scapholunate interosseous ligament. *Journal of Anatomy* **208**(6): 671-679

Miosge N, Sasaki T, Chu ML, Herken R, Timpl R (1998) Ultrastructural localization of microfibrillar fibulin-1 and fibulin-2 during heart development indicates a switch in molecular associations. *Cell Mol Life Sci* **54**(6): 606-613

Miyamoto T, Inoue H, Sakamoto Y, Kudo E, Naito T, Mikawa T, Mikawa Y, Isashiki Y, Osabe D, Shinohara S, Shiota H, Itakura M (2005) Identification of a Novel Splice Site Mutation of the CSPG2 Gene in a Japanese Family with Wagner Syndrome. *Invest Ophthalmol Vis Sci* **46**(8): 2726-2735

Mjaatvedt CH, Yamamura H, Capehart AA, Turner D, Markwald RR (1998) The Cspg2 gene, disrupted in the hdf mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol* **202**(1): 56-66

Mongiat M, Sweeney SM, San Antonio JD, Fu J, Iozzo RV (2003) Endorepellin, a Novel Inhibitor of Angiogenesis Derived from the C Terminus of Perlecan. *J Biol Chem* **278**(6): 4238-4249

Morgelin M, Heinegard D, Engel J, Paulsson M (1994) The cartilage proteoglycan aggregate: assembly through combined protein-carbohydrate and protein-protein interactions. *Biophys Chem* **50**: 113-128

Morgelin M, Paulsson M, Malmstrom A, Heinegard D (1989) Shared and distinct structural features of interstitial proteoglycans from different bovine tissues revealed by electron microscopy. *J Biol Chem* **264**(20): 12080-12090

Moulin V, Tam BYY, Castilloux G, Auger FA, O'Connor-McCourt MD, Philip A, Germain L (2001) Fetal and adult human skin fibroblasts display intrinsic differences in contractile capacity. *Journal of Cellular Physiology* **188**(2): 211-222

Mukaratirwa S, van Ederen AM, Gruys E, Nederbragt H (2004) Versican and hyaluronan expression in canine colonic adenomas and carcinomas: relation to malignancy and depth of tumour invasion. *J Comp Pathol* **131**(4): 259-270

Mukhopadhyay A, Nikopoulos K, Maugeri A, de Brouwer APM, van Nouhuys CE, Boon CJF, Perveen R, Zegers HAA, Wittebol-Post D, van den Biesen PR, van der Velde-Visser SD, Brunner HG, Black GCM, Hoyng CB, Cremers FPM (2006) Erosive Vitreoretinopathy and Wagner Disease Are Caused by Intronic Mutations in CSPG2/Versican That Result in an Imbalance of Splice Variants. *Invest Ophthalmol Vis Sci* **47**(8): 3565-3572

Murphy G, Nagase H (2008) Reappraising metalloproteinases in rheumatoid arthritis and osteoarthritis: destruction or repair? *Nat Clin Pract Rheum* **4**(3): 128-135

Nakashima N, Sobue M, Fukata S, Fukatsu T, Nagasaka T, Ohiwa N, Nara Y, Lai S, Takeuchi J (1990) Immunohistochemical characterization of extracellular matrix components of yolk sac tumors. *Virchows Arch* **58**(4): 309-315

Nara Y, Kato Y, Torii Y, Tsuji Y, Nakagaki S, Goto S, Isobe H, Nakashima N, Takeuchi J (1997) Immunohistochemical localization of extracellular matrix components in human breast tumours with special reference to PG-M/versican. *Histochem J* **29**(1): 21-30

Nara Y, Takeuchi J, Yoshida K, Fukatsu T, Nagasaka T, Kawaguchi T, Meng N, Kikuchi H, Nakashima N (1991) Immunohistochemical characterisation of extracellular matrix components of salivary gland tumours. *Br J Cancer* **64**(2): 307-314

Naso MF, Zimmermann DR, lozzo RV (1994) Characterization of the complete genomic structure of the human versican gene and functional analysis of its promoter. *J Biol Chem* **269**(52): 32999-33008

Ney A, Booms P, Epple G, Morgelin M, Guo G, Kettelgerdes G, Gebner R, Robinson PN (2006) Calcium-dependent self-association of the C-type lectin domain of versican. *The International Journal of Biochemistry & Cell Biology* **38**: 23-29

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88**(2): 277-285

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao J, Sage EH, Folkman J (1994) Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79**: 315-328

Oba-Shinjo SM, Correa M, Ricca TI, Molognoni F, Pinhal MA, Neves IA, Marie SK, Sampaio LO, Nader HB, Chammas R, Jasiulionis MG (2006) Melanocyte transformation associated with substrate adhesion impediment *Neoplasia* **8**(3): 231 – 241

Ogawa H, Oohashi T, Sata M, Bekku Y, Hirohata S, Nakamura K, Yonezawa T, Kusachi S, Shiratori Y, Ninomiya Y (2004) Lp3/HapIn3, a novel link protein that co-localizes with versican and is coordinately upregulated by platelet-derived growth factor in arterial smooth muscle cells. *Matrix Biol* **23**(5): 287-298

Ohno-Jinno A, Isogai Z, Yoneda M, Kasai K, Miyaishi O, Inoue Y, Kataoka T, Zhao J-S, Li H, Takeyama M, Keene DR, Sakai LY, Kimata K, Iwaki M, Zako M (2008) Versican and Fibrillin-1 Form a Major Hyaluronan-Binding Complex in the Ciliary Body. *Invest Ophthalmol Vis Sci*: iovs.07-1488

Okada A, Tomasetto C, Lutz Y, Bellocq J-P, Rio M-C, Basset P (1997) Expression of Matrix Metalloproteinases during Rat Skin Wound Healing: Evidence that Membrane Type-1 Matrix Metalloproteinase Is a Stromal Activator of Pro-Gelatinase A. *J Cell Biol* **137**(1): 67-77

Olin AI, Morgelin M, Sasaki T, Timpl R, Heinegard D, Aspberg A (2001) The proteoglycans aggrecan and Versican form networks with fibulin-2 through their lectin domain binding. *J Biol Chem* **276**(2): 1253-1261

Overall C, Dean R (2006) Degradomics: Systems biology of the protease web. Pleiotropic roles of MMPs in cancer. *Cancer Metastasis Rev* **25**(1): 69-75

Overall CM, Blobel CP (2007) In search of partners: linking extracellular proteases to substrates. *Nat Rev Mol Cell Biol* **8**(3): 245-257

Overall CM, Kleifeld O (2006) Tumour microenvironment - Opinion: Validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* **6**(3): 227-239

Overall CM, Sodek J (1990) Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. *J Biol Chem* **265**(34): 21141-21151

Overall CM, Tam E, McQuibban GA, Morrison C, Wallon UM, Bigg HF, King AE, Roberts CR (2000) Domain Interactions in the Gelatinase A TIMP-2 MT1-MMP Activation Complex. The ectodomain of the 44-kDa form of membrane type-1 matrix metalloproteinase does not modulate gelatinase A activation. *J Biol Chem* **275**(50): 39497-39506

Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* **8**(3): 221-233

Pardo A, Selman M, Kaminski N (2008) Approaching the degradome in idiopathic pulmonary fibrosis. *Int J Biochem Cell Biol* **40**(6-7): 1141-1155

Paris S, Sesboue R, Chauzy C, Maingonnat C, Delpech B (2006) Hyaluronectin modulation of lung metastasis in nude mice. *European Journal of Cancer* **42**(18): 3253-3259

Passi A, Negrini D, Albertini R, Miserocchi G, De Luca G (1999) The sensitivity of versican from rabbit lung to gelatinase A (MMP-2) and B (MMP-9) and its involvement in the development of hydraulic lung edema. *FEBS Lett* **456**(1): 93-96

Paulus W, Baur I, Dours-Zimmermann MT, Zimmermann DR (1996) Differential expression of versican isoforms in brain tumors. *J Neuropathol Exp Neurol* **55**(5): 528-533

Perides G, Asher RA, Lark MW, Lane WS, Robinson RA, Bignami A (1995) Glial hyaluronate-binding protein: a product of metalloproteinase digestion of versican? *Biochem J* **312** (**Pt 2**): 377-384

Perides G, Lane WS, Andrews D, Dahl D, Bignami A (1989) Isolation and partial characterization of a glial hyaluronate-binding protein. *J Biol Chem* **264**(10): 5981-5987

Perides G, Rahemtulla F, Lane WS, Asher RA, Bignami A (1992) Isolation of a large aggregating proteoglycan from human brain. *J Biol Chem* **267**(33): 23883-23887

Perissinotto D, Iacopetti P, Bellina I, Doliana R, Colombatti A, Pettway Z, Bronner-Fraser M, Shinomura T, Kimata K, Morgelin M, Lofberg J, Perris R (2000) Avian neural crest cell migration is diversely regulated by the two major hyaluronan-binding proteoglycans PG-M/versican and aggrecan. *Development* **127**(13): 2823-2842

Perris R, Perissinotto D, Pettway Z, Bronner-Fraser M, Morgelin M, Kimata K (1996) Inhibitory effects of PG-H/aggrecan and PG-M/versican on avian neural crest cell migration. *Faseb J* **10**(2): 293-301

Pirinen R, Leinonen T, Bohm J, Johansson R, Ropponen K, Kumpulainen E, Kosma VM (2005) Versican in nonsmall cell lung cancer: relation to hyaluronan, clinicopathologic factors, and prognosis. *Hum Pathol* **36**(1): 44-50

Ponting JM, Kumar S (1995) Localisation and cellular origin of hyaluronectin. J Anat 187(Pt 2): 331-346

Porter S, Scott SD, Sassoon EM, Williams MR, Jones JL, Girling AC, Ball RY, Edwards DR (2004) Dysregulated expression of adamalysin-thrombospondin genes in human breast carcinoma. *Clin Cancer Res* **10**(7): 2429-2440

Potter-Perigo S, Baker C, Tsoi C, Braun KR, Isenhath S, Altman GM, Altman LC, Wight TN (2004) Regulation of proteoglycan synthesis by leukotriene d4 and epidermal growth factor in bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* **30**(1): 101-108

Prockop DJ, Sieron AL, Li S-W (1998) Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. *Matrix Biol* **16**(7): 399-408

Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MWJ, Doetschman T (1995) Transforming growth factor-[beta]3 is required for secondary palate fusion. *Nat Genet* **11**(4): 409-414

Puente XS, Sanchez LM, Overall CM, Lopez-Otin C (2003) Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* **4**(7): 544-558

Pukkila M, Kosunen A, Ropponen K, Virtaniemi J, Kellokoski J, Kumpulainen E, Pirinen R, Nuutinen J, Johansson R, Kosma V-M (2007) High stromal versican expression predicts unfavourable outcome in oral squamous cell carcinoma. *J Clin Pathol* **60**(3): 267-272

Pukkila MJ, Kosunen AS, Virtaniemi JA, Kumpulainen EJ, Johansson RT, Kellokoski JK, Nuutinen J, Kosma VM (2004) Versican expression in pharyngeal squamous cell carcinoma: an immunohistochemical study. *J Clin Pathol* **57**(7): 735-739

Radisky DC, Kenny PA, Bissell MJ (2007) Fibrosis and cancer: Do myofibroblasts come also from epithelial cells via EMT? *J Cell Biochem* **101**(4): 830-839

Rahmani M, Read JT, Carthy JM, McDonald PC, Wong BW, Esfandiarei M, Si X, Luo Z, Luo H, Rennie PS, McManus BM (2005) Regulation of the versican promoter by the beta-catenin-T-cell factor complex in vascular smooth muscle cells. *J Biol Chem* **280**(13): 13019-13028

Raman R, Sasisekharan V, Sasisekharan R (2005) Structural Insights into Biological Roles of Protein-Glycosaminoglycan Interactions. *Chemistry & Biology* **12**(3): 267-277

Ramaswamy S, Ross KN, Lander ES, Golub TR (2003) A molecular signature of metastasis in primary solid tumors. *Nat Genet* **33**(1): 49-54

Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A (2001) Fibroblasts from Idiopathic Pulmonary Fibrosis and Normal Lungs Differ in Growth Rate, Apoptosis, and Tissue Inhibitor of Metalloproteinases Expression. *Am J Respir Cell Mol Biol* **24**(5): 591-598

Resch A, Xing Y, Modrek B, Gorlick M, Riley R, Lee C (2004) Assessing the Impact of Alternative Splicing on Domain Interactions in the Human Proteome. *J Proteome Res* **3**(1): 76-83

Ricciardelli C, Brooks JH, Suwiwat S, Sakko AJ, Mayne K, Raymond WA, Seshadri R, LeBaron RG, Horsfall DJ (2002) Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer. *Clin Cancer Res* **8**(4): 1054-1060

Ricciardelli C, Mayne K, Sykes PJ, Raymond WA, McCaul K, Marshall VR, Horsfall DJ (1998) Elevated levels of versican but not decorin predict disease progression in early-stage prostate cancer. *Clin Cancer Res* **4**(4): 963-971

Ricciardelli C, Russell DL, Ween MP, Suwiwat S, Byers S, Marshall VR, Tilley WD, Horsfall DJ (2007) Formation of hyaluronan-and versican-rich pericellular matrix by prostate cancer cells promotes cell motility. *J Biol Chem* **282**(14): 10814-10825

Robbins JR, Evanko SP, Vogel KG (1997) Mechanical loading and TGF-beta regulate proteoglycan synthesis in tendon. *Arch Biochem Biophys* **342**(2): 203-211

Roberts CR (1995) Is asthma a fibrotic disease? Chest 107(3 Suppl): 111S-117S

Roberts CR (2003) Versican in the Cell Biology of Pulmonary Fibrosis. In *Proteoglycans in Lung Disease*, Garg HG, Roughley PJ, Hales CA (eds), Vol. 168, pp 191-212. New York, NY: Marcel Dekker

Roberts CR, Burke AK (1998) Remodelling of the extracellular matrix in asthma: proteoglycan synthesis and degradation. *Can Respir J* **5**(1): 48-50

Rocks N, Paulissen G, Quesada Calvo F, Polette M, Gueders M, Munaut C, Foidart JM, Noel A, Birembaut P, Cataldo D (2006) Expression of a disintegrin and metalloprotease (ADAM and ADAMTS) enzymes in human non-small-cell lung carcinomas (NSCLC). *Br J Cancer* **94**(5): 724-730

Rogliani P, Mura M, Assunta Porretta M, Saltini C (2008) Review: New perspectives in the treatment of idiopathic pulmonary fibrosis. *Therapeutic Advances in Respiratory Disease* **2**(2): 75-93

Romeo S, Oosting J, Rozeman LB, Hameetman L, Taminiau AH, Cleton-Jansen AM, Bovee JV, Hogendoorn PC (2007) The role of noncartilage-specific molecules in differentiation of cartilaginous tumors. *Cancer* **110**(2): 385-394

Ruoslahti E (1996) Brain extracellular matrix. Glycobiology 6(5): 489-492

Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, Lopez-Otin C, Shapiro S, Inada M, Krane S, Allen E, Chung D, Weiss SJ (2004) Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *J Cell Biol* **167**(4): 769-781

Sakko AJ, Ricciardelli C, Mayne K, Dours-Zimmermann MT, Zimmermann DR, Neufing P, Tilley WD, Marshall VR, Horsfall DJ (2007) Changes in steroid receptors and proteoglycan expression in the guinea pig prostate stroma during puberty and hormone manipulation. *The Prostate* **67**(3): 288-300

Sakko AJ, Ricciardelli C, Mayne K, Suwiwat S, LeBaron RG, Marshall VR, Tilley WD, Horsfall DJ (2003) Modulation of prostate cancer cell attachment to matrix by versican. *Cancer Res* **63**(16): 4786-4791

Sakko AJ, Ricciardelli C, Mayne K, Tilley WD, Lebaron RG, Horsfall DJ (2001) Versican accumulation in human prostatic fibroblast cultures is enhanced by prostate cancer cell-derived transforming growth factor beta1. *Cancer Res* **61**(3): 926-930

Samiric T, Ilic MZ, Handley CJ (2004) Characterisation of proteoglycans and their catabolic products in tendon and explant cultures of tendon. *Matrix Biol* **23**(2): 127-140

Sandy JD (2006) A contentious issue finds some clarity: on the independent and complementary roles of aggrecanase activity and MMP activity in human joint aggrecanolysis. *Osteoarthritis and Cartilage* **14**(2): 95-100

Sandy JD, Westling J, Kenagy RD, Iruela-Arispe ML, Verscharen C, Rodriguez-Mazaneque JC, Zimmermann DR, Lemire JM, Fischer JW, Wight TN, Clowes AW (2001) Versican V1 proteolysis in human aorta in vivo occurs at the Glu441-Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4. *J Biol Chem* **276**(16): 13372-13378

Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T (1997) TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* **124**(13): 2659-2670

Sato H, Takino T, Miyamori H (2005) Roles of membrane-type matrix metalloproteinase-1 in tumor invasion and metastasis. *Cancer Sci* **96**(4): 212-217

Schmalfeldt M, Bandtlow CE, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR (2000) Brain derived versican V2 is a potent inhibitor of axonal growth. *J Cell Sci* **113** (**Pt 5**): 807-816

Schmalfeldt M, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR (1998) Versican V2 is a major extracellular matrix component of the mature bovine brain. *J Biol Chem* **273**(25): 15758-15764

Schonherr E, Jarvelainen HT, Sandell LJ, Wight TN (1991) Effects of platelet-derived growth factor and transforming growth factor-beta 1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J Biol Chem* **266**(26): 17640-17647

Scott A, Lian O, Roberts CR, Cook JL, Handley CJ, Bahr R, Samiric T, Ilic MZ, Parkinson J, Hart DA, Duronio V, Khan KM (2008) Increased versican content is associated with tendinosis pathology in the patellar tendon of athletes with jumper's knee. *Scandinavian Journal of Medicine & Science in Sports*: in press

Scott PG, Dodd CM, Tredget EE, Ghahary A, Rahemtulla F (1996) Chemical characterization and quantification of proteoglycans in human post-burn hypertrophic and mature scars. *Clin Sci* **90:** 417-425

Selman M, Pardo A (2002) Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder. *Respiratory Research* **3**(1): 3

Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R, Barrios R, Pardo A (2000) TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? *Am J Physiol Lung Cell Mol Physiol* **279**(3): L562-574

Selman M, Talmadge EKJ, Pardo A (2001) Idiopathic Pulmonary Fibrosis: Prevailing and Evolving Hypotheses about its Pathogenesis and Implications for Therapy. *Ann Intern Med* **134**(2): 136-151

Serra M, Miquel L, Domenzain C, Docampo MJ, Fabra A, Wight TN, Bassols A (2005) V3 versican isoform expression alters the phenotype of melanoma cells and their tumorigenic potential. *Int J Cancer* **114**(6): 879-886

Shepard JB, Gliga DA, Morrow AP, Hoffman S, Capehart AA (2008) Versican Knock-Down Compromises Chondrogenesis in the Embryonic Chick Limb. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* **291**(1): 19-27

Shepard JB, Krug HA, LaFoon BA, Hoffman S, Capehart AA (2007) Versican expression during synovial joint morphogenesis. *int J Biol Sci* **3**: 380-384

Shi S, Grothe S, Zhang Y, O'Connor-McCourt MD, Poole AR, Roughley PJ, Mort JS (2004) Link protein has greater affinity for versican than aggrecan. *J Biol Chem* **279**(13): 12060-12066

Shi Y, Niculescu R, Wang D, Ormont M, Magno M, San Antonio JD, Williams KJ, Zalewski A (2000) Myofibroblast involvement in glycosaminoglycan synthesis and lipid retention during coronary repair. *J Vasc Res* **37**(5): 399-407

Shou J, Bull CM, Li L, Qian HR, Wei T, Luo S, Perkins D, Solenberg PJ, Tan SL, Chen XY, Roehm NW, Wolos JA, Onyia JE (2006) Identification of blood biomarkers of rheumatoid arthritis by transcript profiling of peripheral blood mononuclear cells from the rat collagen-induced arthritis model. *Arthritis Res Ther* **8**(1): R28

Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschman T (1992) Targeted disruption of the mouse transforming growth factor-[beta]1 gene results in multifocal inflammatory disease. *Nature* **359**(6397): 693-699

Singer AJ, Clark RAF (1999) Cutaneous Wound Healing. N Engl J Med 341(10): 738-746

Skandalis SS, Kletsas D, Kyriakopoulou D, Stavropoulos M, Theocharis DA (2006a) The greatly increased amounts of accumulated versican and decorin with specific post-translational modifications

may be closely associated with the malignant phenotype of pancreatic cancer. *Biochim Biophys Acta* **1760**(8): 1217-1225

Skandalis SS, Theocharis AD, Papageorgakopoulou N, Vynios DH, Theocharis DA (2006b) The increased accumulation of structurally modified versican and decorin is related with the progression of laryngeal cancer. *Biochimie* **88**(9): 1135-1143

Skandalis SS, Theocharis AD, Theocharis DA, Papadas T, Vynios DH, Papageorgakopoulou N (2004) Matrix proteoglycans are markedly affected in advanced laryngeal squamous cell carcinoma. *Biochim Biophys Acta* **1689**(2): 152-161

Sobue M, Nakashima N, Fukatsu T, Nagasaka T, Fukata S, Ohiwa N, Nara Y, Ogura T, Katoh T, Takeuchi J (1989) Production and immunohistochemical characterization of a monoclonal antibody raised to proteoglycan purified from a human yolk sac tumour. *Histochem J* **21**(8): 455-460

Somerville RP, Longpre JM, Jungers KA, Engle JM, Ross M, Evanko S, Wight TN, Leduc R, Apte SS (2003) Characterization of ADAMTS-9 and ADAMTS-20 as a distinct ADAMTS subfamily related to Caenorhabditis elegans GON-1. *J Biol Chem* **278**(11): 9503-9513

Spicer AP, Joo A, Bowling RA, Jr. (2003) A Hyaluronan Binding Link Protein Gene Family Whose Members Are Physically Linked Adjacent to Chrondroitin Sulfate Proteoglycan Core Protein Genes: The Missing Links. *J Biol Chem* **278**(23): 21083-21091

Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, Little CB, Last K, Farmer PJ, Campbell IK, Fourie AM, Fosang AJ (2005) ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. *Nature* **434**(7033): 648-652

Stetler-Stevenson WG (2008) Tissue Inhibitors of Metalloproteinases in Cell Signaling: Metalloproteinase-Independent Biological Activities. *Sci Signal* **1**(27): re6-

Stetler-Stevenson WG, Hewitt R, Corcoran M (1996) Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. *Semin Cancer Biol* **7**(3): 147-154

Stocker W, Grams F, Baumann U, Reinemer P, Gomis-Ruth FX, McKay DB, Bode W (1995) The metzincins -- Topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. *Protein Sci* **4**(5): 823-840

Strom A, Olin AI, Aspberg A, Hultgardh-Nilsson A (2006) Fibulin-2 is present in murine vascular lesions and is important for smooth muscle cell migration. *Cardiovasc Res* **69**(3): 755-763

Stylianou M, Skandalis SS, Papadas TA, Mastronikolis NS, Theocharis AD, Papageorgakopoulou N, Vynios DH (2008) Stage-related decorin and versican expression in human laryngeal cancer. *Anticancer Res* **28**(1A): 245-251

Sugahara K, Mikami T, Uyama T, Mizuguchi S, Nomura K, Kitagawa H (2003) Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. *Current Opinion in Structural Biology* **13**(5): 612-620

Sullivan KM, Lorenz HP, Meuli M, Lin RY, Adzick NS (1995) A model of scarless human fetal wound repair is deficient in transforming growth factor beta. *Journal of Pediatric Surgery* **30**(2): 198-203

Suwiwat S, Ricciardelli C, Tammi R, Tammi M, Auvinen P, Kosma VM, LeBaron RG, Raymond WA, Tilley WD, Horsfall DJ (2004) Expression of extracellular matrix components versican, chondroitin sulfate, tenascin, and hyaluronan, and their association with disease outcome in node-negative breast cancer. *Clin Cancer Res* **10**(7): 2491-2498

Tam EM, Morrison CJ, Wu YI, Stack MS, Overall CM (2004) Membrane protease proteomics: Isotopecoded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Nat Acad Sci* **101**(18): 6917-6922

Thannickal VJ, Toews GB, White ES, Lynch Iii JP, Martinez FJ (2004) Mechanisms of Pulmonary Fibrosis. *Annual Review of Medicine* **55**(1): 395-417

Theocharis AD (2002) Human colon adenocarcinoma is associated with specific post-translational modifications of versican and decorin. *Biochim Biophys Acta* **1588**(2): 165-172

Theocharis AD, Tsolakis I, Hjerpe A, Karamanos NK (2003a) Versican undergoes specific alterations in the fine molecular structure and organization in human aneurysmal abdominal aortas. *Biomed Chromatogr* **17**(6): 411-416

Theocharis AD, Vynios DH, Papageorgakopoulou N, Skandalis SS, Theocharis DA (2003b) Altered content composition and structure of glycosaminoglycans and proteoglycans in gastric carcinoma. *Int J Biochem Cell Biol* **35**(3): 376-390

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA (2002) Myofibroblasts and mechanoregulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* **3**(5): 349-363 Tortorella MD, Arner EC, Hills R, Gormley J, Fok K, Pegg L, Munie G, Malfait A-M (2005) ADAMTS-4 (aggrecanase-1): N-Terminal activation mechanisms. *Arch Biochem Biophys* **444**(1): 34-44

Touab M, Arumi-Uria M, Barranco C, Bassols A (2003) Expression of the proteoglycans versican and mel-CSPG in dysplastic nevi. *Am J Clin Pathol* **119**(4): 587-593

Touab M, Villena J, Barranco C, Arumi-Uria M, Bassols A (2002) Versican is differentially expressed in human melanoma and may play a role in tumor development. *Am J Pathol* **160**(2): 549-557

Travis WD, King TE, Bateman ED, Lynch DA, Capron F, Center D, Colby TV, Cordier J-F, du Bois RM, Galvin J, Grenier P, Hansell DM, Hunninghake GW, Kitaichi M, Muller NL, Myers JL, Nagai S, Raghu G, Wallabert B (2002) American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias . This Joint Statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS Board of Directors, June 2001 and by The ERS Executive Committee, June 2001. *Am J Respir Crit Care Med* **165**(2): 277-304

Tsara ME, Theocharis AD, Theocharis DA (2002) Compositional and structural alterations of proteoglycans in human rectum carcinoma with special reference to versican and decorin. *Anticancer Res* **22**(5): 2893-2898

Tsujii M, Hirata H, Yoshida T, Imanaka-Yoshida K, Morita A, A. U (2006) Involvement of tenascin-C and PG-M/versican in flexor tenosynovial pathology of idiopathic carpal tunnel syndrome. *Histol Histopathol* **21**(5): 511-518

Turnbull J, Powell A, Guimond S (2001) Heparan sulfate: decoding a dynamic multifunctional cell regulator. *Trends in Cell Biology* **11**(2): 75-82

Uldbjerg N, Malmstrom A (1991) The role of proteoglycans in cervical dilation. *Semin Perinatol* **15**(2): 127-132

Venkatesan N, Roughley PJ, Ludwig MS (2002) Proteoglycan expression in bleomycin lung fibroblasts: role of transforming growth factor-beta(1) and interferon-gamma. *Am J Physiol Lung Cell Mol Physiol* **283**(4): L806-814

Voutilainen K, Anttila M, Sillanpaa S, Tammi R, Tammi M, Saarikoski S, Kosma VM (2003) Versican in epithelial ovarian cancer: relation to hyaluronan, clinicopathologic factors and prognosis. *Int J Cancer* **107**(3): 359-364

Vynios DH, Theocharis DA, Papageorgakopoulou N, Papadas TA, Mastronikolis NS, Goumas PD, Stylianou M, Skandalis SS (2008) Biochemical Changes of Extracellular Proteoglycans in Squamous Cell Laryngeal Carcinoma. *Connect Tissue Res* **49**(3): 239 - 243

Wang W-M, Ge G, Lim NH, Nagase H, Greenspan DS (2006) TIMP-3 inhibits the procollagen N-proteinase ADAMTS-2. *Biochem J* **398**(3): 515-519

Wart HEV, Birkedal-Hansen H (1990) The Cysteine Switch: A Principle of Regulation of Metalloproteinase Activity with Potential Applicability to the Entire Matrix Metalloproteinase Gene Family. *Proceedings of the National Academy of Sciences* **87**(14): 5578-5582

Westergren-Thorsson G, Norman M, Bjornsson S, Endresen U, Stjernholm Y, Ekman G, Malmstrom A (1998) Differential expressions of mRNA for proteoglycans, collagens and transforming growth factorbeta in the human cervix during pregnancy and involution. *Biochim Biophys Acta* **1406**(2): 203-213

Westling J, Gottschall PE, Thompson VP, Cockburn A, Perides G, Zimmermann DR, Sandy JD (2004) ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu405-Gln406 to generate glial hyaluronate binding protein. *Biochem J* **377**(Pt 3): 787-795

Whitby DJ, Ferguson MWJ (1991) The extracellular matrix of lip wounds in fetal, neonatal and adult mice. *Development* **112:** 651-668

Wight TN (2008) Arterial remodeling in vascular disease: a key role for hyaluronan and versican. *Front Biosci* **1**(13): 4933-4937

Wight TN, Lara S, Riessen R, Le Baron R, Isner J (1997) Selective deposits of versican in the extracellular matrix of restenotic lesions from human peripheral arteries. *Am J Pathol* **151**(4): 963-973

Wight TN, Merrilees MJ (2004) Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res* **94**(9): 1158-1167

Will H, Atkinson SJ, Butler GS, Smith B, Murphy G (1996) The Soluble Catalytic Domain of Membrane Type 1Matrix Metalloproteinase Cleaves the Propeptide of Progelatinase A and Initiates Autoproteolytic Activation. Regulation by TIMP-2 and TIMP-3. *J Biol Chem* **271**(29): 17119-17123

Wolf M, Albrecht S, Marki C (2008) Proteolytic processing of chemokines: Implications in physiological and pathological conditions. *Int J Biochem Cell Biol* **40**(6-7): 1185-1198

Wolf YG, Rasmussen LM, Ruoslahti E (1994) Antibodies against transforming growth factor-beta 1 suppress intimal hyperplasia in a rat model. *J Clin Invest* **93**(3): 1172-1178

Wynn TA (2004) Fibrotic disease and the TH1/TH2 paradigm. Nat Rev Immunol 4(8): 583-594

Wynn TA (2007) Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest* **117**(3): 524-529

Xu J, Clark RA (1996) Extracellular matrix alters PDGF regulation of fibroblast integrins. *J Cell Biol* **132**(1): 239-249

Yamagata M, Kimata K (1994) Repression of a malignant cell-substratum adhesion phenotype by inhibiting the production of the anti-adhesive proteoglycan, PG-M/versican. *J Cell Sci* **107 (Pt 9):** 2581-2590

Yamagata M, Saga S, Kato M, Bernfield M, Kimata K (1993) Selective distributions of proteoglycans and their ligands in pericellular matrix of cultured fibroblasts. Implications for their roles in cell-substratum adhesion. *J Cell Sci* **106 (Pt 1):** 55-65

Yamamura H, Zhang M, Markwald RR, Mjaatvedt CH (1997) A heart segmental defect in the anteriorposterior axis of a transgenic mutant mouse. *Developmental Biology* **186**(1): 58-72

Yao LY, Moody C, Schonherr E, Wight TN, Sandell LJ (1994) Identification of the proteoglycan versican in aorta and smooth muscle cells by DNA sequence analysis, in situ hybridization and immunohistochemistry. *Matrix Biol* **14**(3): 213-225

Yeo TK, Brown L, Dvorak HF (1991) Alterations in proteoglycan synthesis common to healing wounds and tumors. *Am J Pathol* **138**(6): 1437-1450

Yoon H, Liyanarachchi S, Wright FA, Davuluri R, Lockman JC, de la Chapelle A, Pellegata NS (2002) Gene expression profiling of isogenic cells with different TP53 gene dosage reveals numerous genes that are affected by TP53 dosage and identifies CSPG2 as a direct target of p53. *Proc Natl Acad Sci U S A* **99**(24): 15632-15637

Yu M, Sato H, Seiki M, Thompson EW (1995) Complex Regulation of Membrane-Type Matrix Metalloproteinase Expression and Matrix Metalloproteinase-2 Activation by Concanavalin A in MDA-MB-231 Human Breast Cancer Cells. *Cancer Res* **55**(15): 3272-3277 Zako M, Shinomura T, Ujita M, Ito K, Kimata K (1995) Expression of PG-M(V3), an alternatively spliced form of PG-M without a chondroitin sulfate attachment region in mouse and human tissues. *J Biol Chem* **270**(8): 3914-3918

Zhang K, Rekhter MD, Gordon D, Phan SH (1994) Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. *Am J Pathol* **145**(1): 114-125

Zhao M, Lu Y, Takata T, Ogawa I, Miyauchi M, Mock D, Nikai H (1999) Immunohistochemical and histochemical characterization of the mucosubstances of odontogenic myxoma: histogenesis and differential diagnosis. *Pathol Res Pract* **195**(6): 391-397

Zhao X, Russell P (2005) Versican splice variants in human trabecular meshwork and ciliary muscle. *Mol Vis* **11:** 603-608

Zheng PS, Wen J, Ang LC, Sheng W, Viloria-Petit A, Wang Y, Wu Y, Kerbel RS, Yang BB (2004) Versican/PG-M G3 domain promotes tumor growth and angiogenesis. *FASEB J* **18**(6): 754-756

Zimmermann DR, Dours-Zimmermann MT, Schubert M, Bruckner-Tuderman L (1994) Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. *J Cell Biol* **124**(5): 817-825

Zimmermann DR, Ruoslahti E (1989) Multiple domains of the large fibroblast proteoglycan, versican. *EMBO J* **8**(10): 2975-2981

CHAPTER 2 – PURIFICATION AND CHARACTERIZATION OF VERSICAN^{*}

2.1. Summary

In development and normal wound healing, versican is highly expressed in association with migrating and proliferating mesenchymal cells and then rapidly cleared during the resolution of remodeling. Versican is also a major component of the provisional matrix in a number of wound healing disorders. Alterations in its metabolism may contribute to the development of pulmonary fibrosis and atherosclerosis, and to cancer growth and metastasis. It is a challenging molecule to characterize biochemically, yet understanding molecular events involving versican metabolism is key to assessing the utility of potential interventions that would target faulty proteolytic pathways. Here I report that versican purified from human fetal lung fibroblast cells contains numerous proteolytic fragments, potentially as a result of a co-purifying proteolytic enzyme. An optimized protocol for versican purification is presented. I characterize a versican fragment with N-terminal sequencing and discuss challenges to characterization, including the use of mass spectrometry.

2.2. Introduction

Versican expression is associated with migrating and proliferating, mesenchymal cells. In development and normal tissue remodeling versican seems to be required for formation of a hydrated matrix that is conducive to cell migration. Versican ablation is lethal due to defects in endocardial cushion formation in heart morphogenesis

⁽Mjaatvedt et al, 1998). During wound healing, activated fibroblast cells synthesize a ^{*} A version of this chapter will be submitted for publication Sean B. Maurice, Clive R. Roberts, Alain Doucet and Christopher M. Overall, Purification and characterization of versican.

versican-rich provisional matrix in which wound closure and tissue remodeling occur. The persistence of versican in the provisional matrix is associated with fibrotic remodeling. Versican is a major component of the provisional matrix in all the major granulomatous and non-granulomatous forms of pulmonary fibrosis (Bensadoun et al, 1996; Bensadoun et al, 1997). Versican is highly expressed in atherosclerotic and restenotic lesions (Evanko et al, 1998; Wight et al, 1997) and transplant arteriopathy (Lin et al, 1996).

Versican expression has been observed surrounding many types of human tumors. In fact versican expression is suggested to be predictive of poor prognosis in oral (Pukkila et al, 2007), breast (Ricciardelli et al, 2002; Suwiwat et al, 2004), prostate (Ricciardelli et al, 1998), cervical (Kodama et al, 2007a), endometrial (Kodama et al, 2007b) and cutaneous (Touab et al, 2003; Touab et al, 2002) cancers. In the context of wound healing disorders, cancer displays several attributes of non-healing wounds (Bissell & Radisky, 2001; Dvorak, 1986). It is possible that versican plays a role in perpetuating certain cancers which is similar to its role in sustaining fibrotic processes in pulmonary fibrosis and vascular remodeling.

As versican expression is essential in development yet correlated with a number of wound healing disorders, its role in aberrant remodeling may involve impaired degradation leading to its persistence. Therefore characterization of molecular events involved in versican metabolism is of great physiological interest. This is prerequisite for the creation of therapies that target the aberrant degradative events without interfering with the appropriate proteolytic events that are necessary for restorative remodeling. We now know from clinical trials that relatively broad protease inhibition is not helpful in cancer and is sometimes even detrimental to patients (Coussens et al,

2002). This is presumably due to the variety of tasks performed by each proteinase and highlights the need for more accurately targeted therapies along with a more thorough understanding of the precise proteolytic events involved (Fingleton, 2008). Improved characterization of versican metabolism is also crucial to allow disease process monitoring with biomarkers.

Several versican cleaving proteases have been described and four cleavage sites have been characterized to date (Jonsson-Rylander et al, 2005; Sandy et al, 2001; Westling et al, 2004). Yet there is evidence that more versican cleavage sites exist than have been described thus far. In order to better understand differences between normal and aberrant versican turnover, there is a need to improve characterization of versican proteolysis.

In addition to maintaining versican in the pericellular matrix, fibroblasts secrete relatively large amounts of versican into culture media providing a suitable source for purification and subsequent studies (Zimmermann et al, 1994). Here I describe purification of versican and versican fragments from human fetal lung fibroblast cells. I characterize several proteolytic fragments of versican and discuss opportunities to improve characterization with tandem mass spectrometry.

2.3. Experimental procedures

2.3.1. Cell culture

Human fetal lung fibroblasts (HFL-1) were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 20 mM HEPES and 10% (v/v) Cosmic Calf Serum (Hyclone, Logan, UT). Cells at low passage number were grown to approximately 70% of confluence

before fixation and staining for microscopy or harvesting of serum-free conditioned medium (CM).

2.3.2. Immunofluorescence staining and microscopy

Cells were fixed for 10-15 minutes with 4% para-formaldehyde in phosphate buffered saline (PBS), pH 7.5, then rinsed with PBS. Cells were permeabilized in TBS-triton (20 mM Tris, pH 7.5, 0.9% NaCl, 0.2 % triton X-100) with 2% (w/v) bovine serum albumin (BSA). Blocking was performed with 5% (v/v) normal goat serum and 2% (w/v) BSA in TBS-triton. Washing was performed in 0.2% (w/v) BSA in TBS-triton and antibodies were diluted in the same buffer. The primary antibody was mouse monoclonal antiversican C-terminal domain, 2B1 (Isogai et al, 2002; Isogai et al, 1996) (Seikagaku, Tokyo, Japan), dilution 1:500. Alexa Fluor 594 goat anti—mouse IgG, highly crossadsorbed secondary antibody was used (Molecular Probes, Eugene, OR). Counterstaining for F-actin was with Alexa Fluor 488 phalloidin stain (Molecular Probes) and nuclear counterstaining was with Hoescht 33342 (Molecular Probes). Stained cells were mounted under coverslips with Prolong Gold antifade reagent (Molecular Probes) and stored at -20°C. Microscopy was performed on a Leica DMRA2 automated microscope (Leica Microsystems GmbH, Wetzlar). In the antibody labeled channel, three-dimensional images were acquired and image stacks were deconvolved using the Nearest Neighbour Deconvolution algorithm (Improvision, Coventry, UK).

2.3.3. Isolation of versican

Cells at 70% of confluence were rinsed in serum-free media followed by incubation in serum-free media for 24 hours. Cells were grown a minimum of 24 hours in serumsupplemented media before incubating in serum-free conditions again. This cycle was performed a maximum of three consecutive times before discarding cultures. Serumfree CM were collected and centrifuged at 1500 x g for 20 minutes to remove cellular debris. Urea was added to 7 M before loading onto Q-Sepharose Fast Flow ion exchange resin (Amersham Biosciences, Piscataway, NJ) at approximately 1 litre CM per 5 mls resin. The column was equilibrated with 0.1 M Tris, pH 7.5, 7M urea and eluted with 0.1 M Tris, pH 7.5, 7M urea, 1.5 M NaCl.

Later purifications optimized to reduce co-purification of proteases and proteolytic fragments were performed at pH 6.0 with additional salt in the start and equilibration buffer. 150 mM NaCl was added to bring the salt concentration to 400 mM before loading onto Q-Sepharose. The column was equilibrated in 0.1 M sodium acetate, pH 6.0, 7 M urea, 0.4 M NaCl and eluted with 0.1 M sodium acetate, pH 6.0, 7 M urea, 1.5 M NaCl.

2.3.4. Electrophoretic techniques

Samples in non-reducing sample buffer (125 mM Tris-HCL, pH 6.8, 2.0% SDS, 2.0 M urea, 0.05% bromophenol blue) were separated on discontinuous SDS-PAGE gels with 4% (stacking) and 10% (separating) acrylamide. Stacking and separating gels were kept during Western blotting to monitor high molecular weight versican aggregates within the stacking gel. Relative molecular mass (measured in kDa) was estimated based on mobility of molecular weight markers: MagicMark XP (Invitrogen)

and Kaleidoscope Prestained (Bio-Rad). Alcian blue staining was used for visualization of sulfated glycosaminoglycans (Krueger & Schwartz, 1987).

2.3.5. Western blotting

Western blotting was performed using the XCell II blot module (Invitrogen) to PVDF membrane (Millipore, Billerica, MA). Blocking was performed with a solution of 2% (w/v) casein, 2% (w/v) bovine serum albumin, 0.5% (w/v) PVP, 20 mM Tris, pH 7.5, 5 mM EDTA, 0.9% NaCl, 1 x PSN antibiotic mixture (Gibco, Grand Island, NY) and 0.3% (v/v) Tween 20. The following antibodies were used: mouse monoclonal anti-versican N-terminal antibody 12C5 (Asher et al, 1991)(obtained from the Developmental Studies Hybridoma Bank (NICHD), the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), 1:500 dilution; anti-versican C-terminal domain 2B1, 1:1000 dilution; rabbit polyclonal anti-versican C-terminal domain recombinant construct LC2 (Pourmalek & Roberts, 2008), 1:10 000 dilution; and rabbit polyclonal anti-PG40 (Brennan et al, 1984; Krusius & Ruoslahti, 1986), 1:500 dilution. Antibodies were diluted in a solution of Tris-BSA with 0.05% (v/v) Tween 20. Highly cross-adsorbed goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugate (Bio-Rad) secondary antibodies were diluted 1:5000. Visualization of the peroxidase was performed with Enhanced Chemiluminescence Plus Western blotting reagents (Amersham Biosciences, Piscataway, NJ) and exposed to X-ray film (Kodak, New Haven, CT) or captured using the ChemiGenius-2 bio-imaging system and Gene Snap software (Perkin Elmer, Woodbridge, ON)

2.3.6. Quantification of chondroitin sulfate concentration with the DMMB assay

1,9-dimethylmethylene blue chloride (DMMB) was used to measure concentration of sulfated glycosaminoglycans, in this case chondroitin sulfate, at a wavelength of 525 nm according to the established protocol (Farndale et al, 1986). Shark cartilage chondroitin sulfate A was used to prepare standard concentrations of chondroitin sulfate for generation of a standard absorbance curve (Seikagaku, Tokyo, Japan). Log₁₀ standard curve of the chondroitin sulfate concentrations was plotted with Graph Pad Prism 5 (La Jolla, CA).

2.3.7. Gel filtration chromatography

Purified versican was analyzed by gel filtration chromatography over a Superose-6 column (Amersham Biosciences) in TBS (20 mM Tris, pH 7.5, 0.9 % NaCl). Column fractions were filter concentrated by a factor of 10 using Amicon centrifugal filter units (Millipore). Concentrated column fractions were treated or not treated with 0.5 U/ml chondroitinase ABC (Sigma) at 37°C for 40 minutes before electrophoresis.

2.3.8. Characterization of proteolytic fragments

Proteolytic fragments were separated by electrophoresis and electroblotted to immobilon-PSQ membrane (Millipore) with CAPS pH 11. Membranes were stained with coomassie R-250 to identify fragments to be excised for sequencing. N-terminal amino acid sequencing was performed directly off PVDF membrane by Edman degradation at the Nucleic Acid Protein Service Unit at the University of British Columbia.

2.3.9. Enzyme incubation

Soluble human MT1-MMP lacking the transmembrane and cytoplasmic tail was expressed and purified as described (Tam et al, 2004). His-tagged recombinant versican C-terminal 'G3' domain construct was expressed in *Escherichia coli*, purified, refolded and verified by fluorescence anisotropy spectroscopy, N-terminal sequencing and mass spectrometry (Pourmalek & Roberts, 2009). Purified versican or versican G3 domain constructs were incubated alone in enzyme buffer or incubated with recombinant MT1-MMP for 0 or 24 hours at 37°C.

2.3.10. Proteomic identification

After enzyme assay or control incubations, proteins were reduced with dithiothreitol and alkylated with iodoacetamide. Differential reductive dimethyl labeling was performed with formaldehyde (37% w/v) or formaldehyde-d₂ (20% w/v)(Sigma) with sodium cyanoborohydride (ALD Coupling Solution, Sterogene Bioseparations, Carlsbad, CA) as catalyst (Hsu et al, 2003). After labeling, proteins were digested with mass spectrometry grade Trypsin Gold (Promega, Madison, WI). Peptides were purified over Sep-Pak Light C18 cartridges (Waters, Milford, MA) before lyophilizing and reconstituting.

Peptides were separated with nanoscale reverse phase high performance liquid chromatography and sprayed into LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Alternately, peptides were sprayed into a QSTAR-XL hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems Inc., Foster City, CA). Peak lists were searched against the SwissProt human protein database (releases 50.0 through 54.0) using Mascot version 2.2 (Matrix Science, London, UK,

www.matrixscience.com). Search parameters included semi-tryptic cleavage, fixed carbamidomethyl cysteine, variable oxidation of methionine, and variable light or heavy (+4) dimethylation of N-terminus and lysine. Peptide and fragment mass tolerances were 0.2 Da, confidence level was greater than 99.5% and ion score cut-off was 10. The scoring scheme used was ESI-TRAP for the LTQ-Orbitrap or ESI-QUAD-TOF for the QSTAR-XL. Peptide chromatograms were manually verified to contain a minimum of three consecutive y-ions.

2.4. Results

2.4.1. Versican purification from human fetal lung fibroblast cells

Versican is synthesized by migrating and proliferating fibroblast cells and is involved in development and wound healing in the lungs. Therefore human fetal lung fibroblast (HFL-1) cells were chosen as a candidate cell line from which to harvest versican. I found these cells to stain for versican in their extracellular matrix with the most abundant staining pericellularly (Fig. 2.1).

I also found that HFL-1 cells secreted adequate quantities of versican to allow purification. Following standard proteoglycan purification techniques, I purified versican over ion exchange resin. In initial work I found that I was able to purify versican but that the preparation was labile in the presence of urea (Fig. 2.2). Versican is present as an aggregate and monomer in the denaturing polyacrylamide gels (Fig. 2.2. large arrows) and numerous proteolytic fragments of versican are detectable (Fig. 2.2. small arrows). The presence of lower molecular weight versican fragments detected by Western blotting could be attributed to degradation products in the medium that were co-purified. This suggested that a protease could be co-purifying with versican.



Figure 2.1. Versican at the cell surface of human fetal lung fibroblasts.

Versican (red) stains strongly in the pericellular matrix associated with proliferating myofibroblasts. *Scale bar* = 25 μ m. Results shown are representative of at least three experiments, each analyzed in triplicate.



Figure 2.2. Purification of versican from human fetal lung fibroblast cells at pH 7.5. Initial purification attempts at pH 7.5 and with no additional salt added to starting material or equilibration buffer resulted in a preparation containing versican or other proteoglycan fragments (Alcian Blue - small arrows) and degradation products of versican (2B1 and LC2 antibodies - small arrows). Position of versican aggregate and monomer are marked with large black arrows. Results shown are representative of at least three experiments, each analyzed in triplicate.

2.4.2. Optimization of versican purification

In order to reduce the possibility of a protease co-purifying with versican and cleaving versican in the preparation, I experimented with conditions to optimize versican stability and selection. Proteinase inhibitors were avoided in favor of obtaining a versican preparation suitable for subsequent enzymatic assays. A pH of 6.0 was chosen to reduce the possibility of a co-purifying enzyme having substantial catalytic activity and to reduce positive charges in weakly acidic proteins that might bind the column resin at neutral pH. Salt was added to the starting material and the wash buffer to select for higher affinity binding to the ion exchange resin and reduce co-purification of a putative versican binding and degrading enzyme.

In order to obtain high concentrations of versican, a dual ion exchange purification method protocol was used. Initially a high volume of culture media was batch bound to a small volume of ion exchange resin. Reactive elution fractions were pooled from multiple runs and then re-purified over a smaller volume ion exchange column. Concentration of sulfated glycosaminoglycans in versican reactive fractions was measured using the DMMB assay (Farndale et al, 1986). In initial purification attempts at pH 7.5, I was able to obtain a maximum concentration of 0.08 mg/ml chondroitin sulfate. With optimized conditions and dual ion exchange runs, a chondroitin sulfate concentration of 0.75 mg/ml was obtained in versican eluting fractions (Fig. 2.3 asterisk). The Roberts lab and others have observed that preparations from proliferating fibroblast cultures such as these contain predominantly versican V0 and V1 (Roberts, C.R., unpublished observations). Since these large isoforms contain approximately twice as much glycosaminoglycan mass as core protein mass, the concentration of versican was estimated to be 1.1 mg/ml or about 1.1 μM versican.



Figure 2.3. Chondroitin sulfate concentration measured by the DMMB assay. Purified versican was analyzed by the DMMB (1,9-dimethylmethylene blue) assay (Farndale et al, 1986). Chondroitin sulfate A was used to make a standard absorbance curve at 525 nm and absorbance of the versican preparation was plotted on this curve to determine the concentration of chondroitin sulfate in the versican preparation. Results shown are representative of three experiments, each analyzed in duplicate.

Under these optimized conditions, versican was purified with only very minor degradation products present.

2.4.3. Gel filtration analysis of versican

I used gel filtration chromatography to further characterize our versican preparation. Versican was separated over Superose-6 high molecular weight gel filtration resin. Versican eluted in the predominant peak at the void volume of the column (Fig. 2.4). Alcian Blue staining and Western blotting showed a high molecular weight proteoglycan that stains for versican with both the N- (2B1) and C-terminal domain (12C5) antibodies, indicating it was intact versican. Versican was present as high molecular weight aggregate and monomer in the alcian blue stain. After chondroitinase digestion, versican was present predominantly as monomers with V0 and V1 isoforms detectable. Silver staining indicated a co-purifying protein in the third lane that had an apparent molecular weight of 120 kDa that is reduced to a doublet of 43 and 45 kDa upon chondroitinase ABC digestion. Since this profile matches that of decorin, previously known as PG40, I performed a Western blot with a PG40 antibody and identified this protein as decorin (Brennan et al, 1984; Krusius & Ruoslahti, 1986).

2.4.4. Characterization of versican degradation

Under our optimized versican purification scheme I occasionally observed two versican degradation products faintly in Western blots with the 2B1 antibody (Fig. 2.5). These fragments were visible with or without incubation, suggesting that they were copurifying with versican from the culture media. The products observed were a doublet with molecular weights of about 42 and 50 kDa. In repeated experiments I attempted






Figure 2.4. Analysis of versican separated by gel filtration.

A. Gel filtration chromatography of purified veriscan detected at 280nm. B. Starting material (S) shows a high concentration of aggregating (Ag) and monomer (Mo) versican by Alcian Blue glycosaminoglycan staining and silver staining. Western blotting with anti-versican C-terminus (2B1) and anti-versican N-terminus (12C5) antibodies after chondroitinase ABC treatment shows versican V0 and V1 isoforms. Silver staining after chondroitinase ABC treatment of samples shows a potential chondroitin sulfate proteoglycan with a double band at 40 kDa. Western blotting with anti-PG40 antibody confirmed the identity of the chondroitin sulfate proteoglycan as decorin which is known to have a molecular weight of 120 kDa and to form a doublet of 43 and 45 kDa after chondroitinase digestion (Brennan et al, 1984; Krusius & Ruoslahti, 1986). Results shown are representative of three experiments, each analyzed in duplicate.

to obtain N-terminal sequencing information to characterize these degradation products. One sequence was obtained for the 50 kDa product with 9 residues detected corresponding to cleavage at alanine 2963 - aspartate 2964 of versican V0 or V1 (Fig. 2.5). This cleavage would release the 432 amino acids of versican compromising the entire C-terminal globular domain.

2.4.5. Mass spectrometric characterization of versican

As chemical N-terminal sequencing was only occasionally successful in producing a definitive sequence, other methods to characterize versican and versican fragments were sought. Tandem mass spectrometry offers much promise as a technique to precisely identify peptide sequences. Versican was analyzed by mass spectrometry before and after enzyme digestion experiments in hopes of characterizing cleavage sites. By dimethyl labeling N-termini with isobaric formaldehyde prior to tryptic digestion (Hsu et al, 2003), it is possible to discriminate between tryptic peptides, proteolytic peptides in the control and proteolytic peptides produced by the experimental conditions. In attempts to reduce sample complexity, removal of tryptic, un-labeled peptides prior to analysis was employed (Keller et al, 2007; Kleifeld et al, 2009). Unfortunately, in repeat experiments only ambiguous sequence assignments were obtained. However it was possible to obtain high confidence sequence data for three tryptic versican peptides (Fig. 2.6). All three of these are proteotypic peptides that have been detected multiple times in the global proteome machine database (www.thegpm.org). As the starting material in these experiments was a purified preparation of versican, the low number of peptides detected underscores the challenges in characterization of this molecule.



Figure 2.5. N-terminal sequencing of versican degradation product.

Purified versican at 0 or 24 hours incubation shows a prominent doublet of products at 42 and 50 kDa. Arrow marks the 50 kDa band which was sequenced. N-terminal sequencing by Edman degradation reveals 9 residues corresponding to a previously uncharacterized C-terminal cleavage between Ala2963 and Asp2964. Result shown was obtained once.

2.4.6. Versican glycosylation and characterization

As versican is substituted with large sulfated glycosaminoglycans and N- and Olinked glycosylations, it is likely that these abundant substitutions interfere with and limit peptide purification. It is also possible that substitutions or fragments that remain after purification would interfere with the elution, ion sorting and detection in the mass spectrometer. To date there are four published versican cleavage sites produced by ADAMTS metalloproteinases (Fig 2.7A) (Jonsson-Rylander et al, 2005; Sandy et al, 2001; Westling et al, 2004). These four cleavage sites, plus the newly identified proteolytic site, all occur in the two glycosaminoglycan attachment domains (Fig. 2.7). It is likely that there are more versican-degrading enzymes and cleavage sites yet to be characterized, but the extent of versican's substitutions continues to hinder efforts.

The global proteome machine database (www.thegpm.org) was queried for versican to analyze detection of different regions of the molecule. All detected versican peptides from approximately 400 experiments were compiled and compared to versican's core protein domains and potential glycosylation sites (Fig. 2.7B)(adapted from (Dours-Zimmermann & Zimmermann, 1994; Zimmermann & Ruoslahti, 1989)). The number of observations of each peptide were complied from all versican detecting experiments in the database (Fig 2.7C). The most frequently detected peptides were all found in the N- and C- terminal globular domains. Throughout the molecule the location of detected peptides largely corresponded with regions lacking potential substitution sites, underscoring the need to remove or otherwise accommodate these glycosylations prior to analysis in order to obtain better characterization.

| Δ | |
|------------|--|
| ^ . | |

| Protein <u>(score)</u> | Mass delta | lon score | Peptide | |
|---------------------------|---------------|--------------|---------------------------------|--|
| Versican | 0.0801 | 30 | <u>R</u> .YEINSLI <u>R</u> .Y | |
| (80) | 0.0756 | 36 | <u>K</u> .LLASDAGLY <u>R</u> .C | |



Figure 2.6. Tryptic versican peptides detected in MS/MS.

A. Analysis of versican tryptic peptides detected three significant peptides. Lys and Arg residues preceeding cleavage sites are underlined. Chromatograms and error plots for ion fragments of tryptic peptide YEINSLIR (B), LLASDAGLYR (C) and LATVGELQAAWR (D). Mass error plots indicate difference between observed and expected mass for ions shown in adjacent chromatograms. Database searching in MASCOT identified peptides and ion fragments as listed. Results shown are representative of four separate experiments.

2.5. Discussion

Based on the ubiquitous expression of versican in remodeling tissues and diseases, there is much interest in characterizing molecular events involved in its metabolism. In addition to being a large, glycosylated, negatively charged, hydrophilic molecule, versican exhibits a pronounced tendency to aggregate. Versican self-aggregation is through core protein interactions that may be calcium dependent interations of its Ctype lectin domains (Morgelin et al, 1989; Ney et al, 2006). Yet despite these biochemical challenges, versican has been purified successfully by several groups.

Standard versican purification schemes exploit versicans negatively charged glycosaminoglycans and large molecular weight to purify by anion exchange and gel filtration chromatography (Sakko et al, 2003; Schmalfeldt et al, 1998). Some more extensive strategies have been reported to reduce trace contaminants (Mazzucato et al, 2002) or separate versican isoforms (Dutt et al, 2006). Here I describe a relatively straightforward but effective strategy that allows purification of high molecular weight versican aggregates with only minor contaminants. The preparation was shown to contain high molecular weight proteoglycans that label with a versican antibody. Analysis by mass spectrometry indicated that only versican is abundant in the preparation with three peptides being identified.

At neutral pH we observed a number of versican proteolytic fragments that co-purify over ion exchange resin. Others have noted that uncharacterized versican fragments are present in the vasculature (Formato et al, 2004; Kenagy et al, 2006; Sandy et al, 2001; Theocharis et al, 2003), eyes (Ohno-Jinno et al, 2008), pancreatic and laryngeal cancer (Skandalis et al, 2006; Stylianou et al, 2008; Vynios et al, 2008). We sequenced a C-terminal fragment of versican that appears to be proteolytically derived





and bound to intact purified versican. There seems to be a spectrum of proteolytic events and fragments that have yet to be characterized and which are likely physiologically significant.

There are several known versican cleavage sites (Jonsson-Rylander et al, 2005; Sandy et al, 2001; Westling et al, 2004). As with the related hyalectan aggrecan, most of the cleavage sites have been identified using a hypothesis driven approach with synthetic peptide substrates. Though cleavage sites have been validated *in vivo*, there remains much evidence of further cleavage sites and relevant enzymes. Gaining a more thorough understanding of versican cleavage events requires new and unbiased approaches.

Future detection by mass spectrometry will need to improve methods to deal with glycosaminoglycans and glycosylations to obtain higher confidence assignments. While mass spectrometry combined with database searching is an extremely powerful tool for identification of unknown peptides, standard procedures rely on the hydrophobicity of most peptides and the reliable masses of unmodified amino acid residues. Wherein versican contains an abundance of both acidic residues and substituted amino acids, improving analysis by mass spectrometry will require methods to deal with these atypical requirements.

2.6. References

Asher R, Perides G, Vanderhaeghen JJ, Bignami A (1991) Extracellular matrix of central nervous system white matter: demonstration of an hyaluronate-protein complex. *J Neurosci Res* **28**(3): 410-421

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1996) Proteoglycan deposition in pulmonary fibrosis. *Am J Respir Crit Care Med* **154**(6 Pt 1): 1819-1828

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1997) Proteoglycans in granulomatous lung diseases. *Eur Respir J* **10**(12): 2731-2737

Bissell MJ, Radisky D (2001) Putting tumours in context. Nat Rev Cancer 1(1): 46-54

Brennan MJ, Oldberg A, Pierschbacher MD, Ruoslahti E (1984) Chondroitin/dermatan sulfate proteoglycan in human fetal membranes. Demonstration of an antigenically similar proteoglycan in fibroblasts. *J Biol Chem* **259**(22): 13742-13750

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix Metalloproteinase Inhibitors and Cancer--Trials and Tribulations. *Science* **295**(5564): 2387-2392

Dours-Zimmermann MT, Zimmermann DR (1994) A novel glycosaminoglycan attachment domain identified in two alternative splice variants of human versican. *J Biol Chem* **269**(52): 32992-32998

Dutt S, Kleber M, Matasci M, Sommer L, Zimmermann DR (2006) Versican V0 and V1 guide migratory neural crest cells. *J Biol Chem* **281**(17): 12123-12131

Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**(26): 1650-1659

Evanko SP, Raines EW, Ross R, Gold LI, Wight TN (1998) Proteoglycan distribution in lesions of atherosclerosis depends on lesion severity, structural characteristics, and the proximity of plateletderived growth factor and transforming growth factor-beta. *Am J Pathol* **152**(2): 533-546

Farndale RW, Buttle DJ, Barrett AJ (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* **883**(2): 173-177

Fingleton B (2008) MMPs as therapeutic targets--Still a viable option? *Seminars in Cell & Developmental Biology* **19**(1): 61-68

Formato M, Farina M, Spirito R, Maggioni M, Guarino A, Cherchi GM, Biglioli P, Edelstein C, Scanu AM (2004) Evidence for a proinflammatory and proteolytic environment in plaques from endarterectomy segments of human carotid arteries. *Arterioscler Thromb Vasc Biol* **24**(1): 129-135

Groffen AJA, Buskens CAF, Van Kuppevelt TH, Veerkamp JH, Monnens LAH, Van den Heuvel LPWJ (1998) Primary structure and high expression of human agrin in basement membranes of adult lung and kidney. *Eur J Biochem* **254**(1): 123-128

Hsu JL, Huang SY, Chow NH, Chen SH (2003) Stable-Isotope Dimethyl Labeling for Quantitative Proteomics. *Anal Chem* **75**(24): 6843-6852

Isogai Z, Aspberg A, Keene DR, Ono RN, Reinhardt DP, Sakai LY (2002) Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. *J Biol Chem* **277**(6): 4565-4572

Isogai Z, Shinomura T, Yamakawa N, Takeuchi J, Tsuji T, Heinegard D, Kimata K (1996) 2B1 antigen characteristically expressed on extracellular matrices of human malignant tumors is a large chondroitin sulfate proteoglycan, PG-M/versican. *Cancer Res* **56**(17): 3902-3908

Jonsson-Rylander AC, Nilsson T, Fritsche-Danielson R, Hammarstrom A, Behrendt M, Andersson JO, Lindgren K, Andersson AK, Wallbrandt P, Rosengren B, Brodin P, Thelin A, Westin A, Hurt-Camejo E, Lee-Sogaard CH (2005) Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican. *Arterioscler Thromb Vasc Biol* **25**(1): 180-185

Keller UAD, Doucet A, Overall CM (2007) Protease research in the era of systems biology. *Biol Chem* **388**(11): 1159-1162

Kenagy RD, Plaas AH, Wight TN (2006) Versican Degradation and Vascular Disease. *Trends in Cardiovascular Medicine* **16**(6): 209-215

Kleifeld O, Doucet A, Keller UAD, Schilling O, Foster L, Kizhakkedathu J, Overall CM (2009) 'terminal amino isotope labeling of substrates' (TAILS). *in preparation*

Koch M, Bernasconi C, Chiquet M (1992) A major oligomeric fibroblast proteoglycan identified as a novel large form of type-XII collagen. *Eur J Biochem* **207**(3): 847-856

Kodama J, Hasengaowa, Kusumoto T, Seki N, Matsuo T, Nakamura K, Hongo A, Hiramatsu Y (2007a) Versican expression in human cervical cancer. *Eur J Cancer* **43**(9): 1460-1466

Kodama J, Hasengaowa, Kusumoto T, Seki N, Matsuo T, Ojima Y, Nakamura K, Hongo A, Hiramatsu Y (2007b) Prognostic significance of stromal versican expression in human endometrial cancer. *Ann Oncol* **18**(2): 269-274

Kridel SJ, Sawai H, Ratnikov BI, Chen EI, Li W, Godzik A, Strongin AY, Smith JW (2002) A unique substrate binding mode discriminates membrane type-1 matrix metalloproteinase from other matrix metalloproteinases. *J Biol Chem* **277**(26): 23788-23793

Krueger RCJ, Schwartz NB (1987) An improved method of sequential alcian blue and ammoniacal silver staining of chondroitin sulfate proteoglycan in polyacrylamide gels. *Anal Biochem* **167**(2): 295-300

Krusius T, Ruoslahti E (1986) Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. *Proceedings of the National Academy of Sciences* **83**(20): 7683-7687

Li D, Clark CC, Myers JC (2000) Basement Membrane Zone Type XV Collagen Is a Disulfide-bonded Chondroitin Sulfate Proteoglycan in Human Tissues and Cultured Cells. *J Biol Chem* **275**(29): 22339-22347

Lin H, Wilson JE, Roberts CR, Horley KJ, Winters GL, Costanzo MR, McManus BM (1996) Biglycan, decorin, and versican protein expression patterns in coronary arteriopathy of human cardiac allograft: distinctness as compared to native atherosclerosis. *J Heart Lung Transplant* **15**(12): 1233-1247

Mazzucato M, Cozzi MR, Pradella P, Perissinotto D, Malmstrom A, Morgelin M, Spessotto P, Colombatti A, De Marco L, Perris R (2002) Vascular PG-M/versican variants promote platelet adhesion at low shear rates and cooperate with collagens to induce aggregation. *Faseb J* **16**(14): 1903-1916

Mjaatvedt CH, Yamamura H, Capehart AA, Turner D, Markwald RR (1998) The Cspg2 gene, disrupted in the hdf mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol* **202**(1): 56-66

Morgelin M, Paulsson M, Malmstrom A, Heinegard D (1989) Shared and distinct structural features of interstitial proteoglycans from different bovine tissues revealed by electron microscopy. *J Biol Chem* **264**(20): 12080-12090

Ney A, Booms P, Epple G, Morgelin M, Guo G, Kettelgerdes G, Gebner R, Robinson PN (2006) Calcium-dependent self-association of the C-type lectin domain of versican. *The International Journal of Biochemistry & Cell Biology* **38**: 23-29 Ohno-Jinno A, Isogai Z, Yoneda M, Kasai K, Miyaishi O, Inoue Y, Kataoka T, Zhao J-S, Li H, Takeyama M, Keene DR, Sakai LY, Kimata K, Iwaki M, Zako M (2008) Versican and Fibrillin-1 Form a Major Hyaluronan-Binding Complex in the Ciliary Body. *Invest Ophthalmol Vis Sci*: iovs.07-1488

Pourmalek S, Roberts CR (2008) Macrophage mediated proteolysis of versican in human pulmonary fibrosis. *in preparation*

Pourmalek S, Roberts CR (2009) Macrophage mediated proteolysis of versican in human pulmonary fibrosis. *in preparation*

Pukkila M, Kosunen A, Ropponen K, Virtaniemi J, Kellokoski J, Kumpulainen E, Pirinen R, Nuutinen J, Johansson R, Kosma V-M (2007) High stromal versican expression predicts unfavourable outcome in oral squamous cell carcinoma. *J Clin Pathol* **60**(3): 267-272

Ricciardelli C, Brooks JH, Suwiwat S, Sakko AJ, Mayne K, Raymond WA, Seshadri R, LeBaron RG, Horsfall DJ (2002) Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer. *Clin Cancer Res* **8**(4): 1054-1060

Ricciardelli C, Mayne K, Sykes PJ, Raymond WA, McCaul K, Marshall VR, Horsfall DJ (1998) Elevated levels of versican but not decorin predict disease progression in early-stage prostate cancer. *Clin Cancer Res* **4**(4): 963-971

Rozanov DV, Strongin AY (2003) Membrane type-1 matrix metalloproteinase functions as a proprotein self-convertase. Expression of the latent zymogen in pichia pastoris, autolytic activation, and the peptide sequence of the cleavage forms. *J Biol Chem* **278**(10): 8257-8260

Sakko AJ, Ricciardelli C, Mayne K, Suwiwat S, LeBaron RG, Marshall VR, Tilley WD, Horsfall DJ (2003) Modulation of prostate cancer cell attachment to matrix by versican. *Cancer Res* **63**(16): 4786-4791

Sandy JD, Westling J, Kenagy RD, Iruela-Arispe ML, Verscharen C, Rodriguez-Mazaneque JC, Zimmermann DR, Lemire JM, Fischer JW, Wight TN, Clowes AW (2001) Versican V1 proteolysis in human aorta in vivo occurs at the Glu441-Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4. *J Biol Chem* **276**(16): 13372-13378

Schmalfeldt M, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR (1998) Versican V2 is a major extracellular matrix component of the mature bovine brain. *J Biol Chem* **273**(25): 15758-15764

Skandalis SS, Kletsas D, Kyriakopoulou D, Stavropoulos M, Theocharis DA (2006) The greatly increased amounts of accumulated versican and decorin with specific post-translational modifications may be closely associated with the malignant phenotype of pancreatic cancer. *Biochim Biophys Acta* **1760**(8): 1217-1225

Stylianou M, Skandalis SS, Papadas TA, Mastronikolis NS, Theocharis AD, Papageorgakopoulou N, Vynios DH (2008) Stage-related decorin and versican expression in human laryngeal cancer. *Anticancer Res* **28**(1A): 245-251

Suwiwat S, Ricciardelli C, Tammi R, Tammi M, Auvinen P, Kosma VM, LeBaron RG, Raymond WA, Tilley WD, Horsfall DJ (2004) Expression of extracellular matrix components versican, chondroitin sulfate, tenascin, and hyaluronan, and their association with disease outcome in node-negative breast cancer. *Clin Cancer Res* **10**(7): 2491-2498

Tam EM, Morrison CJ, Wu YI, Stack MS, Overall CM (2004) Membrane protease proteomics: Isotopecoded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Nat Acad Sci* **101**(18): 6917-6922

Theocharis AD, Tsolakis I, Hjerpe A, Karamanos NK (2003) Versican undergoes specific alterations in the fine molecular structure and organization in human aneurysmal abdominal aortas. *Biomed Chromatogr* **17**(6): 411-416

Tomte LT, Geiser A, Hansen A, Tesche F, Herken R, Miosge N (2004) Collagen types XII and XIV are present in basement membrane zones during human embryonic development. *J Mol Histol* **35**(8): 803-810

Touab M, Arumi-Uria M, Barranco C, Bassols A (2003) Expression of the proteoglycans versican and mel-CSPG in dysplastic nevi. *Am J Clin Pathol* **119**(4): 587-593

Touab M, Villena J, Barranco C, Arumi-Uria M, Bassols A (2002) Versican is differentially expressed in human melanoma and may play a role in tumor development. *Am J Pathol* **160**(2): 549-557

Tzortzaki EG, Koutsopoulos AV, Dambaki KI, Lambiri I, Plataki M, Gordon MK, Gerecke DR, Siafakas NM (2006) Active Remodeling in Idiopathic Interstitial Pneumonias: Evaluation of Collagen Types XII and XIV. *J Histochem Cytochem* **54**(6): 693-700

Vynios DH, Theocharis DA, Papageorgakopoulou N, Papadas TA, Mastronikolis NS, Goumas PD, Stylianou M, Skandalis SS (2008) Biochemical Changes of Extracellular Proteoglycans in Squamous Cell Laryngeal Carcinoma. *Connect Tissue Res* **49**(3): 239 - 243

Walchli C, Koch M, Chiquet M, Odermatt BF, Trueb B (1994) Tissue-specific expression of the fibrilassociated collagens XII and XIV. *J Cell Sci* **107**(2): 669-681

Watt SL, Lunstrum GP, McDonough AM, Keene DR, Burgeson RE, Morris NP (1992) Characterization of collagen types XII and XIV from fetal bovine cartilage. *J Biol Chem* **267**(28): 20093-20099

Westling J, Gottschall PE, Thompson VP, Cockburn A, Perides G, Zimmermann DR, Sandy JD (2004) ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu405-Gln406 to generate glial hyaluronate binding protein. *Biochem J* **377**(Pt 3): 787-795

Wight TN, Lara S, Riessen R, Le Baron R, Isner J (1997) Selective deposits of versican in the extracellular matrix of restenotic lesions from human peripheral arteries. *Am J Pathol* **151**(4): 963-973

Zimmermann DR, Dours-Zimmermann MT, Schubert M, Bruckner-Tuderman L (1994) Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. *J Cell Biol* **124**(5): 817-825

Zimmermann DR, Ruoslahti E (1989) Multiple domains of the large fibroblast proteoglycan, versican. *EMBO J* **8**(10): 2975-2981

CHAPTER 3 – VERSICAN-ADAMTS-2 INTERACTIONS IN HUMAN PULMONARY FIBROSIS^{*}

3.1. Summary

The chondroitin sulfate proteoglycan versican is transiently expressed in developmental and remodeling processes where its expression coincides with cell migration, proliferation and the formation of new tissue. In idiopathic pulmonary fibrosis (IPF), versican is associated with proliferating fibroblasts that synthesize collagen-rich fibrotic matrix. Collagen fibrillogenesis takes place in a versican-rich provisional matrix which is apparently resorbed concomitant with collagen deposition, by a process that is yet to be identified. As the two processes are concurrent, it was hypothesized that the procollagen N-propeptidase ADAMTS-2 (a disintegrin and metalloproteinase with thrombospondin motifs-2) might contribute to versican degradation and thus, resorption of the provisional matrix. In this report I show that: (i) ADAMTS-2 is found in versicanrich areas of normal human lungs; (ii) ADAMTS-2 staining is associated with proliferating fibroblasts in the versican-rich proliferative lesions of IPF; (iii) ADAMTS-2 is released from normal human lung tissue by incubation with chondroitinase ABC; (iv) ADAMTS-2 and versican co-purify in human fetal lung fibroblast culture media; (v) ADAMTS-2 does not auto-degrade in the presence of versican and (vi) versican is a substrate for purified ADAMTS-2 in vitro. This data demonstrates that versican binding may regulate ADAMTS-2 biological activity and thus collagen assembly.

^{*} A version of this chapter will be submitted for publication, Sean B. Maurice and Clive R. Roberts, Versican-ADAMTS-2 interactions in human pulmonary fibrosis: the proteoglycan versican binds the procollagen N-propeptidase ADAMTS-2 and regulates its activity.

3.2. Introduction

Pulmonary fibrosis is a dysregulated repair process that is often associated with but not necessarily perpetuated by inflammation (Thannickal et al, 2004). Idiopathic pulmonary fibrosis (IPF) is distinct from the related idiopathic interstitial pneumonias, exhibiting the histologic pattern usual interstitial pneumonia (UIP) with characteristic fibroblastic foci and is driven largely if not entirely by non-inflammatory mechanisms (Selman et al, 2001; Travis et al, 2002). Median survival of patients with IPF is 3 to 5 years and there is no currently effective therapy (Khalil & O'Connor, 2004).

Previous work in our laboratory has demonstrated a consistent expression of versican within the active fibroproliferative lesions of organizing diffuse alveolar damage associated with adult respiratory distress syndrome, idiopathic bronchiolitis obliterans organizing pneumonia (BOOP), UIP (Bensadoun et al, 1996); and the lesions of sarcoidosis, extrinsic allergic alveolitis and tuberculosis (Bensadoun et al, 1997). The spatial and temporal association of versican with fibroblasts in fibroproliferative lesions suggests that versican plays a specific role in the cell biology of pulmonary remodeling that leads to fibrosis (Roberts, 2003). Since *de novo* procollagen synthesis occurs in fibroblasts surrounded by versican (Bensadoun et al, 1996), collagen fibril formation occurs in a versican-rich matrix. However, these studies showed that versican is absent from areas of mature collagenous fibrosis. Therefore, it was hypothesized that enzymes involved in collagen assembly might degrade versican in the evolution of fibroproliferative lesions.

Versican is a large aggregating 'hyalectan' proteoglycan with hyaluronan-binding and lectin-like domains (lozzo, 1998). Versican was first identified because of its association with skeletal development (Kimata et al, 1986). Versican has a wide tissue distribution (Bode-Lesniewska et al, 1996; Dours-Zimmermann & Zimmermann, 1994)

and exhibits diverse functions associated with migrating and proliferating cells in development and disease (Wight, 2002). Through the reversible binding of water, versican occupies a very large hydrodynamic space influencing chemokine gradients and cell adhesive properties (Kinsella et al, 2004). In the developing lung, versican is the predominant chondroitin sulfate bearing proteoglycan and its expression is associated with alveolar tissue volume changes (Faggian et al, 2007). Alternative splicing of versican m-RNA results in four different gene products with identical N- and C-terminal globular domains but different sized central glycosaminoglycan-attachment domains due to the inclusion of one or both or neither of the GAG α and GAG β domains (Ito et al, 1995; Naso et al, 1994; Zako et al, 1995).

The versican deficient *hdf* mouse dies due to severe cardiac defects by embryonic day 10.5 (Mjaatvedt et al, 1998) and the hyaluronan deficient Has2 -/- (hyaluronan synthase-2) mouse dies at embryonic day 9.5-10 with similar severe cardiac abnormalities (Camenisch et al, 2000). In both phenotypes, cardiac defects appear to result from the absence of the hyaluronan and versican-rich matrix required for cell migration and proliferation, as has been shown for vascular smooth muscle cells *in vitro* (Evanko et al, 1999).

The ADAMTS proteinases are members of the metzincin superfamily of metalloproteinases (Stocker et al, 1995). They are large multi-domain enzymes which exhibit multiple proteolytically processed variants produced through autocatalytic activation as well as processing by other metalloproteinases (Colige et al, 2005; Flannery et al, 2002; Gao et al, 2004; Tortorella et al, 2005).

ADAMTS-1 and -4 have been shown to bind sulfated glycosaminoglycans through thrombospondin-like, cysteine-rich and spacer domain-dependent interactions (Flannery et al, 2002; Kuno & Matsushima, 1998). Substrate recognition and cleavage

of aggrecan by ADAMTS-4 does not occur when the glycosaminoglycan side chains are removed (Tortorella et al, 2000). Similarly, the absence of thrombospondin and ancillary domains in ADAMTS-9 ablates its proteoglycan degrading activity (Somerville et al, 2003).

A preliminary immunohistochemical screen of human IPF tissue sections revealed ADAMTS-2 as a proteinase of interest. In this study, I investigated the localization of versican and ADAMTS-2 in normal and remodeling human lung tissue. The results strongly suggest that ADAMTS-2 is bound to versican in lung matrix and that binding to versican localizes and may regulate the biological activity of ADAMTS-2. ADAMTS-2 - mediated degradation of versican may be an important element in resorption of the provisional matrix in the evolution of fibroproliferative lesions in lung wound healing and fibrosis.

3.3. Experimental procedures

3.3.1. Patient samples

Lung tissues used in this study were obtained as part of a lung fibrosis tissue registry as previously described (Bensadoun et al, 1996; Bensadoun et al, 1997). Lung biopsy tissues were obtained at diagnostic biopsy of patients with a clinical diagnosis of idiopathic pulmonary fibrosis, and were entered into the study following histologic diagnoses of bronchiolitis obliterans organizing pneumonia or usual interstitial pneumonia. Age-matched control tissues were obtained from normal-appearing lung tissue, obtained from lung lobes that were resected from individuals with small localized tumors, as previously described (Bensadoun et al, 1996). Tissues from 6 UIP, 6 BOOP and 6 control patients were studied. From some of the control patients it was possible to obtain small samples of unfixed lung tissue for biochemical studies; these were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

3.3.2. Histology

Lung tissues were fixed overnight in 10% neutral buffered formalin, dehydrated, embedded in paraffin and serially sectioned at a thickness of 5µm. Sections were stained with hematoxylin and eosin to visualize overall architecture, alcian blue to localize glycosaminoglycans and picrosirius red to localize collagen as previously described (Bensadoun et al, 1996).

3.3.3. Immunohistochemistry

Sections were deparaffinized and hydrated in Tris-buffered saline (TBS) for 5 minutes before being immersed in freshly prepared 0.6% hydrogen peroxide in methanol for 40 minutes to block endogenous peroxidase activity. Sections were blocked with 10% normal goat serum in 2% BSA for 4 hours. The following primary antibodies were used: mouse monoclonal anti-versican C-terminal domain, 2B1 (Isogai et al, 2002; Isogai et al, 1996) (Seikagaku, Tokyo, Japan), dilution 1:400; rabbit polyclonal anti-versican (LeBaron et al, 1992) used as previously described (Bensadoun et al, 1996; Bensadoun et al, 1997) dilution 1:500; rabbit polyclonal anti-ADAMTS-2 pro-domain (Chemicon International, Temecula, CA) dilution 1:100; and rabbit polyclonal anti-ADAMTS-2 C-terminus (Chemicon International) dilution 1:400. Antibodies were diluted in 2% bovine serum albumin (BSA) in TBS and washed 4 times in TBS. Antibody labeling was visualized with the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingham, CA) and DAB (3,3'-diaminobenzidine) as substrate (Vector Laboratories) according to the manufacturer's instructions. Sections were counterstained with Gill's Haematoxylin. Negative controls were treated identically with the inclusion of non-immune IgG at the same concentration or with omission of primary antibody. For each antibody and detection system, conditions were established that allowed use of purified antibody or serum at concentrations that generated no staining with non-immune IgG or serum at equivalent concentrations. All sections that were to be compared were processed and stained concurrently. After staining, sections were mounted in Histochoice mounting medium (Amresco, Solon, OH).

3.3.4. Release of ADAMTS-2 from normal lung tissues

Normal human lung tissue (50 mg tissue per ml TBS) was finely diced in TBS (20 mM Tris, pH 7.5, 0.9% NaCl) in the presence of complete mini, EDTA-free proteinase inhibitor cocktail tablets (one tablet per 25 ml)(Roche, Indianapolis, IN) and incubated with or without 0.1 U/ml chondroitinase ABC lyase (MP biomedicals) for one hour at 37°C. Samples were centrifuged to remove tissue, and supernatant was isolated, diluted with different ratios of sample to reducing sample buffer (containing 65 mM dithiothreitol) and analyzed by electrophoresis and Western blotting for versican and ADAMTS-2. Alternatively, normal human lung tissue was finely diced in TBS containing proteinase inhibitor cocktail tablets as above and heated to 95°C for 5 minutes before electrophoretic separation and analysis.

3.3.5. Cell culture

Human fetal lung fibroblasts (HFL-1) were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 20 mM HEPES and 10% (v/v) Cosmic Calf Serum (Hyclone, Logan, UT). Cells at low passage number were grown to approximately 70% of confluence before being rinsed in serum-free media followed by incubation in serum free media for 24 hours. Cells were grown a minimum of 24 hours in serum-supplemented media before incubating in serum-free conditions again. This cycle was performed a maximum of three consecutive times before discarding cultures. Serum-free conditioned media were collected and pooled and used for isolation and purification of versican.

3.3.6. Isolation of versican

Serum-free conditioned medium from fibroblast cultures (CM) was collected and centrifuged at 1500 x g for 20 minutes to remove cellular debris. Urea was added to 7 M and NaCl was added to bring the salt concentration to 400 mM before loading onto Q-Sepharose Fast Flow ion exchange resin (Amersham Biosciences, Piscataway, NJ) at approximately 1 litre CM per 5 mls resin. The column was equilibrated in 0.1 M sodium acetate, pH 6.0, 7 M urea, 0.4 M NaCl and eluted with 0.1 M sodium acetate, pH 6.0, 7 M urea, 1.5 M NaCI. Fractions were monitored for versican content by alcian blue (Sigma, St. Louis, MO) staining of SDS-PAGE gels (Krueger & Schwartz, 1987) and by Western blotting. Versican-containing fractions were pooled and concentrated over a smaller volume Q-sepharose column using the same buffers. Concentration of purified versican was estimated using the dimethylmethylene blue (DMMB)(Serva, Heidelberg) assay to quantify sulfated glycosaminoglycans (Farndale et al, 1986), using known concentrations of chondroitin sulfate C as standards (Seikagaku). Chromatography was performed on an AKTA purifier (Amersham Biosciences) and protein elution was monitored at 215, 229 and 280 nm simultaneously. All procedures were performed at 4°C.

3.3.7. Co-purification of versican and ADAMTS-2

Serum-free CM from fibroblast cultures was collected and centrifuged at 1500 x g for 20 minutes to remove cellular debris. Urea was added to 7 M. Approximately 1 litre of culture media was loaded onto 5 mls ANX-Sepharose Fast Flow ion exchange resin (Amersham Biosciences). The column was equilibrated with 0.1 M Tris, pH 7.5, 7M urea and eluted with 0.1 M Tris, pH 7.5, 7M urea, 1.5 M NaCl. All procedures were performed at 4°C.

3.3.8. Separate elution of ADAMTS-2 and versican

Serum-free CM from fibroblast cultures was collected and centrifuged at 1500 x g for 20 minutes to remove cellular debris. Urea was added to 7 M and EDTA to 10 mM. Approximately 1 litre of culture medium was loaded onto 5 mls ANX-Sepharose and equilibrated in 0.1 M Tris, pH 7.5, 7M urea. The pH elution was performed with 0.1 M Tris, 0.1 M sodium acetate, pH 5.0, 7 M urea. This was followed by a salt elution in 0.1 M Tris, 0.1 M sodium acetate, pH 5.0, 7 M urea, 1.5 M NaCl. Column fractions were concentrated by a factor of 10 using Amicon Ultra-15 centrifugal filter devices with 30,000 molecular weight cut-off (Millipore, Billerica, MA). All procedures were performed at 4°C.

3.3.9. Electrophoretic techniques

Samples in non-reducing (except where stated) sample buffer (125 mM Tris-HCL, pH 6.8, 2.0% SDS, 2.0 M urea, 0.05% bromophenol blue) were separated on discontinuous SDS-PAGE gels with 4% (stacking) and 10 or 12% (separating) acrylamide. Stacking and separating gels were kept during staining and Western blotting to monitor high molecular weight versican aggregates within the stacking gel.

For some experiments, 3-8% NuPage Tris-Acetate gradient gels were used with appropriate sample, running and transfer buffers according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Gels were analyzed by silver staining and coomassie blue staining, and alcian blue and alcian blue-enhanced silver staining for visualization of glycosaminoglycans (Krueger & Schwartz, 1987). Relative molecular mass (measured in kDa) was estimated based on mobility of molecular weight markers: HiMark Prestained (Invitrogen), MagicMark XP (Invitrogen) and Kaleidoscope Prestained (Bio-Rad, Hercules, CA). Western blotting was performed using the XCell II blot module (Invitrogen) to PVDF membrane (Millipore). Blocking was performed with a solution of 2% (w/v) casein, 2% (w/v) bovine serum albumin, 0.5% (w/v) PVP, 20 mM Tris, 5 mM EDTA, 0.9% NaCl, 1 x PSN antibiotic mixture (Gibco, Grand Island, NY) and 0.3% (v/v) Tween 20. The following antibodies were used: anti-versican C-terminal domain 2B1, 1:1000 dilution; anti-ADAMTS-2 pro-domain, 1:500 dilution; anti-ADAMTS-2 C-terminus, 1:2000 dilution; rabbit polyclonal anti-DPEAAE neo-epitope antibody (Sandy et al, 2001) (Affinity Bioreagents, Golden, CO) 1:500 dilution; and mouse monoclonal anti-versican N-terminal antibody 12C5 (Asher et al, 1991) (obtained from the Developmental Studies Hybridoma Bank (NICHD), the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), 1:1000 dilution. Antibodies were diluted in a solution of 2% (w/v) bovine serum albumin, 20 mM Tris, pH 7.5, 0.9 % NaCl and 0.05% (v/v) Tween 20. Highly cross-adsorbed goat anti-mouse horseradish peroxidase-conjugate (Bio-Rad) and highly cross adsorbed goat anti-rabbit horseradish peroxidase-conjugate (Bio-Rad) secondary antibodies were diluted 1:5000. Visualization of the peroxidase was performed with Enhanced Chemiluminescence Plus Western blotting reagents (Amersham Biosciences) and exposed to X-ray film

(Kodak, New Haven, CT) or captured using the ChemiGenius-2 bio-imaging system and Gene Snap software (Perkin Elmer, Woodbridge, ON).

3.3.10. Purification of fetal bovine skin ADAMTS-2

Bovine ADAMTS-2 was purified from fetal bovine skin by potassium chloride extraction, ammonium sulfate precipitation, Concanavalin-A Sepharose (Con-A Sepharose) chromatography and heparin Sepharose chromatography as previously published (Colige et al, 1995). In the previous work, the authors showed that the enzyme was active towards α -I-procollagen-N-propeptides. Based on SDS-PAGE gel silver staining, I estimated the concentration of enzyme purified to be 100 ng/ml or 800 ρ M for the approximately 125 kDa enzyme purified.

3.3.11. Versican digestion and ADAMTS-2 incubations

Purified versican concentration was estimated using the DMMB assay (Farndale et al, 1986) to quantify sulfated glycosaminoglycan. Versican V0 and V1 are the primary mRNA splice variants expressed in proliferating HFL-1 cultures (Roberts, C.R. unpublished observations). The concentration of versican was estimated based on an average of 1.5 mg total proteoglycan per 1 mg sulfated glycosaminoglycan detected with a resultant concentration of 1.12 mg/ml or approximately 1.12 µM versican. Versican and ADAMTS-2 were incubated together at a 1:1 volume ratio (1:1400 - E:S ratio) or individually diluted in the respective buffers from 0 to 48 hours at 37°C. Hyaluronan, heparan sulfate and chondroitin sulfate A, B and C (Seikagaku), were suspended in TBS. ADAMTS-2 was incubated with TBS alone or at a 1:1 volume ratio

with each glycosaminoglycan at final concentrations of 5 nM to 5 μ M. Detection was by Western blotting with ADAMTS-2 C-terminus antibody.

3.4. Results

3.4.1. Versican and ADAMTS-2 co-localize in normal human lungs

In normal lungs, alcian blue and picrosirius red staining showed collagen throughout the airway, alveolar and blood vessel walls, and very little histochemically-apparent glycosaminoglycan present (Fig. 3.1A). Versican staining was associated with smooth muscle of the blood vessel walls and airways (Fig. 3.1B). ADAMTS-2 staining was similarly associated with smooth muscle of the blood vessel and airway walls. Versican was also associated with tips of the alveolar septae and as previously described, there was a strong association between versican staining and α -smooth muscle actin (Bensadoun et al, 1996).

3.4.2. Versican and ADAMTS-2 localization in BOOP

Patient samples exhibiting the pathological pattern bronchiolitis obliterans organizing pneumonia (BOOP) showed characteristic intraluminal buds composed of loose connective tissue adjacent to a thickened interstitium (Fig. 3.1A). Alcian blue staining showed that the intraluminal buds contained a matrix rich in glycosaminoglycans which also stained heavily for versican (Fig. 3.1A & B). This is consistent with our previous demonstration that the glycosaminoglycan is chondroitin sulfate, that versican is the predominant proteoglycan within intraluminal buds and that the histochemical glycosaminoglycan staining and versican staining are highly congruent (Bensadoun et al, 1996). ADAMTS-2 staining of serial sections demonstrated abundant deposition of the enzyme in the collagen-rich thickened interstitium and trace staining within the



Figure 3.1. Versican and ADAMTS-2 localization in normal lungs and in bronchiolitis obliterans organizing pneumonia (BOOP). A. Hematoxylin and eosin staining shows a thickened interstitium and characteristic intraluminal buds (asterisks) in distal airspaces in BOOP, in contrast with normal lung morphology. Alcian blue and picrosirius red staining show glycosaminoglycan rich fibroproliferative regions in blue and collagen in red. BOOP sections stain abundantly for glycosaminoglycans within intraluminal buds and weak collagen staining in these glycosaminoglycan-rich areas. *Scale bars* = 200 μ m. B. The normal lung shows versican and ADAMTS-2 staining (brown) in blood vessel walls (arrows) and in the interstitium of airways and alveoli. The intraluminal buds of BOOP stain strongly for versican (asterisks). ADAMTS-2 staining is minimally detectable within the matrix of the intraluminal buds in BOOP and is more abundant in the collagen rich thickened interstitium. Lower panels show a BOOP intraluminal bud at higher magnification. Versican staining is strong and faint ADAMTS-2 staining is associated with the myofibroblasts (small arrows). *Scale bars* = 200 μ m, *Scale bar* (*high power*) = 30 μ m. Tissues from 6 BOOP and 6 control patients were studied and representative semi-serial sections are shown. matrix of the intraluminal buds in BOOP patient sections (Fig. 3.1B). At higher magnification, ADAMTS-2 staining was weakly detectable in close association with the cells (arrows). Similar data were obtained using an antibody to the C-terminus of ADAMTS-2 (data not shown).

3.4.3. Versican and ADAMTS-2 are localized to remodeling areas in UIP

Sub-epithelial fibroblast foci in usual interstitial pneumonia (UIP) stained heavily for versican. These foci also stained for ADAMTS-2, with the most intense staining closer to the centre of the lesions. Higher magnification of a sub-epithelial fibroblast focus showed ADAMTS-2 staining in association with the fibroblasts (Fig. 3.2 arrows). It was previously documented that a similar staining pattern occurs for intracellular α -1-(I) procollagen in the versican-rich fibroproliferative intraluminal buds of BOOP (Bensadoun et al, 1996) consistent with the studies of Kuhn and McDonald (Kuhn & McDonald, 1991). A consistent observation in the patient samples was that in the fibroproliferative lesions of UIP staining for versican and ADAMTS-2 was strong, and that the versican-rich fibroproliferative lesions of BOOP showed weak staining for ADAMTS-2. The intensity of ADAMTS-2 staining in UIP was variable between different lesions and different locations within a given lesion.

The intima and media of arteries in the lung stained intensely for both versican and ADAMTS-2, with ADAMTS-2 staining being stronger in the media, and versican staining stronger in the intima (Fig. 3.2 arrows).

3.4.4. Release of ADAMTS-2 from normal human lung tissue

The apparent co-localization of versican and ADAMTS-2 in normal lung tissue (Fig. 3.1) led us to hypothesize that binding to the chondroitin sulfate side chains of versican



Figure 3.2. Versican and ADAMTS-2 localization in usual interstitial pneumonia (UIP). Versican staining in UIP is abundant within sub-epithelial fibroblast foci (asterisks) and within blood vessel walls (arrows). ADAMTS-2 staining co-localizes to the blood vessel walls and co-localizes in the sub-epithelial fibroblast foci. Lower panels show a UIP sub-epithelial fibroblast focus at higher magnification. ADAMTS-2 staining is evident associated with the myofibroblasts in the versican-rich sub-epithelial fibroblast focus (small arrows). *Scale bars* = 200 μ m, *Scale bar* (*high power*) = 30 μ m. Tissues from 6 UIP patients were studied and representative semi-serial sections are shown.

could localize ADAMTS-2 in the tissue. ADAMTS-2 release from normal human lung tissues was therefore attempted on 2 patients samples, by treating tissue with chondroitinase ABC, using samples from the same patients as paired controls. Normal human lung tissue diced in TBS with proteinase inhibitor tablets and incubated for 1 hour at 37°C released into the supernatant several faint bands detectable with the ADAMTS-2 pro-domain antibody (Fig. 3.3A lanes 8-10 and 11-13). In contrast, incubation of the tissue samples in chondroitinase ABC for 1 hour at 37°C resulted in an increased release of ADAMTS-2, particularly a band of approximately 42 kDa (Fig. 3.3A lanes 1-3 and 4-6). When tissue was heated to 95°C for 5 minutes in reducing sample buffer, ADAMTS-2 was released from the tissue as a series of species, 150-200 kDa and also a 42 kDa species (Fig. 3.3B). This is consistent with the range of species observed in extracting the enzyme from chick and bovine tissues (Colige et al, 1995; Hojima et al, 1989; Hojima et al, 1994). These data suggest that binding to chondroitin sulfate may localize ADAMTS-2 within human lung tissue.

3.4.5. ADAMTS-2 co-purifies with versican at physiological pH

Versican was purified by ion exchange chromatography at pH 7.5 from freshly harvested conditioned medium of HFL-1 fibroblast cultures. Chromatography and medium processing was done at 4°C. Following purification, all column fractions were filter-concentrated by a factor of 10 and separated by SDS-PAGE. Western blotting with versican and ADAMTS-2 antibodies indicated that ADAMTS-2 and versican co-purified on the anion exchange resin (Fig. 3.4A & B). Later elution fractions to 1.5 M NaCl were negative for both versican and ADAMTS-2. Considerable immunoreactivity for ADAMTS-2 was present in the 4% 'stacking' portion of the discontinuous gel, where high molecular weight versican (1-2 MDa) was present. This is a much higher



Figure 3.3. Release of ADAMTS-2 from normal human lung tissue. Normal human lung tissue from patient 1 & patient 2 was finely diced in TBS with proteinase inhibitor tablets and incubated for 1 hour. A, Chondroitinase ABC lyase (0.1 U/ml) was added to some tissue samples (lanes 1 - 6) but not to controls (lanes 8 - 13). Tissues were incubated for 1 hour before removal of supernatant for ADAMTS-2 detection by Western blotting. 1, 2, or 5 µl sample from each patient (left to right for each sample condition) was added to 4, 3, or 0 µl TBS and 5 µl reducing sample buffer followed by SDS PAGE separation (4/10%) and Western blotting. B, Reducing sample buffer was added directly to diced tissue samples at a 1:1 ratio before heating to 95°C for 5 minutes and then Western blotting. C, Identical Western blot with primary antibody omitted and identical contrast adjustments to show absence of secondary antibody cross-reactivity with tissue sample. Results shown are representative of three experiments, each performed in duplicate.



ADAMTS-2

Figure 3.4. ADAMTS-2 and versican co-purify. Immunoblots of serum-free conditioned media (CM) in 7 M urea purified over ANX-Sepharose anion exchange resin. HFL-1 serum-free conditioned medium (lane 1), unbound sample (lane 2), 0 M NaCl column wash (lane 3) and column fractions eluted with a 0 - 1.5 M NaCl gradient (lanes 4 - 9). Fractions were concentrated by equal amounts with centrifugal filter concentrators and separated by SDS PAGE (4/10%) followed by Western blotting. A, Immunoblot with 2B1 antibody. B, Immunoblot with anti-ADAMTS-2 C-terminus antibody. Results shown are representative of four experiments, each analyzed in duplicate.

molecular weight than full length ADAMTS-2 (177 kDa) indicating that this ADAMTS-2 was present in a protein complex that is stable to denaturing conditions (2.0 % SDS and 2M urea). These observations suggested that ADAMTS-2 was bound to versican and eluted from the column as a complex with versican. This is consistent with the observations of Leung *et al.* (Leung et al, 1979) who found that chick tendon procollagen N-propeptidase appeared to be bound to other non-collagenous molecules in a very high molecular weight complex.

3.4.6. ADAMTS-2 co-purification with versican is pH dependent

I investigated the pH-dependence of versican and ADAMTS-2 co-purification. An ion exchange column was loaded in the same manner as before at pH 7.5. Prior to the salt elution, I performed a gradient from pH 7.5 to 5.0 in the same buffer. I observed minimal release of versican from the column and a substantial release of ADAMTS-2 (Fig. 3.5A & B, lanes 4-6). Whereas ADAMTS-2 co-purified with versican at pH 7.5 was apparent in a high molecular weight protein complex after denaturing electrophoresis, this pH elution released predominantly lower molecular weight species including a strong band near the 177 kDa full size of ADAMTS-2 (Fig. 3.5B lane 6).

When I followed the pH elution immediately with a salt elution from 0 to 1.5 M NaCl at pH 5.0, we saw an abundance of versican released from the column (Fig. 3.5A, Iane 7) but no further ADAMTS-2 released (Fig. 3.5B, Iane 7). This is consistent with versican binding to the ion-exchange column directly and ADAMTS-2 binding to versican in a pH-dependent manner. These properties allowed us to separate versican and ADAMTS-2, and purify ADAMTS-2-free versican as a substrate for enzyme digestion experiments.



Figure 3.5. ADAMTS-2 co-purification with versican is pH dependent. Serum-free CM in 7 M urea was purified over ANX-Sepharose anion exchange resin and eluted with a pH elution from 0.1 M Tris, pH 7.5 to 0.1 M Tris / 0.1 M Acetate, pH 5.0, before being eluted with 0 to 1.5 M NaCl at pH 5.0. Immunoblots of starting sample (lane 1), 0 M NaCl, pH 7.5 column wash (lane 2), first elution from pH 7.5 to pH 5.0 (lanes 3-6), second elution from 0 to 1.5 M NaCl at pH 5.0 (lanes 7 & 8). Analysis was by SDS PAGE (4/10%) and Western blotting with 2B1 antibody (A) or anti-ADAMTS-2 C-terminus antibody (B). Results shown are representative of two experiments performed and each analyzed in duplicate.

3.4.7. Purification of bovine ADAMTS-2

Following the previously published protocol (Colige et al. 1995) and in light of more recent work (Colige et al, 2005; Wang et al, 2003), ADAMTS-2 was purified from fetal bovine skin. Potassium chloride extraction, ammonium sulfate precipitation and Con-A Sepharose chromatography were performed as described (Colige et al, 1995), then samples from each step of the purification were separated by SDS-PAGE and detected by Western blotting with ADAMTS-2 N- and C-terminal antibodies. The N-terminal antibody detected several faint bands present in the starting material and through the first two steps of the purification, but no N-terminal immunoreactivity was present after elution from Con-A Sepharose (data not shown). The C-terminal antibody detected a band of approximately 125 kDa in the initial extract as well as several smaller bands, but only the 125 kDa product remained after chromatography on Con-A Sepharose (Fig. 3.6A). This is consistent with proteolytic processing of the enzyme to yield processed fragments with differing binding activities. Fractions 11-14 were pooled and dialyzed, then loaded onto a heparin-Sepharose column and eluted with a gradient to 1 M KCl giving a greatly increased concentration of the 125 kDa product (Fig. 3.6B). Fractions 7-10 from heparin-Sepharose were pooled and dialyzed for further experiments. Based on SDS-PAGE gel silver staining (Fig. 3.6C), I estimate the concentration of enzyme purified to be 100 ng/ml or 800 ρ M. The product purified is consistent with the molecular weight determined in the original publication which showed the enzyme was catalytically active towards α -I-(I) procollagen N-propertides (Colige et al. 1995) and other recent work showing the activity of the 125 kDa enzyme towards α -I-(III) procollagen N-propeptides (Wang et al. 2003).



Figure 3.6. Purification of bovine ADAMTS-2 from fetal calf skin. Following the previously published protocol (Colige et al, 1995) bovine ADAMTS-2 was purified from fetal calf skin. Samples were analyzed by SDS PAGE (3-8% gradient) and Western blotting with anti-ADAMTS-2 C-terminus antibody. A, Extraction with potassium chloride, ammonium sulfate precipitation and Con A-Sepharose purification. Lane 1, initial homogenate in wash buffer after centrifugation. Lane 2, second wash. Lane 3, centrifuge pellet after two extraction cycles. Lane 4, supernatant from ammonium sulfate precipitation of pooled extracts. Lane 5, re-dissolved ammonium sulfate precipitate. Lane 6, centrifugation pellet. Lane 7, centrifugation supernatant/column start material. Lane 8, sample flow through not bound to Con A-Sepharose column. Lane 9, column wash. Lanes 10-14, column fractions eluted with column buffer containing 0 to 0.5 M α-methyl-D-mannoside and observed at 280 nm absorption. B, Purification of pooled ADAMTS-2 reactive fractions (lanes 11-14 above) over heparin-Sepharose. Lane 1, starting material, pooled lanes 11-14 from Con A-Sepharose elution. Lane 2, sample flow through not bound to column. Lane 3, column wash. Lanes 4-13, fractions of gradient elution from 0 to 1 M KCI. Fractions 7 through 10 were pooled, dialyzed and stored for subsequent experiments. C, Silver stain equivalent to B, above. ADAMTS-2 in pooled fractions enclosed in white box. Results shown are representative of one experiment analyzed in triplicate.

3.4.8. ADAMTS-2 degrades versican

I observed early in these studies that versican purified at pH 7.5 was labile and hypothesized that this could be attributable to its co-purification with ADAMTS-2. A method to isolate versican that was free of ADAMTS-2 was established (see above). To investigate whether ADAMTS-2 might be catalytically active towards versican, the two purified proteins were incubated at 37°C at a range of ratios and times. Optimal detection of proteolytic fragments was found to occur with bovine ADAMTS-2 and human versican at an enzyme to substrate ratio of 1:1400. ADAMTS-2 was found to cleave versican, producing C- and N-terminal fragments (Figs. 3.7A & B respectively). C-terminal cleavage of versican produced a number of high molecular weight products, a prominent doublet close to 50 kDa and another band close to 40 kDa (Fig. 3.7A). Nterminal cleavage resulted in high molecular weight products and two bands at approximately 80 and 70 kDa (Fig. 3.7B). A 70 kDa versican fragment produced by the action of ADAMTS-1 and -4 has previously been described and characterized with an antibody to the DPEAAE neo-epitope (Sandy et al. 2001). To determine whether ADAMTS-2 cleavage of versican generated this same neo-epitope, I analyzed the same samples by Western blotting with the DPEAAE antibody but observed no immunoreactivity in triplicate experiments (data not shown). Therefore, I concluded that the N- and C-terminal cleavages described here have not been previously characterized. For both N- and C-terminal versican antibodies, an increased versican immunoreactivity was detectable in the stacking gel after enzymatic cleavage. Since versican is a high molecular weight (~1.5 MDa) proteoglycan which aggregates to form multimers with hyaluronan, SDS-PAGE does not adequately resolve the high molecular weight aggregates and immunoblotting to a membrane may also be only poorly effective to transfer the aggregate. Therefore, an increase in versican immunoreactivity


Figure 3.7. ADAMTS-2 degrades versican *in vitro.* A, purified human versican (0.56 µM) was incubated alone or with purified bovine ADAMTS-2 (0.4 nM) for 0 to 48 hours at 37°C as indicated, then separated by SDS PAGE (4/12%) followed by Western blotting with 2B1 antibody. B, purified human versican (0.56 µM) alone, purified bovine ADAMTS-2 (0.4 nM) alone, or versican and ADAMTS-2 together, were incubated for 24 hours at 37°C, then separated by SDS PAGE (4/10%) followed by Western blotting with 12C5 antibody. Results shown are representative of three experiments, each analyzed in triplicate.

in the stacking gel may be indicative of proteolytic processing of the core protein which results in disaggregation and altered protein behavior in SDS-PAGE separation and Western blotting. Interestingly, purified versican alone does undergo some degradation and production of a 50 kDa doublet (Fig. 3.7A lane 2). This proteolytic fragment is of a similar size to that produced by purified bovine ADAMTS-2 *in vitro*, though ADAMTS-2 is not detectable in this preparation by Western blotting (Fig. 3.8 lanes 1 & 2).

3.4.9. Versican inhibits autodegradation of ADAMTS-2

I found that ADAMTS-2 when incubated alone underwent autocatalytic processing which degraded the C-terminal epitope within 48 hours (Fig. 3.8A lanes 3-6). I also observed that incubation of ADAMTS-2 with versican resulted in preserved immunoreactivity of the 125 kDa band after 48 hours (Fig. 3.8A lanes 7-10). This experiment was performed in triplicate and a representative experiment is shown. To investigate the specificity of versican for inhibition of ADAMTS-2 autocatalytic degradation, I incubated ADAMTS-2 with 5 different glycosaminoglycans. After 24 hours with no glycosaminoglycan added, ADAMTS-2 autodegradation was evident (Fig. 3.8B) compared with 0 hours incubation (Fig. 3.8A lanes 3 & 5). Addition of hyaluronan had no effect and chondroitin sulfate B (dermatan sulfate) and C (chondroitin-6-sulfate) resulted in mild inhibition of ADAMTS-2 autodegradation. Chondroitin sulfate A (chondroitin-4-sulfate) provided stronger inhibition and heparan sulfate provided the strongest inhibition of autodegradation. Glycosaminoglycan concentrations up to 5 μM had no further effect (data not shown).

As glycosaminoglycans were able to inhibit ADAMTS-2 auto-degradation, this result suggested that versican inhibition of ADAMTS-2 was through glycosaminoglycan binding rather than the availability of a preferred substrate such as versican. Thus,



Figure 3.8. Versican inhibits ADAMTS-2 autodegradation. A, Purified human versican (0.56 μ M) alone, purified bovine ADAMTS-2 (0.4 nM) alone, or versican and ADAMTS-2 together were incubated for 0 to 48 hours at 37°C as indicated, then separated by SDS PAGE (3-8% gradient). B, Purified bovine ADAMTS-2 (0.4 nM) was incubated for 24 hours at 37°C, alone in TBS or with the following glycosaminoglycans in TBS (5 or 50 nM final concentrations): hyaluronan (HA), chondroitin sulfate A (chondroitin-4-sulfate)(CS-A), chondroitin sulfate B (dermatan sulfate)(CS-B), chondroitin sulfate C (chondroitin-6-sulfate)(CS-C) and heparan sulfate (HS). Detection was by Western blotting with anti-ADAMTS-2 C-terminus antibody. Results shown are representative of three experiments, each analyzed in triplicate.

physiological binding of ADAMTS-2 to versican may inhibit autocatalytic degradation of ADAMTS-2 resulting in preserved enzymatic activity. C-terminal processing of ADAMTS enzymes has been to shown to occur through autocatalytic processing as well as processing by other proteases, resulting in altered substrate specificity and catalytic efficiency of the enzymes (Colige et al, 2004; Colige et al, 2005; Flannery et al, 2002; Gao et al, 2004; Somerville et al, 2003; Tortorella et al, 2005). Further work will be needed to clarify the nature of the autocatalytic degradation observed for ADAMTS-2 and its influence on enzyme activity.

3.5. Discussion

The chondroitin sulfate proteoglycan versican is a pericellular matrix molecule that is important in development of the skeleton and cardiovascular system. Versican is believed to influence cell behavior through creation of a pericellular matrix that is permissive for migration and proliferation of distinct cell types. Versican also binds a number of structural macromolecules and bio-active molecules (Wight, 2002). It was previously shown that versican is expressed in remodeling processes in the most prevalent forms of human lung fibrosis, including those associated with nongranulomatous inflammation: Organizing diffuse alveolar damage in patients with adult respiratory distress syndrome; Usual interstitial pneumonia and idiopathic BOOP (Bensadoun et al, 1996). Versican is also expressed in association with fibroblasts in granulomatous forms of lung fibrosis including the lesions of tuberculosis, sarcoidosis and extrinsic allergic alveolitis. In all of these forms of human lung fibrosis, versican is found in association with migratory, proliferating alpha actin-positive fibroblasts (Bensadoun et al, 1997). These are the cells that synthesize type I procollagen and thus are considered to be primarily responsible for the synthesis of new collagenous

matrix (Kuhn & McDonald, 1991). As collagen fibrillogenesis takes place in a versicanrich provisional matrix and as this matrix is apparently resorbed concomitant with collagen deposition, it was hypothesized that a type I collagen-processing enzyme might contribute to versican degradation.

Immunohistochemical staining showed that the procollagen N-propeptidase ADAMTS-2 co-localizes with versican in normal human lungs. Both the proteoglycan and the enzyme are predominantly associated with smooth muscle in normal human lung tissue. Consistent with a functional association between versican and ADAMTS-2, ADAMTS-2 was released from normal tissue by incubation of tissue with chondroitinase ABC. This suggests that ADAMTS-2 is bound to chondroitin sulfate proteoglycans, including versican, in normal human lung tissue. Two pathologic patterns of idiopathic human lung fibrosis were studied in the current study. In remodeling lung tissue, ADAMTS-2 was found in association with the fibroblasts of the subepithelial fibroblast foci and in association with the fibroblasts of the intraluminal buds, in the pathologic patterns usual interstitial pneumonia and in bronchiolitis obliterans organizing pneumonia respectively. Staining for ADAMTS-2 in the remodeling lesions of UIP was consistently stronger than those of BOOP. It is of interest that BOOP is characterized by even-aged lesions that can resolve spontaneously, or following corticosteroid therapy. In contrast, UIP is associated with a higher rate of morbidity and is less responsive to therapy. Versican is clearly a major component of the matrix of these remodeling lesions.

Consistent with a functional interaction between ADAMTS-2 and versican, the two molecules were found to be bound together in human fetal lung fibroblast culture media, in a form that was stable to 7M Urea. I observed that versican preparations were labile, and that spontaneous degradation could be inhibited by procedures that

separated versican from contaminating ADAMTS-2. Purified versican was shown to be a substrate for purified bovine ADAMTS-2 *in vitro* and versican was susceptible to cleavage at a number of sites. Though some degradation products appeared similar to those found naturally in versican preparations, none of the products of ADAMTS-2 activity appeared to correspond to the products of degradation previously shown to arise from ADAMTS-1 and -4 (Sandy et al, 2001). These studies showed ADAMTS-2 to be susceptible to auto-degradation and that addition of versican appeared to protect ADAMTS-2 from auto-degradation more potently than addition of glycosaminoglycans. If this occurs *in vivo*, versican may prolong the biological half-life of this procollagen Npropeptidase.

ADAMTS-2, -3 and -14 are procollagen N-propeptidases with a known substrate profile limited to the amino-propeptides of types I, II, III & V procollagens. This work is apparently the first report of degradation of a non-collagenous substrate by a member of this clade of enzymes. Recent work indicates that multiple processing events may alter catalytic activity and substrate specificity of ADAMTS-2 (Colige et al, 2004; Colige et al, 2005). ADAMTS-2 expression seems disproportionate to apparent rates of collagen biosynthesis in several tissues (Colige et al, 1997). Based on homologies to other proteins that are critical in development and cell signaling, it has been suggested that ADAMTS-2 likely plays important roles in development and cell signaling independent of its role in procollagen processing (Prockop et al, 1998).

In contrast to the procollagen N-propeptidases, ADAMTS-1, -4, & -9 have been shown to cleave versican (Jonsson-Rylander et al, 2005; Sandy et al, 2001; Somerville et al, 2003; Westling et al, 2004). ADAMTS-1, -4, -9, and by homology -5, -8, -15 & -20, are considered to form a group of 'hyalectanases' that process hyalectans

(hyaluronan-binding proteoglycans including aggrecan and versican) and are evolutionarily distinct from the other ADAMTS enzymes (Apte, 2004).

Defects in the ADAMTS-2 gene result in the autosomal recessive connective tissue disorder Ehlers-Danlos (EDS) syndrome type VIIC, characterized by extreme skin fragility and joint laxity (Colige et al, 1999). Collagen fibrils in the skin of these patients retain their N-propeptides and are thin, branched and irregular, appearing 'hieroglyphic' in cross section (Nusgens et al, 1992). ADAMTS-2 null mice develop similar skin fragility and irregular collagen fibril formation, but they also exhibit decreased spermatogenesis and are sterile (Li et al, 2001). The ADAMTS-2 knockout mouse shows altered lung architecture with a decrease in lung surface area due to enlarged distal airspaces. This appears to result from disordered lung development (Le Goff et al, 2006).

Our data suggest that versican binding of ADAMTS-2 may regulate collagen assembly and that ADAMTS-2-mediated degradation of versican may be important in resorption of the provisional matrix following inflammation. ADAMTS-2 binding by versican is potentially important in the fibroproliferative lesions of human pulmonary fibrosis; this may also explain our observation that collagen biosynthesis occurred in cells surrounded by versican, and that versican is essentially absent, as is type 1 collagen synthesis, from areas of mature collagenous fibrosis.

This is the first report of an ancillary role for a procollagen N-propeptidase and the first report of a proteoglycanase activity for ADAMTS-2.

3.6. References

Apte SS (2004) A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motifs: the ADAMTS family. *Int J Biochem Cell Biol* **36**(6): 981-985

Asher R, Perides G, Vanderhaeghen JJ, Bignami A (1991) Extracellular matrix of central nervous system white matter: demonstration of an hyaluronate-protein complex. *J Neurosci Res* **28**(3): 410-421

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1996) Proteoglycan deposition in pulmonary fibrosis. *Am J Respir Crit Care Med* **154**(6 Pt 1): 1819-1828

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1997) Proteoglycans in granulomatous lung diseases. *Eur Respir J* **10**(12): 2731-2737

Bode-Lesniewska B, Dours-Zimmermann MT, Odermatt BF, Briner J, Heitz PU, Zimmermann DR (1996) Distribution of the large aggregating proteoglycan versican in adult human tissues. *J Histochem Cytochem* **44**(4): 303-312

Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro AJ, Kubalak S, Klewer SE, McDonald JA (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* **106**(3): 349-360

Colige A, Beschin A, Samyn B, Goebels Y, Beeumen JV, Nusgens BV, Lapiere CM (1995) Characterization and partial amino acid sequencing of a 107-kDa procollagen I N-proteinase purified by affinity chromatography on immobilized type XIV collagen. *J Biol Chem* **270**(28): 16724-16730

Colige A, Li S-W, Sieron AL, Nusgens BV, Prockop DJ, Lapiere CM (1997) cDNA cloning and expression of bovine procollagen I N-proteinase: A new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. *Proc Natl Acad Sci U S A* **94**(6): 2374-2379

Colige A, Nuytinck L, Hausser I, van Essen AJ, Thiry M, Herens C, Ades LC, Malfait F, Paepe AD, Franck P, Wolff G, Oosterwijk JC, Smitt JH, Lapiere CM, Nusgens BV (2004) Novel types of mutation responsible for the dermatosparactic type of Ehlers-Danlos syndrome (Type VIIC) and common polymorphisms in the ADAMTS2 gene. *J Invest Dermatol* **123**(4): 656-663

Colige A, Ruggiero F, Vandenberghe I, Dubail J, Kesteloot F, Van Beeumen J, Beschin A, Brys L, Lapiere CM, Nusgens B (2005) Domains and maturation processes that regulate the activity of ADAMTS-2, a

metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. *J Biol Chem* **280**(41): 34397-34408

Colige A, Sieron AL, Li SW, Schwarze U, Petty E, Wertelecki W, Wilcox W, Krakow D, Cohn DH, Reardon W, Byers PH, Lapiere CM, Prockop DJ, Nusgens BV (1999) Human Ehlers-Danlos syndrome type VII C and bovine dermatosparaxis are caused by mutations in the procollagen I N-proteinase gene. *Am J Hum Genet* **65**(2): 308-317

Dours-Zimmermann MT, Zimmermann DR (1994) A novel glycosaminoglycan attachment domain identified in two alternative splice variants of human versican. *J Biol Chem* **269**(52): 32992-32998

Evanko SP, Angello JC, Wight TN (1999) Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **19**(4): 1004-1013

Faggian J, Fosang AJ, Zieba M, Wallace MJ, Hooper SB (2007) Changes in versican and chondroitin sulfate proteoglycans during structural development of the lung. *Am J Physiol Regul Integr Comp Physiol* **293**(2): R784-792

Farndale RW, Buttle DJ, Barrett AJ (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* **883**(2): 173-177

Flannery CR, Zeng W, Corcoran C, Collins-Racie LA, Chockalingam PS, Hebert T, Mackie SA, McDonagh T, Crawford TK, Tomkinson KN, LaVallie ER, Morris EA (2002) Autocatalytic cleavage of ADAMTS-4 (Aggrecanase-1) reveals multiple glycosaminoglycan-binding sites. *J Biol Chem* **277**(45): 42775-42780

Gao G, Plaas A, Thompson VP, Jin S, Zuo F, Sandy JD (2004) ADAMTS4 (aggrecanase-1) activation on the cell surface involves C-terminal cleavage by glycosylphosphatidyl inositol-anchored membrane type 4-matrix metalloproteinase and binding of the activated proteinase to chondroitin sulfate and heparan sulfate on syndecan-1. *J Biol Chem* **279**(11): 10042-10051

Hojima Y, McKenzie JA, van der Rest M, Prockop DJ (1989) Type I procollagen N-proteinase from chick embryo tendons. Purification of a new 500-kDa form of the enzyme and identification of the catalytically active polypeptides. *J Biol Chem* **264**(19): 11336-11345

Hojima Y, Morgelin MM, Engel J, Boutillon MM, van der Rest M, McKenzie J, Chen GC, Rafi N, Romanic AM, Prockop DJ (1994) Characterization of type I procollagen N-proteinase from fetal bovine tendon and skin. Purification of the 500-kilodalton form of the enzyme from bovine tendon. *J Biol Chem* **269**(15): 11381-11390

lozzo RV (1998) Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* **67**: 609-652

Isogai Z, Aspberg A, Keene DR, Ono RN, Reinhardt DP, Sakai LY (2002) Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. *J Biol Chem* **277**(6): 4565-4572

Isogai Z, Shinomura T, Yamakawa N, Takeuchi J, Tsuji T, Heinegard D, Kimata K (1996) 2B1 antigen characteristically expressed on extracellular matrices of human malignant tumors is a large chondroitin sulfate proteoglycan, PG-M/versican. *Cancer Res* **56**(17): 3902-3908

Ito K, Shinomura T, Zako M, Ujita M, Kimata K (1995) Multiple forms of mouse PG-M, a large chondroitin sulfate proteoglycan generated by alternative splicing. *J Biol Chem* **270**(2): 958-965

Jonsson-Rylander AC, Nilsson T, Fritsche-Danielson R, Hammarstrom A, Behrendt M, Andersson JO, Lindgren K, Andersson AK, Wallbrandt P, Rosengren B, Brodin P, Thelin A, Westin A, Hurt-Camejo E, Lee-Sogaard CH (2005) Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican. *Arterioscler Thromb Vasc Biol* **25**(1): 180-185

Khalil N, O'Connor R (2004) Idiopathic pulmonary fibrosis: current understanding of the pathogenesis and the status of treatment. *CMAJ* **171**(2): 153-160

Kimata K, Oike Y, Tani K, Shinomura T, Yamagata M, Uritani M, Suzuki S (1986) A large chondroitin sulfate proteoglycan (PG-M) synthesized before chondrogenesis in the limb bud of chick embryo. *J Biol Chem* **261**(29): 13517-13525

Kinsella MG, Bressler SL, Wight TN (2004) The regulated synthesis of versican, decorin, and biglycan: extracellular matrix proteoglycans that influence cellular phenotype. *Crit Rev Eukaryot Gene Expr* **14**(3): 203-234

Krueger RCJ, Schwartz NB (1987) An improved method of sequential alcian blue and ammoniacal silver staining of chondroitin sulfate proteoglycan in polyacrylamide gels. *Anal Biochem* **167**(2): 295-300

Kuhn C, McDonald JA (1991) The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol* **138**(5): 1257-1265

Kuno K, Matsushima K (1998) ADAMTS-1 protein anchors at the extracellular matrix through the thrombospondin type I motifs and its spacing region. *J Biol Chem* **273**(22): 13912-13917

Le Goff C, Somerville RPT, Kesteloot F, Powell K, Birk DE, Colige AC, Apte SS (2006) Regulation of procollagen amino-propeptide processing during mouse embryogenesis by specialization of homologous ADAMTS proteases: insights on collagen biosynthesis and dermatosparaxis. *Development* **133**(8): 1587-1596

LeBaron RG, Zimmermann DR, Ruoslahti E (1992) Hyaluronate binding properties of versican. *J Biol Chem* **267**(14): 10003-10010

Leung MKK, Fessler LI, Greenberg DB, Fessler JH (1979) Separate amino and carboxyl procollagen peptidases in chick embryo tendon. *J Biol Chem* **254**(1): 224-232

Li SW, Arita M, Fertala A, Bao Y, Kopen GC, Langsjo TK, Hyttinen MM, Helminen HJ, Prockop DJ (2001) Transgenic mice with inactive alleles for procollagen N-proteinase (ADAMTS-2) develop fragile skin and male sterility. *Biochem J* **355**(Pt 2): 271-278

Mjaatvedt CH, Yamamura H, Capehart AA, Turner D, Markwald RR (1998) The Cspg2 gene, disrupted in the hdf mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol* **202**(1): 56-66

Naso MF, Zimmermann DR, lozzo RV (1994) Characterization of the complete genomic structure of the human versican gene and functional analysis of its promoter. *J Biol Chem* **269**(52): 32999-33008

Nusgens BV, Verellen-Dumoulin C, Hermanns-Le T, De Paepe A, Nuytinck L, Pierard GE, Lapiere CM (1992) Evidence for a relationship between Ehlers-Danlos type VII C in humans and bovine dermatosparaxis. *Nat Genet* **1**(3): 214-217

Prockop DJ, Sieron AL, Li S-W (1998) Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. *Matrix Biol* **16**(7): 399-408

Roberts CR (2003) Versican in the Cell Biology of Pulmonary Fibrosis. In *Proteoglycans in Lung Disease*, Garg HG, Roughley PJ, Hales CA (eds), Vol. 168, pp 191-212. New York, NY: Marcel Dekker

Sandy JD, Westling J, Kenagy RD, Iruela-Arispe ML, Verscharen C, Rodriguez-Mazaneque JC, Zimmermann DR, Lemire JM, Fischer JW, Wight TN, Clowes AW (2001) Versican V1 proteolysis in human aorta in vivo occurs at the Glu441-Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4. *J Biol Chem* **276**(16): 13372-13378

Selman M, Talmadge EKJ, Pardo A (2001) Idiopathic Pulmonary Fibrosis: Prevailing and Evolving Hypotheses about its Pathogenesis and Implications for Therapy. *Ann Intern Med* **134**(2): 136-151

Somerville RP, Longpre JM, Jungers KA, Engle JM, Ross M, Evanko S, Wight TN, Leduc R, Apte SS (2003) Characterization of ADAMTS-9 and ADAMTS-20 as a distinct ADAMTS subfamily related to Caenorhabditis elegans GON-1. *J Biol Chem* **278**(11): 9503-9513

Stocker W, Grams F, Baumann U, Reinemer P, Gomis-Ruth FX, McKay DB, Bode W (1995) The metzincins -- Topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. *Protein Sci* **4**(5): 823-840

Thannickal VJ, Toews GB, White ES, Lynch Iii JP, Martinez FJ (2004) Mechanisms of Pulmonary Fibrosis. *Annual Review of Medicine* **55**(1): 395-417

Tortorella M, Pratta M, Liu RQ, Abbaszade I, Ross H, Burn T, Arner E (2000) The thrombospondin motif of aggrecanase-1 (ADAMTS-4) is critical for aggrecan substrate recognition and cleavage. *J Biol Chem* **275**(33): 25791-25797

Tortorella MD, Arner EC, Hills R, Gormley J, Fok K, Pegg L, Munie G, Malfait A-M (2005) ADAMTS-4 (aggrecanase-1): N-Terminal activation mechanisms. *Arch Biochem Biophys* **444**(1): 34-44

Travis WD, King TE, Bateman ED, Lynch DA, Capron F, Center D, Colby TV, Cordier J-F, du Bois RM, Galvin J, Grenier P, Hansell DM, Hunninghake GW, Kitaichi M, Muller NL, Myers JL, Nagai S, Raghu G, Wallabert B (2002) American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias . This Joint Statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS Board of Directors, June 2001 and by The ERS Executive Committee, June 2001. *Am J Respir Crit Care Med* **165**(2): 277-304 Wang WM, Lee S, Steiglitz BM, Scott IC, Lebares CC, Allen ML, Brenner MC, Takahara K, Greenspan DS (2003) Transforming growth factor-beta induces secretion of activated ADAMTS-2. A procollagen III N-proteinase. *J Biol Chem* **278**(21): 19549-19557

Westling J, Gottschall PE, Thompson VP, Cockburn A, Perides G, Zimmermann DR, Sandy JD (2004) ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu405-Gln406 to generate glial hyaluronate binding protein. *Biochem J* **377**(Pt 3): 787-795

Wight TN (2002) Versican: a versatile extracellular matrix proteoglycan in cell biology. *Curr Opin Cell Biol* **14**(5): 617-623

Zako M, Shinomura T, Ujita M, Ito K, Kimata K (1995) Expression of PG-M(V3), an alternatively spliced form of PG-M without a chondroitin sulfate attachment region in mouse and human tissues. *J Biol Chem* **270**(8): 3914-3918

CHAPTER 4 – VERSICAN DEGRADATION AT THE CELL SURFACE BY MMP-2 AND MT1-MMP^{*}

4.1. Summary

Versican is a pericellular proteoglycan that is associated with proliferating mesenchymal cells in development and a number of disease processes that involve tissue remodeling. In normal wound healing, versican is an abundant element of the provisional matrix in which fibroblast cell proliferation occurs. The provisional matrix is apparently degraded, concomitant with fibroblast apoptosis and a return to normal tissue architecture. The persistence of the versican-rich matrix is seen in pulmonary fibrosis and atherosclerosis; and in the pericellular matrix of many cancers. The process of versican degradation is poorly understood, but as versican is a pericellular molecule, physiological degradation likely involves cell surface proteolysis. Using concanavalin A (ConA) induction of fibroblasts as a model of matrix degradation I found that both pericellular versican and secreted versican were degraded concomitant with ConA induced fibroblast cell apoptosis. MT1-MMP and MMP-2 were apparently mobilized to the cell surface and MMP-2 was activated on ConA treatment. Microarray analysis was used to investigate expression of possible versican-degrading enzymes and their inhibitors, expressed in response to ConA. Metalloproteinase inhibitors prinomastat (AG3340) and GM6001 (Ilomastat/Galardin) impeded versican degradation, filamentous actin degradation and MMP-2 activation to a greater degree than an inhibitor of apoptosis, Z-VAD-FMK. Recombinant MMP-2 and MT1-MMP were capable of degrading versican *in vitro* and MT1-MMP exhibited a more subtle activity against versican within the C-terminal domain. These data suggest that resolution of

^{*} A version of this chapter will be submitted for publication Sean B. Maurice, Reinhild Kappelhoff, Alain Doucet, Christopher M. Overall and Clive R. Roberts. Versican degradation at the cell surface by MMP-2 and MT1-MMP.

the versican-rich provisional matrix in tissue remodeling occurs through MMP-2 and MT1-MMP dependent proteolysis.

4.2. Introduction

Versican is a large proteoglycan of the pericellular matrix surrounding fibroblast cells. It is highly expressed in tissue remodeling events of development, wound healing and cancer. In development, versican is crucial for formation of a hydrated extracellular space in which cell migration and differentiation can occur. Deletion of the versican gene prevents successful formation of the endocardial cushion swellings necessary for proper segmentation of the mouse heart and is lethal in development (Mjaatvedt et al, 1998). Versican is a member of the 'hyalectan' family of hyaluronan-binding proteoglycans with lectin-like domains (Iozzo, 1998).

In normal wound healing, versican is expressed as part of a provisional matrix in which collagen synthesis and wound contraction occur, followed by degradation of the versican-rich matrix and a return to normal tissue architecture. High levels of versican in the provisional matrix are associated with all the major forms of pulmonary fibrosis arising from granulomatous and non-granulomatous processes (Bensadoun et al, 1996; Bensadoun et al, 1997). Similarly, versican deposition is associated with lesion severity in atherosclerosis and restenosis (Evanko et al, 1998; Wight et al, 1997). Versican is highly expressed in numerous cancers and is associated with poor prognosis in oral, breast, prostate, cervical, endometrial and cutaneous cancers (Kodama et al, 2007a; Kodama et al, 2007b; Pukkila et al, 2007; Ricciardelli et al, 2002; Ricciardelli et al, 1998; Suwiwat et al, 2004; Touab et al, 2003; Touab et al, 2002).

Versican is a multifunctional regulator of the pericellular matrix that alters the physical and chemical properties surrounding fibroblast cells. By virtue of its size and

glycosylation, high levels of versican may create substantial steric resistance to certain binding interactions and thus regulate cell signaling and survival (Roberts, 2003).

Normal wound healing requires the regulated deposition of collagen to restore tissue architecture. Excess deposition of collagen is associated with fibrosis. In the resolution of normal wound healing, myofibroblasts disappear by apoptosis (Desmouliere et al, 1995). The persistence of the myofibroblasts appears to be critical to sustaining fibrogenesis and may be related to the persistence of the versican-rich pericellular matrix. Characterization of normal and dysregulated versican degradation is therefore critical to understanding aberrant events that contribute to fibroproliferative remodeling in pulmonary fibrosis, atherosclerosis, and cancer stroma. As regulated ECM remodeling often occurs through cell surface focalized proteolysis (Basbaum & Werb, 1996), versican turnover may involve cell surface-associated proteolysis.

MMP-2, TIMP-2 and MT1-MMP form a tightly regulated cell surface proteolytic complex (Overall et al, 2000; Strongin et al, 1995; Will et al, 1996). Under normal conditions MMP-2 and MT1-MMP are synthesized by fibroblasts but remain predominantly in their inactive zymogen form. During remodeling, MMP-2 and MT1-MMP are activated and participate in the regulated proteolysis of the ECM. MMP-2 and MT1-MMP are expressed at high levels in resolving granulation tissue (Madlener et al, 1998) and in idiopathic pulmonary fibrosis (Garcia-Alvarez et al, 2006; Selman et al, 2000). MMP-2 is activated in tumor cells and appears to be involved in tumor invasion and metastasis (Brown et al, 1993a; Brown et al, 1993b). MT1-MMP is expressed on the surface of invasive tumor cells (Sato et al, 1994), is correlated with invasiveness (Gilles et al, 1996; Nakamura et al, 1999; Ueno et al, 1997; Yamamoto et al, 1996) and is critical in cancer cell migration (Sabeh et al, 2004).

Concanavalin-A (ConA) is a lectin from the jack bean plant known to induce fibroblast cell apoptosis, inducing synthesis of metalloproteinases and a matrix degradative phenotype (Overall & Sodek, 1990). ConA binds cell surface α-D-glucose and α-D-mannose residues (Smith & Goldstein, 1967) causing receptor clustering and cell agglutination (Kulkarni & McCulloch, 1995). ConA induces three modes of protease up-regulation including the rapid activation of MT1-MMP from intracellular compartments (Jiang et al, 2001) and corresponding MMP-2 activation (Zucker et al, 2002). Upregulation of MT1-MMP occurs post-translationally (Yu et al, 1995) and MMP-2 and MT1-MMP are also transcriptionally up-regulated (Overall & Sodek, 1990; Zucker et al, 2002). As the provisional matrix in normal wound healing is degraded concomitant with fibroblast cell apoptosis, ConA stimulation of fibroblast cells offers a tissue culture model of matrix degradation that may mimic certain physiological events relevant to the proper resolution of wound healing.

Here I show that ConA treatment of fibroblasts induces versican degradation, concomitant with up-regulation of MMP-2 and MT1-MMP. I show that versican degradation is reduced by metalloproteinase inhibitors with a corresponding inhibition of morphological events of apoptosis. MMP-2 and MT1-MMP are shown to cleave versican in vitro. I suggest that MMP-2 and MT1-MMP may be critical proteases involved in resolution of the versican-rich matrix in tissue remodeling.

4.3. Experimental procedures

4.3.1. Tissue culture

Human fetal lung fibroblasts (HFL-1) were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 20 mM HEPES and 10% (v/v) Cosmic Calf Serum (Hyclone, Logan,

UT). Cells were seeded at 2 x 10⁴ cells per ml and grown to approximately 70% confluence in Lab-Tek II chamber slides (Nunc, Rochester, NY), incubated one hour in serum free medium before addition of ConA diluted in serum free medium (Sigma, St. Louis, MO). Inhibitors were diluted in serum free medium and added for one hour prior to ConA treatment. The following inhibitors were used: Z-FA-FMK (Carboxybenzyl-phenylalanine, alanine, fluoromethylketone)(Calbiochem, San Diego, CA), Z-VAD-FMK (Carboxybenzyl-valine, alanine, aspartate, O-methylated fluoromethylketone) (Calbiochem), GM6001 (Ilomastat/Galardin) (Calbiochem), prinomastat (AG3340) (Agouron Pharmaceuticals, Inc., a Pfizer Company, La Jolla, CA). Versican released into conditioned medium was concentrated by a factor of 10 using Amicon centrifugal filter units (Millipore, Billerica, MA) and retentate was treated with 0.5 U/ml chondroitinase ABC (Sigma) at 37°C for 40 minutes before electrophoresis.

4.3.2. Immunofluorescence staining and microscopy

Treated cells were fixed for 10-15 minutes with 4 % para-formaldehyde in phosphate buffered saline (PBS), pH 7.5, then rinsed with PBS. Cells were permeabilized in TBStriton (20 mM Tris, pH 7.5, 0.9 % NaCl, 0.2 % triton X-100) with 2% (w/v) bovine serum albumin (BSA). Blocking was performed with 5 % (v/v) normal goat serum and 2% (w/v) BSA in TBS-triton. Washing was performed in 0.2% (w/v) BSA in TBS-triton and antibodies were diluted in the same buffer. The following primary antibodies were used: mouse monoclonal anti-versican C-terminal domain, 2B1 (Isogai et al, 2002; Isogai et al, 1996) (Seikagaku, Tokyo, Japan), dilution 1:500; rabbit polyclonal anti-MMP-2 C-domain, α 72Ex12 (Overall et al, 1999), dilution 1:400; and mouse monoclonal anti-MT1-MMP catalytic domain, clone 5H2 (R&D Systems, Minneapolis, MN), dilution 1:400. Alexa Fluor 594 goat anti—rabbit IgG and goat anti—mouse IgG,

highly cross-adsorbed secondary antibodies were used (Molecular Probes, Eugene, OR). Counterstaining for F-actin was with Alexa Fluor 488 phalloidin stain (Molecular Probes) and nuclear counterstaining was with Hoescht 33342 (Molecular Probes). Stained cells were mounted under coverslips with Prolong Gold antifade reagent (Molecular Probes) and stored at -20°C. Microscopy was performed on a Leica DMRA2 automated microscope (Leica Microsystems GmbH, Wetzlar). In the antibody labeled channel, three-dimensional images were acquired and image stacks were deconvolved using the Nearest Neighbour Deconvolution algorithm (Improvision, Coventry, UK). Exposure and deconvolution settings were identical for each fluorescent channel in each experiment, throughout the different concentrations of ConA.

4.3.3. SDS-PAGE

Samples in non-reducing sample buffer (125 mM Tris-HCL, pH 6.8, 2.0% SDS, 2.0 M urea, 0.05% bromophenol blue) were separated on discontinuous SDS-PAGE gels with 4% (stacking) and 10% (separating) acrylamide. Stacking and separating gels were kept during Western blotting to monitor high molecular weight versican aggregates within the stacking gel. For some experiments, 4-15% Tris-HCl precast gradient gels were used (Bio-Rad, Hercules, CA). Relative molecular mass (measured in kDa) was estimated based on mobility of molecular weight markers: HiMark Prestained (Invitrogen), MagicMark XP (Invitrogen) and Kaleidoscope Prestained (Bio-Rad). Western blotting was performed as previously described (Maurice & Roberts, 2008).

4.3.4. Gelatin zymography

SDS-PAGE gels were cast as above with the addition of 0.1% gelatin to the 10% acrylamide, separating portion of the gel. Gels were incubated in exchange buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM CaCl₂, 2.5 % Triton X-100) for one hour then washed three times in incubation buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM CaCl₂) and incubated 16 hours in the same buffer at 37°C. Staining and destaining was performed with Coomassie Blue R-250 following standard protocol. The following antibodies were used: mouse monoclonal anti-versican N-terminal antibody 12C5 (Asher et al, 1991)(obtained from the Developmental Studies Hybridoma Bank (NICHD), the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), 1:500 dilution; anti-versican C-terminal domain 2B1, 1:1000 dilution; rabbit polyclonal anti-versican C-terminal domain recombinant construct LC2 (Pourmalek & Roberts, 2009), 1:10 000 dilution; and rabbit polyclonal anti-PG40 (Brennan et al, 1984; Krusius & Ruoslahti, 1986), 1:500 dilution.

4.3.5. RNA preparation

Following incubations with or without ConA, RNA was prepared and labeled as described (Kappelhoff & Overall, 2007). Cells were washed three times with phosphate-buffered saline (PBS) to remove all serum components. RNA was extracted, purified and homogenized with RNeasy Mini Kits and QIAshredder spin columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was determined by running samples on a 1.2% native agarose gel and inspecting for distinct 18S and 28S ribosomal RNA bands at an approximate 2:1 ratio. RNA quantity was determined by A₂₆₀ measurement. Samples were maintained at 4°C

and used immediately or frozen at –80°C until use. RNA was reverse transcribed and purified using MessageAmp II aRNA amplification kit (Ambion, Austin, TX) according to the manufacturer's instructions. Amplified RNA (aRNA) was fluorescently labelled with the universal linkage system (Kreatech Diagnostics, Amsterdam, Netherlands) according to the manufacturer's instructions. Control and test samples were separately labeled with Cy3- or Cy5-ULS and then purified. Labeling efficiency was monitored at A₂₆₀ and A₅₅₀ (Cy3) or A₂₆₀ and A₆₅₀ (Cy5) using the NanoDrop spectrophotometer microarray measurement tool to ensure successful labeling (Thermo Fisher Scientific, Waltham, MA). All procedures were performed in an RNase-free environment.

4.3.6. Microarrays

The CLIP-CHIP aminosilane microarray slides contain 70-mer oligonucleotides to all human protease, inhibitor and homologue genes (http://www.clip.ubc.ca)(Kappelhoff & Overall, 2007). Microarray slides were prehybridized and oligonucleotides denatured as described (Kappelhoff & Overall, 2007) before hybridizing with Cy3- and Cy5-labeled aRNA. After hybridization, slides were washed under stringent conditions before scanning with an Affymetrix 428 microarray laser scanner (Affymetrix, Santa Clara, CA) using 532 nm for Cy3 and 635 nm for Cy5. Three biological replicates were performed and analyzed on separate slides containing duplicate arrays for a total of six arrays. Image files were acquired in Imagene 6.1 (Biodiscovery, El Segundo, CA). Data were normalized in CARMAweb using 'normexp' for background correction, print tip loess for within array normalization and the quantile algorithm for normalization between arrays (https://carmaweb.genome.tugraz.at/carma/). Two dimensional SAM (significance analysis of microarrays) analysis with a 1.5 fold cutoff and paired T-tests

were performed in TM4 MultiExperiment Viewer (Saeed et al, 2003) (http://www.tm4.org/mev.html).

4.3.7. Enzyme assays

Versican was purified from HFL-1 cells at approximately 70% of confluence. Cultures were rinsed in serum-free media followed by incubation in serum-free media for 24 hours. Serum-free CM were collected and centrifuged at 1500 x g for 20 minutes to remove cellular debris. Urea was added to 7 M, pH 6.0 and 150 mM NaCl was added to bring the salt concentration to 400 mM before loading onto Q-Sepharose Fast Flow ion exchange resin (Amersham Biosciences, Piscataway, NJ) at approximately 1 litre CM per 5 mls resin. The column was equilibrated in 0.1 M sodium acetate, pH 6.0, 7 M urea, 0.4 M NaCl and eluted with 0.1 M sodium acetate, pH 6.0, 7 M urea, 1.5 M NaCl. Versican reactive fractions were pooled and purified a second time on a smaller volume column with the same protocol.

Concentration of purified versican was estimated using the dimethylmethylene blue (DMMB)(Serva, Heidelberg) assay to quantify sulfated glycosaminoglycans (Farndale et al, 1986), using known concentrations of chondroitin sulfate A as standards (Seikagaku). Recombinant human MMP-2 was expressed in mammalian cell cultures as previously described (Bigg et al, 2001). Soluble human MT1-MMP lacking the transmembrane and cytoplasmic tail was expressed and purified as described (Tam et al, 2004). His-tagged recombinant versican C-terminal 'G3' domain construct was expressed in *Escherichia coli*, purified, refolded and verified by fluorescence anisotropy spectroscopy, N-terminal sequencing and mass spectrometry (Pourmalek & Roberts, 2009). Purified versican or versican G3 domain constructs were incubated alone in

enzyme buffer or incubated with recombinant MMP-2 or MT1-MMP for 0 or 24 hours at 37°C.

4.4. Results

4.4.1. ConA induces degradation of versican.

As ConA is known to induce a matrix degradative phenotype accompanying an apoptotic response in fibroblast cells (Overall & Sodek, 1990), I investigated whether versican was degraded in response to ConA. Under normal conditions, versican stains strongly in the pericellular matrix of human fetal lung (HFL-1) fibroblasts (Fig. 4.1A). F-actin staining is strong and organized into elongated stress fibers. Nuclei are mostly oval shaped with a regular granular pattern of staining. In the presence of 40 µg/ml ConA for 16 hours, versican staining is dramatically reduced, f-actin staining is reduced, actin stress fibers are lost and nuclei are irregular, condensed and fragmented. In addition to maintaining cell-associated versican, HFL-1 fibroblasts also secrete versican into the media (Fig. 4.1B). In response to increasing concentrations of ConA, reactivity to versican 12C5 antibody (N-terminal domains) is lost from the media. These data indicate that both pericellular and secreted versican are degraded concomitant with apoptosis in the matrix degradative phenotype induced by ConA in HFL-1 fibroblasts.

4.4.2. ConA induces changes in MMP-2 and MT1-MMP localization.

MMP-2 and MT1-MMP immunofluorescent staining increased dramatically in response to ConA (Fig. 4.2). MT1-MMP consistently stained very strongly at 4 hours (Fig. 4.2D) followed by a decrease at 16 hours (Fig. 4.2F). This was correlated with moderate staining for MMP-2 at 4 hours (Fig. 4.2C) which increased at 16 hours (Fig. 4.2C).



Figure 4.1. Concanavalin-A induces degradation of versican concomitant with fibroblast cell apoptosis. A. Human fetal lung fibroblasts (HFL-1) at sub-confluence were incubated with or without 40 μ g/ml ConA in serum free media for 16 hours. DNA, F-actin and versican (using the antibody 2B1) were visualized by fluorescence microscopy. Nuclear condensation and fragmentation, loss of organized actin stress fibers and substantial loss of versican staining are apparent in response to ConA. Exposure and deconvolution settings were identical in the antibody channel at the different concentrations of ConA. *Scale bars* = 25 μ m. B. HFL-1 conditioned media after 16 hours treatment with 0, 10, 20 or 40 μ g/ml ConA was filter concentrated and treated with chondroitinase ABC before separation by SDS-PAGE and Western blotting for versican with 12C5 antibody. Results shown are representative of three experiments, each analyzed in duplicate.



Figure 4.2. Concanavalin-A induced changes in metalloproteinase expression and localization. Immunofluorescent staining of HFL-1 cells for MMP-2 (A, C, E) and MT1-MMP (B, D, F). Cells were treated for 0 (A & B), 4 (C & D) or 16 hours (E & F) with 40 μ g/ml ConA before fixing and staining. Exposure and deconvolution settings were identical for each antibody at the different time points. *Scale bars* = 25 μ m. G. Gelatin zymogram showing MMP-2 zymogen (pro-), intermediate and active forms in HFL-1 conditioned media over time in the presence of 40 μ g/ml ConA. Results shown are representative of three experiments, each analyzed in duplicate.

4.2E). Both transcriptional and post-translational up-regulation of MMP-2 and MT1-MMP likely contributed to the observed staining (Overall & Sodek, 1990; Yu et al, 1995; Zucker et al, 2002). Concomitant with this activation and the ensuing fibroblast cell apoptosis was a dramatic reduction in pericellular versican (Fig. 4.1A), suggesting that these enzymes could play a role in degrading versican in ConA stimulated HFL-1 cells. Consistent with the immunofluorescent staining observed, secreted MMP-2 was observed in its active form in the culture media, detected by gelatin zymography (Fig. 4.2G).

4.4.3. Microarrays

Based on the observations of rapid cell surface presentation of MT1-MMP and activation of MMP-2, I next investigated corresponding gene expression changes for all MMPs using the CLIP-CHIP complete human protease, inhibitor and homologue oligonucleotide microarray. It is well known that a net proteolytic phenotype is induced by ConA (Overall & Sodek, 1990). Stringent hybridization and wash conditions have been previously established to provide optimum specificity of hybridization and minimal background noise (Kappelhoff & Overall, 2007).

Microarray spots intensities were corrected against the background, normalized within each array and normalized between arrays before analysis. Three biological replicates were employed with two arrays per chip, giving 6 total arrays per condition. Two dimensional SAM (significance analysis of microarrays) analysis was performed according to Tusher *et al* (Tusher et al, 2001) using a 1.5 fold change cutoff and *p*-values were obtained by paired students T-test. MMP-11 was found to exhibit the highest expression change in response to ConA. This was followed by increased expression for MMP-1 and MT1-MMP respectively (Fig. 4.3). In addition to MMP-11,



Figure 4.3. Microarray analysis of differentially expressed fibroblast proteases and inhibitors in response to Concanavalin-A. HFL-1 cells were treated with 40 µg/ml ConA (Cy5) or control serum free media (Cy3) for 4 or 16 hours. Normalized data were analyzed by two dimensional SAM analysis and genes with significant expression changes are shown. Fold change of expression between 4 and 16 hour timepoints is shown. *P*-values were determined with a paired T-test. All 8 tubulin positive control spots showed significantly altered expression. Results shown represent three biological replicates each performed on duplicate arrays. several genes not previously associated with ConA that may have contributed to the matrix degradative phenotype included cathepsin L, plasminogen and tissue-type plasminogen activator. MMP-2 and MMP-7 up-regulation did not meet the significance threshold employed, but they were up-regulated with p=0.014608 and 0.000648 respectively (Appendix 1). The complete list of proteases, inhibitors, homologues and control spots printed on the chip is in Appendix 1 with significant genes highlighted and remaining genes that did not meet the significance threshold (either in fold change in expression or probability) in shades of grey. All 8 tubulin control spots were significantly down-regulated.

4.4.4. Caspase and MMP inhibition alters the apoptotic response of fibroblast cells to ConA.

To investigate the relationship between degradation of the versican-rich matrix and the intracellular events induced by ConA, I inhibited caspase and metalloproteinase activities. With the pan-caspase inhibitor Z-VAD-FMK (200 μ M), nuclei were irregular but not condensed or fragmented; some filamentous actin staining was present and there was minimal versican staining compared to the negative control caspase inhibitor Z-FA-FMK (Fig. 4.4A). Inhibition with GM6001 (20 μ M) provided protection to filamentous actin and cell morphology; and increased protection of versican staining compared to Z-VAD-FMK. Nuclei were partially irregular and condensed or fragmented. Whereas GM6001 is a broad spectrum (hydroxamate-based) MMP inhibitor, prinomastat is a structure based MMP inhibitor designed to potently and selectively inhibit MMP-2, -3, -9, -13 and MT1-MMP with Ki = 0.05, 0.3, 0.26, 0.03 and 0.33 nM respectively, having weaker affinities for MMP-1 and -7 of 8.3 and 54 nM respectively (Shalinsky et al, 1999). Prinomastat (20 μ M) gave the strongest staining



Figure 4.4. Caspase and MMP inhibition alters the apoptotic response of fibroblast cells to Concanavalin-A. A. HFL-1 cells were treated with Z-VAD-FMK poly-caspase inhibitor and Z-FA-FMK as a negative control, GM6001 broad spectrum matrix metalloproteinase inhibitor or Prinomastat MMP-2, -3, -9, -13 and MT1-MMP inhibitor, prior to treatment with 40 μ g/ml ConA. Exposure and deconvolution settings were identical in the antibody channel at the different concentrations of ConA. *Scale bars* = 25 μ m. B. MMP-2 activity in the presence of Z-FA-FMK, Z-VAD-FMK, GM6001 or Prinomastat. Results shown are representative of three experiments each analyzed in duplictae.

for versican and filamentous actin; and reduced the number of nuclei that were condensed or fragmented.

Gelatin zymograms show MMP-2 in the zymogen (pro-) form, intermediate and active form with Z-FA-FMK as well as Z-VAD-FMK; whereas with GM6001 and prinomastat, MMP-2 is exclusively in the zymogen form, indicating inhibition of activation (Fig. 4.4B).

4.4.5. MMP-2 and MT1-MMP cleave versican in vitro.

As prinomastat inhibits versican degradation subsequent to ConA stimulation most effectively and is a potent inhibitor of MMP-2 and MT1-MMP, I investigated their proteolytic capacity toward versican in vitro. Versican incubated alone was found to produce minor auto-degradation products of 42 and 50 kDa as detected by 2B1 (Fig. 4.5A & B). In the presence of MMP-2, versican degradation was apparent with a loss of high molecular weight protein and numerous proteolytic fragments detected, especially in the 30 – 60 kDa range as detected by 2B1, indicating cleavage of the Cterminal globular domain (Fig. 4.5A). Versican incubated with MT1-MMP did not show any cleavage products or any loss of high molecular weight protein as detected by 2B1 (Fig. 4.5B). However, Western blotting with the LC2 polyclonal antibody did detect a more specific cleavage, a single fragment at approximately 100 kDa. Both 2B1 and LC2 detect epitopes in versican's C-terminal G3 domain, therefore a difference in epitope accessibility must have been responsible for the difference in immunoreactivity. It is also evident that intact high molecular weight versican was not detected well by LC2, which was raised against a recombinant G3 domain construct containing the lectin-like and complement regulatory-like domains (Pourmalek & Roberts, 2009).



Figure 4.5. MMP-2 and MT1-MMP cleave versican in vitro. A. Purified versican (0.45 mg/ml) from HFL-1 conditioned medium was not incubated (lane 1), incubated alone (lane 2), incubated with recombinant human MMP-2 (10 μ g/ml) or incubated with recombinant human MMP-2 (20 μ g/ml) at 37° C for 24 hours before SDS-PAGE separation and Western blotting with 2B1 antibody. B. The same incubation was performed without or with recombinant human MT1-MMP (7 μ g/ml) and (14 μ g/ml) before SDS-PAGE separation and Western blotting with anti-versican antibodies 2B1 and LC2. Results shown are representative of three experiments, each analyzed in triplicate.

4.4.6. MT1-MMP cleaves and disaggregates recombinant versican G3.

To further investigate the nature of versican proteolysis, a recombinant versican Cterminal G3 domain construct was incubated with MMP-2 and MT1-MMP. The Histagged G3 construct was determined to have the expected 37.5 kDa molecular weight by MALDI-TOF MS (data not shown). Under denaturing but non-reducing conditions in SDS-PAGE, the constructs were detected as an aggregate at the top of the gel (Fig. 4.6 & B) consistent with the self-association that has been described for this domain (Ney et al, 2006). When incubated with MMP-2, there was no change in the appearance of the aggregated protein and no production of proteolytic fragments detected (Fig. 4.6A). With MT1-MMP there was an increase in immunoreactivity at the top of the gel, numerous proteolytic products detected from 60 – 180 kDa, as well as a prominent band near the 37.5 kDa monomer molecular weight (Fig. 4.6B). These data indicate that through a cleavage event that has a limited effect on the molecular weight of the G3 domain construct, MT1-MMP is able to disaggregate the protein aggregates into monomers and several smaller multimers.

4.5. Discussion

ConA is known to induce a matrix degrading phenotype in human fibroblasts with an increase in metalloproteinase expression and a concomitant decrease in tissue inhibitor of metalloproteinase expression (Overall & Sodek, 1990). Since versican is degraded from the provisional matrix accompanying fibroblast apoptosis in normal tissue remodeling, it was hypothesized that ConA induced apoptosis of HFL-1 fibroblasts would provide a useful tissue culture model of versican degradation. Indeed both pericellular and secreted versican are degraded from the matrix in this model which also stimulates expression and activation of MMP-2 and MT1-MMP. By microarray



Figure 4.6. MT1-MMP cleaves and disaggregates recombinant versican G3. A. Recombinant human versican His-G3 constructs were incubated with 0, 4, 8, 12, 24, 50, 100 μ g/ml recombinant human MMP-2 for 24 hours, followed by SDS-PAGE separation and detection by Western blotting with LC2 antibody. B. His-G3 constructs were incubated with 0, 4, 8, 12, 24, 50, 100 μ g/ml recombinant human MT1-MMP for 24 hours, followed by SDS-PAGE separation and detection by Western blotting with LC2 antibody. Results shown are representative of three experiments, each analyzed in triplicate. analysis I was able to confirm expression changes in MMP-1 and MT1-MMP that were consistent with previous reports (Overall & Sodek, 1990; Yu et al, 1995; Zucker et al, 2002). Though MMP-2 up-regulation did not meet the significance threshold in SAM analysis in these experiments, immunofluorescent staining confirmed the significant mobilization of both MMP-2 and MT1-MMP at the cell surface. This may indicate a greater effect of ConA on MMP-2 localization and activation rather than expression changes.

I observed substantial ConA induced expression of MMP-11 (stromelysin 3) that has not been previously reported. MMP-11 has a broad normal distribution, is expressed in normal wound healing and is associated with breast, cutaneous and colorectal carcinomas (Basset et al, 1990; Porte et al, 1995; Wolf et al, 1992). MMP-11 contributes to cancer progression and poor patient prognosis through rapid temporal expression of the active enzyme that contributes to inhibition of cancer cell apoptosis (Rio, 2005). It would be interesting to discern the role of MMP-11 in matrix resorption in response to ConA.

Moderate expression of Cathepsin L, plasminogen and t-plasminogen activator were also observed. Cathepsin L, along with MT1-MMP, is suggested to be a promising target for cancer treatment (Lah et al, 2006). Cathepsin L has recently been shown to cleave extracellular endorepellin and is associated with activated caspase-3 in apoptotic endothelial cells (Cailhier et al, 2008). Plasmin(ogen) is implicated in activating numerous MMPs (Ra & Parks, 2007) and thus along with expression of its activator (tissue-type plasminogen activator), likely contributes directly to the proteolytic phenotype.

As ConA induced apoptosis requires the sustained presence of ConA (Wang et al, 1983) I wondered whether the early and late events were separable through protease

inhibition. Caspases are intracellular proteases that are required for apoptosis, regardless of apoptotic stimuli (Reed, 2000; Salvesen & Dixit, 1997). It was predicted that caspase inhibition would alter only later stage events in ConA induced apoptosis. As expected, caspase inhibition substantially protected nuclei from condensation and fragmentation, however minimal protection from versican degradation and cytoskeletal changes was observed. The metalloproteinase inhibitors protected versican from degradation more robustly and also limited changes to the cytoskeleton. I have shown that inhibition of matrix degrading enzymes subsequent to ConA stimulation significantly preserves the actin cytoskeleton and cell morphology. This appears to partially protect cells from apoptotic nuclear changes, suggesting a link between matrix degradation and apoptosis. The concept of ECM changes affecting gene expression is termed "dynamic reciprocity" and involves ECM communication with the nucleus through membrane receptors and cytoskeletal changes, which invoke gene expression that further regulates cell morphology (Bissell et al, 1982; Nelson & Bissell, 2006). It may be that ConA induction of matrix degradation stimulates cytoskeletal changes that in turn effect gene expression and the induction of apoptosis through mechanisms of dynamic reciprocity. As versican is a major component of the pericellular matrix of HFL-1 cells, its degradation could contribute to cell signaling pathways leading to apoptosis.

The inhibition of versican degradation provided by prinomastat suggested a more specific inhibition of versicanase enzymes. While MMP-1 and MMP-7 are transcriptionally up-regulated in response to ConA (Overall & Sodek, 1990), prinomastat inhibits MMP-2, -3, -9, -13 and MT1-MMP much more potently than MMP-1 or -7. Thus, the inhibition of versican degradation and apoptotic changes observed in

the presence of prinomastat is likely due to the more potent and selective inhibition of MMP-2 and MT1-MMP.

MMP-2 has previously been shown to process a versican preparation (Passi et al, 1999). With purified versican and recombinant MMPs, I was able to better characterize cleavage by MMP-2 and show that MT1-MMP also cleaves versican, apparently more specifically. MT1-MMP was also shown to play a role in disaggregation of versican's G3 domains, the selectin-like domain that may bind a number of ECM ligands and/or an as yet undescribed cell surface receptor. Proteolytic release of versican's G3 binding interactions could have dramatic effects on the mechanical and biochemical properties of the pericellular matrix. Through releasing C-terminal binding interactions and maintaining the bulk of the protein intact, MT1-MMP could signal subsequent events in versican degradation and could alter the ways in which versican contributes to the steric hindrance and cytokine binding capacity of the pericellular matrix (Roberts, 2003).

Knockdown of MT1-MMP produces a more substantial phenotype than any other MMP knockdown to date. MT1-MMP deficient mice develop numerous connective tissue defects including soft tisue fibrosis, delayed endochondral ossification and defects in angiogenesis (Holmbeck et al, 1999; Zhou et al, 2000). These defects have been attributed to decreased collagen turnover (Holmbeck et al, 1999) and certainly altered collagen turnover contributes as MT1-MMP is critical for collagen phagocytosis (Lee et al, 2006). Yet another factor contributing to this phenotype might be that MT1-MMP deficiency contributes to the persistence of the versican-rich matrix that promotes collagen deposition.

Taken together, these data suggest that proteolytic degradation of the versican-rich matrix is associated with the loss of actin stress fibers and subsequent apoptotic
changes in cell morphology, and that degradation of the versican-rich matrix occurs at least in part through MMP-2 and MT1-MMP dependent processes.

4.6. References

Asher R, Perides G, Vanderhaeghen JJ, Bignami A (1991) Extracellular matrix of central nervous system white matter: demonstration of an hyaluronate-protein complex. *J Neurosci Res* **28**(3): 410-421

Basbaum CB, Werb Z (1996) Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. *Curr Opin Cell Biol* **8**(5): 731-738

Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC, Chambon P (1990) A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* **348**(6303): 699-704

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1996) Proteoglycan deposition in pulmonary fibrosis. *Am J Respir Crit Care Med* **154**(6 Pt 1): 1819-1828

Bensadoun ES, Burke AK, Hogg JC, Roberts CR (1997) Proteoglycans in granulomatous lung diseases. *Eur Respir J* **10**(12): 2731-2737

Bigg HF, Morrison CJ, Butler GS, Bogoyevitch MA, Wang Z, Soloway PD, Overall CM (2001) Tissue inhibitor of metalloproteinases-4 inhibits but does not support the activation of gelatinase A via efficient inhibition of membrane type 1-matrix metalloproteinase. *Cancer Res* **61**(9): 3610-3618

Bissell MJ, Hall HG, Parry G (1982) How does the extracellular matrix direct gene expression? *J Theor Biol* **99**(1): 31-68

Brennan MJ, Oldberg A, Pierschbacher MD, Ruoslahti E (1984) Chondroitin/dermatan sulfate proteoglycan in human fetal membranes. Demonstration of an antigenically similar proteoglycan in fibroblasts. *J Biol Chem* **259**(22): 13742-13750

Brown PD, Bloxidge RE, Anderson E, Howell A (1993a) Expression of activated gelatinase in human invasive breast carcinoma. *Clin Exp Metastasis* **11**(2): 183-189

Brown PD, Bloxidge RE, Stuart NSA, Galler KC, Carmichael J (1993b) Association Between Expression of Activated 72-Kilodalton Gelatinase and Tumor Spread in Non-Small-Cell Lung Carcinoma. *J Natl Cancer Inst* **85**(7): 574-578

Cailhier J-F, Sirois I, Laplante P, Lepage S, Raymond M-A, Brassard N, Prat A, Iozzo RV, Pshezhetsky AV, Hebert M-J (2008) Caspase-3 Activation Triggers Extracellular Cathepsin L Release and Endorepellin Proteolysis. *J Biol Chem* **283**(40): 27220-27229

Desmouliere A, Redard M, Darby I, Gabbiani G (1995) Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* **146**(1): 56-66

Evanko SP, Raines EW, Ross R, Gold LI, Wight TN (1998) Proteoglycan distribution in lesions of atherosclerosis depends on lesion severity, structural characteristics, and the proximity of plateletderived growth factor and transforming growth factor-beta. *Am J Pathol* **152**(2): 533-546

Farndale RW, Buttle DJ, Barrett AJ (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* **883**(2): 173-177

Garcia-Alvarez J, Ramirez A, Sampieri CL, Nuttall RK, Edwards DR, Selman M, Pardo A (2006) Membrane type-matrix metalloproteinases in idiopathic pulmonary fibrosis. *Sarcoidosis Vasc Diffuse Lung Dis* **23**: 13-21

Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Gehron Robey P, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H (1999) MT1-MMP-Deficient Mice Develop Dwarfism, Osteopenia, Arthritis, and Connective Tissue Disease due to Inadequate Collagen Turnover. *Cell* **99**(1): 81-92

Iozzo RV (1998) Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* **67**: 609-652

Isogai Z, Aspberg A, Keene DR, Ono RN, Reinhardt DP, Sakai LY (2002) Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. *J Biol Chem* **277**(6): 4565-4572

Isogai Z, Shinomura T, Yamakawa N, Takeuchi J, Tsuji T, Heinegard D, Kimata K (1996) 2B1 antigen characteristically expressed on extracellular matrices of human malignant tumors is a large chondroitin sulfate proteoglycan, PG-M/versican. *Cancer Res* **56**(17): 3902-3908

Jiang A, Lehti K, Wang X, Weiss SJ, Keski-Oja J, Pei D (2001) Regulation of membrane-type matrix metalloproteinase 1 activity by dynamin-mediated endocytosis. *PNAS* **98**(24): 13693-13698

Kappelhoff R, Overall C (2007) The CLIP-CHIP oligonucleotide microarray: dedicated array for analysis of all protease, nonproteolytic homolog, and inhibitor gene transcripts in human and mouse. *Curr Protoc Protein Sci* **Chapter 21:** Unit 21.19

Kodama J, Hasengaowa, Kusumoto T, Seki N, Matsuo T, Nakamura K, Hongo A, Hiramatsu Y (2007a) Versican expression in human cervical cancer. *Eur J Cancer* **43**(9): 1460-1466

Kodama J, Hasengaowa, Kusumoto T, Seki N, Matsuo T, Ojima Y, Nakamura K, Hongo A, Hiramatsu Y (2007b) Prognostic significance of stromal versican expression in human endometrial cancer. *Ann Oncol* **18**(2): 269-274

Krusius T, Ruoslahti E (1986) Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. *Proceedings of the National Academy of Sciences* **83**(20): 7683-7687

Kulkarni GV, McCulloch CA (1995) Concanavalin A induced apoptosis in fibroblasts: the role of cell surface carbohydrates in lectin mediated cytotoxicity. *J Cell Physiol* **165**(1): 119-133

Lah TT, Alonso MBD, Van Noorden CJF (2006) Antiprotease therapy in cancer: hot or not? *Expert Opin Biol Ther* **6**(3): 257-279

Lee H, Overall CM, McCulloch CA, Sodek J (2006) A Critical Role for the Membrane-type 1 Matrix Metalloproteinase in Collagen Phagocytosis. *Mol Biol Cell* **17**(11): 4812-4826

Madlener M, Parks WC, Werner S (1998) Matrix Metalloproteinases (MMPs) and Their Physiological Inhibitors (TIMPs) Are Differentially Expressed during Excisional Skin Wound Repair. *Exp Cell Res* **242**(1): 201-210

Maurice SB, Roberts CR (2009) Versican-ADAMTS-2 interactions in human pulmonary fibrosis: the proteoglycan versican binds the procollagen N-propeptidase ADAMTS-2 and regulates its activity. *In preparation.*

Mjaatvedt CH, Yamamura H, Capehart AA, Turner D, Markwald RR (1998) The Cspg2 gene, disrupted in the hdf mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol* **202**(1): 56-66

Nelson CM, Bissell MJ (2006) Of Extracellular Matrix, Scaffolds, and Signaling: Tissue Architecture Regulates Development, Homeostasis, and Cancer. *Annu Rev Cell Dev Biol* **22**(1): 287-309

Ney A, Booms P, Epple G, Morgelin M, Guo G, Kettelgerdes G, Gebner R, Robinson PN (2006) Calcium-dependent self-association of the C-type lectin domain of versican. *The International Journal of Biochemistry & Cell Biology* **38**: 23-29

Overall CM, King AE, Sam DK, Ong AD, Lau TTY, Wallon UM, DeClerck YA, Atherstone J (1999) Identification of the tissue inhibitor of metalloproteinases-2 (TIMP-2) binding site on the hemopexin carboxyl domain of human gelatinase A by site-directed mutagenesis. The hierarchical role in binding TIMP-2 of the unique cationic clusters of hemopexin modules III AND IV. *J Biol Chem* **274**(7): 4421-4429 Overall CM, Sodek J (1990) Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. *J Biol Chem* **265**(34): 21141-21151

Overall CM, Tam E, McQuibban GA, Morrison C, Wallon UM, Bigg HF, King AE, Roberts CR (2000) Domain Interactions in the Gelatinase A TIMP-2 MT1-MMP Activation Complex. The ectodomain of the 44-kDa form of membrane type-1 matrix metalloproteinase does not modulate gelatinase A activation. *J Biol Chem* **275**(50): 39497-39506

Passi A, Negrini D, Albertini R, Miserocchi G, De Luca G (1999) The sensitivity of versican from rabbit lung to gelatinase A (MMP-2) and B (MMP-9) and its involvement in the development of hydraulic lung edema. *FEBS Lett* **456**(1): 93-96

Porte H, Chastre E, Prevot S, Nordlinger B, Empereur S, Basset P, Chambon P, Gespach C (1995) Neoplastic progression of human colorectal cancer is associated with overexpression of the stromelysin-3 and BM-40/SPARC genes. *Int J Cancer* **64**(1): 70-75

Pourmalek S, Roberts CR (2009) Macrophage mediated proteolysis of versican in human pulmonary fibrosis. *in preparation*

Pukkila M, Kosunen A, Ropponen K, Virtaniemi J, Kellokoski J, Kumpulainen E, Pirinen R, Nuutinen J, Johansson R, Kosma V-M (2007) High stromal versican expression predicts unfavourable outcome in oral squamous cell carcinoma. *J Clin Pathol* **60**(3): 267-272

Ra H-J, Parks WC (2007) Control of matrix metalloproteinase catalytic activity. *Matrix Biol* **26**(8): 587-596

Reed JC (2000) Mechanisms of Apoptosis. Am J Pathol 157(5): 1415-1430

Ricciardelli C, Brooks JH, Suwiwat S, Sakko AJ, Mayne K, Raymond WA, Seshadri R, LeBaron RG, Horsfall DJ (2002) Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer. *Clin Cancer Res* **8**(4): 1054-1060

Ricciardelli C, Mayne K, Sykes PJ, Raymond WA, McCaul K, Marshall VR, Horsfall DJ (1998) Elevated levels of versican but not decorin predict disease progression in early-stage prostate cancer. *Clin Cancer Res* **4**(4): 963-971

Rio MC (2005) From a unique cell to metastasis is a long way to go: clues to stromelysin-3 participation. *Biochimie* **87:** 299-306

Roberts CR (2003) Versican in the Cell Biology of Pulmonary Fibrosis. In *Proteoglycans in Lung Disease*, Garg HG, Roughley PJ, Hales CA (eds), Vol. 168, pp 191-212. New York, NY: Marcel Dekker

Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, Lopez-Otin C, Shapiro S, Inada M, Krane S, Allen E, Chung D, Weiss SJ (2004) Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *J Cell Biol* **167**(4): 769-781

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J (2003) TM4: A Free, Open-Source System for Microarray Data Management and Analysis. *Biotechniques* **34**(2): 374-378

Salvesen GS, Dixit VM (1997) Caspases: intracellular signaling by proteolysis. Cell 91(4): 443-446

Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M (1994) A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* **370**(6484): 61-65

Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R, Barrios R, Pardo A (2000) TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? *Am J Physiol Lung Cell Mol Physiol* **279**(3): L562-574

Shalinsky DR, Brekken J, Zou H, Mcdermott CD, Forsyth P, Edwards D, Margosiak S, Bender S, Truitt G, Wood A, Varki NM, Appelt K (1999) Broad Antitumor and Antiangiogenic Activities of AG3340, a Potent and Selective MMP Inhibitor Undergoing Advanced Oncology Clinical Trials. *Ann N Y Acad Sci* **878:** 236-270

Smith EE, Goldstein IJ (1967) Protein-carbohydrate interaction. V. Further inhibition studies directed toward defining the stereochemical requirements of the reactive sites of concanavalin A. *Arch Biochem Biophys* **121**(1): 88-95

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI (1995) Mechanism Of Cell Surface Activation Of 72-kDa Type IV Collagenase. *J Biol Chem* **270**(10): 5331-5338

Suwiwat S, Ricciardelli C, Tammi R, Tammi M, Auvinen P, Kosma VM, LeBaron RG, Raymond WA, Tilley WD, Horsfall DJ (2004) Expression of extracellular matrix components versican, chondroitin

sulfate, tenascin, and hyaluronan, and their association with disease outcome in node-negative breast cancer. *Clin Cancer Res* **10**(7): 2491-2498

Tam EM, Morrison CJ, Wu YI, Stack MS, Overall CM (2004) Membrane protease proteomics: Isotopecoded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Nat Acad Sci* **101**(18): 6917-6922

Touab M, Arumi-Uria M, Barranco C, Bassols A (2003) Expression of the proteoglycans versican and mel-CSPG in dysplastic nevi. *Am J Clin Pathol* **119**(4): 587-593

Touab M, Villena J, Barranco C, Arumi-Uria M, Bassols A (2002) Versican is differentially expressed in human melanoma and may play a role in tumor development. *Am J Pathol* **160**(2): 549-557

Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Nat Acad Sci* **98:** 5116-5121

Wang H-M, Hurum S, Sodek J (1983) Con A stimulation of collagenase synthesis by human gingival fibroblasts. *J Periodontal Res* **18**(2): 149-155

Wight TN, Lara S, Riessen R, Le Baron R, Isner J (1997) Selective deposits of versican in the extracellular matrix of restenotic lesions from human peripheral arteries. *Am J Pathol* **151**(4): 963-973

Will H, Atkinson SJ, Butler GS, Smith B, Murphy G (1996) The Soluble Catalytic Domain of Membrane Type 1Matrix Metalloproteinase Cleaves the Propeptide of Progelatinase A and Initiates Autoproteolytic Activation. Regulation by TIMP-2 and TIMP-3. *J Biol Chem* **271**(29): 17119-17123

Wolf C, Chenard MP, Durand de Grossouvre P, Bellocq JP, Chambon P, Basset P (1992) Breastcancer-associated stromelysin-3 gene is expressed in basal cell carcinoma and during cutaneous wound healing. *J Invest Dermatol* **99:** 870-872

Yu M, Sato H, Seiki M, Thompson EW (1995) Complex Regulation of Membrane-Type Matrix Metalloproteinase Expression and Matrix Metalloproteinase-2 Activation by Concanavalin A in MDA-MB-231 Human Breast Cancer Cells. *Cancer Res* **55**(15): 3272-3277

Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K (2000) Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proc Nat Acad Sci* **97**(8): 4052-4057 Zucker S, Hymowitz M, Conner CE, DiYanni EA, Cao J (2002) Rapid trafficking of membrane type 1matrix metalloproteinase to the cell surface regulates progelatinase A activation. *Lab Invest* **82**(12): 1673-1684

CHAPTER 5 – CONCLUDING REMARKS AND FUTURE DIRECTIONS

This thesis has focused on elucidating novel mechanisms of versican proteolysis that may be relevant to normal and pathological versican turnover. My studies shed light on versican synthesis and processing by human fetal lung cells in culture. I have successfully demonstrated *in vitro* cleavage by three cell surface-associated metalloproteinases that may be involved in versican processing *in vivo*. These findings advance our understanding of versican proteolytic processing and move the field further toward a complete understanding of physiological turnover of versican in normal and pathological remodeling.

ADAMTS-2 has historically been considered highly specific based on its sensitivity to substrate conformation. Heat denaturation of type I or II collagen ablates N-propeptide cleavage (Tuderman et al, 1978). This finding has since been taken to imply that ADAMTS-2 processes only procollagens. Yet an alternative explanation for the specificity of ADAMTS-2 is that the enzyme performs a quality control function, only allowing properly folded procollagens to be assembled into fibrils (Prockop et al, 1998). In addition to types I and II collagen, ADAMTS-2 is now known to also process collagen types III and V (Colige et al, 2005; Wang et al, 2003). Based on domain composition ADAMTS-2 appears to have be designed for functions in addition to procollagen processing (Colige et al, 1997; Prockop et al, 1998). Expression levels also suggest the enzyme may have functions other than collagen processing (Colige et al, 1997). Surely a better understanding of the activation states of the enzyme will help immeasurably in further analysis of its capacity to cleave non-collagen substrates (Colige et al, 2005).

While the specificity of ADAMTS-2 toward exclusively types I and II collagen was dogma in the field for over a decade, we now know that type III collagen is in fact processed by the same enzyme (Greenspan & Wang, 2005). The procollagen C-propeptidases (bone morphogenetic protein-1 and the tolloid-like metalloproteinases) are well known to be involved in signaling events that occur in morphogenic and homeostatic events in the ECM (Ge & Greenspan, 2006). Likewise, it was predicted that ADAMTS-2 not only processes versican but may have other actions that regulate tissue remodeling. I have shown that versican is processed by ADAMTS-2 in vitro and provided strong evidence for a binding interaction between the two. This work lays the ground work for future studies necessary to further characterize the pattern of colocalization and cleavage events described here.

Further work will need to confirm cleavage with a recombinant enzyme and investigate the effects of the 7 different enzyme activation states on its activity toward versican (Colige et al, 2005). The use of ADAMTS-2 null mice could potentially be used to validate the *in vivo* significance of our claims but interpreting results would be a challenge. ADAMTS-2 null mice have lungs that do not develop properly and resemble an emphysematous phenotype (Le Goff et al, 2006). Additionally, there is not a good model for inducing chronic pulmonary fibrosis. The most common model of pulmonary fibrosis is bleomycin induced fibrosis which denudes epithelium and induces a rapid, acute inflammatory response that is very different from human pulmonary fibrosis.

The current 'standard of care' therapy for idiopathic pulmonary fibrosis is of limited therapeutic benefit and the same is true for most forms of fibrosis. Historically the deposition of collagen rich scar tissue was considered irreversible. In the last decade numerous reports have shown a degree of reversibility in animal models. However, the increased speed of disease progression in animal models may not accurately mimic the

human pathologies which can advance over a much more prolonged period (Friedman, 2007). One problematic aspect of the more prolonged human disease may be the crosslinking of matrix molecules, particularly collagen, that occurs in more mature scars.

MMP-2 has previously been shown to cleave versican (Passi et al, 1999). Thus as MT1-MMP activates MMP-2 in a cell surface activation complex (Will et al, 1996), I queried both MMP-2 and MT1-MMP cleavage of versican. I found several detectable products of versican produced by MMP-2 and an apparently more specific cleavage by MT1-MMP. In combination there are several newly identified products of versican that need to be further studied to determine if these products are produce *in vivo* and if they have physiological significance. The singular cleavage event induced by MT1-MMP seems to affect versican's G3 domain aggregation. If this is an *in vivo* role of MT1-MMP, it could have dramatic effects on the properties of the ECM.

MT1-MMP has a unique substrate binding preference from the other MMPs (Kridel et al, 2002). It appears to play a dramatic role in cancer metastasis (Sato et al, 2005) and has developmental roles independent of MMP-2 activation (Oblander et al, 2005). MT1-MMP null mice have a more dramatic phenotype than any of the knockout mice to date and exhibit numerous skeletal abnormalities including soft connective tissue fibrosis (Holmbeck et al, 1999). This connective tissue fibrosis is blamed on defective collagenolysis which is likely at least part of the phenotype. But if MT1-MMP cleavage of versican is required to signal an end to the fibroproliferative phase, then MT1-MMP deficiency could lead to persistent versican and persistent collagen deposition that causes fibrosis.

In order to validate the *in vivo* significance of the *in vitro* proteolytic events described here, future work will need to characterize the N-terminal proteolytic sequences described here and determine the presence of these putative processing sites *in vivo*. Cleavage site neo-epitopes are difficult to determine with proteoglycan substrates, presumably because of interference from the glycosylations and glycosaminoglycans. I have made numerous attempts to characterize cleavage products by Edman Degradation chemical sequencing but these attempts have been mostly frustrated by the concentration of protein transferred to sequencing membrane, the charged and glycosylated nature of versican's side chains or possible binding interactions that are not solubilized by urea and SDS.

In order to circumvent the challenges inherent in chemical sequencing of proteoglycans, I have employed mass spectrometry based techniques to characterize versican degradation. The results have shown some promise, but unfortunately are still confronted with difficulties that are presumably again related to the difficult nature of the protein and its substitutions. However, with appropriate improvements in sample preparation that can accommodate this challenging substrate, it is possible that mass spectrometry will soon be able to offer great advances in proteoglycan characterization.

Mass spectrometry based proteomics is becoming a very powerful tool for biological and medical research. As instruments and protocols improve and become more affordable, it is likely that this technology will in the future be used for clinical diagnosis. Thus, improved detection of versican is not only relevant to medical research and therapeutic avenues, but also to diagnosis and treatment. Once pathways and consequences of versican processing are more fully elucidated, it may be possible to identify biomarkers of disease prognosis and progression, as is suggested for ECM metabolites in general (Moseley et al, 2004).

I have investigated degradation of versican in a model of apoptosis associated with extracellular matrix proteolysis. While the expression of several metalloproteinases is well characterized in this model, including the putative versican-degrading enzymes MMP-2 and MT1-MMP, it is unclear what relative contribution these known enzymes make to the total matrix degradation observed. I have attempted to investigate the complete repertoire of proteases induced by ConA in Human Fetal Lung fibroblasts. Additionally this work has identified several new candidate proteases that may be important in degrading versican in this model of apoptosis.

To date, versican cleavage has been documented by MMP-1, -2, -3, -7 and -9; and ADAMTS-1, -4, & -9 (Halpert et al, 1996; Jonsson-Rylander et al, 2005; Passi et al, 1999; Perides et al, 1995; Sandy et al, 2001; Somerville et al, 2003; Westling et al, 2004). I have been able to expand this repertoire by two enzymes and provide some characterization of their cleavage products. This is beginning to look like a long list of potential *in vivo* versican processing enzymes, yet the list is likely not yet complete, nor do we fully understand all the processing events of each of these enzymes. Recent work has predicted that many more enzymes and cleavages were likely to occur than the current state of knowledge describes (Kenagy et al, 2006). Indeed much work has been done to identify a key aggrecan degrading enzyme and though no such enzyme has yet been identified, much insight into the complexity of turnover of this large proteoglycan has been gained (Fosang et al, 2008; Sandy, 2006). Thus a

understand aberrant events in proteolytic pathways.

In addition to the complexity inherent in multiple proteases performing multiple cleavages, protease regulation is complex. Though the search for a single protease to target for therapeutic intervention will remain tempting, it is increasingly clear that all

large challenge lies ahead to inventory all proteases and cleavages before we can fully

proteases are part of webs and cascades of interconnected pathways, often leading to pleiotropic roles for a given protease (McCawley & Matrisian, 2001; Overall & Dean, 2006; Overall & Kleifeld, 2006). This does not mean that identification of a single critical enzyme that is dysregulated and therefore targetable in a certain disease is not possible. But, in order to find this elusive target, we need to first understand the whole web that it is a part of.

It is now abundantly clear from the many clinical trials of MMP inhibition in cancer and arthritis that broad spectrum MMP inhibition isn't an therapeutic option (Coussens et al, 2002; Egeblad & Werb, 2002; Murphy & Nagase, 2008). In addition to needing more specific inhibitors and a more detailed understanding of the proteases that are targets or anti-targets in cancer and other diseases, an understanding of protease balance in normal remodeling is also required. Whereas all proteases presumably have a normal function and many are implicated in normal wound healing, it is perhaps the imbalance of proteases and inhibitors that is detrimental in pathological wound healing (Wynn, 2007).

In addition to the need to characterize the complete repertoire of proteases acting in complex physiological processes, it is also critical to understand the different paths of physiological remodeling that can lead to complete regeneration, partial scarring, or perpetuative scarring disorders. These diverse but related processes may involve subtle alterations in expression and activity of the same proteases. Interestingly, turnover of the cartilage proteoglycan aggrecan appears to involve different subsets of metalloproteinases that contribute to distinct remodeling processes (Sandy, 2006). Likewise, versican turnover under different conditions could involve different proteolytic pathways.

Though the field is complex, progress is being made and I feel confident that the work contained in this thesis will make a valuable contribution to our understanding of versican turnover and lead to more answers in the future. As progress is made with new techniques like mass spectrometry, I expect we will be able to gather much more detailed information about precise cleavage events and in turn gain a more thorough understanding of molecular events of normal and aberrant remodeling. This work represents one more step toward better diagnosis and the eventual rational design of therapies for dysregulated events of versican turnover.

5.1. References

Colige A, Li S-W, Sieron AL, Nusgens BV, Prockop DJ, Lapiere CM (1997) cDNA cloning and expression of bovine procollagen I N-proteinase: A new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. *Proc Natl Acad Sci U S A* **94**(6): 2374-2379

Colige A, Ruggiero F, Vandenberghe I, Dubail J, Kesteloot F, Van Beeumen J, Beschin A, Brys L, Lapiere CM, Nusgens B (2005) Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. *J Biol Chem* **280**(41): 34397-34408

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix Metalloproteinase Inhibitors and Cancer--Trials and Tribulations. *Science* **295**(5564): 2387-2392

Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* **2**(3): 161-174

Fosang AJ, Rogerson FM, East CJ, Stanton H (2008) ADAMTS-5: the story so far. *Eur Cell Mater* **5**(15): 11-26

Friedman S (2007) Reversibility of hepatic fibrosis and cirrhosis—is it all hype? *Nat Clin Pract Gastroenterol Hepatol* **4**: 236-237

Ge G, Greenspan DS (2006) Developmental roles of the BMP1/TLD metalloproteinases. *Birth Defects Res C Embryo Today* **78**(1): 47-68

Greenspan DS, Wang W-M (2005) Overview of ADAMTS proteinases and ADAMTS-2. In *The ADAM family of proteases*, Hooper NM, Lendeckel U (eds), Vol. 4, pp 261-282. Netherlands: Springer

Halpert I, Sires UI, Roby JD, Potter-Perigo S, Wight TN, Shapiro SD, Welgus HG, Wickline SA, Parks WC (1996) Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. *Proc Natl Acad Sci U S A* **93**(18): 9748-9753

Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Gehron Robey P, Poole AR, Pidoux I, Ward JM, Birkedal-Hansen H (1999) MT1-MMP-Deficient Mice Develop Dwarfism, Osteopenia, Arthritis, and Connective Tissue Disease due to Inadequate Collagen Turnover. *Cell* **99**(1): 81-92

Jonsson-Rylander AC, Nilsson T, Fritsche-Danielson R, Hammarstrom A, Behrendt M, Andersson JO, Lindgren K, Andersson AK, Wallbrandt P, Rosengren B, Brodin P, Thelin A, Westin A, Hurt-Camejo E, Lee-Sogaard CH (2005) Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican. *Arterioscler Thromb Vasc Biol* **25**(1): 180-185

Kenagy RD, Plaas AH, Wight TN (2006) Versican Degradation and Vascular Disease. *Trends in Cardiovascular Medicine* **16**(6): 209-215

Kridel SJ, Sawai H, Ratnikov BI, Chen EI, Li W, Godzik A, Strongin AY, Smith JW (2002) A unique substrate binding mode discriminates membrane type-1 matrix metalloproteinase from other matrix metalloproteinases. *J Biol Chem* **277**(26): 23788-23793

Le Goff C, Somerville RPT, Kesteloot F, Powell K, Birk DE, Colige AC, Apte SS (2006) Regulation of procollagen amino-propeptide processing during mouse embryogenesis by specialization of homologous ADAMTS proteases: insights on collagen biosynthesis and dermatosparaxis. *Development* **133**(8): 1587-1596

McCawley LJ, Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* **13**: 534-540

Moseley R, Stewart JE, Stephens P, Waddington RJ, Thomas DW (2004) Extracellular matrix metabolites as potential biomarkers of disease activity in wound fluid: lessons learned from other inflammatory diseases? *British Journal of Dermatology* **150**(3): 401-413

Murphy G, Nagase H (2008) Reappraising metalloproteinases in rheumatoid arthritis and osteoarthritis: destruction or repair? *Nat Clin Pract Rheum* **4**(3): 128-135

Oblander SA, Zhou Z, Galvez BG, Starcher B, Shannon JM, Durbeej M, Arroyo AG, Tryggvason K, Apte SS (2005) Distinctive functions of membrane type 1 matrix-metalloprotease (MT1-MMP or MMP-14) in lung and submandibular gland development are independent of its role in pro-MMP-2 activation. *Developmental Biology* **277**(1): 255-269

Overall C, Dean R (2006) Degradomics: Systems biology of the protease web. Pleiotropic roles of MMPs in cancer. *Cancer Metastasis Rev* **25**(1): 69-75

Overall CM, Kleifeld O (2006) Tumour microenvironment - Opinion: Validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* **6**(3): 227-239

Passi A, Negrini D, Albertini R, Miserocchi G, De Luca G (1999) The sensitivity of versican from rabbit lung to gelatinase A (MMP-2) and B (MMP-9) and its involvement in the development of hydraulic lung edema. *FEBS Lett* **456**(1): 93-96

Perides G, Asher RA, Lark MW, Lane WS, Robinson RA, Bignami A (1995) Glial hyaluronate-binding protein: a product of metalloproteinase digestion of versican? *Biochem J* **312** (**Pt 2**): 377-384

Prockop DJ, Sieron AL, Li S-W (1998) Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. *Matrix Biol* **16**(7): 399-408

Sandy JD (2006) A contentious issue finds some clarity: on the independent and complementary roles of aggrecanase activity and MMP activity in human joint aggrecanolysis. *Osteoarthritis and Cartilage* **14**(2): 95-100

Sandy JD, Westling J, Kenagy RD, Iruela-Arispe ML, Verscharen C, Rodriguez-Mazaneque JC, Zimmermann DR, Lemire JM, Fischer JW, Wight TN, Clowes AW (2001) Versican V1 proteolysis in human aorta in vivo occurs at the Glu441-Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4. *J Biol Chem* **276**(16): 13372-13378

Sato H, Takino T, Miyamori H (2005) Roles of membrane-type matrix metalloproteinase-1 in tumor invasion and metastasis. *Cancer Sci* **96**(4): 212-217

Somerville RP, Longpre JM, Jungers KA, Engle JM, Ross M, Evanko S, Wight TN, Leduc R, Apte SS (2003) Characterization of ADAMTS-9 and ADAMTS-20 as a distinct ADAMTS subfamily related to Caenorhabditis elegans GON-1. *J Biol Chem* **278**(11): 9503-9513

Tuderman L, Kivirikko KI, Prockop DJ (1978) Partial Purification and Characterization of a Neutral Protease Which Cleaves the N-Terminal Propertides from Procollagen. *Biochemistry* **17**(15): 2948-2954

Wang WM, Lee S, Steiglitz BM, Scott IC, Lebares CC, Allen ML, Brenner MC, Takahara K, Greenspan DS (2003) Transforming growth factor-beta induces secretion of activated ADAMTS-2. A procollagen III N-proteinase. *J Biol Chem* **278**(21): 19549-19557

Westling J, Gottschall PE, Thompson VP, Cockburn A, Perides G, Zimmermann DR, Sandy JD (2004) ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu405-Gln406 to generate glial hyaluronate binding protein. *Biochem J* **377**(Pt 3): 787-795 Will H, Atkinson SJ, Butler GS, Smith B, Murphy G (1996) The Soluble Catalytic Domain of Membrane Type 1Matrix Metalloproteinase Cleaves the Propeptide of Progelatinase A and Initiates Autoproteolytic Activation. Regulation by TIMP-2 and TIMP-3. *J Biol Chem* **271**(29): 17119-17123

Wynn TA (2007) Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest* **117**(3): 524-529

Appendix 1. Complete data set of CLIP-CHIP proteinase, inhibitor and control spot fold changes and *p*-values.

Significantly regulated genes from Figure 4.3 are highlighted in orange or green for up- or downregulation respectively. Non-significant genes are shaded grey with fold changes greater than one light and fold changes less than one dark. Normalized data were analyzed by two dimensional SAM (significance analysis of microarrays) analysis with fold change cutoff of 1.5 and *p*-values were determined with paired T-tests. A, Proteinases, B, Inhibitors, C, Control spots.

A. Proteinases

| RefSeq | Abbreviation | Description | Fold change | <i>p</i> -value |
|-----------|--------------|------------------------------------|-------------|-----------------|
| NM_022060 | ABHD4 | abhydrolase dom. containing 4 | 1.454261 | 0.000254 |
| NM_001097 | ACR | acrosin | 1.043162 | 0.473111 |
| NM_001640 | APEH | acylaminoacyl-peptidase | 0.884535 | 0.009870 |
| NM_021794 | ADAM30 | ADAM 30 | 0.919071 | 0.071365 |
| NM_145004 | ADAM32 | ADAM 32 | 1.403959 | 0.230837 |
| AL117415 | ADAM33 | ADAM 33 | 1.134082 | 0.052602 |
| NM_001110 | ADAM10 | ADAM10 | 1.134054 | 0.084147 |
| NM_002390 | ADAM11 | ADAM11 | 1.278375 | 0.167937 |
| NM_003474 | ADAM12 | ADAM12 | 1.029362 | 0.497379 |
| NM_003815 | ADAM15 | ADAM15 | 0.879478 | 0.003346 |
| NM_003183 | ADAM17 | ADAM17 | 1.042539 | 0.330290 |
| NM_014237 | ADAM18 | ADAM18 | 0.942399 | 0.367394 |
| NM_033274 | ADAM19 | ADAM19 | 1.062181 | 0.363387 |
| NM_001464 | ADAM2 | ADAM2/Fertilin-b | 1.048863 | 0.316374 |
| NM_003814 | ADAM20 | ADAM20 | 1.002430 | 0.985725 |
| NM_003813 | ADAM21 | ADAM21 | 0.577993 | 6.96E-07 |
| NM_021723 | ADAM22 | ADAM22 | 1.048896 | 0.205405 |
| NM_003812 | ADAM23 | ADAM23 | 1.075683 | 0.211801 |
| NM_014265 | ADAM28 | ADAM28 | 1.133435 | 0.116771 |
| NM_014269 | ADAM29 | ADAM29 | 0.844513 | 0.285773 |
| AF215824 | ADAM7 | ADAM7 | 1.306727 | 0.175068 |
| NM_001109 | ADAM8 | ADAM8 | 0.988350 | 0.799768 |
| NM_003816 | ADAM9 | ADAM9 | 0.833627 | 0.011397 |
| NM_006988 | ADAMTS1 | ADAMTS1 | 0.824290 | 0.000376 |
| AF163762 | ADAMTS10 | ADAMTS10 | 1.143656 | 0.027877 |
| NM_030955 | ADAMTS12 | ADAMTS12 | 0.715973 | 0.000259 |
| AB069698 | ADAMIS13 | ADAMIS13 | 1.019927 | 0.611/18 |
| NM_080722 | ADAMIS14 | ADAMIS14 | 0.890824 | 0.03/1/2 |
| NM_139055 | ADAMIS15 | ADAMIS15 | 1.359314 | 0.001548 |
| NM_139056 | ADAMIS16 | ADAMIS16 | 1.101893 | 0.088858 |
| NM_139057 | ADAMIS1/ | ADAMIS1/ | 1.118995 | 0.015415 |
| NM_139054 | ADAMIS18 | ADAMIS18 | 0.961064 | 0.463806 |
| NM_133638 | ADAMIS19 | ADAMIS19 | 1.415382 | 0.195874 |
| NM_014244 | ADAMIS2 | ADAMIS2 | 1.098380 | 0.075114 |
| NM_025003 | ADAMIS20 | ADAMIS20 | 0.547807 | 0.873311 |
| AB002364 | ADAMIS3 | ADAMIS3 | 1.398798 | 0.030722 |
| NM_005099 | ADAMIS4 | ADAMIS4 | 1.155083 | 0.036036 |
| NM_007038 | ADAMIS5 | ADAMIS5/11 | 1.152815 | 0.024532 |
| NM_014273 | ADAMIS6 | ADAMIS | 0.931969 | 0.060252 |
| NM_014272 | ADAMIS/ | ADAMIS/ | 1.180135 | 0.004580 |
| NM_007037 | ADAMTS8 | | 1.268601 | 0.001373 |
| ABU37733 | ADAIVIT59 | ADAIVIT59 | 1.144522 | 0.327950 |
| NM_001129 | AEBPI | adipocyte-ennancer binding prot. 1 | 1.056484 | 0.301068 |
| NM_006796 | AFG3L2 | Algo-like protein 2 | 1.109291 | 0.095558 |
| NM_004262 | HAI | airway-trypsin-like protease | 1.003150 | 0.960339 |
| NM_000666 | ACY1 | aminoacylase | 1.086611 | 0.062162 |

| NM_001977 | ENPEP | aminopeptidase A | 0.996591 | 0.896260 |
|-----------------------|------------------------------|--|--|--|
| NM 020216 | RNPEP | aminopeptidase B | 0.997609 | 0.844104 |
| NM_018226 | RNPEPL1 | aminopeptidase B-like 1 | 1.011218 | 0.791509 |
| NM 022350 | AMPEP | aminopeptidase MAMS | 0.974787 | 0.662580 |
| NM_001150 | ANPEP | aminopeptidase N | 1.000585 | 0.930189 |
| NM_032823 | AOPEP | aminopeptidase O | 1.007141 | 0.946547 |
| NM 022098 | PEPP | aminopeptidase P homologue | 0.871418 | 0.000921 |
| BC007579 | XPNPEP1 | aminopeptidase P1 | 1.010884 | 0.962761 |
| AB011097 | ARTS1 | aminopeptidase PILS | 0.924429 | 0.522424 |
| AK075131 | AQPEP | aminopeptidase Q | 0.920071 | 0.750169 |
| NM 024663 | NPEPL1 | aminopeptidase-like 1 | 1.093503 | 0.201465 |
| NM_006463 | AMSH | AMSH | 0 701548 | 0.001395 |
| AB037794 | AMSH2 | AMSH 2 | 1 589992 | 0.338583 |
| NM 000789 | ACE | angiotensin-converting enzyme 1 | 1.772955 | 0.002470 |
| NM 021804 | ACE2 | angiotensin-converting enzyme 2 | 0.976711 | 0.421599 |
| NM_002770 | PRSS2 | anionic trypsin (II) | 1 120854 | 0.066294 |
| NM_005577 | | anolinonrotein | 0 979589 | 0.961044 |
| NM 133463 | | Archeometzincin 1 | 1 062921 | 0.550218 |
| NM_016627 | | Archeometzincin 2 | 0.969020 | 0.643988 |
| NM_000049 | ASPA/ACY-2 | aspartoacylase-2 | 1 076493 | 0.260786 |
| BC008689 | | aspartoacylase-3 | 1 230206 | 0.005368 |
| NM 012100 | | aspartul aminopentidase | 1.200200 | 0.000581 |
| NM_004003 | | aspartyl annihopepticase | 1.020045 | 0.665557 |
| XM 045705 | | atavin-3 like | 1.025545 | 0.0000007 |
| NM 013325 | | | 1.073047 | 0.2201 |
| NM_052026 | | autophagin-1 | 1.013331 | 0.009911 |
| NIM_022850 | AUTL2 | autophagin-2 | 1.002071 | 0.913473 |
| NIM_022052 | AUTL3 | autophagin-5 | 1.230341 | 0.177552 |
| NM_001700 | AUTL4 | autopriagin-4 | 1.070013 | 0.279442 |
| NM_022850 | | | 0.622009 | 0.063914 |
| RC014040 | | | 1.026164 | 0.107233 |
| BC014049 | | | 1.020104 | 0.452129 |
| DG/42/3 | | beta lastamasa | 0.964424 | 0.221574 |
| NIVI_032657 | LACID | | 0.004424 | 0.107739 |
| NIVI_012104 | BACE2 | beta appretana 2 | 0.072120 | 0.400203 |
| AF 170332 | | bleamuein budreleee | 1.069627 | 0.952415 |
| NIM_000300 | | brein serine preteinese 2 | 1.000027 | 0.323703 |
| NIM_024222 | C6 1 A | | 0.931212 | 0.150799 |
| NM_005186 | | colorin 1 | 0.779339 | 0.051575 |
| NM_022087 | | | 1 408405 | 0.150050 |
| NM_007058 | | | 1.400400 | 0.034203 |
| XM 200840 | | | 0.736004 | 0.028707 |
| ΔK027176 | | calpain 12 | 0.730004 | 0.020707 |
| AK02277 | | calpain 10 | 1 107906 | 0.134747 |
| NM 005632 | CAPN15 | calpain 15/Sol protein | 1.107500 | 0.107625 |
| NM_001748 | CAPN2 | calpain 19/001 protein | 0.810171 | 0.137023 |
| NM_000070 | CAPN3 | calpain 2 | 1 268147 | 0.309727 |
| NM_004055 | CAPN5 | calpain 5 | 0 944898 | 0.130228 |
| NM 01/289 | | calpain 6 | 1 030702 | 0.626466 |
| NM 01/206 | CAPN7 | calpain 7 | 1 067843 | 0.367554 |
| ΔΔ0/13003 | CAPNS | calpain 8 | 1 857022 | 0.180051 |
| NM 006615 | | calpain 0 | 1 000505 | 0.051106 |
| NM_001868 | CPA1 | carbovypentidese A1 | 0.990094 | 0.715732 |
| NM_001960 | CPA2 | carboxypeptidase A1 | 1 021822 | 0.655100 |
| NM_001009 | CPA2 | carboxypeptidase A2 | 0.808208 | 0.032564 |
| NM_016252 | CPA3 | carboxypeptidase As | 0.090200 | 0.030304 |
| 14101_010352 | | | | |
| VE38/662 | CPA4 CPA5 | carboxypeptidase A4 | 1 222602 | 0.003035 |
| AF384667 | CPA4 CPA5 | carboxypeptidase A5 | 1.233693 | 0.003033 |
| AF384667 NM_020361 | CPA4 CPA5 CPA6 CPB1 | carboxypeptidase A5 carboxypeptidase A6 carboxypeptidase B | 0.837495 1.233693 0.973282 0.927342 | 0.003030 0.004121 0.587763 0.452585 |

| NM 001304 | CPD | carboxypeptidase D | 1.166176 | 0.008111 |
|-----------|-----------|-------------------------|----------|----------|
| NM_001873 | CPE | carboxypeptidase E | 1.163725 | 0.042171 |
| AF368463 | CPM | carboxypeptidase M | 0.998476 | 0.977612 |
| NM 001308 | CPN | carboxypeptidase N | 1.052441 | 0.461802 |
| NM 173077 | CPO | carboxypeptidase O | 1,493100 | 0.031803 |
| NM_001872 | CPB2 | carboxypeptidase U | 1 277515 | 0 120754 |
| NM_019609 | CPX1 | carboxypeptidade 8 | 1 021360 | 0.558988 |
| XM_058409 | CPX2 | carboxypeptidase X1 | 0.97/808 | 0.534689 |
| NM_003652 | | carboxypeptidase 72 | 1 117024 | 0.000525 |
| NM_022202 | | | 0.001156 | 0.000525 |
| NM 022077 | | | 1 796967 | 0.090000 |
| ND 000025 | | caspase-10 | 1./0000/ | 0.157123 |
| NR_000035 | CASPIZ | caspase-12 | 1.528438 | 0.163008 |
| NM_012114 | CASP14 | caspase-14 | 1.093792 | 0.686632 |
| AF098666 | CASP14L | caspase-14-like | 0.885609 | 0.025528 |
| NM_032982 | CASP2 | caspase-2 | 0.825651 | 0.007925 |
| NM_004346 | CASP3 | caspase-3 | 1.036027 | 0.589779 |
| NM_033306 | CASP4 | caspase-4/11 | 1.128637 | 0.924746 |
| NM_004347 | CASP5 | caspase-5 | 1.842041 | 0.086213 |
| NM_001226 | CASP6 | caspase-6 | 0.903534 | 0.100255 |
| NM_033339 | CASP7 | caspase-7 | 0.697202 | 0.177442 |
| NM 033357 | CASP8 | caspase-8 | 1.095961 | 0.186674 |
| NM_001229 | CASP9 | caspase-9 | 1.102391 | 0.243970 |
| Y14039 | CFLAR | casper | 1.451308 | 0.110366 |
| NM 001908 | CTSB | cathepsin B | 1.238410 | 0.002583 |
| NM 001814 | CTSC | cathensin C | 0.968233 | 0 460786 |
| NM_001909 | CTSD | cathensin D | 1 257334 | 0.001232 |
| NM_001910 | CTSE | cathensin F | 0.989282 | 0.806077 |
| NM_003703 | CTSE | cathopsin E | 1 122/62 | 0.154566 |
| NM 001011 | CTSC | | 0.071204 | 0.154500 |
| NM_004200 | CTSU | | 0.971304 | 0.005914 |
| NM_004390 | CISH | | 0.044747 | 0.005614 |
| NM_000396 | CISK | | 1.101728 | 0.051209 |
| NM_001912 | CISL | | 1.634475 | 0.002243 |
| NM_001333 | CTSL2 | cathepsin L2 | 0.976291 | 0.772144 |
| NM_001334 | CISO | cathepsin O | 1.134489 | 0.118/64 |
| AK024855 | CTSS | cathepsin S | 1.186685 | 0.095733 |
| NM_001335 | CTSW | cathepsin W | 1.347526 | 0.198029 |
| NM_001336 | CTSZ | cathepsin Z | 0.875334 | 0.171538 |
| NM_002769 | PRSS1 | cationic trypsin | 0.870892 | 0.127983 |
| NM_020205 | CEZANNE | Cezanne | 0.902312 | 0.192495 |
| AJ430383 | LOC161725 | Cezanne-2 | 1.117649 | 0.350853 |
| NM_016006 | CGI-58 | CGI-58 | 0.871740 | 0.040012 |
| NM 016014 | CGI-67 | CGI-67 protein | 0.845476 | 0.395027 |
| NM_031213 | CGI-67L1 | CGI-67-like protease-1 | 1.218480 | 0.002672 |
| AL390079 | CGI-67L2 | CGI-67-like protease-2 | 1.069017 | 0.220878 |
| AL 137441 | CGI77 | CGI-77 | 0.913404 | 0.322111 |
| NM 001836 | CMA1 | chymase | 1 014554 | 0 721889 |
| Δ 1272212 | CTRI | chymonasin | 1.086110 | 0.880373 |
| NM 001006 | CTPB1 | chymotrypsin B | 0.040463 | 0.000070 |
| NM_007272 | CTRC | chymotrypsin C | 1 10/0/2 | 0.403403 |
| NM 000122 | | chymolitypsin C | 1.134342 | 0.205224 |
| NM_000133 | F7 | | 0.064472 | 0.000829 |
| | | coagulation factor Via | 0.904173 | 0.000000 |
| NM_000504 | F 10 | | 1.1526/1 | 0.003286 |
| NM_000128 | F11 | coagulation factor XIa | 1.029300 | 0.477540 |
| NM_000505 | F12 | coagulation factor XIIa | 1.013373 | 0.807388 |
| NM_002421 | MMP1 | collagenase 1 | 1.649192 | 0.000323 |
| NM_002424 | MMP8 | collagenase 2 | 0.971386 | 0.560481 |
| NM_002427 | MMP13 | collagenase 3 | 1.197408 | 0.009373 |
| NM_016546 | C1RL | complement C1r-homolog | 1.136744 | 0.260458 |
| NM_000063 | C2 | complement component 2 | 1.237043 | 0.102853 |

| AK024951 | C1R | complement component C1ra | 0.871277 | 0.026977 |
|---------------------|----------|-------------------------------------|----------|----------|
| NM 001734 | C1S | complement component C1sa | 1.200853 | 0.250167 |
| NM 001710 | BF | complement factor B | 0.832811 | 0.001992 |
| NM_001928 | DE | complement factor D | 0.955309 | 0.467658 |
| XM 115647 | DE2 | complement factor D-like | 1 208070 | 0.032828 |
| NM_000204 | | complement factor L | 1.230070 | 0.002020 |
| NIVI_000204 | | | 1.005059 | 0.295363 |
| NNI_000033 | CUPS0 | COPSo | 0.740200 | 2.59E-00 |
| NM_006587 | PRSC | corin | 1.051322 | 0.263489 |
| NM_015247 | CYLD1 | cylindromatosis protein | 0.920750 | 0.434999 |
| NM_006310 | NPEPPS | cytosol alanyl aminopeptidase | 0.963657 | 0.555967 |
| AK055994 | DDI-RP | DDI-related protease | 1.007998 | 0.797404 |
| NM_014479 | ADAMDEC1 | DECYSIN | 1.024712 | 0.551656 |
| NM 014058 | DESC1 | DESC1 protease | 1.469035 | 0.223924 |
| NM_021044 | DHH | desert hedgehog protein | 1.114139 | 0.097488 |
| NM 004341 | CAD | dihvdroorotase | 0.762871 | 0.002296 |
| NM_001385 | DPYS | dihydropyrimidinase | 1 061531 | 0 132688 |
| NM_001313 | CRMP1 | dihydropyrimidinase-related prot 1 | 1 110940 | 0 140675 |
| 1107105 | | dihydropyrimidinaso related prot. 1 | 1.045365 | 0.140070 |
| 09/103 NM 001207 | DEVOLO | dihydropyrinidinase-related prot. 2 | 0.741700 | 0.415555 |
| NNI_001367 | DPTSL3 | dihydropyrimidinase-related prot. 5 | 0.741723 | 0.070001 |
| NM_006426 | DPYSL4 | dinydropyrimidinase-related prot. 4 | 1.429597 | 0.010371 |
| AK022795 | DPYSL5 | dihydropyrimidinase-related prot. 5 | 2.159467 | 0.224888 |
| NM_004826 | ECEL1 | DINE peptidase | 1.045482 | 0.471216 |
| AB040925 | DPP10 | dipeptidyl-peptidase 10 | 0.897500 | 0.852863 |
| NM_001935 | DPP4 | dipeptidyl-peptidase 4 | 1.293671 | 1.15E-06 |
| NM 001936 | DPP6 | dipeptidyl-peptidase 6 | 1.030660 | 0.691847 |
| NM 017743 | DPP8 | dipeptidvl-peptidase 8 | 0.949749 | 0.514363 |
| BC000970 | DPP9 | dipeptidyl-peptidase 9 | 0.844169 | 0.008445 |
| NM 013379 | DPP7 | dipeptidyl-peptidase II | 1.029468 | 0.406123 |
| NM_005700 | DPP3 | dipentidyl-pentidase III | 1 079052 | 0.099389 |
| NM_007262 | | | 0.645218 | 1.85E_06 |
| | | DU-1 | 0.045210 | 0.804250 |
| AKU93330 | | DNA-damage inducible protein | 0.790767 | 0.094259 |
| BIN000122 | | DINA-damage inducible protein 2 | 1.230150 | 0.282234 |
| NM_022450 | EGFR-RS | EGF Receptor Related Sequence | 1.438846 | 0.020862 |
| NM_004771 | MMP20 | enamelysin | 0.481213 | 0.317351 |
| NM_006012 | CLPP | endopeptidase Clp | 1.000976 | 0.988273 |
| NM_001397 | ECE1 | endothelin-converting enzyme 1 | 1.136857 | 0.081053 |
| NM_014693 | ECE2 | endothelin-converting enzyme 2 | 1.042765 | 0.355675 |
| NM 002772 | PRSS7 | enteropeptidase | 1.083461 | 0.442237 |
| BQ638967 | PRSS7L | enteropeptidase-like | 0.984907 | 0.840640 |
| BN000134 | ESSPL | epidermis-specific SP-like | 0.809350 | 0.080004 |
| NM 032950 | MMP28 | epilysin | 1,126129 | 0.076767 |
| NM_005656 | TMPRSS2 | enitheliasin | 0 903374 | 0.646202 |
| NM_000120 | | epoxide bydrolase | 1 000713 | 0.040202 |
| NM 17267 | | epoxide hydrolase | 1.000713 | 4 765 05 |
| NIVI_173007 | | epoxyde nydrolase related protein | 1.412447 | 4.76E-05 |
| NM_003756 | EIF353 | eukar. translation Initiation F353 | 1.064378 | 0.401685 |
| NM_003754 | EIF3S5 | eukar. translation initiation F3S5 | 0.863917 | 0.435274 |
| XM_062387 | EIF3S5B | eukar. translation initiation F3S5B | 0.963789 | 0.541981 |
| NM_005857 | FACE1 | FACE-1/ZMPSTE24 | 0.929754 | 0.034499 |
| NM_005133 | FACE2 | FACE-2/RCE1 | 0.878942 | 0.493130 |
| NM 002569 | PCSK3 | furin | 1.356860 | 0.001526 |
| NM_003878 | GGH | gamma-glutamyl hydrolase | 0.925619 | 0.047417 |
| NM 013421 | GGT1 | gamma-glutamyltransferase 1 | 0.969636 | 0.664871 |
| M30474 | GGT2 | gamma-glutamyltransferase 2 | 1.093889 | 0.004378 |
| NM 004121 | GGTLA1 | gamma-glutamyltransferase 5 | 1 105785 | 0 208916 |
| NM 152228 | GGT6 | aamma-alutamyltransferase 6 | 1 449491 | 0.085111 |
| NM 090920 | GGTLA | gamma glutamylitansierase m 2 | 0.064244 | 0.000111 |
| NM_050000 | CCTL2 | gamma glutamyltransferase like 2 | 1 010000 | 0.202200 |
| NIM_002830 | GGTL3 | | 7.445700 | 0.051485 |
| NM_002652 | PIP | GCDFP15 | 7.145798 | 0.160822 |
| NM_004530 | MMP2 | gelatinase A | 1.244619 | 0.014608 |

| NM_004994 | MMP9 | gelatinase B | 1.013671 | 0.715787 |
|-------------|--------|------------------------------------|----------|-----------|
| NM_004994 | MMP9 | gelatinase B | 0.935106 | 0.603452 |
| NM 002056 | GFPT1 | GIn-fructose-6-P transamidase 1 | 0.781841 | 0.291443 |
| NM_005110 | GFPT2 | Gln-fructose-6-P transamidase 2 | 1.051759 | 0.178847 |
| XM_115871 | GFPT3 | GIn-fructose-6-P transamidase 3 | 1,130827 | 0.264770 |
| U00238 | PPAT | GIn-PRPP amidotransferase | 0 533084 | 0 145170 |
| AK024471 | CPGI | du-carboxypentidase-like 1 | 0.867341 | 0.001391 |
| NM 032649 | CPGL2 | glu-carboxypeptidase-like 2 | 1 0/0153 | 0.408566 |
| NM 004476 | | glutamata carboxypontidaso II | 1.043100 | 0.400000 |
| NM 012412 | OPCT | | 2 020007 | 0.0009952 |
| NM 017650 | | | 2.029997 | 0.020002 |
| NIM_000007 | | | 0.907000 | 0.040403 |
| NIM_000027 | AGA | giycosylasparaginase | 0.927215 | 0.677721 |
| NM_025080 | ASRGLI | giycosylasparaginase-2 | 0.961729 | 0.565542 |
| NM_017714 | AGA3 | giycosylasparaginase-3 | 1.146179 | 0.179608 |
| NM_006144 | GZMA | granzyme A | 1.153341 | 0.342180 |
| NM_004131 | GZMB | granzyme B | 0.952086 | 0.525466 |
| NM_033423 | GZMH | granzyme H | 0.920577 | 0.200595 |
| NM_002104 | GZMK | granzyme K | 1.214102 | 0.247507 |
| NM_005317 | GZMM | granzyme M | 1.073179 | 0.614885 |
| AK055872 | HP | haptoglobin-1 | 1.001206 | 0.985733 |
| NM 020995 | HPR | haptoglobin-related protein | 0.961388 | 0.335165 |
| BN000133 | HATL1 | HAT-like 1 | 1.060462 | 0.233544 |
| NM 207407 | HATL4 | HAT-like 4 | 1.140300 | 0.320269 |
| XM 068002 | HATL5 | HAT-like 5 | 0.956217 | 0.585807 |
| NM 182559 | HATRP | HAT-related protease | 0 976751 | 0 996477 |
| AK056446 | HSPCA | heat shock 90kDa protein 1 alpha | 0.820919 | 0.020186 |
| NM 007355 | HSPCB | heat shock 90kDa protein 1, heta | 0.995373 | 0.872926 |
| NM 016292 | | heat shock protein 75 | 0.865612 | 0.001331 |
| V16222 | | henatocyte growth factor | 1 862170 | 0.0015145 |
| NIM 001529 | HCEAC | hepatocyte growth factor activator | 0.057072 | 0.124110 |
| NIM_0001526 | | | 0.957075 | 0.124110 |
| NM_015281 | HPN | | 0.937720 | 0.049480 |
| NIM_015281 | HEIFL | Helf-like | 1.020152 | 0.684515 |
| AF022913 | PIGK | nGP18 | 0.828745 | 0.199609 |
| NM_017493 | HSHIN1 | Hin-1 | 0.853778 | 0.071094 |
| NM_024810 | HSHIN2 | Hin-2 | 0.931537 | 0.109526 |
| XM_170950 | HSHIN3 | Hin-3 | 0.951775 | 0.422968 |
| XM_166659 | HSHIN4 | Hin-4 | 1.621677 | 0.006825 |
| XM_054098 | HSHIN5 | Hin-5 | 1.026979 | 0.471303 |
| XM_066765 | HSHIN6 | Hin-6 | 1.101814 | 0.046186 |
| BI829009 | HSHIN7 | Hin-7 | 0.834376 | 0.586693 |
| XM_072402 | HMRALP | HmrA-like protease | 0.966737 | 0.728342 |
| XM_495898 | ICEYH | homologue ICEY | 1.256028 | 0.253802 |
| AB067478 | USP38 | HP43.8KD | 0.905724 | 0.653267 |
| NM 013247 | HTRA2 | HTRA2 | 1.204027 | 0.001415 |
| AY040094 | HTRA3 | HTRA3 | 1.813415 | 0.012818 |
| NM 153692 | HTRA4 | HTRA4 | 0.897795 | 0.032727 |
| NM_004132 | HABP2 | hvaluronan-binding ser-protease | 0.917983 | 0.327131 |
| NM_014263 | YME1L1 | i-AAA protease | 0.980993 | 0 709621 |
| NM 0310/3 | IED38 | IFD38 | 1 11/173 | 0.980057 |
| XM 200345 | | | 0.837753 | 0.300037 |
| 1 38517 | | indian hedgebog pretein | 0.031099 | 0.219770 |
| | | | 1 210240 | 0.019190 |
| NM_000007 | CORE | | 0.954500 | 0.304742 |
| NIVI_006837 | | | 0.851506 | 0.231860 |
| AB067502 | JAMML1 | Jammin-like protease 1 | 0.979801 | 0.741203 |
| NM_032868 | JAMML2 | jammin-like protease 2 | 1.025238 | 0.560262 |
| NM_014876 | JSPH1 | Josephin-1 | 1.124937 | 0.348931 |
| NM_138334 | SBBI54 | Josephin-2 | 0.924007 | 0.051575 |
| NM_002257 | KLK1 | kallikrein hK1 | 1.014014 | 0.755107 |
| NM_002776 | KLK10 | kallikrein hK10 | 1.150840 | 0.072977 |

| NM_006853 | KLK11 | kallikrein hK11 | 1.469602 | 0.479766 |
|-------------------------------------|--|---|--|--|
| NM 019598 | KLK12 | kallikrein hK12 | 0.880613 | 0.008479 |
| NM 015596 | KLK13 | kallikrein hK13 | 1.041709 | 0.664638 |
| NM 022046 | KLK14 | kallikrein hK14 | 0.866821 | 0.021344 |
| NM 017509 | KLK15 | kallikrein hK15 | 1.047758 | 0.333588 |
| AF188747 | KLK2 | kallikrein hK2 | 1.040085 | 0.934142 |
| NM 001648 | KLK3 | kallikrein hK3 | 1.598505 | 0.000569 |
| NM 004917 | KLK4 | kallikrein hK4 | 1,130866 | 0.131797 |
| NM 012427 | KLK5 | kallikrein hK5 | 1 015698 | 0 708888 |
| NM_002774 | KI K6 | kallikrein hK6 | 1 252756 | 0 184855 |
| NM 005046 | KI K7 | kallikrein hK7 | 0.394917 | 0 174768 |
| NM_007196 | KI K8 | kallikrein hK8 | 0 970183 | 0.596052 |
| NM_012315 | KI K9 | kallikrein hK9 | 1 098943 | 0.090614 |
| NM_000420 | KEI | Kell blood-group protein | 1.050540 | 0.000014 |
| NM 002343 | ITE | lactotransferrin | 1 236181 | 0.103103 |
| BC008004 | | legumain | 0.9/9136 | 0.209814 |
| NM 033020 | | leishmanolysin-2 | 1 105515 | 0.200014 |
| NM_015007 | | | 0.01/205 | 0.040409 |
| NM_005575 | | leucyl-cystinyl aminopentidase | 1 403500 | 0.000923 |
| NM_000805 | | leukotriene A4 hydrolase | 1.403500 | 0.007507 |
| NM_000209 | | lucesomal earboxupentidees A | 1.022013 | 0.009000 |
| NM_005040 | | lysosomal Dro X sorbow/pontidase | 1.120002 | 0.012300 |
| NM_00040 | | | 1.020909 | 0.701323 |
| NIVI_002426 | | macrophage elastase | 1.025370 | 0.583689 |
| NM_020998 | MSP | macrophage-stimulating protein | 1.514111 | 0.019433 |
| AF282732 | TLL | mammalian tolloid-like 1 protein | 0.939397 | 0.298330 |
| AB023149 | ILL2 | mammalian tolloid-like 2 protein | 1.559307 | 0.022867 |
| AK055576 | MPN | marapsin | 0.971473 | 0.290059 |
| BN000131 | | marapsin 2 | 1.200812 | 0.925842 |
| D17525 | MASP1/3 | MASP1/3 | 1.092833 | 0.080067 |
| NM_000010 | MASP2 | MASP2 | 0.999048 | 0.950861 |
| XIVI_003840 | | masun | 1.223391 | 0.468394 |
| NIM_002423 | | matrilysin | 1.439942 | 0.000648 |
| NM_021801 | MMP26 | matrilysin-2 | 1.091954 | 0.185303 |
| NM_021978 | MISP1 | matriptase | 1.170041 | 0.087936 |
| AL022314 | TMPRSS6 | matriptase-2 | 0.937635 | 0.361323 |
| BN000125 | IMPRSS/ | matriptase-3 | 1.238426 | 0.916249 |
| NM_004413 | DPEP1 | membrane dipeptidase | 1.124531 | 0.046123 |
| NM_022355 | DPEP2 | membrane dipeptidase 2 | 1.149583 | 0.142254 |
| NM_022357 | DPEP3 | membrane dipeptidase 3 | 1.179464 | 0.001996 |
| NM_032046 | MSPL | membrane-type mosaic Ser-prot. | 1.215649 | 0.083991 |
| NM_005588 | MEP1A | meprin alpha subunit | 1.232047 | 0.498529 |
| NM_005925 | MEP1B | meprin beta subunit | 0.934135 | 0.139786 |
| NM_002402 | MEST | mesoderm specific transcript hom. | 0.386115 | 0.000161 |
| NM_002771 | PRSS3 | mesotrypsin | 0.734651 | 0.048182 |
| NM_145243 | MPRP-1 | metalloprotease related protein 1 | 0.918582 | 0.020607 |
| D42084 | METAP1 | methionyl aminopeptidase I | 1.064125 | 0.249254 |
| NM_006838 | METAP2 | methionyl aminopeptidase II | 1.033054 | 0.250483 |
| XM_171022 | METAPL1 | methionyl aminopeptidase-like 1 | 1.061225 | 0.413293 |
| NM_032549 | IMMP2L | mitoc. inner membrane protease 2 | 0.851981 | 0.034280 |
| NM_005932 | MIPEP | mitochondrial intermediate peptidase | 0.971591 | 0.668752 |
| NM_004279 | PMPCB | mitochondrial processing peptidase beta-subunit | 0.959081 | 0.226366 |
| D50913 | INPP5E | mitochondrial processing protease | 1.048021 | 0.270769 |
| AK057788 | IMMP1 | mitochondrial signal peptidase | 0.664368 | 0.345235 |
| NM_022790 | | MMD10 | 1 1 2 2 1 2 2 | 0 990510 |
| NIM 1/7101 | MMP19 | MIMP 19 | 1.120133 | 0.009510 |
| 11111_147131 | MMP19 MMP21 | MMP19 MMP21 | 1.005056 | 0.941916 |
| NM_004659 | MMP19 MMP21 MMP23A/B | MMP19 MMP21 MMP23A/B | 1.005056 0.998273 | 0.941916 |
| NM_004659 NM_022122 | MMP19 MMP21 MMP23A/B MMP27 | MMP19 MMP21 MMP23A/B MMP27 | 1.005056 0.998273 0.861858 | 0.941916 0.928795 0.853438 |
| NM_004659 NM_022122 NM_004995 | MMP19 MMP21 MMP23A/B MMP27 MMP14 | MMP19 MMP21 MMP23A/B MMP27 MT1-MMP | 1.120133 1.005056 0.998273 0.861858 2.163458 | 0.883310 0.941916 0.928795 0.853438 0.018383 |

| NM_005941 | MMP16 | MT3-MMP | 0.812033 | 0.001099 |
|-------------|----------|--|----------|----------|
| NM_016155 | MMP17 | MT4-MMP | 1.190632 | 0.255853 |
| NM_006690 | MMP24 | MT5-MMP | 1.111633 | 0.443901 |
| NM 006690 | MMP24 | MT5-MMP | 0.911841 | 0.029017 |
| NM_022468 | MMP25 | MT6-MMP | 0.945224 | 0.945125 |
| NM 022468 | MMP25 | MT6-MMP | 0.432952 | 0.244066 |
| NM 005467 | NAALAD2 | NAALADASE II | 1.037426 | 0.621333 |
| XM 173084 | NAALAD3 | NAALADASE III | 1.222454 | 0.023891 |
| NM_005468 | NAALADI | NAALADASE L peptidase | 0.908969 | 0.049063 |
| NM_004851 | NAP1 | nansin A | 1 082102 | 0.237901 |
| NM_002525 | NRD1 | nardilysin | 0.966886 | 0.597463 |
| NM_007289 | MME | nenrilysin | 0.500905 | 0.072480 |
| NM_033467 | MMEL 2 | neprilysin-2 | 1 118694 | 0.086336 |
| AB033052 | | neurolysin | 1 020092 | 0.641340 |
| NM 003619 | DDSS12 | neurotrypsin | 0.516020 | 0.04104 |
| NM_001972 | FLA2 | neutrophil elastase | 0.964628 | 0.583718 |
| NM_030808 | | nuclear distribution element-like-oligonentidase | 1 585110 | 0.000710 |
| NM_017668 | NDE1 | nuclear distribution-oligopentidase | 1 103807 | 0.060491 |
| NIM_021474 | | Nuclear recent interacting prot 2 | 1.103097 | 0.000491 |
| NM_020645 | | Nuclear recept. Interacting prot. 2 | 0.905127 | 0.00430 |
| NIVI_020043 | | Nuclean recept. Interacting prot. 5 | 1.250546 | 0.034707 |
| NIVI_005387 | NUP98 | | 1.300040 | 0.164170 |
| NIVI_032582 | 05P32 | NY-REN-60 | 1.119869 | 0.321096 |
| NM_017807 | USGEP | | 0.959080 | 0.311587 |
| AK027836 | OSGEP2 | O-slaloglycoprotein endopeptidase 2 | 1.019548 | 0.544832 |
| AB058718 | OJP | Ojeda peptidase | 1.043205 | 0.465846 |
| NM_002775 | HIRA1 | osteoblast serine protease | 1.484490 | 0.000301 |
| NM_017670 | OTUB1 | Otubain-1 | 1.017346 | 0.602179 |
| NM_023112 | OTUB2 | Otubain-2 | 1.301934 | 0.104560 |
| BI061462 | OVCN | Ovastacin | 0.904816 | 0.036898 |
| BN000120 | OVTN | oviductin-like | 1.212961 | 0.311264 |
| BN000128 | OVCH | ovochymase-like | 1.429653 | 0.000457 |
| NM_002570 | PCSK6 | PACE4 proprotein convertase | 1.017082 | 0.767492 |
| NM_005805 | POH1 | Pad1-homolog | 0.963119 | 0.812916 |
| NM_033440 | ELA2A | pancreatic elastase II (IIA) | 1.037992 | 0.472482 |
| NM_015849 | ELA2B | pancreatic elastase II form B | 1.124211 | 0.440893 |
| NM_005747 | ELA3A | pancreatic endopeptidase E (A) | 1.138915 | 0.019980 |
| NM_007352 | ELA3B | pancreatic endopeptidase E (B) | 1.064688 | 0.562000 |
| U28727 | PAPPA | pappalysin-1 | 1.009101 | 0.850082 |
| AF342989 | PLAC3 | pappalysin-2 | 1.038529 | 0.603443 |
| NM_006785 | MALT1 | paracaspase | 1.392879 | 0.057095 |
| NM_003119 | SPG7 | paraplegin | 1.345894 | 0.026691 |
| AJ223812 | PGA3/4/5 | pepsin A | 1.034250 | 0.890539 |
| NM_002630 | PGC | pepsin C | 1.109538 | 0.077240 |
| NM_000444 | PHEX | PHEX endopeptidase | 1.096208 | 0.624305 |
| NM_004793 | PRSS15 | PIM1 endopeptidase | 0.796414 | 0.030515 |
| NM_006875 | PIM2 | PIM2 endopeptidase | 1.014907 | 0.720152 |
| NM_014889 | PITRM1 | pitrilysin metalloproteinase 1 | 0.753610 | 0.358606 |
| NM_006102 | PGCP | plasma Glu-carboxypeptidase | 0.998208 | 0.947075 |
| NM_000892 | KLKB1 | plasma kallikrein | 1.514657 | 0.084405 |
| XP_116753 | KLKBL1 | plasma-kallikrein-like 1 | 1.170515 | 0.044606 |
| XM_114622 | KLKBL2 | plasma-kallikrein-like 2 | 1.132022 | 0.623932 |
| AL136785 | KLKBL4 | plasma-kallikrein-like 4 | 0.978601 | 0.498375 |
| NM_000301 | PLG | plasminogen | 1.809249 | 0.041620 |
| AJ488946 | TMPRSS9 | Polyserase I | 1.065656 | 0.743825 |
| NM 007318 | PSEN1 | presenilin 1 | 1.033506 | 0.403327 |
| NM 000447 | PSEN2 | presenilin 2 | 1.161450 | 0.116783 |
| AL110147 | PSH1 | presenilin homolog 1/SPPL3 | 0.988963 | 0.717937 |
| XM 091623 | PSH2 | presenilin homolog 2 | 1.131264 | 0.181913 |
| BC008959 | PSH3 | presenilin homolog 3/SPP | 1.032244 | 0.413360 |

| AB040965 | PSH4 | presenilin homolog 4/SPPL2B | 1.082414 | 0.478373 |
|-------------|---------|--------------------------------------|-----------|----------|
| NM 032802 | PSH5 | presenilin homolog 5 | 1.389466 | 0.197330 |
| NM 018622 | PARL | Presenilins associated rhomboid like | 0.808326 | 0.009104 |
| NM_002768 | PCOLN3 | procol III N-endopentidase | 0 943641 | 0.522468 |
| NM_006129 | RMP1 | procollagen C-proteinase | 1 009797 | 0.844182 |
| NM_006101 | | protoinagen o-proteinase | 0.736682 | 0.000327 |
| NM 002726 | | proliteration-association protein 1 | 0.750002 | 0.000327 |
| AD007906 | | prolytoligopeptidase | 0.304000 | 0.229092 |
| AB007896 | PREP2 | prolyi-oligopepildase 2 | 0.030032 | 0.035987 |
| NM_000439 | PCSK1 | proprotein convertase 1 | 1.015411 | 0.833113 |
| NM_002594 | PCSK2 | proprotein convertase 2 | 1.059467 | 0.569288 |
| AK057235 | PCSK4 | proprotein convertase 4 | 1.041521 | 0.461231 |
| NM_006200 | PCSK5 | proprotein convertase 5 | 0.828836 | 0.140023 |
| NM_004716 | PCSK7 | proprotein convertase 7 | 0.976692 | 0.971112 |
| NM_174936 | PCSK9 | proprotein convertase 9 | 0.887406 | 0.016149 |
| NM_002773 | PRSS8 | prostasin | 0.976496 | 0.290852 |
| NM 173502 | PSTL1 | prostasin-like 1 | 0.989622 | 0.767593 |
| NM_024006 | PSTL2 | prostasin-like 2 | 1.010635 | 0.811424 |
| NM 002786 | PSMA1 | proteasome alpha 1 subunit | 0.802332 | 0.000258 |
| NM_002787 | PSMA2 | proteasome alpha 2 subunit | 1 049705 | 0 253316 |
| NM_002788 | PSMA3 | proteasome alpha 3 subunit | 0 797633 | 0.000971 |
| AK055714 | | proteasome alpha o subunit | 0.845025 | 0.005051 |
| NM 002700 | | proteasome alpha 5 subunit | 1 1113/18 | 0.000001 |
| NIVI_002790 | POMAG | proteasome alpha 5 subunit | 0.795520 | 0.040027 |
| A09417 | PSIVIAO | proteasome alpha o subunit | 0.760020 | 0.000566 |
| NM_002792 | PSMA/ | proteasome alpha 7 subunit | 0.894487 | 0.207568 |
| NM_144662 | PSMA8 | proteasome alpha 8 subunit | 0.865805 | 0.353937 |
| AK023290 | PSMB1 | proteasome beta 1 subunit | 0.860735 | 0.000279 |
| NM_002794 | PSMB2 | proteasome beta 2 subunit | 0.978726 | 0.726067 |
| NM_002795 | PSMB3 | proteasome beta 3 subunit | 1.091757 | 0.147756 |
| NM_002796 | PSMB4 | proteasome beta 4 subunit | 0.936015 | 0.154752 |
| XM 063287 | LMP7L | proteasome beta subunit LMP7-like | 0.924460 | 0.164743 |
| BF698890 | PSMB6 | proteasome catalytic subunit 1 | 0.916387 | 0.064043 |
| NM 002800 | PSMB9 | proteasome catalytic subunit 1i | 0.852746 | 0.022004 |
| NM_002799 | PSMB7 | proteasome catalytic subunit 2 | 1 195697 | 0 139885 |
| NM_002801 | PSMB10 | proteasome catalytic subunit 2 | 0 974389 | 0.512615 |
| NM_002797 | PSMB5 | proteasome catalytic subunit 3 | 1 026158 | 0.453165 |
| NM_00/159 | PSMB8 | proteasome catalytic subunit 3 | 1 107383 | 0.400100 |
| NM 000212 | | protein C | 0.070992 | 0.000023 |
| AK027944 | PROCI | protein C like | 1.055244 | 0.010247 |
| AKU27041 | PROCL | protein C-like | 1.055244 | 0.436177 |
| NM_003891 | PROZ | protein Z | 1.055624 | 0.834986 |
| NM_002777 | PRIN3 | proteinase 3 | 0.958978 | 0.416603 |
| NM_006445 | PRPF8 | PRPF8 | 0.967341 | 0.966338 |
| NM_002811 | PSMD7 | PSMD7 | 0.818327 | 0.061244 |
| NM_017712 | PGPEP1 | pyroglutamyl peptidase I | 1.162120 | 0.008243 |
| XM_085243 | PGPEP2 | pyroglutamyl-peptidase II | 1.064520 | 0.982954 |
| NM_013381 | TRHDE | pyroglutamyl-peptidase II | 0.801618 | 0.288159 |
| NM 005045 | RELN | Reelin | 1.044203 | 0.944445 |
| NM 000537 | REN | renin | 1.533507 | 0.366429 |
| NM_003961 | RHBDL | rhomboid-like protein 1 | 1.222101 | 0.001525 |
| NM 017821 | RHBDL2 | rhomboid-like protein 2 | 0.846682 | 0.249048 |
| NM 138328 | RHBDI 4 | rhomboid-like protein 4 | 1 774088 | 0 161399 |
| NM_032276 | RHBDL5 | rhomboid-like protein 5 | 0.922198 | 0 137353 |
| NM_024599 | RHBDI 6 | rhomboid-like protein 6 | 1 131907 | 0.090738 |
| NM_020694 | | rhomboid-like protein 7 | 1 270725 | 0.011077 |
| NNA_045004 | | | 1.270765 | 0.011977 |
| NN_015884 | NBTP52 | SZF protease | 1.191144 | 0.102409 |
| NM_006590 | 05P39 | SADT | 1.239193 | 0.001094 |
| NM_014554 | SENP1 | sentrin/SUMO protease 1 | 1.108648 | 0.517573 |
| AB037752 | SENP2 | sentrin/SUMO protease 2 | 0.948542 | 0.572702 |
| NM_015670 | SENP3 | sentrin/SUMO protease 3 | 1.031751 | 0.526853 |
| BC008589 | SENP5 | sentrin/SUMO protease 5 | 2.752479 | 0.054450 |

| NM_015571 | SENP6 | sentrin/SUMO protease 6 | 0.938460 | 0.504407 |
|--------------|-----------|---|----------|----------|
| AL136599 | SENP7 | sentrin/SUMO protease 7 | 0.847125 | 0.179761 |
| AY008293 | SENP8 | sentrin/SUMO protease 8 | 1.096534 | 0.237534 |
| NM 012291 | ESPI 1 | separase | 1 017833 | 0 908275 |
| NM_004460 | EAD | Seprase | 1.05/007 | 0.202252 |
| NM 021626 | | Sepiase | 1.034337 | 0.202252 |
| NINI_021020 | | | 1.070023 | 0.230520 |
| NIVI_014300 | SPC 10 | | 0.023709 | 0.019556 |
| NM_033280 | SPC21 | signalase 21 kDa component | 1.058889 | 0.062658 |
| NM_144966 | SPCL1 | signalase-like 1 | 1.049542 | 0.//15/1 |
| BC016840 | SASP | similar to Arabidopsis Ser-prot. | 1.066196 | 0.124099 |
| NM_153362 | SPUVE2 | similar to SPUVE | 1.214683 | 0.184805 |
| NM_003791 | MBTPS1 | site-1 protease | 0.962031 | 0.434660 |
| NM 000193 | SHH | sonic hedgehog protein | 1.192162 | 0.328709 |
| NM 030770 | TMPRSS5 | spinesin | 0.937140 | 0.587066 |
| NM_002422 | MMP3 | stromelysin 1 | 1.156270 | 0.027624 |
| NM 002425 | MMP10 | stromelysin 2 | 0.259647 | 0.843173 |
| NM 005940 | MMP11 | stromelysin 3 | 3 436513 | 3 26E-07 |
| NM_007192 | SUPT16H | suppressor of Ty 16 homolog | 0.022230 | 0.384692 |
| NM_000020 | | t plasminogen estivator | 2.045029 | |
| NM 002194 | | | 2.945956 | 4.12E-10 |
| NIVI_003184 | TAF2 | TBP-associated factor 2 | 0.850099 | 0.058997 |
| AJ544583 | TESSP2 | testis serine protease 2 | 0.981679 | 0.846803 |
| BN000137 | TESSP5 | testis serine protease 5 | 1.010511 | 0.905798 |
| NM_013270 | TSP50 | testis-specific protein tsp50 | 1.502202 | 0.078141 |
| NM_006799 | PRSS21 | testisin | 0.929528 | 0.094963 |
| NM_003249 | THOP1 | thimet oligopeptidase | 1.023426 | 0.695655 |
| NM 000506 | F2 | thrombin | 3.580076 | 0.236128 |
| NM_005865 | PRSS16 | thymus-specific serine peptidase | 1.139589 | 0.304958 |
| NM_022164 | LCN7 | TINAG related protein | 1,104531 | 0.372483 |
| NM_006290 | TNFAIP3 | TNEa-induced protein $3/A20$ | 1 117219 | 0.025918 |
| A 1252060 | TRABID | TRAF-binding protein domain | 0.962997 | 0.020010 |
| NM 003227 | | transforrin recentor 2 protein (transforrin recentor 2) | 1 324253 | 0.400200 |
| NM 002227 | TEDO | transferrin receptor 2 protein (transferrin receptor 2) | 0.022970 | 0.000007 |
| NNI_003234 | THERE | transferrin receptor protein (transferrin receptor) | 0.933070 | 0.009004 |
| NM_032401 | TMPR553 | transmembrane Ser-protease 3 | 1.270711 | 0.369986 |
| NM_019894 | IMPRSS4 | transmembrane Ser-protease 4 | 1.056896 | 0.054228 |
| NM_000391 | CLN2 | tripeptidyl-peptidase l | 1.213880 | 0.002512 |
| NM_003291 | TPP2 | tripeptidyl-peptidase II | 1.130411 | 0.008832 |
| XM_059830 | TRYX2 | trypsin X2 | 0.708824 | 0.835672 |
| NM_003294 | TPS/TPSB1 | tryptase alpha/beta 1 | 0.975988 | 0.734864 |
| NM 024164 | TPSB2 | tryptase beta 2 | 0.850545 | 0.260166 |
| NM_012217 | TPSD1 | tryptase delta 1 | 1.111942 | 0.115072 |
| NM_012467 | TPSG1 | tryptase gamma 1 | 1.326345 | 0.008683 |
| NM 152891 | FOS | tryptase homolog 2 | 1 015734 | 0 953286 |
| BN000124 | TESSP1 | tryptase homolog 3 | 1.066833 | 0.522966 |
| NM 014464 | TINAG | tubulointerstitial pophritic antigon | 0.830403 | 0.800713 |
| NM 002200 | | tumor rejection entigen (gp06) | 0.039493 | 0.039713 |
| NM 000050 | | unior rejection antigen (gp90) | 0.070270 | 0.040994 |
| NIM_002058 | PLAU | | 0.949939 | 0.625004 |
| NM_004656 | BAP1 | ubiquitin C-term. hydrolase BAP1 | 1.170622 | 0.179090 |
| NM_004181 | UCHL1 | ubiquitin C-terminal hydrolase 1 | 1.061961 | 0.325957 |
| NM_006002 | UCHL3 | ubiquitin C-terminal hydrolase 3 | 0.887849 | 0.047070 |
| NM_015984 | UCHL5 | ubiquitin C-terminal hydrolase 5 | 0.887117 | 0.030883 |
| NM_003365 | UQCRC1 | UCR1 | 1.116582 | 0.007519 |
| NM 003366 | UQCRC2 | UCR2 | 1.026141 | 0.447849 |
| NM 007173 | SPUVE | umbelical vein proteinase | 0.434380 | 2.72E-06 |
| NM_003368 | USP1 | USP1 | 0.672616 | 0.007092 |
| AI 162049 | USP10 | USP10 | 0.973712 | 0.787306 |
| NM 004651 | | LISP11 | 0.026187 | 0 176778 |
| AE022790 | | | 1 002746 | 0.054422 |
| NM 002040 | | | 1 16740 | 0.954455 |
| NM_003940 | USP13 | U0P13 | 1.15/484 | 0.267612 |
| INIVI_005151 | 05P14 | USP 14 | 0.769938 | 0.015344 |

| NINA 000040 | | | 0.005000 | 0.407500 |
|--------------|---------|----------------------------------|----------|----------|
| INIVI_000313 | USP 15 | USP 15 | 0.000900 | 0.407500 |
| NM_006447 | USP16 | USP16 | 1.123125 | 0.181230 |
| XM_172439 | USP17 | USP17 | 1.340944 | 0.192792 |
| BN000116 | USP17L | USP17-like | 0.915872 | 0.567893 |
| NM 017414 | USP18 | USP18 | 1.164220 | 0.017610 |
| AB020698 | USP19 | USP19 | 1.180446 | 0.279487 |
| AK057225 | USP2 | USP2 | 1 547674 | 0 199746 |
| NM 006676 | LISP20 | LISP20 | 0.846658 | 0.007529 |
| NM_016572 | | | 1 168330 | 0.007020 |
| AD020006 | | | 1.100555 | 0.010000 |
| AD020900 | | | 0.000015 | 0.435309 |
| ABU20900 | 03P24 | U3P24 | 0.909215 | 0.175551 |
| AF170562 | USP25 | USP25 | 0.938393 | 0.512111 |
| NM_031907 | USP26 | USP26 | 1.028510 | 0.504775 |
| AW851065 | USP27 | USP27 | 0.939221 | 0.202904 |
| NM_020886 | USP28 | USP28 | 1.037381 | 0.543870 |
| NM_020903 | USP29 | USP29 | 1.473793 | 0.089794 |
| NM_006537 | USP3 | USP3 | 1.191413 | 0.011935 |
| NM_032663 | USP30 | USP30 | 1.031572 | 0.760051 |
| AB033029 | USP31 | USP31 | 0.659025 | 0.028016 |
| AK023845 | LISP34 | USP34 | 0.869625 | 0 528234 |
| AR023043 | | | 1 104436 | 0.020204 |
| AD037793 | USF 33 | | 0.000405 | 0.144595 |
| AB040886 | USP30 | USP30 | 0.990405 | 0.867364 |
| AB046814 | USP37 | USP37 | 0.817328 | 0.245052 |
| NM_003363 | USP4 | USP4 | 1.066400 | 0.888168 |
| NM_018218 | USP40 | USP40 | 0.841031 | 0.136884 |
| XM_036729 | USP41 | USP41 | 1.706400 | 0.023639 |
| XM 166526 | USP42 | USP42 | 1.281985 | 0.219238 |
| AK055188 | USP43 | USP43 | 1.035240 | 0.394008 |
| NM 032147 | USP44 | USP44 | 0.745347 | 0.007580 |
| NM_032929 | USP45 | USP45 | 0.920439 | 0.061584 |
| NM_022832 | USP46 | USP46 | 1 241866 | 0 123345 |
| AK027362 | | | 0.808842 | 0.038003 |
| NM 018301 | | | 1 222462 | 0.000000 |
| NM_019561 | | | 1.223403 | 0.002013 |
| NIN_010501 | 03P49 | 00749 | 1.203904 | 0.306250 |
| NM_003481 | USP5 | USP5 | 1.104538 | 0.719374 |
| AI990110 | USP50 | USP50 | 0.832580 | 0.495060 |
| BF741256 | USP51 | USP51 | 1.459642 | 0.055035 |
| NM_014871 | USP52 | USP52 | 1.137918 | 0.257128 |
| XM_052597 | USP53 | USP53 | 0.566877 | 0.002066 |
| NM_152586 | USP54 | USP54 | 1.175494 | 0.007097 |
| NM 004505 | USP6 | USP6 | 0.764128 | 0.284748 |
| NM_003470 | USP7 | USP7 | 1.126001 | 0.175068 |
| NM 005154 | USP8 | USP8 | 0.917845 | 0 745830 |
| NM_004652 | USP9X | USP9X | 0 849442 | 0.020622 |
| NM_004654 | LISP9Y | LISP9Y | 0.934081 | 0.635807 |
| NIM_025054 | VCIP125 | VCP(p07)/p47 interacting protein | 1 315525 | 0.421102 |
| AB020020 | | | 1.006005 | 0.421193 |
| AB029020 | 05P33 | | 1.006095 | 0.885476 |
| NM_031311 | | vitellogenic carboxypeptidase-L. | 1.041653 | 0.664587 |
| NM_000285 | PEPD | X-Pro dipeptidase | 1.070165 | 0.134920 |
| NM 003399 | XPNPEP2 | X-prolyl aminopeptidase 2 | 1.052772 | 0.348058 |

B. Inhibitors

| RefSeq | Abbreviation | Description | Fold change | <i>p</i> -value |
|-------------|--------------|----------------------------------|-------------|-----------------|
| NM_000014 | A2M | a-2-macroglobulin | 2.729719 | 0.016373 |
| NM_003381 | VIP | a-2-macroglobulin-family VIP | 1.113748 | 0.200719 |
| AK057908 | A2ML | a-2-macroglobulin-like | 0.979668 | 0.863725 |
| NM_001085 | SERPINA3 | a1-antichymotrypsin | 1.115762 | 0.119054 |
| NM_175739 | SERPINA11 | a1-antitrypsin member 11 | 0.967817 | 0.685694 |
| NM_173850 | SERPINA12 | a1-antitrypsin member 12 | 0.949620 | 0.347089 |
| NM_006220 | SERPINA2 | a1-antitrypsin member 2 | 0.963738 | 0.501943 |
| NM_175739 | SERPINA9 | a1-antitrypsin member 9 | 1.180897 | 0.010430 |
| AF113676 | SERPINA1 | a1-antitrypsin/a1-PI | 2.069461 | 0.540468 |
| NM_001633 | AMBP | a1-microglobulin/bikunin | 0.963393 | 0.647342 |
| NM_000934 | SERPINF2 | a2-antiplasmin | 1.058816 | 0.539507 |
| NM_001622 | AHSG | a2-HS-glycoprotein/fetuinA | 1.131796 | 0.298448 |
| BC032003 | BUSI2/SPINK6 | acrosin inhibitor | 1.234841 | 0.282044 |
| NM_016519 | AMBN | ameloblastin | 1.156183 | 0.318906 |
| NM_000484 | APP | amyloid-b precursor protein | 1.221408 | 0.014118 |
| NM_005166 | APLP1 | amyloid-b precursor-like prot. 1 | 1.217549 | 0.017650 |
| NM_001642 | APLP2 | amyloid-b precursor-like prot. 2 | 1.373346 | 5.78E-05 |
| NM_000029 | SERPINA8 | angiotensinogen/AGT | 1.363918 | 1.30E-06 |
| NM_003064 | SLPI | antileukoproteinase | 1.241500 | 0.085895 |
| NM_000488 | SERPINC1 | antithrombin III | 0.820918 | 0.411764 |
| NM_016252 | BIRC6 | apollon | 1.069461 | 0.279690 |
| NM_005024 | SERPINB10 | bomapin | 1.422769 | 0.880646 |
| NM_000062 | SERPING1 | C1 inhibitor | 0.990278 | 0.811730 |
| NM_001750 | CAST | calpastatin | 1.150194 | 0.039511 |
| NM_001166 | BIRC2 | cIAP1 | 0.840867 | 0.184977 |
| AF070674 | BIRC3 | cIAP2 | 0.691155 | 0.303716 |
| NM_004369 | COL6A3 | collagen, type VI, alpha 3 | 1.058041 | 0.537182 |
| NM_000094 | COL7A1 | collagen, type VII, alpha 1 | 1.582294 | 0.005767 |
| NM_001235 | SERPINH1 | colligin/CBP1 | 1.392081 | 0.217024 |
| NM_000064 | C3 | complement component 3 | 0.916612 | 0.420624 |
| NM_000715 | C4 | complement component 4 | 0.994905 | 0.840848 |
| NM_001735 | C5 | complement component 5 | 1.274596 | 0.019929 |
| BG568135 | CPLP | complement prec. like protein | 0.989554 | 0.705651 |
| NM_001756 | SERPINA6 | corticosteroid-binding glob. | 1.557266 | 0.043683 |
| AL096677 | CST11 | CRES2/cystatin 11 | 0.777972 | 0.692066 |
| XM_166592 | CVL2 (BMPER) | crossveinless 2 | 1.051923 | 0.214969 |
| AF494536 | CST9 | cystatin 9/CLM | 1.104737 | 0.148970 |
| NM_005213 | CSTA | cystatin A | 1.110940 | 0.031630 |
| NM_000100 | CSTB | cystatin B | 0.952614 | 0.403644 |
| NM_000099 | CST3 | cystatin C | 1.229179 | 0.003679 |
| NM_001900 | CST5 | cystatin D | 0.972687 | 0.607470 |
| NM_001323 | CS16 | cystatin E/M | 1.003441 | 0.930285 |
| NM_003650 | CST/ | cystatin F/leukocystatin | 0.966097 | 0.673878 |
| NM_005492 | CS18 | cystatin G | 1.188810 | 0.366838 |
| AK056477 | CSTL1 | cystatin L1 | 1.010096 | 0.843235 |
| NM_001322 | CS12 | cystatin SA | 1.083803 | 0.115995 |
| NM_001898 | CSTI | cystatin SN | 1.139818 | 0.093672 |
| NIM_002638 | PI3 | | 1.030232 | 0.746807 |
| AF419955 | SERPINBTI | epipin | 0.980262 | 0.777802 |
| NM_020398 | SPINLW1 | eppin | 0.975148 | 0.924577 |
| NIVI_032566 | | esophagus cancer-rel. Prot. 2 | 1.296855 | 0.000104 |
| NIM_014375 | FETUB | | 1.043486 | 0.765349 |
| NIM_007005 | | Tomstatin felliatetia like 1 | 0.309850 | 9.03E-08 |
| NM_004550 | FSILT | | 0.667555 | 1.04E-06 |
| NIM_001553 | | | 0.774831 | 0.000173 |
| NM 122402 | FSIL3 | Ionistatin-like 3 | 1.002908 | 0.980355 |
| NIVI_133493 | | Gov platelet alloantigens | 1.173000 | 0.000910 |
| AJ001090 | JERFINDIS | neaupin/nurpin | 1.490/10 | 0.100010 |

| NM_000185 | SERPIND1 | heparin cofactor II | 1.080161 | 0.334504 |
|--------------|-----------|--|----------|----------|
| NM_004684 | SPARCL1 | hevin | 1.096283 | 0.057894 |
| NM_003710 | SPINT1 | HGF activator inhibitor 1 | 1.109062 | 0.491511 |
| NM_002159 | HTN1 | histatin 1 | 1.155191 | 0.089019 |
| NM_000200 | HTN3 | histatin 3 | 1,578399 | 0.070932 |
| NM_000412 | HRG | histidine-rich alvcoprotein | 1 029859 | 0 640591 |
| NM_002864 | P7P | human pregnanzy-zone prot | 1 394909 | 0.133821 |
| NM_000596 | | IGE binding protein 1 | 1.004000 | 0.100021 |
| NM_000507 | | ICE binding protein 2 | 1.200220 | 0.100337 |
| NIVI_000397 | | IOF binding protein 2 | 1.10//00 | 0.004274 |
| IVI35878 | IGFBP3 | IGF binding protein 3 | 1.011298 | 0.771358 |
| NM_033341 | BIRC8 | | 0.967029 | 0.719820 |
| NM_000599 | IGFBP5 | Insulin-like growth factor binding protein 5 | 1.073942 | 0.170618 |
| NM_002178 | IGFBP6 | insulin-like growth factor binding protein 6 † | 0.738959 | 0.000108 |
| NM_006215 | SERPINA4 | kallistatin | 0.998705 | 0.885007 |
| NM_020116 | FSTL5 | kazal, EF-hand and Ig protein | 1.013316 | 0.914660 |
| NM_000893 | KNG | kininogen | 1.191082 | 0.140830 |
| NM_020169 | LXN | latexin/tissue caboxpep. Inhibitor | 0.711593 | 0.182445 |
| NM_002639 | SERPINB5 | maspin | 0.889059 | 0.064913 |
| NM 003784 | SERPINB7 | megsin | 0.879306 | 0.152962 |
| NM 004355 | CD74 | MHC II invariant gamma chain | 1.008040 | 0.816759 |
| BC007758 | KAZALD1 | MIG30-like protease inhibitor | 0.960607 | 0.591541 |
| NM 022161 | BIRC7 | MI -IAP | 0.900314 | 0.061127 |
| A 1001403 | MUC5AC | mucin type 5A/C | 0.948732 | 0.068525 |
| 106711 | MUC5B | mucin type 5R | 0.071023 | 0.530027 |
| NM 004526 | | | 0.071923 | 0.061294 |
| NIVI_004030 | | | 0.905095 | 0.001204 |
| NM_002508 | | nidogon | 1.090020 | 0.003110 |
| NINI_002006 | | nidogen | 1.049992 | 0.200303 |
| NM_007361 | NID2 | nidogen 2 | 1.016664 | 0.706755 |
| NM_003118 | SPARC | osteonectin | 0.892947 | 0.057646 |
| XM_495909 | OVOS | ovostatin | 0.755928 | 0.567147 |
| XM_495917 | OVOS2 | ovostatin-2 | 0.810840 | 0.507534 |
| NM_006217 | SERPINI2 | pancpin | 0.516027 | 0.133038 |
| BC042057 | PAPLN | papilin | 1.058672 | 0.340063 |
| NM_002615 | SERPINF1 | PEDF | 1.043587 | 0.546737 |
| NM_021102 | SPINT2 | placental bikunin | 1.253444 | 0.000317 |
| NM_002575 | SERPINB2 | Plg activator inhibitor-2 | 0.965416 | 0.915980 |
| NM 000602 | SERPINE1 | plg-activator inhibitor-1 | 0.911202 | 0.673847 |
| NM 002728 | PRG2 | Pro-eosinophil major basic protein | 1.000214 | 0.848812 |
| NM_013271 | PCSK1N | proSAAS | 1.037607 | 0.548793 |
| NM_002567 | PBP | prostatic binding protein | 1.032255 | 0.540805 |
| NM_030666 | SERPINB1 | protease inhibitor 2 | 0.897819 | 0.147236 |
| AK057138 | SERPINB6 | protease inhibitor 6/CAP | 0.920037 | 0 077848 |
| NM 002640 | SERPINB8 | protease inhibitor 8/CAP2 | 1 025011 | 0.680374 |
| BC002538 | SERPINBO | protease inhibitor 9/CAP3 | 1 106084 | 0.230701 |
| BC002000 | SERDINA5 | protein C inhibitor | 0.002072 | 0.200701 |
| NM 016186 | | protein 7 dependent Pl | 1 202672 | 0.013031 |
| NIM_005979 | SERFINATO | protein 2-dependent PT | 1.203072 | 0.134204 |
| NIVI_003070 | DECK | | 1.347003 | 0.554026 |
| | | | 0.971043 | 0.703321 |
| NM_002888 | RARREST | retinoic acid receptor responder | 0.963711 | 0.899784 |
| NM_006919 | SERPINB3 | SCC antigen 1 | 1.661538 | 0.152534 |
| NM_002974 | SERPINB4 | SCC antigen 2 | 0.859302 | 0.151886 |
| NM_003020 | SGNE1 | secretogranin V/7B2 | 1.020087 | 0.751839 |
| X77166 | SPINT3 | ser. protease inh., Kunitz 3 | 1.216085 | 0.429040 |
| NM_003122 | SPINK1 | serine PI Kazal type 1 | 0.913402 | 0.802950 |
| NM_021114 | SPINK2 | serine PI Kazal type 2 | 0.619857 | 0.003004 |
| NM_014471 | SPINK4 | serine PI Kazal type 4 | 1.273783 | 0.031096 |
| NM_006846 | SPINK5 | serine PI Kazal type 5 | 1.356567 | 0.103273 |
| BC032033 | SPINK5L1 | serine PI Kazal type 5-like 1 | 1.111233 | 0.368456 |
| NM_001001325 | SPINK5L2 | serine PI Kazal type 5-like 2 | 1.132326 | 0.024687 |

| XM 376433 | SPINK5L3 | serine PI Kazal type 5-like 3 | 1.212333 | 0.008169 |
|-----------|--------------|---------------------------------------|----------|----------|
| AJ249900 | SMOC | SPARC related mod. Ca binding 1 | 0.952066 | 0.246799 |
| AJ420521 | SMOC2 | SPARC related mod. Ca binding 2 | 0.957090 | 0.457938 |
| NM_004598 | SPOCK | sparc/osteonectin, testican | 1.463729 | 0.106900 |
| NM_014767 | SPOCK2 | sparc/osteonectin, testican-2 | 1.090887 | 0.046304 |
| NM_016950 | SPOCK3 | sparc/osteonectin, testican-3 | 0.639993 | 0.656148 |
| NM_003154 | STATH | statherin | 1.031822 | 0.711237 |
| NM_001168 | BIRC5 | survivin | 0.977001 | 0.493230 |
| NM_005422 | TECTA | tectorin a | 1.069791 | 0.251197 |
| NM_080610 | CST9L | testatin | 0.982269 | 0.795178 |
| NM_003235 | TG | thyroglobulin | 1.314124 | 0.218070 |
| NM_000354 | SERPINA7 | thyroxine-binding globulin | 1.195770 | 0.496546 |
| NM_006287 | TFPI | tissue factor path. inhibitor | 1.072906 | 0.099817 |
| NM_006528 | TFPI2 | tissue factor path. inhibitor-2 | 0.900887 | 0.341419 |
| NM_003254 | TIMP1 | tissue inhibitor of metalloprotease-1 | 1.146030 | 0.055343 |
| NM_003255 | TIMP2 | tissue inhibitor of metalloprotease-2 | 1.226753 | 0.001573 |
| NM_000362 | TIMP3 | tissue inhibitor of metalloprotease-3 | 0.829834 | 0.003184 |
| NM_003256 | TIMP4 | tissue inhibitor of metalloprotease-4 | 1.065226 | 0.154352 |
| NM_003256 | TIMP4 | tissue inhibitor of metalloprotease-4 | 0.994201 | 0.960888 |
| NM_003692 | TMEFF1 | TMEFF1 | 1.165063 | 0.019164 |
| NM_016192 | TMEFF2 | TMEFF2 | 1.212011 | 0.178170 |
| BN000366 | VTLGL1 | vitellogenin-like 1 | 1.306186 | 0.126148 |
| NM_000552 | VWF | von Willebrand factor | 1.072439 | 0.228383 |
| NM_080753 | WFDC10A | WAP four-disulfide core 10A | 1.103681 | 0.415616 |
| NM_172006 | WFDC10B | WAP four-disulfide core 10B | 0.344524 | 0.224507 |
| NM_147197 | WFDC11 | WAP four-disulfide core 11 | 1.008892 | 0.854191 |
| NM_080869 | WFDC12 | WAP four-disulfide core 12 | 1.054683 | 0.082567 |
| NM_172005 | WFDC13 | WAP four-disulfide core 13 | 0.831007 | 0.905993 |
| NM_006103 | WFDC2 | WAP four-disulfide core 2 | 0.864907 | 0.006063 |
| AL591713 | WFDC3 | WAP four-disulfide core 3 | 0.888224 | 0.416020 |
| NM_145652 | WFDC5 (WAP1) | WAP four-disulfide core 5 | 1.298514 | 0.009845 |
| AL031663 | WFDC6 | WAP four-disulfide core 6 | 0.927344 | 0.389578 |
| NM_080827 | WFDC8 | WAP four-disulfide core 8 | 0.712735 | 0.315655 |
| NM 147198 | WFDC9 | WAP four-disulfide core 9 | 1.066751 | 0.279610 |
| XM_086637 | WFDCL1 | WAP four-disulfide core-like 1 | 1.016788 | 0.715165 |
| NM_053284 | WFIKKN | WAP,FS,Ig,KU,NTR-containing prot. | 1.306077 | 0.238757 |
| NM_175575 | WFIKKNRP | WFIKKNRP-related protein | 0.945046 | 0.147954 |
| NM_001167 | BIRC4 | XIAP | 1.076452 | 0.004785 |
| AF411191 | SERPINB12 | yukopin | 0.881319 | 0.299544 |

C. Controls

| RefSeq | Abbreviation | Des | cription | Fold change | <i>p</i> -value |
|--------|--------------|----------------------|----------|-------------|-----------------|
| | | 3xSSC-Buffer-Control | | 9.101044 | 0.491734 |
| | | 3xSSC-Buffer-Control | | 2.153620 | 0.053697 |
| | | 3xSSC-Buffer-Control | | 2.117839 | 0.212673 |
| | | 3xSSC-Buffer-Control | | 1.683767 | 0.062998 |
| | | 3xSSC-Buffer-Control | | 1 682604 | 0 609299 |
| | | 3xSSC-Buffer-Control | | 1 541443 | 0.039585 |
| | | 3xSSC-Buffer-Control | | 1 522581 | 0.166693 |
| | | 3xSSC Buffer Control | | 1.504644 | 0.100055 |
| | | 2xSSC Buffer Control | | 1.004044 | 0.370031 |
| | | SXSSC-Buller-Control | | 1.407030 | 0.010120 |
| | | 3xSSC-Buller-Control | | 1.400009 | 0.251017 |
| | | 3xSSC-Buller-Control | | 1.443690 | 0.564168 |
| | | 3xSSC-Buffer-Control | | 1.401066 | 0.137379 |
| | | 3xSSC-Buffer-Control | | 1.394853 | 0.217646 |
| | | 3xSSC-Buffer-Control | | 1.350338 | 0.335351 |
| | | 3xSSC-Buffer-Control | | 1.333502 | 0.323246 |
| | | 3xSSC-Buffer-Control | | 1.321818 | 0.124892 |
| | | 3xSSC-Buffer-Control | | 1.318924 | 0.401541 |
| | | 3xSSC-Buffer-Control | | 1.293731 | 0.275640 |
| | | 3xSSC-Buffer-Control | | 1.269646 | 0.459571 |
| | | 3xSSC-Buffer-Control | | 1.265324 | 0.337944 |
| | | 3xSSC-Buffer-Control | | 1.255772 | 0.443341 |
| | | 3xSSC-Buffer-Control | | 1.253593 | 0.403036 |
| | | 3xSSC-Buffer-Control | | 1.213508 | 0.317620 |
| | | 3xSSC-Buffer-Control | | 1.201503 | 0.754413 |
| | | 3xSSC-Buffer-Control | | 1.200071 | 0.324825 |
| | | 3xSSC-Buffer-Control | | 1,199639 | 0.528563 |
| | | 3xSSC-Buffer-Control | | 1 198666 | 0.357011 |
| | | 3xSSC-Buffer-Control | | 1 184350 | 0 318411 |
| | | 3xSSC-Buffer-Control | | 1.104000 | 0.290846 |
| | | 3xSSC-Buffer-Control | | 1 175003 | 0.230040 |
| | | 3xSSC-Buffer-Control | | 1.175005 | 0.207300 |
| | | 3xSSC Buffer Control | | 1.105400 | 0.364807 |
| | | 2xSSC Buffer Control | | 1.155500 | 0.534697 |
| | | 2xSSC-Buffer Centrel | | 1.130230 | 0.374331 |
| | | 2xSSC-Buller-Control | | 1.147013 | 0.437094 |
| | | 3x55C-Buller-Control | | 1.132709 | 0.413412 |
| | | 3XSSC-Buller-Control | | 1.131731 | 0.039070 |
| | | 3xSSC-Buller-Control | | 1.120790 | 0.875669 |
| | | 3xSSC-Buffer-Control | | 1.119219 | 0.407661 |
| | | 3xSSC-Buffer-Control | | 1.111363 | 0.492707 |
| | | 3xSSC-Buffer-Control | | 1.109306 | 0.352026 |
| | | 3xSSC-Buffer-Control | | 1.108/98 | 0.749334 |
| | | 3xSSC-Buffer-Control | | 1.108515 | 0.399319 |
| | | 3xSSC-Buffer-Control | | 1.104813 | 0.415468 |
| | | 3xSSC-Buffer-Control | | 1.093869 | 0.708624 |
| | | 3xSSC-Buffer-Control | | 1.086596 | 0.754336 |
| | | 3xSSC-Buffer-Control | | 1.085606 | 0.496637 |
| | | 3xSSC-Buffer-Control | | 1.081740 | 0.725755 |
| | | 3xSSC-Buffer-Control | | 1.074953 | 0.713016 |
| | | 3xSSC-Buffer-Control | | 1.074158 | 0.525540 |
| | | 3xSSC-Buffer-Control | | 1.070059 | 0.649994 |
| | | 3xSSC-Buffer-Control | | 1.069545 | 0.502262 |
| | | 3xSSC-Buffer-Control | | 1.069191 | 0.852549 |
| | | 3xSSC-Buffer-Control | | 1.068030 | 0.654231 |
| | | 3xSSC-Buffer-Control | | 1.065620 | 0.640266 |
| | | 3xSSC-Buffer-Control | | 1.060773 | 0.667300 |
| | | 3xSSC-Buffer-Control | | 1.054381 | 0.801154 |
| | | 3xSSC-Buffer-Control | | 1.049149 | 0.738078 |
| | | 3xSSC-Buffer-Control | | 1.048573 | 0.764230 |

| 3xSSC-Buffer-Control | 1.048339 | 0.726058 |
|----------------------|----------|----------|
| 3xSSC-Buffer-Control | 1.045175 | 0.582461 |
| 3xSSC-Buffer-Control | 1 045149 | 0.815620 |
| 3xSSC-Buffer-Control | 1.040140 | 0.504428 |
| 3xSSC Buffer Control | 1.037000 | 0.869471 |
| 3x33C-Dullel-Control | 1.030005 | 0.000471 |
| 3x35C-Dullel-Control | 1.030245 | 0.070102 |
| 3XSSC-Buller-Control | 1.024650 | 0.788535 |
| | 1.024094 | 0.521891 |
| 3xSSC-Buffer-Control | 1.023334 | 0.888356 |
| 3xSSC-Buffer-Control | 1.018369 | 0.852364 |
| 3xSSC-Buffer-Control | 1.016294 | 0.732374 |
| 3xSSC-Buffer-Control | 1.012211 | 0.678951 |
| 3xSSC-Buffer-Control | 1.011647 | 0.755222 |
| 3xSSC-Buffer-Control | 1.009960 | 0.717156 |
| 3xSSC-Buffer-Control | 1.002063 | 0.889794 |
| 3xSSC-Buffer-Control | 1.001977 | 0.795394 |
| 3xSSC-Buffer-Control | 0.998393 | 0.757535 |
| 3xSSC-Buffer-Control | 0.992013 | 0.998461 |
| 3xSSC-Buffer-Control | 0.981882 | 0.627969 |
| 3xSSC-Buffer-Control | 0.979601 | 0.652281 |
| 3xSSC-Buffer-Control | 0.979103 | 0.685563 |
| 3xSSC-Buffer-Control | 0.973698 | 0.715474 |
| 3xSSC-Buffer-Control | 0.969745 | 0.584018 |
| 3xSSC-Buffer-Control | 0.964005 | 0.822468 |
| 3xSSC-Buffer-Control | 0.958943 | 0.809208 |
| 3xSSC-Buffer-Control | 0.958912 | 0.801799 |
| 3xSSC-Buffer-Control | 0.958092 | 0.706562 |
| 3xSSC-Buffer-Control | 0.000002 | 0.858589 |
| 3xSSC-Buffer-Control | 0.344343 | 0.562575 |
| 2xSSC-Duffer-Control | 0.930911 | 0.002070 |
| 3x33C-Dullel-Control | 0.905564 | 0.920803 |
| 3X55C-Dullel-Colliol | 0.902727 | 0.410000 |
| 3X3SC-Dullel-Collion | 0.902456 | 0.922249 |
| 3XSSC-Buller-Control | 0.899661 | 0.362540 |
| | 0.898053 | 0.634489 |
| 3xSSC-Buffer-Control | 0.895706 | 0.611872 |
| 3xSSC-Buffer-Control | 0.890558 | 0.788501 |
| 3xSSC-Buffer-Control | 0.881565 | 0.482852 |
| 3xSSC-Buffer-Control | 0.876999 | 0.827741 |
| 3xSSC-Buffer-Control | 0.844306 | 0.213852 |
| 3xSSC-Buffer-Control | 0.828721 | 0.227371 |
| 3xSSC-Buffer-Control | 0.825767 | 0.490169 |
| 3xSSC-Buffer-Control | 0.821133 | 0.388954 |
| 3xSSC-Buffer-Control | 0.817605 | 0.651503 |
| 3xSSC-Buffer-Control | 0.816332 | 0.559286 |
| 3xSSC-Buffer-Control | 0.814497 | 0.286290 |
| 3xSSC-Buffer-Control | 0.813750 | 0.437126 |
| 3xSSC-Buffer-Control | 0.811158 | 0.562574 |
| 3xSSC-Buffer-Control | 0.800972 | 0.494220 |
| 3xSSC-Buffer-Control | 0.798177 | 0.219879 |
| 3xSSC-Buffer-Control | 0.790439 | 0.648159 |
| 3xSSC-Buffer-Control | 0.781922 | 0.192736 |
| 3xSSC-Buffer-Control | 0.781826 | 0.700627 |
| 3xSSC-Buffer-Control | 0.781793 | 0.281725 |
| 3xSSC-Buffer-Control | 0 777017 | 0.302385 |
| 3xSSC-Buffer-Control | 0.767380 | 0.817716 |
| 3vSSC_Buffer_Control | 0.761909 | 0.261408 |
| 3vSSC_Buffer_Control | 0.760820 | 0.089047 |
| 3vSSC Buffer Control | 0.700029 | 0.000047 |
| | 0.740231 | 0.121/1/ |
| SXSSC-Duller-Collubi | 0.132131 | 0.120131 |

| | 3xSSC-Buffer-Control | 0.720524 | 0.044995 |
|----------------|-----------------------------------|----------|----------|
| | 3xSSC-Buffer-Control | 0.691510 | 0.232009 |
| | 3xSSC-Buffer-Control | 0.687026 | 0.214044 |
| | 3xSSC-Buffer-Control | 0.656844 | 0.146509 |
| | 3xSSC-Buffer-Control | 0.654457 | 0.057955 |
| | 3xSSC-Buffer-Control | 0.648200 | 0.071157 |
| | 3xSSC-Buffer-Control | 0.589694 | 0.023626 |
| | 3xSSC-Buffer-Control | 0.576692 | 0.167650 |
| | 3xSSC-Buffer-Control | 0.562008 | 0.165418 |
| | 3xSSC-Buffer-Control | 0.554861 | 0.521925 |
| | 3xSSC-Buffer-Control | 0.441577 | 0.701282 |
| NM 001101 ACTB | Actin cytoplasmic 1 (beta-actin) | 1 320158 | 0.076795 |
| NM 001101 ACTB | Actin, cytoplasmic 1 (beta-actin) | 1.260563 | 0.126537 |
| NM 001101 ACTB | Actin, cytoplasmic 1 (beta-actin) | 1.214199 | 0.173790 |
| NM 001101 ACTB | Actin, cytoplasmic 1 (beta-actin) | 1.163747 | 0.093199 |
| NM 001101 ACTB | Actin, cytoplasmic 1 (beta-actin) | 1.143629 | 0.327883 |
| NM 001101 ACTB | Actin, cytoplasmic 1 (beta-actin) | 1.108609 | 0.439689 |
| NM 001101 ACTB | Actin cytoplasmic 1 (beta-actin) | 1 048575 | 0.566668 |
| NM 001101 ACTB | Actin, cytoplasmic 1 (beta-actin) | 0.990517 | 0.954436 |
| NM_001658_ARE1 | ADP-ribosylation factor 1 | 1.301575 | 1.97E-05 |
| NM 001658 ARF1 | ADP-ribosylation factor 1 | 1,179632 | 0.003460 |
| NM 001658 ARF1 | ADP-ribosylation factor 1 | 1,158393 | 0.000788 |
| NM_001658_ARF1 | ADP-ribosylation factor 1 | 1.120414 | 0.015584 |
| NM_001658_ARE1 | ADP-ribosylation factor 1 | 1 109894 | 0.054697 |
| NM_001658_ARE1 | ADP-ribosylation factor 1 | 1 107241 | 0 145408 |
| NM_001658_ARE1 | ADP-ribosylation factor 1 | 1.079506 | 0 140435 |
| NM_001658_ARE1 | ADP-ribosylation factor 1 | 1.061861 | 0 178591 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.960322 | 0.591693 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.888141 | 0.015703 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.882977 | 0.006216 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.858743 | 0.024970 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.000740 | 0.000582 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.734323 | 0.000360 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.758082 | 0.000500 |
| NM_001916_CYC1 | Cytochrome c-1 | 0.688509 | 0.000010 |
| | Empty | 1 3/2889 | 0.010104 |
| | Empty | 1 316/70 | 0.273144 |
| | Empty | 1 272894 | 0.283970 |
| | Empty | 1 269845 | 0.200070 |
| | Empty | 1 261/16 | 0.201032 |
| | Empty | 1 251900 | 0.070200 |
| | Empty | 1 202748 | 0.255801 |
| | Empty | 1 193655 | 0.331574 |
| | Empty | 1 113334 | 0.511300 |
| | Empty | 1 111840 | 0.535238 |
| | Empty | 1.1110-0 | 0.550250 |
| | Empty | 1.032046 | 0.078316 |
| | Empty | 1.052040 | 0.855747 |
| | Empty | 1.006018 | 0 794495 |
| | Empty | 0.989327 | 0.958162 |
| | Empty | 0.975999 | 0.396827 |
| | Empty | 0.966479 | 0.978768 |
| | Empty | 0.930263 | 0.490321 |
| | Empty | 0.914253 | 0.674592 |
| | Empty | 0.906395 | 0.479707 |
| | Empty | 0.890632 | 0.318396 |
| | Empty | 0.882011 | 0.760864 |
| | Empty | 0.880530 | 0.746988 |
| | Empty | 0.879583 | 0.512697 |

| Empty | 0.871275 | 0.230929 |
|-------|----------|----------|
| Empty | 0.870609 | 0.250286 |
| Empty | 0.869799 | 0.388612 |
| Empty | 0.869308 | 0.503993 |
| Empty | 0.859441 | 0.220139 |
| Empty | 0.852364 | 0.736347 |
| Empty | 0.847178 | 0.389354 |
| Empty | 0.839135 | 0.274108 |
| Empty | 0 838974 | 0.972312 |
| Empty | 0.834134 | 0.972503 |
| Empty | 0.830354 | 0.202315 |
| Empty | 0.824056 | 0.258456 |
| Empty | 0.024000 | 0.200400 |
| Empty | 0.021014 | 0.284541 |
| Empty | 0.010000 | 0.204041 |
| Empty | 0.010921 | 0.427722 |
| Empty | 0.009213 | 0.493912 |
| Emply | 0.000970 | 0.002220 |
| Emply | 0.790010 | 0.244291 |
| Emply | 0.794539 | 0.187829 |
| Emply | 0.793571 | 0.709491 |
| Empty | 0.792932 | 0.795721 |
| Empty | 0.785398 | 0.145544 |
| Empty | 0.782756 | 0.289911 |
| Empty | 0.779207 | 0.148956 |
| Empty | 0.767169 | 0.114927 |
| Empty | 0.762504 | 0.261767 |
| Empty | 0.754913 | 0.423930 |
| Empty | 0.747020 | 0.369506 |
| Empty | 0.743294 | 0.684023 |
| Empty | 0.741315 | 0.351065 |
| Empty | 0.738735 | 0.272198 |
| Empty | 0./19/05 | 0.097783 |
| Empty | 0.719635 | 0.045603 |
| Empty | 0.718907 | 0.425930 |
| Empty | 0.704479 | 0.037835 |
| Empty | 0.702649 | 0.688181 |
| Empty | 0.700777 | 0.218482 |
| Empty | 0.683730 | 0.183219 |
| Empty | 0.676628 | 0.436179 |
| Empty | 0.665640 | 0.097736 |
| Empty | 0.663862 | 0.029969 |
| Empty | 0.652267 | 0.148114 |
| Empty | 0.649508 | 0.067682 |
| Empty | 0.640181 | 0.482898 |
| Empty | 0.628502 | 0.181282 |
| Empty | 0.626553 | 0.066332 |
| Empty | 0.624301 | 0.158246 |
| Empty | 0.619147 | 0.151247 |
| Empty | 0.613382 | 0.064076 |
| Empty | 0.611611 | 0.029449 |
| Empty | 0.604340 | 0.070956 |
| Empty | 0.596538 | 0.213151 |
| Empty | 0.585420 | 0.062434 |
| Empty | 0.582423 | 0.013252 |
| Empty | 0.581263 | 0.142787 |
| Empty | 0.579439 | 0.098209 |
| Empty | 0.579194 | 0.017104 |
| Empty | 0.578505 | 0.068985 |
| Empty | 0.572952 | 0.126854 |
| Empty | 0.570977 | 0.133955 |
|-------------|-----------|-----------|
| Empty | 0.566039 | 0.005847 |
| Empty | 0.551470 | 0.019429 |
| Empty | 0.538511 | 0.034281 |
| Empty | 0.531330 | 0.250666 |
| Empty | 0.518457 | 0.006085 |
| Empty | 0.507123 | 0.145761 |
| Empty | 0.496518 | 0.003269 |
| Empty | 0.479337 | 0.010037 |
| Empty | 0.453828 | 0.102864 |
| Empty | 0.413143 | 0.005362 |
| Empty | 0.410812 | 0.002063 |
| Empty | 0.055740 | 0.143100 |
| GFP-Control | 1.373794 | 0.041312 |
| GFP-Control | 1.362032 | 0.380011 |
| GFP-Control | 1.227926 | 0.595134 |
| GFP-Control | 1.221479 | 0.020548 |
| GFP-Control | 1.214157 | 0.027285 |
| GEP-Control | 1 211399 | 0 164803 |
| GEP-Control | 1 199223 | 0.002935 |
| GEP-Control | 1 193315 | 0.056545 |
| GEP-Control | 1 189702 | 0 126065 |
| GEP-Control | 1 177525 | 0.231654 |
| GEP-Control | 1 169067 | 0.263240 |
| GEP-Control | 1 164519 | 0.030689 |
| GEP-Control | 1 15/1077 | 0.0000000 |
| GEP-Control | 1 1//335 | 0.330825 |
| GEP-Control | 1 139/60 | 0.375250 |
| GEP-Control | 1 136747 | 0.573230 |
| GEP-Control | 1 136351 | 0.110585 |
| GEP-Control | 1 133607 | 0.066904 |
| GEP-Control | 1 126970 | 0.307614 |
| GEP-Control | 1 126735 | 0 353548 |
| GEP-Control | 1 126402 | 0 384632 |
| GEP-Control | 1 124167 | 0.778590 |
| GEP-Control | 1 115585 | 0 165799 |
| GEP-Control | 1 103227 | 0.602188 |
| GEP-Control | 1 099573 | 0.310374 |
| GEP-Control | 1.008660 | 0.805795 |
| GEP-Control | 1 094874 | 0 277425 |
| GEP-Control | 1 085744 | 0.511572 |
| GEP-Control | 1 084966 | 0.361320 |
| GEP-Control | 1 081926 | 0 196239 |
| GEP-Control | 1.069167 | 0 481475 |
| GEP-Control | 1 065749 | 0.536470 |
| GEP-Control | 1 064340 | 0 290710 |
| GEP-Control | 1.062529 | 0 383850 |
| GEP-Control | 1.002020 | 0.558894 |
| GEP-Control | 1.056287 | 0.382762 |
| GEP-Control | 1.050203 | 0 477599 |
| GEP-Control | 1.000200 | 0.559015 |
| GEP-Control | 1 045609 | 0.555001 |
| GEP-Control | 1 04/303 | 0.413408 |
| GEP-Control | 1 043899 | 0 701141 |
| GEP-Control | 1 042501 | 0 727120 |
| GEP-Control | 1 041707 | 0.596280 |
| GEP-Control | 1 030078 | 0.544586 |
| GEP-Control | 1 018080 | 0 746441 |
| GEP-Control | 1 014789 | 0 701207 |
| Off Control | 1.01-103 | 0.101201 |

| | | GFP-Control | 1.010149 | 0.958673 |
|-------------|-------|---|----------|----------|
| | | GFP-Control | 1.006125 | 0.875940 |
| | | GFP-Control | 1.005197 | 0.899216 |
| | | GFP-Control | 1.004508 | 0.891268 |
| | | GFP-Control | 0.996329 | 0.890316 |
| | | GFP-Control | 0.990736 | 0.813793 |
| | | GFP-Control | 0.990362 | 0.994875 |
| | | GEP-Control | 0.989534 | 0.968705 |
| | | GEP-Control | 0.987680 | 0.981875 |
| | | GEP-Control | 0.984609 | 0.905031 |
| | | GEP-Control | 0.001000 | 0.836310 |
| | | GEP-Control | 0.300034 | 0.000010 |
| | | GEP-Control | 0.0730// | 0.864026 |
| | | GER Control | 0.373344 | 0.004020 |
| | | CER Control | 0.971139 | 0.097307 |
| | | GFP-Control | 0.900959 | 0.000000 |
| | | GFP-Control | 0.962044 | 0.478087 |
| | | GFP-Control | 0.961799 | 0.934211 |
| | | GFP-Control | 0.959449 | 0.951717 |
| | | GFP-Control | 0.951498 | 0.480183 |
| | | GFP-Control | 0.949018 | 0.744483 |
| | | GFP-Control | 0.947818 | 0.612266 |
| | | GFP-Control | 0.940700 | 0.444380 |
| | | GFP-Control | 0.938334 | 0.178634 |
| | | GFP-Control | 0.936164 | 0.748720 |
| | | GFP-Control | 0.935277 | 0.518793 |
| | | GFP-Control | 0.933481 | 0.389017 |
| | | GFP-Control | 0.932744 | 0.908251 |
| | | GFP-Control | 0.930030 | 0.414937 |
| | | GFP-Control | 0.925838 | 0.669011 |
| | | GFP-Control | 0.919144 | 0.770480 |
| | | GFP-Control | 0.913763 | 0.353311 |
| | | GFP-Control | 0.912675 | 0.508359 |
| | | GFP-Control | 0.911817 | 0.875077 |
| | | GFP-Control | 0.907427 | 0.343631 |
| | | GFP-Control | 0.903184 | 0.114940 |
| | | GFP-Control | 0.899543 | 0.537866 |
| | | GFP-Control | 0.894978 | 0.399149 |
| | | GEP-Control | 0.869033 | 0.076159 |
| | | GEP-Control | 0.867638 | 0.419373 |
| | | GEP-Control | 0.850792 | 0.052546 |
| | | GEP-Control | 0.846430 | 0.002573 |
| | | GEP-Control | 0.040400 | 0.227671 |
| | | GEP-Control | 0.014000 | 0.227071 |
| | | GEP-Control | 0.804644 | 0.020020 |
| | | GER Control | 0.686506 | 0.197/95 |
| | | GEP Control | 0.000500 | 0.107403 |
| NM 002046 | САРОН | Giveoraldohydo 3 phosphata dohydragonaso | 1.006744 | 0.012090 |
| NM 002040 | | Clyceraldehyde 2 phosphate dehydrogenase | 1.000744 | 0.990347 |
| NM 002046 | GAPDH | Giveraldehyde 3 pheephate dehydrogenase | 0.075115 | 0.900102 |
| NIVI_002046 | GAPDH | Giveraldehyde-3-phosphate dehydrogenase | 0.975115 | 0.752235 |
| NM 002046 | CAPDH | Chippenaldehyde 2 phoephate dehydrogenase | 0.974302 | 0.093841 |
| NIM 002046 | CAPDI | Chierardehyde 2 phoephate dehydrogenase | 0.951271 | 0.00109 |
| NIM_002046 | GAPDH | Giveraldenvde 3 phoophate denvdrogenase | 0.857829 | 0.171651 |
| NM_002046 | GAPDH | Giyceraldenyde-3-phosphate dehydrogenase | 0.855477 | 0.103813 |
| NM_002046 | GAPDH | Giveraidenyde-3-phosphate dehydrogenase | 0.824674 | 0.044534 |
| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 1.038948 | 0.495769 |
| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 1.003586 | 0.961562 |
| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 0.968885 | 0.711352 |
| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 0.923480 | 0.124930 |
| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 0.896015 | 0.084222 |

| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 0.854087 | 0.010235 |
|----------|-------|--|----------|-----------|
| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 0.824989 | 0.010652 |
| AF218029 | H3F3B | H3 histone, family 3B (H3.3B) | 0.775796 | 0.005712 |
| M15035 | apt | MPA - Xanthine guanine phosphoribosyltransferase | 1.213503 | 0.207676 |
| M15035 | apt | MPA - Xanthine quanine phosphoribosvltransferase | 1.164360 | 0.600562 |
| M15035 | apt | MPA - Xanthine quanine phosphoribosvltransferase | 1.059769 | 0.548813 |
| M15035 | apt | MPA - Xanthine guanine phosphoribosyltransferase | 1.020576 | 0.539109 |
| M15035 | apt | MPA - Xanthine guanine phosphoribosyltransferase | 0.962912 | 0.946339 |
| M15035 | apt | MPA - Xanthine quanine phosphoribosyltransferase | 0.873381 | 0 797657 |
| M15035 | apt | MPA - Xanthine guanine phosphoribosyltransferase | 0.866626 | 0.375594 |
| M15035 | apt | MPA - Xanthine quanine phosphoribosyltransferase | 0.686178 | 0 409494 |
| NDK1 | | NDK1 | 1 842349 | 0 191870 |
| | | NDK1 | 1 713619 | 0.102483 |
| | | NDK1 | 1.521871 | 0.102403 |
| | | NDK1 | 1 313271 | 0.403030 |
| | | NDK1 | 1.010271 | 0.506352 |
| | | | 1.270237 | 0.030002 |
| | | | 1.222047 | 0.0000000 |
| | | | 1.214170 | 0.052524 |
| | | | 1.032023 | 0.957795 |
| | | | 1.01/2/0 | 0.805858 |
| | | | 1.010801 | 0.956049 |
| NDKT | NDK1 | NDK1 | 0.920441 | 0.717328 |
| NDK1 | NDK1 | NDK1 | 0.872578 | 0.289445 |
| NDK1 | NDK1 | NDK1 | 0.794864 | 0.754110 |
| NDK1 | NDK1 | NDK1 | 0.665884 | 0.271131 |
| NDK1 | NDK1 | NDK1 | 0.619484 | 0.084678 |
| NDK1 | NDK1 | NDK1 | 0.521094 | 0.932313 |
| NC_N1 | NC_N1 | Negative Control_N1 | 1.564936 | 0.705817 |
| NC_N1 | NC_N1 | Negative Control_N1 | 1.025611 | 0.688093 |
| NC_N1 | NC_N1 | Negative Control_N1 | 1.005630 | 0.848389 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.966209 | 0.391972 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.964266 | 0.875656 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.940094 | 0.163284 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.920840 | 0.091896 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.914413 | 0.385506 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.911967 | 0.293816 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.898627 | 0.006943 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.885450 | 0.033359 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.883065 | 0.036959 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.876633 | 0.007467 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.864734 | 0.228948 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.862219 | 0.001460 |
| NC_N1 | NC_N1 | Negative Control_N1 | 0.842646 | 0.020060 |
| NC_N2 | NC_N2 | Negative Control_N2 | 1.041449 | 0.731445 |
| NC_N2 | NC_N2 | | 1.041330 | 0.534733 |
| NC_N2 | NC_N2 | Negative Control_N2 | 1.014790 | 0.724225 |
| NC_N2 | NC_N2 | Negative Control_N2 | 1.014354 | 0.769509 |
| NC_N2 | NC_N2 | Negative Control_N2 | 0.968201 | 0.687824 |
| NC_N2 | NC_N2 | | 0.890440 | 0.202514 |
| NC_N2 | NC_N2 | | 0.885558 | 0.265160 |
| NC_N2 | NC_N2 | | 0.869849 | 0.708348 |
| NC_N2 | | Negative Control_N2 | 0.833630 | 0.170708 |
| NC_N2 | NC_N2 | Negative Control_N2 | 0.821774 | 0.307062 |
| NC_N2 | NC_N2 | | 0.790588 | 0.202369 |
| NC_N2 | NC_N2 | Negative Control_N2 | 0.775770 | 0.137188 |
| NC_N2 | NC_N2 | Negative Control_N2 | 0.768202 | 0.372975 |
| NC_N2 | NC_N2 | Negative Control_N2 | 0.747658 | 0.009641 |
| NC_N2 | NC_N2 | Negative Control_N2 | 0.734347 | 0.080367 |
| NC_N2 | NC_N2 | Negative Control_N2 | 0.621109 | 0.038855 |

| NC N3 | NC N3 | Negative Control N3 | 1.221637 | 0.205127 |
|-----------|---------|---|----------|----------|
| NC N3 | NC N3 | Negative Control N3 | 1.161713 | 0.608263 |
| NC N3 | NC N3 | Negative Control N3 | 1.143881 | 0.377324 |
| NC N3 | NC N3 | Negative Control N3 | 0.964219 | 0.568178 |
| NC N3 | NC N3 | Negative Control N3 | 0.950491 | 0 868757 |
| NC N3 | NC N3 | Negative Control N3 | 0 927004 | 0.888828 |
| NC N3 | NC N3 | Negative Control_N3 | 0.027004 | 0.000020 |
| | | Negative Control_N3 | 0.020022 | 0.000072 |
| | | Negative Control_N3 | 0.914373 | 0.094014 |
| | | Negative Control_NS | 0.906233 | 0.902994 |
| | | Negative Control_NS | 0.004407 | 0.220095 |
| NC_N3 | | Negative Control_N3 | 0.823245 | 0.093444 |
| NC_N3 | NC_N3 | Negative Control_N3 | 0.809570 | 0.356488 |
| NC_N3 | NC_N3 | Negative Control_N3 | 0.803589 | 0.120472 |
| NC_N3 | NC_N3 | Negative Control_N3 | 0.787581 | 0.140850 |
| NC_N3 | NC_N3 | Negative Control_N3 | 0.773114 | 0.133649 |
| NC_N3 | NC_N3 | Negative Control_N3 | 0.603433 | 0.421886 |
| NC_N5 | NC_N5 | Negative Control_N5 | 1.443052 | 0.715605 |
| NC_N5 | NC_N5 | Negative Control_N5 | 1.352722 | 0.135082 |
| NC_N5 | NC_N5 | Negative Control_N5 | 1.270996 | 0.455543 |
| NC N5 | NC N5 | Negative Control N5 | 1.229399 | 0.253185 |
| NC N5 | NC N5 | Negative Control N5 | 1.166800 | 0.377805 |
| NC N5 | NC N5 | Negative Control N5 | 1.130391 | 0.346664 |
| NC N5 | NC N5 | Negative Control N5 | 1.011218 | 0.770438 |
| NC N5 | NC N5 | Negative Control N5 | 0.960458 | 0 694140 |
| NC N5 | NC N5 | Negative Control_N5 | 0.952509 | 0.813063 |
| NC N5 | NC N5 | Negative Control_N5 | 0.002000 | 0.812088 |
| NC_N5 | NC N5 | Negative Control_N5 | 0.920450 | 0.012000 |
| NC_N5 | | Negative Control_N5 | 0.910459 | 0.995510 |
| NC_N5 | | Negative Control_N5 | 0.910951 | 0.072901 |
| | | Negative Control_N5 | 0.01/000 | 0.240898 |
| | | | 0.654316 | 0.274503 |
| NC_N5 | NC_N5 | Negative Control_N5 | 0.525622 | 0.138899 |
| NC_N5 | NC_N5 | Negative Control_N5 | 0.514356 | 0.346240 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 1.4261/4 | 0.252390 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 1.386859 | 0.293967 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 1.227263 | 0.718116 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 1.102149 | 0.880148 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 1.008823 | 0.358213 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 0.884944 | 0.890223 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 0.790242 | 0.928064 |
| AF264723 | neo | NEOMYCIN - Aminoglycoside-3'-phosphotransferase | 0.654853 | 0.246601 |
| NM 016553 | NUP62 | Nucleoporin 62kD | 1.643939 | 0.185881 |
| NM_016553 | NUP62 | Nucleoporin 62kD | 1.100782 | 0.134747 |
| NM 016553 | NUP62 | Nucleoporin 62kD | 1.097558 | 0.179589 |
| NM_016553 | NUP62 | Nucleoporin 62kD | 1.092638 | 0.087445 |
| NM_016553 | NUP62 | Nucleoporin 62kD | 1 006540 | 0 810547 |
| NM_016553 | NUP62 | Nucleoporin 62kD | 0.971338 | 0.604648 |
| NM_016553 | NUP62 | Nucleoporin 62kD | 0.071000 | 0.677006 |
| NM 016553 | NUID62 | Nucleoporin 62kD | 0.000710 | 0.366862 |
| M25246 | 1101 02 | DUDINVCIN N Acetyltransforace (S. albanigar) | 2 657761 | 0.500002 |
| N25240 | pac | PURIMYCIN N-Acetyltransferase (S. alboniger) | 1 029002 | 0.012093 |
| IVI25340 | pac | PURIMYCIN N-Acetyltransierase (S. alboniger) | 1.020002 | 0.015300 |
| W25346 | pac | PURINI CIN N-Acetyltransferase (S. alboniger) | 1.026649 | 0.901336 |
| NI25346 | pac | PURINIYCIN N-Acetyltransferase (S. alboniger) | 0.893954 | 0.253321 |
| W25346 | pac | PURINIYCIN N-Acetyltransferase (S. alboniger) | 0.808827 | 0.006837 |
| M25346 | pac | PURIMYCIN N-Acetyltransferase (S. alboniger) | 0.800525 | 0.023850 |
| M25346 | pac | PURIMYCIN N-Acetyltransferase (S. alboniger) | 0.765104 | 0.024010 |
| M25346 | pac | PURIMYCIN N-Acetyltransferase (S. alboniger) | 0.728418 | 0.006156 |
| AK023419 | RPL37a | Ribosomal protein L37A | 0.958284 | 0.527895 |
| AK023419 | RPL37a | Ribosomal protein L37A | 0.932828 | 0.180346 |
| AK023419 | RPL37a | Ribosomal protein L37A | 0.904768 | 0.109153 |

| AK023419 | RPL37a | Ribosomal protein L37A | 0.877932 | 0.054668 |
|----------------------|----------------|--|---------------|----------|
| AK023419 | RPL37a | Ribosomal protein L37A | 0.863956 | 0.029053 |
| AK023419 | RPL37a | Ribosomal protein L37A | 0.861970 | 0.001551 |
| AK023419 | RPL37a | Ribosomal protein L37A | 0.849491 | 0.016032 |
| AK023419 | RPL37a | Ribosomal protein L37A | 0.768271 | 0.002747 |
| NM 021104 | RPL41 | Ribosomal protein L41 (60S, HG12) | 1.036171 | 0.629180 |
| NM 021104 | RPL41 | Ribosomal protein L41 (60S, HG12) | 0.929887 | 0.120827 |
| NM_021104 | RPL41 | Ribosomal protein L41 (60S, HG12) | 0.919265 | 0.206726 |
| NM 021104 | RPL41 | Ribosomal protein L41 (60S, HG12) | 0.917678 | 0.038296 |
| NM 021104 | RPL41 | Ribosomal protein L41 (60S, HG12) | 0.899487 | 0.113507 |
| NM 021104 | RPI 41 | Ribosomal protein L41 (60S, HG12) | 0 897576 | 0.057351 |
| NM 021104 | RPI 41 | Ribosomal protein L41 (60S, HG12) | 0 793321 | 0.061383 |
| NM 021104 | RPI 41 | Ribosomal protein L41 (60S, HG12) | 0 792411 | 0.005852 |
| NM_001021 | RPS17 | Ribosomal protein S17 | 0 909104 | 0.230369 |
| NM_001021 | RPS17 | Ribosomal protein S17 | 0.899736 | 0.105351 |
| NM_001021 | RPS17 | Ribosomal protein S17 | 0.89/875 | 0.007540 |
| NM_001021 | PPS17 | Ribosomal protein S17 | 0.883006 | 0.655064 |
| NM_001021 | RDS17 | Ribosomal protein S17 | 0.863176 | 0.028946 |
| NM_001021 | PPS17 | Ribosomal protein S17 | 0.834702 | 0.020940 |
| NM_001021 | DDS17 | Pibesomal protein S17 | 0.054702 | 0.002045 |
| NM_001021 | | Ribosomal protein S17 | 0.730372 | 0.000310 |
| AL 024270 | RFOIT | Ribosomal protein S17 | 1 1 2 2 2 0 7 | 0.000270 |
| AL034379 | DDC27 | Pibesomal protein S27 | 1.120007 | 0.102001 |
| AL034379 | DDS27 | Ribosomal protein S27 | 1.000045 | 0.190301 |
| AL034379 | RF3ZI | Ribosomal protein S27 | 1.047300 | 0.576065 |
| AL034379 | RP327 | Ribosomal protein 527 | 1.042013 | 0.303200 |
| AL034379 | RPOZI | Ribosomal protein 527 | 0.076216 | 0.711324 |
| AL034379 | RPOZI DDC07 | Ribosomal protein 527 | 0.970310 | 0.010300 |
| AL034379 | | Ribosomal protein 527 | 0.902430 | 0.470200 |
| AL034379 | RF3ZI | Ribosomal protein S27 | 0.030390 | 0.010095 |
| NM_001013 | RF39 | Ribosomal protein S9 | 0.900912 | 0.009755 |
| NM_001013 | RF39 | Ribosomal protein S9 | 0.951073 | 0.394342 |
| NM_001013 | RF39 DDS0 | Ribosomal protein S9 Pibosomal protein S9 | 0.809374 | 0.040201 |
| NM_001013 | RF39 | Ribosomal protein S9 | 0.04/9/0 | 0.000033 |
| NM_001013 | RF39 | Ribosomal protein S9 | 0.020070 | 0.002960 |
| NM_001013 | | Ribosomal protein S9 | 0.010042 | 0.001444 |
| NM_001013 | RF39 | Ribosomal protein S9 | 0.799754 | 0.000100 |
| AK001212 | | Ribosomal protein Jargo P0 | 1 1 2 8 / 2 / | 0.003671 |
| AK001313 | | Pibesomal protein, large, P0 | 1.150454 | 0.441391 |
| AK001313 | | Ribosomal protein, large, PU | 1.009097 | 0.229309 |
| AK001313 | | Ribosomal protein, large, PU | 1.042102 | 0.279000 |
| AK001313 | | Ribosomal protein, large, PU | 1.020000 | 0.433711 |
| AK001313 | | Ribosomal protein, large, P0 | 0.009579 | 0.711921 |
| AK001313 | | Ribosomal protein, large, P0 Pibesomal protein, large, P0 | 0.990070 | 0.997023 |
| AK001313 | | Ribosomal protein, large, P0 Pibesomal protein, large, P0 | 0.970122 | 0.034104 |
| AKUU1313 DI100247 | | Ribosomal protein, large, PU | 1 251752 | 0.210200 |
| DI 190347 | | Ribosomal protein, large, P1 | 1.301703 | 0.372900 |
| DI 190347 | | Ribosomal protein, large, P1 | 1.320017 | 0.100125 |
| DI190347 | | Ribosomal protein, large, P1 | 1.223939 | 0.127324 |
| DI 190347 | | Ribosomal protein, large, P1 | 1.207411 | 0.414771 |
| DI 190347 | | Ribosomal protein, large, P1 | 1.110001 | 0.401144 |
| DI 190347 | | Ribosomal protein, large, P1 | 1.072302 | 0.707395 |
| BI109347 | | Ribosomal protein, large, P1 | 0.801050 | 0.944200 |
| AK055076 | | Thumosin bots 4. X chromosome | 0.091939 | 0.330029 |
| AK055970 | | Thymosin, beta 4, X chromosome | 0.0502417 | 0.751051 |
| AK055970 | | Thymosin, beta 4, X chromosome | 0.959042 | 0.470002 |
| AK055976 | | Thymosin, beta 4, X chromosome | 0.952760 | 0.397004 |
| AK055976 | | Thymosin, beta 4, X chromosome | 0.930307 | 0.317250 |
| AK055976 | | Thymosin, beta 4, X chromosome | 0.909007 | 0.400190 |
| HI0000910 | INSD4A | mymosin, beta 4, A chitomosome | 0.091040 | 0.052155 |

| AK055976 | TMSB4X | Thymosin, beta 4, X chromosome | 0.825296 | 0.072870 |
|-----------|-----------|--------------------------------|----------|----------|
| AK055976 | TMSB4X | Thymosin, beta 4, X chromosome | 0.819117 | 0.042763 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.516300 | 7.00E-05 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.454935 | 8.06E-05 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.410225 | 0.000244 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.370580 | 2.90E-09 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.369174 | 1.96E-07 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.364405 | 2.44E-08 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.363009 | 1.39E-08 |
| NM_006082 | K-ALPHA-1 | Tubulin, alpha, ubiquitous | 0.357775 | 7.04E-08 |