VERSICAN IN THE WOUND HEALING MATRIX: CELLULAR INTERACTIONS AND DEGRADATION BY MATRIX METALLOPROTEINASES

by

Saloumeh Pourmalek

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In wound healing, versican is a component of the provisional matrix laid down at
the site of injury by proliferating myofibroblasts. Versican interacts with a variety
of matrix molecules and is believed to interact with the cell surface. The
mechanism of interaction of versican with the cell surface, however, is not well
documented. Return to normal tissue structure, at late stages of wound healing,
involves degradation of versican and concomitant fibroblast apoptosis.
Macrophage enzymes are candidates for versican degradation; however, the
mechanisms of actions of these enzymes on versican and the rates and cleavage
sites are not yet known.

This thesis tests several hypotheses: 1) Versican interacts with cell surface
receptors of myofibroblasts and macrophages; 2) Versican influences
myofibroblast cell morphology during wound contraction; and 3) Macrophage
matrix metalloproteinases degrade versican during wound resolution.

We first attempted to identify macrophage and fibroblast versican
binding cell surface ligands. Using biotinylated constructs of the C-terminal domain of
versican as baits, we identified versican and versican fragments as the main
ligands for the C-terminal construct. However, we found that most versican could
be released from the cell surface by hyaluronidase treatment, and concluded that
versican is held at the fibroblast cell surface mainly through its interaction with
hyaluronan.

Next, we examined the influence of versican and hyaluronan on the physical
properties of a collagenous matrix, and the cells embedded within the matrix,
using a novel 3-dimensional collagen/versican/hyaluronan matrix model. We
found that fibroblast cells in matrices containing versican express smooth
muscle actin and take on a contractile morphology.

Finally, we hypothesized that macrophage metalloproteinases degrade versican.
The macrophage matrix metalloproteinases (MMPs), MMP-2, MMP-7, and
MMP-12 were chosen as candidate enzymes, which we localized to the resolving
phase of wound healing in the human lung. We found that MMP-7 and MMP-12
cleave versican at multiple sites in vitro, whereas MMP-2 cleaves versican at a
limited number of sites. These macrophage enzymes may be important in
clearing versican in vivo.

A better understanding of the mechanism of versican degradation could enable
therapeutic modification of the disease process in fibrosis, cancer, and nervous
system regeneration.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>α-Smooth Muscle Actin</td>
</tr>
<tr>
<td>3D</td>
<td>Three Dimensional</td>
</tr>
<tr>
<td>AGG</td>
<td>Aggrekan</td>
</tr>
<tr>
<td>ARDS</td>
<td>Adult Respiratory Disease Syndrome</td>
</tr>
<tr>
<td>BOOP</td>
<td>Bronchiolitis Obliterans Organizing Pneumonia</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C Ligand</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CRP</td>
<td>Complementary Repeat Protein</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin Sulfate</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin Sulfate Proteoglycan</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC Chemokine Ligand</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>G1</td>
<td>N-terminal Domain of Lecticans</td>
</tr>
<tr>
<td>G2</td>
<td>Link Binding Modules in Aggrekan</td>
</tr>
<tr>
<td>G3</td>
<td>C-terminal Domain of Lecticans</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte monocyte colony stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>Has</td>
<td>Hyaluronan Synthase</td>
</tr>
<tr>
<td>Hdf</td>
<td>Heart Defect</td>
</tr>
<tr>
<td>HFL</td>
<td>Human Fetal Lung</td>
</tr>
<tr>
<td>HisG3</td>
<td>Histidine-tagged G3 Domain</td>
</tr>
<tr>
<td>HisLC</td>
<td>Histidine-tagged LC domain</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic Pulmonary Fibrosis</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>NCC</td>
<td>Neural Crest Cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinase</td>
</tr>
<tr>
<td>UIP</td>
<td>Usual Interstitial Pneumonia</td>
</tr>
<tr>
<td>VC</td>
<td>Versican</td>
</tr>
</tbody>
</table>
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To My Beloved Parents
CO-AUTHORSHIP STATEMENT

Early immunohistochemical studies in our laboratory, under the supervision of Clive R. Roberts, showed the accumulation of macrophages around versican-rich lesions in fibrotic lung disease through immunohistochemistry. Observation that versican degradation occurs concomitantly with fibroblast cell apoptosis lead to the hypothesis that macrophage enzymes may be involved in the degradation of versican. I purified versican expressed by human lung fibroblast cells and performed the degradation assays with macrophage matrix metalloproteinases (MMPs). MMP-2 and MMP-12 were kind gifts from Chris Overall laboratory. The manuscript was written by me, and edited by my supervisor, Clive Roberts.

Clive Roberts

Saloumeh Pourmalek
1. LITERATURE REVIEW

1.1 Wound Healing

Wound healing is an intricate mechanism involving a myriad of factors and cell types. The wound healing process is classically divided into four phases which include homeostasis, inflammation, proliferation and remodeling (reviewed in\cite{1,2}). Homeostasis is marked by vasoconstriction of injured blood vessels and activation of intrinsic coagulation pathway that leads to formation of a blood clot and release of growth factors and cytokines by platelets. In the inflammatory phase, vasodilation and platelet disaggregation allow neutrophils to enter the wound site and remove bacteria and damaged tissue from the extracellular matrix. Macrophages, which are attracted to the wound site by chemoattractants, continue the process of phagocytosis and recruit fibroblast cells by adding to the pool of growth factors already present at the site. Once the matrix is cleared out, inflammation is turned off and the process of epithelialization and matrix formation is initiated by proliferating fibroblasts. Fibroblasts migrate into the cleared site and lay down a provisional matrix rich in proteoglycan versican, glycosaminoglycan hyaluronan, fibronectin, tenascin, and a number of other proteins. PDGF and activated TGF-β, released mainly by macrophages, are the main triggers for production of this transient matrix. These two growth factors, along with other granulation tissue components, signal fibroblast differentiation into contractile myofibroblast which are the main cell type responsible for wound closure. In the final remodeling phase of wound healing, fibroblasts express high levels of type I collagen, and an organized collagenous matrix replaces proteoglycan and fibronectin. As the provisional matrix is degraded by matrix metalloproteinases (MMPs), myofibroblasts go into apoptosis and the anatomy and function of the tissue is restored.

Repeated insult and the ensuing inflammation result in prolonged myofibroblast proliferation and excessive granulation tissue synthesis at the site of injury. The accumulation of non-functional and excessive scar tissue, or fibrosis, is associated with many clinical problems such as keloid or hypertrophic scar formation in the skin, delayed nervous system regeneration, lung and liver dysfunction, and atherosclerosis\cite{3,4}.
My study centers around wound healing and fibrosis in the lungs. Replacement of normal lung architecture with collagenous matrix causes decreased lung air space volume and obstruction of gas exchange, which can result in considerable loss of lung function and ultimately respiratory failure and death. Persistence and magnitude of initial injury, multiple modes of tissue injury and slower than normal clot resolution are all possible causes that may lead to pulmonary fibrosis instead of normal wound healing. Whatever the cause, however, heavy proteoglycan deposition in association with proliferating myofibroblasts is central to the persistence of active lesions in most fibrotic lung diseases. What follows is a review of the cellular and molecular mechanisms involved in wound contraction and granulation tissue remodeling, and the pathophysiology of wound healing and pulmonary fibrosis.

1.1.1 Molecular Mechanisms of Wound Contraction and Remodeling

The deposition of a transient granulation tissue and its subsequent maturation and remodeling is an important phase of wound healing. Multiple growth factors and cytokines regulate the process of new matrix synthesis and allow it to proceed in an orderly fashion. Platelet-derived growth factor (PDGF) and Transforming growth factor beta (TGF-β) are the two main growth factors expressed by macrophages and fibroblasts during this phase of wound healing. In response to PDGF, fibroblasts begin synthesizing a provisional matrix rich in glycosaminoglycans, fibronectin and collagen type III. PDGF stimulates hyaluronan synthesis, versican synthesis, and smooth muscle cell proliferation. TGF-β is involved in organizing the extracellular matrix, scar remodeling and wound contracture. TGF-β enhances matrix deposition by fibroblasts through increasing the expression of collagen, hyaluronan, versican, and fibronectin. This growth factor also prevents matrix degradation by lowering production of matrix degrading enzymes (MMPs) and increasing expression of tissue inhibitors of metalloproteinases (TIMPs). Major TGF-β subtypes involved in wound healing are TGF-β1 and TGF-β2, although there are no known major differences in terms of function. In response to this newly synthesized matrix, and under the influence of TGF-β and PDGF, fibroblasts differentiate into a distinct cell type known as myofibroblasts.
Differentiated contractile myofibroblasts are recognizable by their \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) expression in stress fibers\(^{24}\), and are responsible for contracting the granulation tissue and closing the wound\(^{25}\). Studies conducted in different tissues suggest that resident myofibroblasts, arising from a population of tissue specific fibroblasts activated in response to injury, are the primary cell type found in wound matrix\(^{26}\). Several mechanisms have been suggested for the differentiation of fibroblasts to myofibroblasts. Combined effects of TGF-\(\beta\)1 and mechanical tension, in particular, have been the focus of multiple reviews in recent years\(^{4,26,27}\). TGF-\(\beta\)1 induces \(\alpha\)-smooth muscle actin expression in granulation tissue myofibroblasts on stiff 2-dimentional culture substrate\(^{28,29}\), but not on compliant substrate void of tension\(^{30}\). This phenomenon is also observed in 3-dimensional collagen matrices, where TGF-\(\beta\)1 induces myofibroblast differentiation when gels are mechanically restrained\(^{31}\), but not in free-floating and relaxed gels\(^{24}\). On the other hand, mechanical stress alone does not seems to induce myofibroblast differentiation in the absence of active TGF-\(\beta\)1\(^{31,32}\).

Although it is unclear how mechanical stress and TGF-\(\beta\)1 signaling converge to promote increased \(\alpha\)-SMA expression and myofibroblast differentiation, one theory suggests that mechanical tension may regulate TGF-\(\beta\)1 activation by releasing it from its large latent complex\(^{33,34}\). Latent complex provides a reservoir of latent TGF-\(\beta\)1 in the ECM by binding to other ECM components like fibrillin-1 and fibronectin\(^{35-37}\). It is noteworthy, however, that other mechanisms for activation of TGF-\(\beta\)1 have previously been reported. For example thrombospondin, a component of the provisional matrix, is also able to activate TGF-\(\beta\)1\(^{38}\), as can integrin \(\alpha_\text{v}\beta_6\)\(^{39}\) which releases TGF-\(\beta\)1 from its latency complex at remodeling sites.

Another explanation for the cumulative effects of TGF-\(\beta\)1 and mechanical tension on myofibroblast differentiation and \(\alpha\)-SMA expression may lie in the expression of multitude of other ECM proteins that are produced in response to both TGF-\(\beta\)1 and mechanical tension. A comparison of all the major factors found in the wound environment shows that the same factors that induce myofibroblast differentiation and \(\alpha\)-SMA expression also increase versican expression and the expression of proteins that associate with versican (Table 1.1).
### Table 1.1 Different Factors that Influence Matrix Deposition and Myofibroblast Phenotype

<table>
<thead>
<tr>
<th>Effectors / Stimulants</th>
<th>Increased Versican Expression</th>
<th>Stress Fiber Accumulation</th>
<th>Induced α-SMA Expression</th>
<th>Fibroblast to Myofibroblast differentiation</th>
<th>Increased Expression of Other ECM Components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TGF-β1</strong></td>
<td>• Fibroblasts co-cultured with Keratinocytes&lt;sup&gt;40&lt;/sup&gt;</td>
<td>• Granulation tissue myofibroblasts &amp; quiescent cultured Fibroblasts&lt;sup&gt;28&lt;/sup&gt;</td>
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<td>• Quiescent human breast gland Fibroblasts&lt;sup&gt;32&lt;/sup&gt;</td>
<td>• Gingival Fibroblasts&lt;sup&gt;47&lt;/sup&gt;</td>
<td>• HA&lt;sup&gt;17&lt;/sup&gt;</td>
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<td>• Cultured Fibroblasts&lt;sup&gt;41&lt;/sup&gt;</td>
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<td>• CHO Cells&lt;sup&gt;74&lt;/sup&gt;</td>
<td>• Hepatic Stellate Cells&lt;sup&gt;16&lt;/sup&gt;</td>
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<td>• activated Fibroblasts&lt;sup&gt;65&lt;/sup&gt;</td>
<td>• Collagen&lt;sup&gt;71&lt;/sup&gt;</td>
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<td><strong>ET-1</strong></td>
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<td>• CHO Cells&lt;sup&gt;74&lt;/sup&gt;</td>
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<td>• Granulation Tissue Myofibroblast&lt;sup&gt;78&lt;/sup&gt;</td>
<td>• Lung Fibroblasts&lt;sup&gt;73&lt;/sup&gt;</td>
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<tr>
<td><strong>GM-CSF</strong></td>
<td>• Monocytes in Myocardial Infarction&lt;sup&gt;82&lt;/sup&gt;</td>
<td>• Myofibroblasts&lt;sup&gt;83&lt;/sup&gt;</td>
<td>• Asthmatic SMC via induction of TGFβ1 Receptor&lt;sup&gt;84&lt;/sup&gt; &amp; TGFβ&lt;sup&gt;86&lt;/sup&gt;</td>
<td></td>
<td>• Colonic myofibroblast&lt;sup&gt;79&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>• Myofibroblasts&lt;sup&gt;83&lt;/sup&gt;</td>
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<td></td>
<td>• Myofibroblasts&lt;sup&gt;80&lt;/sup&gt;</td>
<td>• HA/CD44 in VSMC&lt;sup&gt;81&lt;/sup&gt;</td>
</tr>
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</table>
**Versican** is a regulatory proteoglycan and is thought to influence cell adhesion, proliferation, migration and extracellular matrix assembly\(^\text{87, 88}\). Versican induces neuronal differentiation and neurite outgrowth\(^\text{89}\), differentiation of preadipocytes\(^\text{90}\), and is essential for pre-cartilage aggregation and subsequent cartilage differentiation\(^\text{91, 92}\). There is a growing body of evidence that associates granulation tissue proteoglycans with myofibroblast cell differentiation and survival. The structural, mechanical, and biochemical properties of versican-rich matrix and its influence on myofibroblast cell phenotype are further investigated in this thesis; and the results have lead me to develop a new hypothesis for the role of proteoglycan versican in wound healing. My hypothesis implicates versican as more than just a structural support in the temporary scaffold of granulation tissue. A detailed description of versican and its binding partners, and an examination of their role in wound healing is given in the next section of this chapter.

In the later stages of wound remodeling, differentiated myofibroblasts lay down a collagenous matrix that replaces the provisional proteoglycan-rich matrix and shapes the fibrous tissue generally observed in fibrosis\(^\text{93-95}\). Once the process of wound contraction by differentiated myofibroblasts is completed, \(\alpha\)-SMA expressing myofibroblasts disappear from the scar\(^\text{25}\) through the process of apoptosis\(^\text{96}\). Although it is well established that regression of granulation tissue occurs by apoptosis\(^\text{42, 97, 98}\), factors and mechanisms that lead to myofibroblast apoptosis are not well understood. One hypothesis considers release of mechanical tension, cytoskeletal disruptions, and growth factor withdrawal as key stimulants of fibroblast apoptosis in an *in vitro* model of granulation tissue\(^\text{99}\). As granulation tissue proteoglycans, such as versican can greatly influence myofibroblast cell phenotype, and as myofibroblast apoptosis occurs concomitantly with granulation tissue degradation *in vivo*; the possible role of proteoglycan degradation in myofibroblast apoptosis is worthy of further investigation.

**Matrix metalloproteinases (MMPs),** which are involved in all aspects of wound healing, are prime candidates for reshaping the matrix and influencing cell morphology. MMPs regulate inflammation through processing of chemokines and cytokines, mediating cell-cell and cell-matrix interactions during re-epithelialization, and remodeling the scar extracellular matrix either directly by proteolytic degradation of
proteins or indirectly by influencing cell behavior (reviewed in\textsuperscript{100,101}). In the granulation tissue, macrophages and fibroblasts release MMPs that can activate TGF-β, which in turn stimulates further fibroblast proliferation and matrix deposition. TGF-β causes the release of \textbf{tissue inhibitors of metalloproteinases (TIMPs)}, and downregulates expression of MMPs known to degrade components of the provisional matrix\textsuperscript{22} (reviewed in\textsuperscript{1,5}).

Although the large MMP family consists of 24 secreted and membrane-bound enzymes that are known to act on a variety of bioactive molecules and other substrates in the extracellular matrix, substrate specificity and compartmentalization allow them to function as regulators of extracellular matrix remodeling and cell morphology in many aspects of normal physiology and pathology\textsuperscript{102}. For example, MMP-2 and MMP-9 of the gelatinase family act on cleaved collagen better than other MMPs\textsuperscript{103}, MMP-7 (Matrilysin) is a more potent proteoglycanase than MMP-3 or MMP-9\textsuperscript{104}, and MMP-12 (Macrophage Metalloelastase) is the most elastolytic enzyme of the MMP family\textsuperscript{105} capable of efficiently degrading fibronectin and chondroitin sulfate chains of proteoglycans\textsuperscript{106}. Also, cells do not release these proteases indiscriminately. Anchoring of MMPs to the cell membrane targets their catalytic activity to specific substrates within the pericellular space. For example, MMP-2 binds to α\textsubscript{v}β\textsubscript{3} integrin\textsuperscript{107}, MMP-9 interacts with CD44\textsuperscript{108}, and MMP-7 binds to cell surface proteoglycans\textsuperscript{109}. The structure and function of MMPs and their role in wound healing is more extensively described in a following section of this chapter.

\textbf{1.1.2 Pulmonary Fibrosis: An Example of Aberrant Wound Healing}

Extracellular matrix in the lungs is composed of basal lamina, underlying the epithelium, and the interstitial matrix. The epithelium of the lung acts as a barrier that allows regulated passage of inflammatory cells into the airways. The basal lamina underlies the epithelium, and provides attachment for epithelial cells through anchorage molecules such as laminin, fibronectin, and type IV collagen\textsuperscript{110, 111}. The basal lamina is also connected, through its components, to the deeper connective tissues such as elastic fibers\textsuperscript{112}. Some constituents of the extracellular matrix, such as collagens I, II, III and IV, elastic fibers, and proteoglycans maintain tissue structure, while others such as laminin
and fibronectin are involved in cell attachment and signaling\textsuperscript{113, 114}. The interstitium of the lung contains many cell types including fibroblasts, myofibroblasts, smooth muscle cells, macrophages, and some undifferentiated fibroblast-like cells.

Significance of intact basal lamina for tissue repair was shown by a study which demonstrated that regeneration and function can be restored if basal lamina remains intact\textsuperscript{115}. In the lungs, for example, major damage to basal lamina results in altered architecture and the degree of remodeling depends on severity of original injury\textsuperscript{116}. Injury to the epithelium causes the release of interstitial content through the basement membrane into the alveolar space of the lung\textsuperscript{117}. Inflammation is marked by an increase in the number of a variety of immune cells including alveolar macrophages, neutrophils, eosinophils, T-cells, B-cells, basophils and mast cells. Constituents of normal ECM, both molecular and cellular, cause the recruitment of many of these immune cells through the formation and release of cytokines and chemokines\textsuperscript{118}. The immune cells that are recruited to the site of injury in turn enhance the inflammatory process by releasing more chemokines\textsuperscript{7, 8}.

There are many parallels between wound healing and pulmonary fibrosis\textsuperscript{119}. Similar to wound healing, PDGF\textsuperscript{7, 120, 121} and TGF-\( \beta \)\textsuperscript{8, 9} are the two main growth factors that are localized to centers of fibroblast proliferation in fibrotic lung. PDGF stimulates hyaluronan synthesis\textsuperscript{11}, smooth muscle cell proliferation\textsuperscript{13}, and versican synthesis\textsuperscript{12}. TGF-\( \beta \) promotes growth and differentiation of connective tissue cells. Fibroblasts differentiate into contractile, smooth muscle actin-rich myofibroblasts as their expression of alpha actin protein is induced by TGF-\( \beta \)\textsuperscript{29}. TGF-\( \beta \) also induces expression of hyaluronan\textsuperscript{16, 17}, versican\textsuperscript{12, 17}, and protease inhibitors that prevent matrix degradation\textsuperscript{122}. Altered cellular and molecular ECM composition leads to the creation of a ‘provisional’ matrix where mesenchymal cell proliferation occurs. Overall, there is an increase in the amount of fibronectin\textsuperscript{123} and proteoglycans versican, biglycan, and the glycosaminoglycan hyaluronan\textsuperscript{93, 94} in the active lesions, and an eventual increase in collagen type I, III, and VI\textsuperscript{124-128} in the fibrotic lung. Thus, pulmonary fibrosis is a disease process driven by chronic inflammation and matrix degradation, cellular migration and
proliferation, that ultimately leads to the accumulation of collagenous matrix in the interstitium and alveolar spaces of the lung\textsuperscript{129,130}.

Regardless of the cause of pulmonary fibrosis, degradation of granulation tissue components along with concomitant myofibroblast apoptosis leaves a collagenous-rich matrix that causes thickening of the alveoli walls and filling up the alveolar spaces\textsuperscript{119}. The expression of several matrix metalloproteinases (MMPs) also increases in pulmonary fibrotic diseases as a result of injury. Production of MMPs may contribute to the removal of versican-rich provisional matrix, a process that must occur in the evolution of the lesions to collagen-rich and proteoglycan-poor fibrotic tissue\textsuperscript{93,94}. Persistence of granulation tissue, rich in proteoglycans and contractile myofibroblasts, and excessive deposition of collagenous matrix is a hallmark of fibrosis in different tissues in the body. The similarity of the process in different tissues suggests universal functions for these proteoglycans in the provisional matrix. Our study centers around pulmonary fibrosis and the role of proteoglycan versican, its effects on myofibroblast cell morphology and its subsequent degradation, in the aberrant process leading to fibrosis.

\textbf{1.1.3 Study of Wound Healing \textit{In Vitro}}

Three dimensional (3D) collagen or fibrin matrices, containing cultured fibroblasts, have become popular as \textit{in vitro} models of wound contraction\textsuperscript{132}. Early experiments with collagen\textsuperscript{133} and fibrin\textsuperscript{134} matrices, floating in culture media, showed that fibroblast cells cultured in 3D matrices phenotypically resemble cells found in the wound matrix more closely than cells grown in a planer culture plate. Three models of collagen matrix contraction are currently in use, and they are floating matrix, anchored matrix, and initially anchored and subsequently released “stress-relaxation” matrix (Figure 1.1). Although contraction occurs in all three types of matrices\textsuperscript{135} as a consequence of motile activity of migrating cells\textsuperscript{136}, the fibroblast phenotype that develops as a result of this contraction differs depending on the matrix mechanics.
Figure 1.1  Contraction of Floating versus Anchored Collagen Matrices

Contraction of floating collagen matrices results in a tissue resembling the dermis, in which fibroblasts develop long processes and a cytoskeletal meshwork\textsuperscript{132, 137} and loose their ability to proliferate\textsuperscript{138-140}. Decreased collagen biosynthesis and increased collagenase production is another hallmark of cells in floating matrices\textsuperscript{14, 141-143}. Anchored collagen gels, on the other hand, develop into a stressed tissue resembling granulation tissue, with a tensional force comparable to that of contracting skin wound\textsuperscript{144-146}. In these stiff matrices, fibroblast cell orient along lines of tension\textsuperscript{147, 137}, and develop prominent stress fibers resembling myofibroblasts\textsuperscript{31, 148, 149}. Cells in anchored matrices continue to proliferate\textsuperscript{138, 139} and synthesize collagen. Ensuing fibroblast cell contraction resembles that of a smooth muscle cell requiring intact stress fibers, regulated by serum factors\textsuperscript{148}, retraction of cell pseudopodia and collapse of actin filament bundles\textsuperscript{148, 149}. In the third model, the reaction of cells to stress release or matrix relaxation may represent an \textit{in vitro} model of the transition from granulation to scar tissue that happens in the later stages of wound healing and fibrosis\textsuperscript{135}. Cell proliferation and collagen synthesis decline rapidly as cells switch from an activated to resting phenotype\textsuperscript{149, 150}.

In summary, studies of the 3D models suggest that mechanical tension in anchored matrices leads to intracellular tension and formation of stress fibers as the fibroblast cells differentiate first into proto-myofibroblasts with organized stress fibers and then into
myofibroblasts with α-SMA decorated stress fibers\textsuperscript{24}. Increased expression of other matrix components, such as fibronectin and collagen I, and inhibition of MMPs also accompany myofibroblast contractile phenotype in response to outside stress. Once the gels are contracted or released from their support (free-floating gels), cells go into a quiescent state\textsuperscript{135, 151} and the process of apoptosis begins\textsuperscript{152}. It is important to acknowledge, however, the dependence of contractile cell phenotype on extracellular factors in these matrices. Collagen matrix contraction requires serum\textsuperscript{153, 154}, whose activity can be replaced or enhanced by purified growth factors. TGF-β, for example, stimulates contraction of both floating and anchored collagen matrices\textsuperscript{14, 155, 156}. PDGF also stimulates matrix contraction\textsuperscript{157, 158} by a mechanism independent of TGF-β\textsuperscript{159}. Mechanical tension combined with extracellular factors join forces to transform quiescent fibroblasts into contractile myofibroblasts. How the downstream signaling mechanisms of growth factors and mechanical tension converge to stimulate this transformation is not known yet. In this thesis, we will focus on one such possible converging factor downstream of both growth factor and mechanical tension stimulation, namely versican.
1.2 Versican

Versican, a large proteoglycan with an estimated molecular mass of more than 1000 kDa, is a member of the lectican, also known as hyalectan, family of proteoglycans which includes aggregcan (abundant in cartilage), brevican and neurocan (nervous system proteoglycans; reviewed in 87, 160). These proteoglycans share structural similarities at both the genomic and protein level. Their tri-domain structure consists of globular N- and C-termini, which is homologous among the members of this family, and a central glycosaminoglycan (GAG) binding region (Figure 1.2). The name hyalectan is attributed to the interactions of their N-terminal domain with hyaluronan and the interactions of C-terminal lectin-like domain with a variety of other ECM molecules that helps to stabilize the matrix through formation of supra-molecular aggregates. Although lecticans share a very similar N-terminal hyaluronan binding domain and a common C-terminal domain, differences in the number and type of GAG chains bound to the core protein adds diversity to the structure and function of proteoglycans. GAGs are composed of repeating disaccharide units161, and variations in sulfation patterns affects the charge density on GAG chains, and thus the shape and properties of the proteoglycan.
Figure 1.2 Structure of Lectican Family of Proteoglycans

The N-terminal domain (G1 domain) of lecticans consists of an immunoglobulin (Ig)-like loop and two copies of a hyaluronan-binding motif, or link modules (also called the proteoglycan tandem repeats). Only aggrecan has an additional globular domain (G2 domain) near the N-terminal, which consists of two more link modules. In cartilage,
aggrecan forms a ternary complex with hyaluronan and the link protein. The link protein plays a crucial role in stabilizing the complex by binding to both aggrecan and hyaluronan\textsuperscript{162}. HA binding properties are shown to be present in all lecticans including versican\textsuperscript{163}. Similar to aggrecan, interactions between the versican G1 domain, link protein and HA have been reported\textsuperscript{164}.

The C-terminal globular domain (G3 domain) consists of one or two epidermal growth factor-like (EGF) repeats, a C-type lectin domain and a complementary repeat protein (CRP)-like domain. The C-terminal domain of lecticans interact with a variety of ligands both \textit{in vivo} and \textit{in vitro}. Binding partners for the G3 domain of versican will be discussed in more detail in another section of this chapter.

Although their N- and C-terminal domains are highly conserved, the central domain of lecticans is quite diverse in terms of size and sequence. Unlike the N-terminal and C-terminal domains of lecticans, the central domain lacks any cysteine residues and probably has a highly extended three dimensional structure due to the fact that most of the GAG attachment sites are present in the central domain. The numbers of potential GAG attachment sites are quite variable among lecticans, with about 120 chondroitin sulfate attachment sites for aggrecan and 24 chondroitin sulfate chains for versican. In addition, aggrecan carries keratan sulfate chains as well as the mentioned chondroitin sulfate chains\textsuperscript{165,166}. The two smallest members of lectican family, namely neurocan and brevican, contain 3-7 and 1-3 chondroitin sulfate chains respectively. Because of the negatively charged sulfate or carboxyl groups, chondroitin sulfate chains can attract various positively charged molecules such as certain growth factors, cytokines, and chemokines\textsuperscript{167-169}. This interaction in the extracellular matrix or on the cell surface is important in the formation of immobilized gradients of these factors, their protection from proteolytic cleavage, and their presentation to specific cell-surface receptors\textsuperscript{170-172}. Another property of negatively charged GAGs, observed most clearly with aggrecan in cartilage, is their interaction with water molecules and large hydrodynamic volume when subjected to compressive forces.
1.2.1 Lectican Family of Proteoglycans

The expression of lecticans varies widely with respect to time and space, in development and disease. The role of lecticans as growth inhibitory molecules in the central nervous system (CNS) has been well documented. **Neurocan**, one of the brain specific lecticans, interacts with the neural cell adhesion molecules *in vitro*\(^{173}\), and inhibits neuronal adhesion and neurite outgrowth following injury to the CNS. It has also been suggested that neurocan, and other chondroitin sulfate proteoglycans, may act as a barrier to neurite extension in developing retina\(^{174}\). The effects of neurocan, however, depends on the nature of the neuron and the precise molecular context in which it is embedded.

**Brevican**, another small neural-specific lectican of the brain extracellular matrix, is expressed by neuronal and glial cells in the terminally differentiated CNS\(^{175}\). Unlike neurocan, brevican is strongly up-regulated during postnatal development and its level peaks particularly after terminal differentiation of the brain\(^{176}\). As a part-time proteoglycan, brevican has bifunctional effects on neurite outgrowth. Brevican can be found both in the matrix in secreted form and on the cell membrane in glycosyl-phosphatidylinositol (GPI) anchored form. The reason for brevican’s bifunctionality has been linked to both its chondroitin sulfate (CS) chains and core protein features. For example, brevican is able to inhibit cerebellar neurite outgrowth on laminin through its CS chains and promote outgrowth of hippocampal neurons via its protein core\(^{177, 178}\). Thus, presence of GAGs have a great influence in determining the function of this lectican. On the other hand, the growth promoting effect of brevican appears to come from its lectin domain\(^ {178}\), suggesting that other lecticans are capable of producing the same effects through their lectin domains.

**Aggrecan**, discovered as a major structural component of cartilage, is present mostly as a CS proteoglycan in the CNS and seems to strongly inhibit neurite extension on a variety of substrata, *in vitro*\(^ {179}\). The effects of aggrecan on neurite outgrowth seems to be influenced by both the composition of the surrounding matrix and the ability of neurons to modulate cellular receptors in response to aggrecan\(^{180}\). The anti-adhesive and anti-migratory effect of chondroitin sulfate proteoglycan (CSPG) aggrecan, from juvenile
or adult cartilage, was demonstrated by addition of this molecule to the ECM in vitro\textsuperscript{181, 182}. It was also shown that the inhibitory effect of PGs were sensitive to chondroitinase ABC digestion, but not to hyaluronidase, suggesting the involvement of CSPG\textsuperscript{183}.

\textbf{Versican} is unique in that it can be expressed as one of 4 distinct structural variants which differ in their potential number of glycosaminoglycan attachment sites\textsuperscript{184, 185}. The structural and functional diversity of versican is increased by this variation in glycosaminoglycan content, and this topic is further investigated in this thesis, under section 1.2.4. Another interesting observation is that although versican and aggrecan appear to share a close N- and C-terminal structural homology, they are different in that aggrecan contains far more glycosaminoglycan attachment sites and a greater variety of GAGs than versican. Not surprisingly, their expression pattern and proposed functions suggest opposing roles for these two lecticans in a number of different processes such as neural crest cell (NCC) migration, chondrogenesis, and development of the heart.

Functional differences between versican and aggrecan have been extensively studied in the context of NCC migration\textsuperscript{180, 186, 187}. For example, in the larvae of white mutant axolotl unlike the normal dark ones, migration of trunk pigment cells is restricted\textsuperscript{188, 189}. Developmental studies revealed a decrease in the quantity of versican mRNA in the mutant embryo compared to the wild type\textsuperscript{190}. Also, the synthesis of versican during NCC migration was shown to be critical in the wild type axolotl embryo and antibodies against versican stained the subepidermal matrix of dark axolotl embryos. A study of NCC migration alongside embedded micromembranes which were coated with either versican or aggrecan discovered that in contrast to versican-coated micromembranes, those covered with aggrecan completely inhibit the movement of NCCs\textsuperscript{191}. Permissiveness of versican to NCC movement has also been illustrated in a collagen type I-versican substrata, a movement which is not permitted through a three-dimensional collagen type I-aggrecan substrata \textit{in vitro}\textsuperscript{191}. Difference in the inhibitory effects of aggrecan and versican may be as a result of the different CS chains bound to each molecule and the presence of KS chains in aggrecan but not versican. Aggrecan seems to bind the cell
surface through HA and inhibit NCC movement through its KS chains, possibly by influencing integrin function\textsuperscript{182}.

A number of other experiments have centered around the role of proteoglycans in the development of the heart. In 1998, Mjaatvedt mapped the recessive lethal heart defect (hdf) gene in mouse to versican\textsuperscript{192}. The disrupted gene was shown to be important in the formation of right ventricle and the endocardial cushions. Immunohistochemistry studies with an antibody against a GAG epitope on versican confirmed the absence of versican in the homozygous mutant embryos when compared to the normal wild-type embryos. \textit{In situ} hybridization and immunohistochemistry on sectioned mouse embryos show that the mRNA and protein for versican are expressed in a dynamic pattern during development of the heart\textsuperscript{193}. From these studies, it is possible to infer that versican is involved in specification of the ventricular chambers, in growth and fusion of the atrial and ventricular septa, and in the transformation from epithelium to mesenchyme that characterizes development of the endocardial cushions. In 1999, another group of researchers mapped versican to the myocardium and the myocardial basement membrane\textsuperscript{194}.

Finally, in developing cartilage, versican is transiently expressed at a high level in the mesenchymal condensation area and rapidly disappears during cartilage development\textsuperscript{195, 196}. Recent immunohistochemical studies on developing limb bud cartilage revealed that an area positive for versican is gradually replaced by an area positive for aggrecan. These reciprocal patterns of versican and aggrecan expression suggest that versican serves as a temporary framework in developing cartilage matrix. Although the aggrecan aggregate is the major component of cartilage ECM and versican has not been detected by immunohistochemical studies\textsuperscript{197}, constitutive low level transcription of the versican gene is reported in cartilage\textsuperscript{198} and chondrocytes\textsuperscript{198, 199}. In addition, extracts of human adult articular cartilage contain versican\textsuperscript{200}, suggesting its distinct role in that tissue. An investigation on versican expression, localization, and aggregate formation in cartilage showed that versican is mainly localized in the interterritorial zone of the articular surface, whereas aggrecan is rather diffused, especially with dense staining in the territorial zone of pre-hypertrophic chondrocytes\textsuperscript{201}.  

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Although transcription of the versican gene dramatically decreases after birth, versican remains in the articular cartilage in the form of the proteoglycan aggregate. Based on these observations, it has been suggested that the versican aggregate is present in the articular surface and may provide ECM properties distinct from deeper zones where aggrecan aggregates are abundant. Above are three examples illustrating opposing functions for versican and aggrecan in different tissues during development. We also present data showing different effects of these two proteoglycans in the context of myofibroblast cell morphology in a 3D model of wound contraction, and we will look back at these functional differences in more detail in chapter 3.

1.2.2 Expression Profile

Although versican is mainly expressed during embryonic development in various tissues which includes the nervous system, it is also expressed in some adult tissues, such as the heart, blood vessels, and brain. Versican is constitutively expressed and serves as a structural macromolecule of the ECM. Smooth muscle cells of blood vessels, epithelial cells of skin, and the cells of central and peripheral nervous system are a few examples of cell types that express versican physiologically. The role of versican in cell adhesion, migration, proliferation, and differentiation has also been studied in development and disease. Versican is involved in guiding the migration of embryonic cells involved in the formation of the heart and outlining the path for neural crest cell migration in development. Versican expression is also high in the developing mesenchyme during limb development and is subsequently down-regulated during mesenchymal condensation as aggrecan replaces versican in the pre-chondrogenic core. In disease, versican is a key factor in inflammation through interactions with adhesion molecules on the surfaces of inflammatory leukocytes and interactions with chemokines that are involved in recruiting inflammatory cells. Increased versican expression is often observed in association with proliferating cells within remodeling tissue in lung and cardiovascular diseases and in cancer. For example, versican is heavily deposited in the lung interstitium during the development of many forms of lung diseases, including asthma, adult respiratory disease syndrome (ARDS), and idiopathic pulmonary fibrosis (IPF). Versican is involved in tumor growth in tissues such as breast.
brain\textsuperscript{214}, ovary\textsuperscript{215}, gastrointestinal tract\textsuperscript{216}, prostate\textsuperscript{217, 218}, and melanoma\textsuperscript{219}, Sarcoma\textsuperscript{210}, and mesothelioma\textsuperscript{220}. Versican can also inhibit nervous system regeneration and axonal growth following an injury to the central nervous system\textsuperscript{221-223,176,224}.

### 1.2.3 Structure and Interactions of Versican with Other Matrix Components

The **N-terminal (G1) globular domain** consists of Ig-like loop and two link module, and has HA binding properties. The N-terminal of versican is thought to be involved in maintaining the integrity of the ECM by interacting with hyaluronan (HA)\textsuperscript{163}. Its interactions with the link protein has also been studied\textsuperscript{164}. Hyaluronan plays an important role in homeostasis and its expression is increased in epidermal injury\textsuperscript{225, 226}, along with its cell surface receptor CD44\textsuperscript{227}. Formation of versican-hyaluronan complex at the cell surface is essential for proliferation and migration of smooth muscle cells *in vitro*\textsuperscript{59, 60}. Versican-hyaluronan pericellular coat also promotes cancer cell motility\textsuperscript{228}. Hyaluronan is required for matrix expansion and initiation of cell migration in the developing heart\textsuperscript{229, 230}. The heart defects in the hyaluronan synthase-2 (*has-2*), which is the most widely expressed hyaluronan synthase during mid-gestation in the mouse\textsuperscript{230}, are very similar to that of the *hdf* mutant (versican deficient) mouse\textsuperscript{231}. This is a good indication that both versican and HA are necessary in the migration of cardiac NCC.

The **C-terminal (G3) globular domain** consists of one or two EGF repeats, a C-type lectin domain and complement regulatory protein (CRP)-like domain. In recent years, a direct role for versican in cell proliferation has been suggested based on the interaction of its C-terminal EGF modules with the cell surface EGF-receptors\textsuperscript{232}. The C-terminal domain also binds a variety of ligands in ECM which contribute significantly to the functions of versican. One important family of ligands is the tenascin family which due to their complex multi-domain structure can possess both growth promoting and inhibitory activities on the same molecule\textsuperscript{233}, and are reported to have anti-adhesive properties\textsuperscript{234}. A study of tenascin knockout mouse identified tenascin as a significant player in corneal wound healing under mechanical stress\textsuperscript{235}. The C-lectin domain of versican interacts with **tenascin-R** through its fibronectin type III (FnIII) repeat 3-5 domain in a calcium dependant manner, *in vivo*\textsuperscript{236} and *in vitro*\textsuperscript{237}. Different tenascin domains interact with a
wide range of cellular receptors, including integrins, cell adhesion molecules and members of the syndecan and glypican proteoglycan families. Tenascin-C, a major versican binding partner during tissue repair, is a transient glycoprotein that promotes fibroblast migration and differentiation in injured tissue and at the tumor invasion front of cancers. Full length tenascin-C also promotes fibroblast migration within fibrin-fibronectin 3D matrices, and tenascin and fibronectin promote corneal fibroblast migration and adhesion. Yet another C-terminal binding partner for versican, fibronectin, plays an important role in cellular proliferation and migration and modulates cell-matrix interactions during tissue repair. ED-A fibronectin splice variant, a crucial factor in myofibroblast differentiation by TGF-β1, is a major component of myofibroblast matrix. ED-A fibronectin is expressed by fibroblastic cells in culture and vascular SMC in vivo and in vitro. Fibulin is another ligand for versican’s C-lectin domain, a protein whose expression is associated with that of versican in the developing heart. Fibulin is also implicated in wound healing and development. The C-lectin domain of Versican also interacts with fibrillin. The interaction with fibrillin maintains the structural integrity of the matrix and links extracellular microfibrils to other connective tissue networks.

The central GAG-binding domain of versican is decorated with chondroitin sulfate glycosaminoglycans (GAGs). The structural and functional diversity of versican is increased by variations in GAG sulfation patterns and the type of GAG chains bound to the core protein. Although there is only a single versican gene, alternative splicing of its mRNA produces 4 distinct versican isoforms that differ in their potential number of GAG chains (Figure 1.3). All isoforms have homologous N-terminal (HA binding) and C-terminal (lectin-like) domains. The central domain of versican V0 contains both the GAG-α and GAG-β domains. V1 isoform has the GAG-β domain, V2 has the GAG-α domain, and V3 is void of any GAG attachment domains, and only consists of the N-terminal and C-terminal globular domains. The GAGs, being composed of repeating disaccharide units, contribute to the negative charge and many other properties of versican. The binding of versican with leukocyte adhesion molecules L-selectin, P-selectin, and CD44 is mediated by the interaction of CS chains of versican with the
carbohydrate-binding domain of these molecules\textsuperscript{167}. Both CD44 and L-selectin have been implicated in leukocyte trafficking\textsuperscript{252, 253}. The ability of versican to bind a large panel of chemokines and the biological consequences of such binding has also been examined\textsuperscript{207}. It has been suggested that versican can bind specific chemokines through its CS chains and this interaction down-regulates the chemokines function\textsuperscript{207}. Recently, in lights of results that V1 and V2 isoforms of versican have opposite effects on cell proliferation, glycosaminoglycan domain GAG-β has been implicated in versican-enhanced cell proliferation and versican-induced reduction of cell apoptosis\textsuperscript{254}.
1.2.4 Versican Function in Pulmonary Fibrosis

Many functions of versican have already been discussed in the context of its tissue specific expression pattern in development or disease, the interactions with its binding partners, and in case of gene deletions that influenced versican’s expression and cell function. It is becoming more apparent that versican affects cell and tissue function in a number of different ways. In addition to serving as a structural molecule affecting mechanics of tissue, versican can interact with a number of different ECM ligands and
possibly with the cell surface and thus regulate processes such as cell adhesion, proliferation, and migration.

Versican’s ability to bind hyaluronan and form highly hydrated, supra-molecular aggregates, can contribute to swelling pressure and thus influence the mechanical properties of matrices such as blood vessel walls or remodeling tissue which are under pressure. This function of versican resembles the role of its family member, aggrecan, in resisting compressive forces put on cartilage. In the lungs, degradation of human airway smooth muscle-associated matrix is associated with decreased passive tension and alterations in smooth muscle contractility. The large size (>1000 kDa) and hydration capability of versican, may also sterically hinder the interaction of integrins (large family of cell adhesion molecules) with their cell surface receptors. Along with its anti-adhesive binding partners, hyaluronan and tenascin-C, versican is expressed in the active fibroproliferative lesions of the remodeling lung and may additionally function as a scaffold on which growth factors and chemokines concentrate.

Versican association with proliferating cells has been observed in a number of tissues and cell types, including atherosclerotic lesions, stromal reaction to tumors, proliferating vascular smooth muscle cells, contractile α-SMA positive cells, and elastic fibers in the airway of normal lung. The spatial and temporal association of large splice-variants of versican, namely V0 and V1, with proliferating and contracting myofibroblasts (as defined by α-SMA and collagen type-I expression) has also been observed in all fibrotic lung diseases. Versican can be found in association with fibroblasts that migrate into airspaces, the hyperplastic epithelium, and in the alveolar wall thickening in the very early stage of idiopathic pulmonary fibrosis.

Research shows that versican may play a direct and an indirect role in cell proliferation. Indirectly, interactions of G1 domain of versican with HA results in the formation of a pericellular matrix that is required for the proliferation of arterial smooth muscle cells. Dissolution of the pericellular matrix by treatment of the cells with HA oligosaccharidates inhibits SMC proliferation. CD44, the main cell receptor for hyaluronan, also interacts
with versican through its chondroitin-sulfate chains\textsuperscript{167}, and versican interaction may complement or modulate CD44 mediated adhesion and migration.

Evidence for a direct role of versican in cellular proliferation is more controversial, and relies upon vector-driven overexpression of versican constructs or deletion of segments within such constructs (Comprehensively examined in Chapter 2 of this thesis). For example, an engineered chimeric molecule named “mini-versican” has been shown to modestly stimulate NIH 3T3 cell proliferation through its EGF-like modules in the G3 domain\textsuperscript{232}. Deletion of the G3 domain or the EGF-like repeats eliminated the effect of overexpression or addition of versican products on cell proliferation. This group has also reported that addition of antisense against EGF receptor could block the effect of added versican, although versican has not been shown to bind or activate the EGF receptor. In contrast, studies of rat SMC that were retrovirally transduced to express versican V3, which lacks the GAG binding domains, showed decreased cell proliferation and migration and increased cell adhesion\textsuperscript{260}. It has been suggested that the effects of V3 on SMC indicate that over-expression of versican G3 domain constructs do not universally promote cell proliferation.

Effects of V3 on cell behavior may be as a result of competition for binding to cell surface-associated endogenous versican (V0/V1) ligands, such as HA\textsuperscript{87}. This suggestion is supported by the observation that the formation of the HA-versican V0 and V1 pericellular matrix is inhibited in cells that express versican V3. This implies that the anti-adhesive chondroitin sulfate GAG chains of V0 and V1 versican isoforms may be most influential in regulating cellular phenotype through binding growth factors, inhibiting cell-cell cell-matrix interactions, or influencing the mechanical properties of the matrix. The precise roles of versican in wound healing and fibrosis in the lung remain open to further investigation, and I hope my present research sheds some light on the cellular biology and biochemistry of versican in association with myofibroblast cell type.
1.3 Metalloproteinases

There are eight clans and forty families in the Metalloproteinase class based on the three dimensional protein folding and evolutionary relations\textsuperscript{275}. These endopeptidases depend on a zinc or calcium ion in their active site, and include the metzincins (serralysins, astacins, adamalysins) and matrixins (matrix metalloproteinases or MMPs) family. Considering the increasing interest on the function of adamalysins (ADAM – a disintegrin and metalloproteinase domain) and matrixins in recent years, my review will focus on these two families of enzymes.

There are more than 20 matrix metalloproteinases known to this day, all of which depend on zinc at their active site for catalytic function\textsuperscript{274}. The common names for MMPs are based on their substrate specificity, and thus traditionally, MMPs are classified as “collagenases”, “gelatinases”, “stromelysin”, “membrane-type MMPs”, and “other MMPs”. All of the members of this family contain a consensus motif of three histidines (HExxHxxGxxH) that bind zinc at the catalytic site and a conserved “Met-turn” motif below the active site zinc\textsuperscript{276}. Most MMPs contain five basic domains: a pre-domain or signal sequence to direct secretion from the cell, a latency or pro-domain, a zinc-binding catalytic domain, and a hinge region followed by a hemopexin domain with sequence homology to both hemopexin, a plasma heme-binding protein, and vitronectin, a cell adhesive protein. The propeptide region contains the ‘cysteine switch’, within the sequence PRCG(V/N)PD, in which the cysteine residue binds the catalytic Zn\textsuperscript{2+} ion in the proenzyme. Latency is maintained through coordination of the active site zinc with the thiol of this conserved cysteine in the pro-domain\textsuperscript{277}.

ADAMs contain a disintegrin or integrin-binding domain, and a metalloproteinase domain that is similar to the conserved MMP zinc-binding catalytic domain\textsuperscript{396}. The primary function of these transmembrane proteases is to cleave extracellular domains of many membrane proteins from the cell surface\textsuperscript{396, 397}. ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) is a recently described family of proteinases closely related to the ADAM family, except that the ADAMTSs are secreted enzymes capable of binding to the ECM\textsuperscript{398, 399}. Several members of the ADAMTS have
been shown to cleave versican N-terminal domain, leading to the generation of the well-known glial hyaluronate-binding protein (GHAP) among other fragments. GHAP was first identified as a 60kDa hyaluronate-binding fragment in the brain white matter, with protein sequence identity to the hyaluronate-binding region of versican\textsuperscript{400, 401}. In this context, it was suggested to be a naturally occurring versican degradation product, possibly generated by the action of MMP-1, MMP-2, and/or MMP-3\textsuperscript{402} on the V1 versican variant. With the knowledge that V2 is the major versican variant of the brain\textsuperscript{403}, it was shown that native GHAP is actually generated from versican V2 core by digestion with ADAMTS-4 and that the cleavage site is at Glu405-Gln406 in the GAG-alpha domain of versican V2\textsuperscript{404}. This discovery followed similar results of versican V0 and V1 degradation by ADAMTS-1 and -4 in aorta\textsuperscript{405}, and others have shown versican N-terminal cleavage by ADAMTS-9\textsuperscript{406} and ADAMTS-1 in atherosclerotic lesions\textsuperscript{407}.

1.3.1 Matrix Metalloproteinases

There are some differences within the family between individual MMPs. The structures of three members of this family, that will be discussed extensively in this thesis, namely MMP-2 MMP-7 and MMP-12 are illustrated in the following figure (Figure 1.4).

![Figure 1.4 Structure of MMP-2, MMP-12, and MMP-7](image)

There are notable differences in the structure of these three MMPs. MMP-7 is one of the few MMPs that is synthesized without a hemopexin-like C-terminal domain, and thus it is comparably small in size. Although the hemopexin-C domain is void of any catalytic activity, it plays an important role in substrate recognition, conferring specificity, and in
binding to the tissue inhibitors of metalloproteinases (TIMPs).\textsuperscript{278} MMP-2, a member of the gelatinase subgroup, contains three additional fibronectin-type II modules which are hydrophobic in nature and are thought to be involved in substrate binding.\textsuperscript{279, 280} The most important difference is, however, among the catalytic domains of MMPs and the size of the S1’ specificity pocket, which will be discussed in more detail for each of MMP-7, MMP-12 and MMP-2 in the sections that follow.

Proteolysis of the extracellular matrix and cell surface molecules is a critical requirement in processes such as morphogenesis, regeneration, tumorigenesis, and wound healing. The family of matrix metalloproteinases (MMPs) plays a major role in remodeling of ECM in many physiological processes such as embryonic development\textsuperscript{264, 265}, skeletal growth\textsuperscript{266} and ovulation\textsuperscript{267}. In disease, the imbalance of these proteases and their corresponding inhibitors, tissue inhibitor of metalloproteinases (TIMPS), could result in the break down of ECM and development of pathological diseases such as cancer invasion and metastasis\textsuperscript{268}, arthritis\textsuperscript{269}, and cardiovascular diseases.\textsuperscript{270} MMPs promote tumor progression and metastasis in invasive cancers by degrading ECM comprised of many proteins such as laminin-5, proteoglycans, entactin, osteonectin, and collagen IV. In addition to their role in matrix degradation, recent research has shown that MMPs are capable of performing highly specific and limited cleavages of a number of bioactive molecules to modulate many aspects of cell behavior (reviewed in\textsuperscript{271, 100, 272}) such as cell proliferation, differentiation, migration, apoptosis, angiogenesis, bone morphogenesis and the immune response.\textsuperscript{272, 273}

The proteolytic activity of MMPs is strictly regulated through a number of mechanisms. Their expression is controlled at the level of transcription, post-transcriptional modification, and protein secretion by several growth factors and cytokines. Among other growth factors, PDGF is a strong inducer of MMP expression in many cell types, while TGF-β can both induce and suppress MMP expression.\textsuperscript{282} The extracellular matrix can also regulate MMP gene expression through binding cell receptors and activating intracellular signaling pathways. For example, collagen type I stimulates the expression of MMP-1\textsuperscript{283}, while fragments of laminin-5\textsuperscript{284} and fibronectin\textsuperscript{285} upregulate MMP-1 and MMP-13 respectively. Tenascin-fibronectin complexes also stimulate
secretion of MMPs by fibroblasts. Proenzyme activation and release from latency, and the action of natural tissue inhibitors of MMPs (TIMPs) provide other means of regulating MMPs. Another important regulatory mechanism is how these enzymes are anchored outside the cell. Anchoring MMPs to the cell surface or extracellular matrix could not only provide a reservoir by preventing them from rapidly diffusing away, but also enable the cell to keep them under close regulatory control.

In vivo study of wound healing in different tissues have implicated MMPs and their natural tissue inhibitors (TIMPs) in normal wound repair (Reviewed in ). Multiple cell types including fibroblasts, keratinocytes, and macrophages release MMPs at the wound site and contribute to ECM remodeling. The expression of most MMPs and TIMPs are perturbed among cells involved in skin repair. MMPs are also induced in a number of diseases that leads to pulmonary fibrosis. Levels of MMP-8, MMP-2 and MMP-9 are all elevated in human idiopathic pulmonary fibrosis. Cancer drug, bleomycin, can induce MMP-2, MMP-9, and MMP-12 in the lungs. Epithelial derived MMP-7 is also highly upregulated in human idiopathic pulmonary fibrosis. Recently, it was shown that the levels of MMP-2, MMP-7, MMP-9, MMP-12 and MMP-13 increase in a pulmonary fibrosis model caused by asbestos injury, and it was suggested that MMP inhibition protects against asbestos-induced fibrosis. We have also studied MMP expression in lung fibrosis, and based on the results of our research and findings of others will focus here on three of these enzymes, namely MMP-7, MMP-12 and MMP-2.

1.3.2 MMP-7 (Matrilysin)

MMP-7, also known as matrilysin, is the smallest of the MMPs which was first identified and purified from involuting rat uterus. The molecular weights of the latent and active form of this enzyme are 28 kDa and 19 kDa, respectively. MMP-7 has a simple structure consisting of a propeptide and a catalytic domain, and lacks a hemopexin domain which is rather a rarity among members of the MMP family. Since MMP-7 has a limited sized substrate pocket, the enzyme prefers residues with aliphatic or aromatic side chain. This enzyme is responsible for the degradation of an array of molecules.
(complete list\textsuperscript{306}), including components of basement membrane such as collagen IV, entactin\textsuperscript{307}, fibronectin and laminin, and components of extracellular matrix such as aggrecan\textsuperscript{308}, versican\textsuperscript{104}, proteoglycan link protein\textsuperscript{309}, fibulin-2\textsuperscript{310}, and tenasin-C. In fact, MMP-7 is a more potent proteoglycanase than other MMPs, including Stromelysin-1\textsuperscript{309} 307 or MMP-2\textsuperscript{104}. It can also activate proMMP-2\textsuperscript{311-313} and is involved in the auto-cleavage of proMMP-7\textsuperscript{314, 315}.

In adult human lung, MMP-7 is an epithelial cell product that tends to be released lumenally\textsuperscript{316, 317} in peribronchial and conducting airways. MMP-7 is also produced by blood monocytes\textsuperscript{318} and by tissue macrophages in atherosclerotic lesions\textsuperscript{104}. Besides tissue-specific expression of MMP-7, specific cell surface interactions such as binding of MMP-7 to cell-surface proteoglycans\textsuperscript{109} localizes MMP-7 even further. Such anchoring of proMMP-7 to either cell surface or nearby basement membrane would retain the enzyme near the cell for proteolytic activation. This regulated compartmentalization may allow the proteinase to serve multiple functions by acting on spatially distinct substrates.

Although there are no known functions for MMP-7 during embryonic development\textsuperscript{319, 320}, its expression in healthy adult tissue signifies its role in tissue homeostasis and innate immunity. All tissues with constitutive expression of MMP-7 are open to the environment and vulnerable to bacterial exposure. For example, expression of MMP-7 by exocrine and mucosal epithelial cells lining peribronchial glands and conducting airways\textsuperscript{316} is upregulated in response to bacterial exposure in the adult lung\textsuperscript{317}. MMP-7 can also activate the pro-form of $\alpha$-defensins\textsuperscript{321}, a class of secreted antimicrobial peptides expressed by specialized epithelial cells in small intestine\textsuperscript{321} which can kill bacteria by membrane disruption\textsuperscript{322}.

The full capacity of MMP-7 in wound healing is perhaps best observed in knock-out mutant mice phenotype. The mutant mouse phenotype is not lethal, though it does present innate immunity defects in response to bacteria\textsuperscript{321}, inability to repair mucosal epithelial wounds due to decreased re-epithelialization after lung injury\textsuperscript{316}, and reduced tumorigenesis\textsuperscript{323}. Let us look at each of these phenotypes in more detail. In MMP-7
knockout mice, absence of the active form of α-defensins otherwise processed by MMP-7, results in impaired bacteriocidal activity\textsuperscript{321}. Also in the absence of MMP-7, neutrophils migration past the epithelial barrier is attenuated due to a lack of the neutrophil attractant chemokine (Keratinocyte-derived chemokine or CXCL1) in the fluid of the alveolar lumens\textsuperscript{324}. Another role of MMP-7 is in re-epithelialization in the injured lungs\textsuperscript{316, 325} and other mucosal epithelia in stomach and intestinal ulcers, injured epithelial cells in the kidney, and basal epithelial cells during corneal wound healing\textsuperscript{328-330}. The process of re-epithelialization of tracheal wounds is almost completely abrogated due to impaired cleavage of E-cadherin and thus epithelial migration in mice lacking MMP-7\textsuperscript{316, 325}. On the other hand, over-expression of constitutively active MMP-7 in lung epithelial cells \textit{in vitro} leads to E-cadherin shedding and increased epithelial cell migration\textsuperscript{325}.

Although expression of MMP-7 in adult human tissue is clearly regulated by bacterial exposure, the production of this enzyme is also induced in response to injury. Impaired airway epithelial cell differentiation in a human tracheal xenograft model has confirmed a role for this MMP during later stages of wound healing, when epithelial cell differentiation occurs\textsuperscript{333}. Expression of MMP-7 is also seen in migrating airway and alveolar epithelial cells at sites of overt damage in several lung diseases such as emphysema, lungs with diffuse alveolar damage, idiopathic pneumonia syndrome, and cystic fibrosis\textsuperscript{316}. MMP-7 is upregulated during the fibrotic phase in bleomycin-induced\textsuperscript{299, 326}, silica-induced\textsuperscript{327}, and asbestos-induced models of pulmonary fibrosis\textsuperscript{300}. It has been suggested that, at this capacity, MMP-7 may contribute to further inflammation by regulating chemokine activity and establishing a chemotactic gradient, thus inducing influx of neutrophils into the lung and airspaces\textsuperscript{324}. This is mediated by the cleavage of syndecan-1, which releases CXCL1 bound to the heparan sulfate glycosaminoglycan chains on syndecan-1, thus establishing a chemotactic gradient for neutrophils\textsuperscript{316, 324}.

The mechanism of how matrilysin facilities repair is not known. In the absence of bacteria, wound-induced matrilysin is released basally towards the underlying matrix, suggesting that matrilysin may act on components of extracellular matrix. Furthermore,
matrilysin may not be the only MMP involved in repair of airway epithelial wounds\textsuperscript{331,332}. MMP-2 is also expressed by injured epithelial cells in distal airways, and deficiency of this MMP leads to excessive bronchiolization\textsuperscript{334}. In addition, the activity of MMP-2 is required for the migration of isolated airway epithelial cells over a matrix substratum\textsuperscript{335}. Several MMPs may thus act concurrently on different substrates to facilitate repair.

1.3.3 MMP-12 (Macrophage Metalloelastase)

MMP-12, also known as macrophage metalloelastase, was first identified as an elastolytic metalloproteinase secreted by inflammatory macrophages 30 years ago\textsuperscript{336, 337}. As a macrophage metalloelastase, its expression is appropriately restricted to macrophages\textsuperscript{338}. MMP-12 has a general structure and is composed of a propeptide which maintains enzyme latency, a catalytic zinc binding domain, the linker region, and a hemopexin-like module which is involved in substrate specificity. The latent enzyme (54 kDa) is self-activating and produces the 45 kDa and 22 kDa active forms of the enzyme after autolytic processing\textsuperscript{339, 340}. The major substrate for MMP-12 is elastin, a highly proteinase-resistant molecule abundant in the lung and arterial wall\textsuperscript{341} that normally lasts a life time\textsuperscript{342}. MMP-12 is also capable of degrading a broad spectrum of other extracellular matrix components, including type IV collagen, fibronectin, laminin, vitronectin, proteoglycans, chondroitin sulfate, and myelin basic protein\textsuperscript{338} (reviewed in\textsuperscript{306}). In addition to degrading its extracellular substrates, another important function of active MMP-12 \textit{in vivo} is its ability to activate other MMPs such as MMP-2 and MMP-3, through which MMP-12 may exaggerates the cascade of proteolytic processes\textsuperscript{343} or play a role in innate immunity\textsuperscript{344}. Abnormal regulation of MMP-12 expression has been implicated in abdominal aortic aneurysm\textsuperscript{345}, atherosclerosis\textsuperscript{346}, and emphysema\textsuperscript{347}.

Studies in MMP-12 mutant mice show key roles for this enzyme in tissue invasion and extracellular degradation\textsuperscript{338}. Although not lethal, MMP-12 deficiency results in diminished recovery from spinal cord injury\textsuperscript{348}, increased angiogenesis due to decreased angiostatin\textsuperscript{349}, delayed wound repair in cut ligament due to an inability to recruit macrophages to the injured site\textsuperscript{350}, reduced elastolytic activity by macrophages\textsuperscript{341, 351},
reduced ability of macrophages to migrate through the matrix\textsuperscript{341, 351}, and reduced protection from smoke induced emphysema\textsuperscript{347}. Other studies have also implicated MMP-12 in the development of emphysema\textsuperscript{347, 352}, and have reported increased expression after lung injury in bleomycin\textsuperscript{123, 298} and asbestos treated fibrotic lung\textsuperscript{300}. Collagen replaces the elastin content of the lung parenchyma in emphysematous patients\textsuperscript{353}. Several other MMPs are also produced in human emphysema\textsuperscript{354, 355}. For example, the expression of MT1-MMP, involved in activation of MMP-2, and the expression of MMP-2 markedly increase in pneumocytes, fibroblasts and alveolar macrophages of emphysematous lung\textsuperscript{355}.

1.3.4 MMP-2 (Gelatinase A)

The nascent form of MMP-2, also known as gelatinase A, contains an N-terminal signal sequence (pre-domain) followed by a pro-domain that maintains enzyme-lateness, and a catalytic domain that contains the conserved zinc-binding region. A hemopexin and vitronectin-like domain is connected to the catalytic domain by a hinge or linker region. The hemopexin domain is involved in TIMP binding\textsuperscript{356} and membrane activation in the case of MMP-2. Though unlike other MMPs, the hemopexin C domain of MMP-2 is not involved in substrate binding\textsuperscript{358, 359}. Instead, the three fibronectin type II modules within the catalytic domain which resemble the collagen-binding type II repeats of fibronectin bind and cleave denatured collagen\textsuperscript{360}. MMP-2 proteolytically digests gelatin\textsuperscript{103} (denatured collagen) better than other MMPs, and is capable of degrading collagen type IV, V, VII, IX and X, and several chemokines including CCL7 and CXCL12\textsuperscript{357}.

The regulation of MMP-2 activity occurs at many levels. Pro-MMP-2 activation is seen by complex signaling induced by ECM proteins like osteopontin, various cytokines, and other factors. Specific cell–MMP interactions, such as the binding of MMP-2 to the integrin \(\alpha_\nu\beta_3\)\textsuperscript{107}, have also been reported in recent years. Another intricate regulatory mechanism is the interaction of latent MMP-2 with tissue inhibitor of metalloproteinases (TIMP)-2 and MT1-MMP on the cell surface, and formation of a trimeric complex, essential for activation of this gelatinase\textsuperscript{361, 362}.

Physiologically, MMP-2 plays a role in normal tissue remodeling events such as
embryonic development, angiogenesis, ovulation, mammary gland involution and wound healing. MMP-2 knockout mice, although not lethal, exhibit reduced body size, reduced neovascularization, decreased primary ductal invasion in the mammary gland, reduced lung saccular development, and reduced angiogenesis and tumor growth.

The role of MMP-2 in allergen induced inflammation is well documented. MMP-2 establishes chemokine gradients and recruits neutrophils and eosinophils, as observed with the immune cells recruited from the lung parenchyma into the airway. On the other hand, MMP-2 can also participate in a regulatory loop that dampens allergic inflammation. MMP-2 protects against inflammation of the brain and spinal cord, and in asthma models, eosinophils from MMP2-deficient mice fail to migrate into the airways and accumulate in the interstitium. The interstitial eosinophil accumulation have been explained by the disruption of transepithelial chemokine gradients. MMP-2 also processes neutrophil- and macrophage-specific chemokines that are found in the bronchioalveolar fluid of asthmatic mice. In addition, truncation of macrophage-derived chemokine, CCL7, by MMP-2 results in the formation of peptides that can bind to the CC chemokine receptor and function as antagonists. In other experiments, a screen for MMP-2 substrates lead to the discovery of monocyte chemotactic protein-3 (MCP-3), which when cleaved acts as a general chemokine antagonist and dampens inflammation.

Elevated expression of MMP-2 is usually seen in invasive and highly tumorigenic cancers such as colorectal tumors, gastric carcinoma, pancreatic carcinoma, breast cancer, and breast cancer metastasis to lungs, oral cancer, melanoma, malignant gliomas, Chondrosarcoma, and osteosarcoma. MMPs promote tumor progression and metastasis in invasive cancers by degradation of the ECM, which consists of two main components: basement membranes and interstitial connective tissue. MMP-2 efficiently degrades collagen IV and laminin-5 which are components of the basement membrane, thereby assisting the metastatic cancerous cells to pass through it. MMP-2, also promotes angiogenesis, a critical process required for tumor cell survival and restoration of healing wound.
MMP-2 is also considered as a key enzyme in tissue remodeling during inflammation and wound healing\textsuperscript{391, 392}. MMP-2 deficient mice have reduced inflammation in their airspaces compared to wild-type mice in an asthma model of lung disease, indicating a role for this MMP in pulmonary inflammation \textsuperscript{371}. In addition, MMP-2 is expressed by fibroblasts and has been hypothesized to play a role in the migration of fibroblasts in the lung (reviewed in \textsuperscript{393}) which may contribute to fibrosis. MMP-2 can also have negative effects on cell proliferation (reviewed in \textsuperscript{100}). For example, MMP-2 produced by cartilage from the trachea and bronchus decreases respiratory epithelial cell proliferation \textit{in vitro}\textsuperscript{394} and addition of a broad spectrum MMP inhibitor rescues epithelial cell proliferation\textsuperscript{394}. It has also been suggested that interaction between keratinocytes and fibroblasts, which occurs during wound healing, can regulate MMP-2 expression by these cells\textsuperscript{392}.

Although both keratinocytes and fibroblasts express MMP-2 in culture\textsuperscript{392}, a co-culture of both keratinocytes and fibroblasts leads to increased MMP-2 expression. Due to prolonged and sustained MMP-2 expression during dermal wound repair, a role for MMP-2 in angiogenesis and matrix remodeling has also been suggested\textsuperscript{292, 300, 395}. In an asbestos model of lung inflammation and fibrosis, it was shown that MMP-2 strongly associates with time points revealing development of fibrosis\textsuperscript{300}, and fibroblast production of MMP-2 assists in fibroblast migration and perhaps fibrosis\textsuperscript{300}. The importance of MMP-2 in cell proliferation and fibrosis suggests that roles for MMP-2, outside of inflammation, should not be overlooked.
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2. VERSICAN INTERACTION WITH HUMAN LUNG FIBROBLAST AND MACROPHAGE CELL SURFACE

2.1 Introduction

Increased versican expression is often observed in association with proliferating cells within remodeling tissue in many diseases including pulmonary fibrosis, cardiovascular diseases, and cancer. In many forms of pulmonary fibrosis, such as organizing diffuse alveolar damage and idiopathic pulmonary fibrosis, the early accumulation of versican\(^1,2\) and its binding partner hyaluronan\(^3\) occurs in association with proliferating myofibroblasts prior to deposition of a collagenous matrix\(^4,5\). Versican accumulates in atherosclerotic lesions (reviewed in\(^6\)), and is found in association with proliferating vascular and arterial smooth muscle cells\(^7\). Versican is also associated with tumor growth in tissues such as breast\(^8-11\), brain\(^12\), ovary\(^13\), gastrointestinal tract\(^14\), prostate\(^15,16\), melanoma\(^17\), and mesothelioma\(^18\).

Versican, a large proteoglycan with an estimated molecular mass of more than 1000 kDa, is a member of the hyalectan (hyaluronan and lectican binding) family of proteoglycans which also includes aggrecan, abundant in cartilage, and brevican and neurocan, the two nervous system proteoglycans (reviewed in\(^19-21\)). Although there is a single versican gene, alternative splicing of its mRNA produces 4 distinct versican isoforms that carry different number of glycosaminoglycan (GAG) chains\(^22,23\). All isoforms have homologous N-terminal (HA binding) and C-terminal (lectin-like) domains. The central domain of versican V0 contains both the GAG-α and GAG-β GAG-bearing protein domains. The V1 isoform has the GAG-β domain, V2 has the GAG-α domain, and V3 is devoid of any GAG domains and only consists of the N-terminal and C-terminal globular domains. Versican is consistently found in association with its binding partners: hyaluronan, fibronectin, and tenascin\(^24-27\). The N-terminal domain of versican interacts with hyaluronan, and its C-terminal lectin-like domain may interact with a variety of other extracellular matrix molecules. These interactions may help to stabilize the matrix through formation of supra-molecular aggregates\(^26-28\).

\(^{1}\) A version of this chapter will be submitted for publication. Pourmalek, S, and Roberts, C. Versican interaction with human lung fibroblast and macrophage cell surface.
The N-terminal (G1) globular domain consists of an Immunoglobulin-like loop and two link module with hyaluronan binding properties. It has been suggested that the N-terminal domain of versican may play a role in maintaining the integrity of the extracellular matrix by interacting with both hyaluronan\(^{29}\) and link protein\(^{30}\). In epidermal injury, the expression of hyaluronan\(^{31, 32}\) along with its cell surface receptor CD44\(^{33}\) is increased. It has been shown that hyaluronan-CD44 signaling can lead to actin cytoskeleton reorganization\(^{34-36}\) and cell proliferation\(^{37}\) in tumor cells. Versican colocalization with hyaluronan and CD44 in pericellular matrix of cultured fibroblasts\(^{38}\), in lesions of atherosclerosis and restenosis\(^{6, 39}\), and in a variety of fibrotic lung diseases\(^{1, 2, 40}\) suggests a possible role for this proteoglycan in the signaling process that leads to migration and proliferation of these different cell types. In addition, versican synthesis is upregulated by proliferating smooth muscle cells \(\textit{in vitro}\)^{41}, and it has been suggested that the formation of versican-hyaluronan complex at the cell surface may facilitate the migration and proliferation of smooth muscle cells\(^{42, 43}\).

The C-terminal (G3) globular domain of versican consists of one or two EGF repeats, a C-type lectin domain and complement regulatory protein (CRP)-like domain. The C-terminal domain binds to a variety of ligands in ECM which may contribute significantly to the functions of versican. A recent study has shown that complexes of G3, fibronectin and vascular endothelial growth factor enhance proliferation and angiogenesis of astrocytoma cells\(^{44}\). Moreover, it has been shown that versican G3 motif is involved in the formation of intermolecular disulfide bonds that stabilize the matrix, and disruption of these interactions can affect cell adhesion and cell-matrix stability\(^{45}\). Studies using a chimeric construct of versican have indicated a direct role for versican in cell proliferation based on the interaction of EGF-like module in the C-terminal domain of versican with the EGF receptors of fibroblasts\(^{46}\). However, versican has not been shown to bind or activate the EGF receptors, and other studies using versican V3 isoform seem to suggest otherwise.

The central glycosaminoglycan-containing domain of versican is decorated with covalently-bound chondroitin sulfate glycosaminoglycan chains. The structural and functional diversity of versican is increased by variations in the number of
glycosaminoglycan chains bound to the core protein, as observed with four versican isoforms that exist due to alternate splicing of their glycosaminoglycan binding region. Versican’s chondroitin sulfate chains can interact with and localize a variety of growth factors\textsuperscript{47, 48} and cytokines in the ECM, which can indirectly regulate cellular behavior\textsuperscript{21, 49}. Studies of self-assembling\textsuperscript{50} hyaluronan molecules have shown that hyaluronan, in association with aggregating chondroitin sulfate proteoglycans, increases the viscosity of the matrix\textsuperscript{51, 52} and adds swelling pressure through the negatively-charged chondroitin sulfate chains\textsuperscript{53, 54}. Chondroitin sulfate proteoglycan aggregation can potentially stiffen the hyaluronan network\textsuperscript{55} and influence cell behavior.

The receptors for three of the four major components of provisional matrix, all of which interact with versican, have been identified. To name a few, $\alpha_9\beta_1$ integrin interacts with tenascin\textsuperscript{56}, $\alpha(2-5)\beta_1$ integrins are receptors for fibronectin\textsuperscript{57}, and CD44 for hyaluronan\textsuperscript{33}. However, there is no evidence for direct interaction between versican and a cell surface ligand. In this study, we tested the hypothesis that versican interacts with the cell surface of fibroblasts and macrophages through its C-terminal domain, considering the N-terminal of versican is generally found in association with hyaluronan and link protein \textit{in vivo}. Our aim was to identify possible versican cell surface receptors using modified G3 constructs as ligands.

We employed the following techniques in achieving the results presented in this study:

i. Biotinylated G3 constructs were used as ligands in “far western blotting” experiments (refer to sections 2.3.3 – 2.3.5).

ii. G3 bound to magnetic beads was used as a tool to pull down interacting proteins in the cell membrane fraction of fibroblast and macrophage cells (2.3.6).

iii. G3-bound magnetic beads were incubated with fibroblast cells to determine any interactions with the cell surface (2.3.6).

We were unable to identify a cell surface receptor that directly interacts with C-terminal domain of versican. However, we have shown that: G3 domain of versican homodimerizes and forms aggregates; G3 interacts with versican and that these interactions are dependant on disulfide bond formation; and G3-coated beads bind to the
surface of fibroblast cells. We have also identified hyaluronan, bound to fibroblast cell surface, as the main ligand for versican and have shown that versican-hyaluronan interaction at the cell surface is important for the survival of fetal lung fibroblasts in vitro. In conclusion, we were unable to detect a versican-specific receptor against a background of these interactions.

2.2 Materials and Methods

Expression and Purification of Versican – Human fetal lung (HFL1) fibroblast cells American Type Culture Collection (Manassas, VA) were cultured in 75-cm² flasks (Sarstedt; Quebec, Canada) in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT) to 80% confluence. Cells from two confluent 75-cm² flasks were trypsinized and transferred to a 850-cm² tissue culture roller bottle (Becton Dickinson) with 200ml of DMEM and incubated at 37°C in a BELCO Biotechnology Roll-in incubator. Serum free conditioned medium from fibroblast cultures (CM) was collected and centrifuged at 1500 X g for 15 minutes to remove cellular debris. Then, Urea and salt concentrations in HFL1 conditioned media were adjusted to 7M Urea and 0.4M NaCl and loaded onto Q-Sepharose Fast Flow ion exchange resin (Amersham Biosciences, Piscataway, NJ) at approximately 1 litre CM per 5 mls resin. The column was washed with a 10 fold bed volume of 7M Urea, 0.4M NaCl, 0.1M NaOAc, pH 6.0 before elution with 7M Urea, 1.5M NaCl, 0.1M NaOAc, pH 6.0. Peak fractions were pooled and dialyzed against PBS (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM NaH₂PO₄, pH7.4) exhaustively, then flash frozen with liquid nitrogen and stored at -70°C. Fractions were monitored for versican content by alcian blue (Sigma, St. Louis, MO) staining of SDS-PAGE gels and by Western blotting. Purified versican concentration was estimated using the dimethylmethylene blue (DMMB) assay (Serva, Heidelberg) to quantify sulfated glycosaminoglycan using known concentrations of chondroitin sulfate C as standards (Seikagaku). 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.


**Generation of Recombinant Constructs (HisL, HisLC, HisG3)** – Before ligating the His-tagged C-terminal construct cDNA to the expression vector pGYMXC, the PCR product was amplified using the pPCR-Script strategy. Briefly, cDNA (generated from an HFL-1 mRNA library and amplified from PCR) was purified using a QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario). The DNA fragment was ligated into the pPCR-Script Cam SK(+) plasmid (Stratagene, CA, USA). The ligation mixture contained 1µl of 10X reaction buffer, 1µl of Srf I restriction enzyme (5U), 1µl of T4DNA ligase (4U), 0.5µl of 10mM rATP, 3µl of the HisLC insert (190ng), and 1µl of the cloning vector (10ng) for a 100:1 molar ratio of insert to cloning vector. The mixture was diluted to 10 µl with ddH2O, gently mixed, and incubated at room temperature for 1 hr before heating at 65°C for 10min. Two µl of the ligation mixture was used for heat shock transformation of supercompetent E. coli strain DH5α. The bacteria was transferred to 50µl of 2X YT media (1.0% w/v yeast extract, 1.6%w/v tryptone, 0.5% w/v NaCl, pH 7.5) and agitated at 275rpm for 30min at 37°C before plating on LB agar plates containing 30µg/ml chloramphenicol. Plates were incubated for 16hrs at 37°C. Colonies were selected and grown in 5ml of terrific broth media (1.2% w/v tryptone, 2.4% w/v yeast extract, 0.231% w/v KH2PO4, 1.254% w/v K2HPO4, 0.4% glycerol) with 30µg/ml chloramphenicol at 37°C for 16hrs at 275rpm. The plasmid was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario). The insert was removed from the CAM SK(+) plasmid by Hind III and NheI restriction enzyme digestion and purified by QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario). The identity of the new HisLC pGYMX construct was confirmed by Hind III and Nhe I restriction enzyme digestion and by DNA sequencing performed by Nucleic Acids-Protein Service (NAPS) at the University of British Columbia.

**Transformation, Expression and Purification of Engineered Versican C-terminal Domain Constructs (HisL, HisLC, and HisG3)**– The pGYMX HisG3 expression vector was transformed into E.coli BL21(DE3) competent cells at a ratio of 1µl of cDNA into 50µl BL21 Gold E.coli cells. Briefly, the mixture was iced for about an hour before a 90 seconds heat shock at 42°C. The mixture was iced for 2 minutes, and incubated at 37°C for 30 min in shaker with 50µl of 2XYT media. The cells were then plated on Luria–
Bertani (LB) agar plates containing 100µg/ml ampicillin. 5ml of superbroth (0.8% w/v yeast extract, 1.0%w/v tryptone, 0.5% w/v NaCl, 0.1% glycerol, pH 7.5) and incubated in 37°C shaker for 24 hours. Next, another plate of agar was inoculated with a single colony and incubated at 37°C for 16 hrs at 275rpm. Aliquots of this log phase seed culture were used to inoculate 3.5L of superbroth in a1:1000 (v/v) ratio with 100µg/ml ampicillin and incubated at 37°C for 24hrs at 275rpm. Collected cells were washed with 500ml of NET buffer (100mM NaCl, 1mM EDTA, 20mM Tris-HCl, pH 8.0) then lysed in 250ml of lysis buffer (50mM NaCl, 1mM EDTA, 20mM Na₂HPO₄, 1mg/ml lysozyme, 1mM PMSF, pH 8.0) for 2hrs at 37°C and 275rpm and sonicated with 5 sec bursts. The inclusion bodies were washed with 500ml of NET buffer then dissolved in a solubilization buffer (8M Urea, 10mM Tris-HCl, 100mM Na₂HPO₄, pH 8.0) for 16hrs at 4°C. Dissolved inclusion bodies were centrifuged at 20000rpm for 1hr and purified using a 30ml Ni²⁺- charged chelating sepharose column (Amersham Pharmacia) equilibrated in column buffer (8M Urea, 0.5M NaCl, 20mM Na₂HPO₄, pH 7.4). The column was washed in succession with 10-fold bed volume of column buffer, column buffer with 1M NaCl, column buffer with 1M NaCl, pH 6.0, and again with column buffer. Proteins with non-specific interactions to the Ni-chelate column were pre-eluted by a 10-fold bed volume of column buffer with 200mM imidazole. HisLC fusion protein was then eluted by a 200mM to 1M imidazole gradient over a 10-fold bed. Fractions were analyzed by SDS-PAGE and stored at −20°C.

**Refolding of HisG3 Recombinant Protein** – Peak fractions obtained from the imidazole gradient were pooled and diluted 20-fold before dialysis in equal volume of refolding buffer (18.2mM Na₂CO₃, 24mM NaHCO₃, 125mM NaCl, 1:10 ration of 3mM Cysteine/Cystine, pH 10.0) with aeration at room temperature. Refolding buffer was changed every 2 hrs for 8 hrs before exhaustive dialysis with refolding buffer minus the redox pair of Cysteine/Cystine for complete removal of urea. Because the pooled fractions were diluted 20 fold before refolding, a 10ml Ni-chelate column equilibrated in 18.2mM Na₂CO₃, 24mM NaHCO₃, 125mM NaCl, pH 10.0 was used to concentrate the diluted pool. HisLC was eluted with 18.2mM Na₂CO₃, 24mM NaHCO₃, 125mM NaCl, 500mM imidazole, pH 10.0. Peak fractions were pooled and dialyzed against excess
volume of 18.2mM Na$_2$CO$_3$, 24mM NaHCO$_3$, 125mM NaCl, pH 10.0. The protein pool was aliquoted into 1ml fractions, flash frozen with liquid nitrogen, and stored at –70°C.

**HisLC Antibody Preparation** – New Zealand white rabbits (3months old, 3kg each) were injected with 1ml of HisLC (0.263 mg/ml, expressed and purified in our laboratory) mixed with Freund’s Incomplete Adjuvant. In total, 64µg of HisLC was injected into each rabbit. 10ml of pre-bleed was collected from each rabbit before HisLC injection. A booster shot of the same amount was repeated at 3 weeks, and rabbits were fully bled at 1 month after initial injection. Blood was incubated at 37°C for 1 hour to promote clotting. Blood clot was removed and the rest of the mixture was centrifuged at 3000rpm for 6 minutes. The supernatant was removed and incubated at 4°C for 3 hours. Serial dilutions of supernatant (1:4 – 1:4096) were prepared in an ELISA plate that had been coated with 200µg/well HisLC and were blocked with 2.5% BSA at 4°C overnight. Each well was washed with TBS plus 0.05% Tween and incubated in 1:2000 GAR-AP for 1 hour at room temperature. The plate was washed again and developed with 100µl/well p-nitrophenyl phosphate (pnPP) substrate, in Tris buffer, to estimate the concentration of antibody in serum.

**Electrophoretic Techniques** – Samples in nonreducing 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 12.5% (separating) acrylamide. Stacking and separating gels were kept during staining and 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: 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 2.5% (w/v) bovine serum albumin, 20 mM Tris, 5 mM EDTA, 0.9%NaCl, and 0.3% (v/v) Tween 20. Anti-versican 2B1(Seikagaku Corporation, Tokyo, Japan), 1:500 dilution, was used for detection of versican at an epitope near the C-terminal domain; anti-G3 antibody (anti-HisLC “LC2”), 1:10,000 dilution, for detection of the C-terminal domain of versican; and biotinylated HisLC and biotinylated HisG3 (prepared
in our laboratories), 1:20 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); highly cross adsorbed goat anti-rabbit horseradish peroxidase-conjugate (Bio-Rad) and Streptavidin Alkaline Phosphate HRP were diluted to 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).

**Release of Versican from Fibroblast Cell Surface Using Hyaluronidase**– Human fetal lung fibroblast cell line (HFL1) was 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) in cell culture flasks. Once cells were at 80% confluence, they were trypsinized and transferred into 4well chamber slides. Cells were allowed to spread on the slides in serum enriched media at 37°C until 80% confluent. Cells were subsequently treated with hyaluronidase (Type X, from Leech, 2000 units/ml, Sigma Chemical Co. Louis, MO, USA) in phosphate buffer saline, at different concentrations (0, 3.3µg/ml, 33.3µg/ml, and 333.3µg/ml) and for different lengths of time (30 minutes, 1 hour, 4 hours, and 8 hours). At the end of each time point, the media containing hyaluronidase enzyme and its cleavage products was collected for further analysis, and the cellular behavior was analyzed using fluorescent staining and microscopy. Products of hyaluronidase treatment in the media were analyzed with silver stained SDS-PAGE and western blotting.

**Cytochemistry** – At the end of each time point, cells were fixed in 4% paraformaldehyde in PBS for 15 minutes in 37°C incubator. Fixed cells were permeabilized with 0.1% Triton X-100 and PBS for 1 minute and washed with PBS before being blocked by freshly prepared 1% bovine serum albumin (BSA) solution in PBS for 5 minutes. Versican was detected with LeBaron rabbit polyclonal antibody, a kind gift from Dr. Richard LeBaron and Dr. Erkki Ruoslahti (La Jolla Cancer Research Foundation, CA, USA), 1:250 dilution; and 1:250 Alexafluor-488 anti-rabbit antibody.
Molecular Probes, Eugene, Oregon, USA). After being washed with PBS, cells were incubated in Hoechst stain (33342, Invitrogen) at 1:2000 dilution in 1% BSA/PBS, for 10 minutes. Cells were then washed with PBS and stored with ProLong Gold antifade reagent (Invitrogen, Molecular Probes) on a covered slide.

**Fluorescent Light Microscopy** – Fixed and stained cells were viewed using a Leica CTR fluorescent light microscope on monochrome setting. Images were captured at 40X magnification, in layers of 0.75µm thickness using the multi-channel z-stack capture option using Q-Imagine Retiga Exi mounted camera and OpenLab software (4.0.2). Emission wavelengths were set to best view each stained organelle: actin filaments were viewed at 520nm; nuclei at 456nm (DAPI), and mitochondrion at 594nm (Texas Red). Once images were captured, each image was deconvoluted using the Nearest Neighbor DCI function to remove unfocussed light. All layered images taken of the same coordinates but at different points along the z-axis (height) were merged to create one 3-dimensional image of cells spread in the collagen matrix. The appropriate color of the stain was then applied to the image, and images captured under different emission wavelength of different organelles were merged into one composite. The scale bar was set at 95µm.

**Biotinylation of Versican C-terminal Constructs** – Versican C-terminal constructs were biotinylated using EZ-Link NHS-PEO Solid Phase Biotinylation Kit (Pierce, 21440, Rockford, IL) following the manufacturer’s instructions. In short, HisG3 protein constructs were bound to a charged Nickel column. The biotin solution at an appropriate concentration for the amount of protein being biotinylated was applied to column and allowed to completely immerse the capped gel bed. The biotin solution was incubated with the gel bed for 30 minutes at room temperature and then removed by allowing it to flow through the column. The biotinylated protein was eluted from the column by 0.2M Imidazole in PBS, and stored at 4°C.

**Production of Magnetic Beads** – Protein constructs were dialyzed (Slide-A-Lyzer dialysis cassette, Pierce, Canada) in a buffer free of any amine or carboxyl groups, with an appropriate pH (4 – 6) for covalent attachment of proteins to magnetic beads. BioMag
Plus (BP618) with carboxyl-terminated surface (Bangs Laboratories, Inc., 6445) was used, and manufacture’s instructions were followed, in the production of HisG3-bound magnetic beads. In short, the beads were washed and activated with EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] and coupled with the protein solution of interest (HisLC, HisG3, or with Glycine for production of control beads). After 24 hours of incubation, the unbound protein solution (supernatant) was removed and saved for binding efficiency calculations, and the reaction was stopped by adding the quenching solution (1.0M Glycine, pH 8.0). The protein-bound beads (with a particle concentration of 5mg/ml) were washed and stored in a favorable buffer (HEPES, pH 7.0) at 4°C. This procedure yielded protein-bound magnetic beads with a 90.9% efficiency. The binding efficiency of proteins to the beads were calculated by measuring the absorption (A_{280}) of protein solution before and after binding to the beads, using the following formula:

\[(\text{Abs}_{\text{before binding}} \times \text{dilution}) - (\text{Abs}_{\text{after binding}} \times \text{dilution}) / (\text{Abs}_{\text{before binding}} \times \text{dilution}) \times 100\%\]

**Examination of G3-Coated Magnetic Bead Binding to Fibroblast Cell Surface** – For visualization of HisG3-bound magnetic beads interaction with HFL1 fibroblast cell surface using phase microscopy, fibroblast cells were plated on 12 well chamber slides. Cells were grown to 50% confluence, and then, HisG3-bound magnetic beads or Glycine-bound control beads (50 µg/ml; diluted in HEPES buffer) were added to the cells. After 20 minutes of incubation at 37°C, cells were fixed with 40% formaldehyde solution for 20 minutes and then observed under a differential interference contrast (DIC) microscope at different magnifications (10X, 20X, 40X). In the case of detecting cell membrane proteins which may interact with HisG3-bound magnetic beads, the solution, containing either HFL1 fibroblast or U937 macrophage cell membrane protein fraction, was incubated with HisG3-bound magnetic beads overnight at 4°C. The beads were magnetically separated from the solution of unbound proteins and the supernatant was saved for electrophoretic analysis. The HisG3 binding proteins were eluted with DTT containing sample buffer and analyzed with silver stain after SDS-PAGE.

**Preparation of cell membrane fraction from cell lysate** – Protein fraction from HFL1 fibroblast and U937 macrophage cell membranes were separated from the cell lysate using nitrogen-disruption bomb (Parr Instrument Company, 4635, Moline, IL,
USA) following manufacturer’s instructions. In summary, cells at a concentration of 1x10^6 cells/ml were centrifuged and washed in an appropriate buffer. The cells in a falcon tube were assembled in the nitrogen flask, and the flask was pressurized with nitrogen gas from a nitrogen tank to 500psi. The cells were incubated under pressure for 30 minutes before the pressure was released slowly and the lysed cells were collected in a second tube. The cell lysate, in appropriate buffer with proteinase inhibitors, was centrifuged at 2700 rpm for 10 minutes to separate the pellet of heavier cell solids from the solubilized membrane proteins in the supernatant. The supernatant was then centrifuged at 18,500 rpm for 1 hour to separate the membrane protein pellet from the solution. The membrane protein pellet was homogenized in buffer at 11,000 rpm for 5 seconds and stored at 4°C.

2.3 Results

Histidine-tagged C-terminal constructs of versican, alternatively called the HisG3 and HisLC domain (figure 2.1), were expressed in *E.coli* and purified in our laboratory. These C-terminal constructs were used to investigate specific interactions between versican and the cell surface through versican’s C-terminal domain. The results presented here are representative of at least three different trials for each given experiment.
2.3.1 Purification and Characterization of Versican from HFL1 Fibroblast Conditioned Media

Versican was purified from the serum-free conditioned media of Human fetal lung (HFL1) fibroblast cells. Serum free media from 80% confluent fibroblast cultures were collected and centrifuged at 1500g for 15 minutes to remove cellular debris. Then, Urea and salt concentrations in HFL1 conditioned media were adjusted to 7M Urea and 0.4M NaCl and loaded onto Q-Sepharose Fast Flow ion exchange resin at approximately 1 litre culture media per 5 mls resin (Figure 2.2, FT). The column was washed with a 10 fold bed volume of 7M Urea, 0.4M NaCl, 0.1M NaOAc, pH 6.0 (Figure 2.2, W) before elution with 7M Urea, 1.5M NaCl, 0.1M NaOAc, pH 6.0 (Figure 2.2, Eluted Vc). Fractions were monitored for versican content by silver staining and alcian blue staining of SDS-PAGE gels (Figure 2.2). The identity of versican as the predominant proteoglycan was established by western blotting and mass spectrometry (data not shown). Purified versican concentration was estimated using the dimethylmethylene blue (DMMB) assay to quantify sulfated glycosaminoglycan using known concentrations of chondroitin sulfate C as standards. 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 $\mu$M versican. The pattern left by high-molecular weight versican and versican aggregates on the polyacrylamide gel is signatory, and is observed in all data presented in this thesis. Versican leaves rippling marks on the larger pored stacking gel, and hardly penetrates the smaller pored separating gel.
Figure 2.2 Versican Purified from HFL1 Cell Culture Form Aggregates in Solution
Serum-free conditioned media of Human fetal lung (HFL1) fibroblast cells was loaded onto Q-Sepharose Fast Flow ion exchange resin and allowed to flow through the column (FT). The column was washed with column buffer (W) before elution with higher NaCl concentrations (Eluted Vc). Fractions were monitored for versican content by OD$_{280}$ trace, silver staining and alcian blue staining of polyacrylamide gels. The fraction representing each lane on the gel is also marked on the OD$_{280}$ trace, and lane 4 represent a pool of all wash fractions. Versican is observed as ripples through the stacking gel, and concentrates at the top of separating gel.
2.3.2 Purified Versican C-terminal (LC) Constructs Form Aggregates in Solution

The HisLC expression vector was transformed into *E. coli* competent cells, and cells were grown in LB media. Cells were lysed and the inclusion bodies were separated through centrifugation, washed, then dissolved in a solubilization buffer (Figure 2.3, L). Dissolved inclusion bodies were centrifuged and purified using a Ni^{2+} charged chelating sepharose column equilibrated in column buffer. The column was washed in succession with 10-fold bed volume of column buffer, column buffer with 1M NaCl, column buffer with 1M NaCl, pH 6.0, and again with column buffer (Figure 2.3, FT). Proteins with non-specific interactions to the Ni-chelate column were pre-eluted by a 10-fold bed volume of column buffer with 200mM imidazole (Figure 2.3, W). HisLC fusion protein was then eluted by a 200mM to 1M imidazole gradient over a 10-fold bed (Figure 2.3, Eluted HisLC).

Peak fractions obtained from the imidazole gradient were pooled and diluted 20-fold before dialysis in equal volume of refolding buffer with 1:10 ratio of 3mM Cysteine/Cystine and aeration at room temperature. Refolding buffer was changed frequently before exhaustive dialysis with refolding buffer minus the redox pair of Cysteine/Cystine for complete removal of urea. Because the pooled fractions were diluted 20 fold before refolding, a 10ml Ni-chelate column was used to concentrate the diluted pool. HisLC was eluted, and peak fractions were pooled and dialyzed against excess volume of Voller’s buffer, based on a test of at least 48 buffers and conditions. The full length C-terminal domain (HisG3) of versican was purified following the same procedures (by Haidi Kai, data not shown). HisLC formed higher molecular aggregates in solution which made the refolding process particularly difficult. HisG3 was refolded and verified by binding to fluorescein-heparin, by fluorescence anisotropy spectroscopy, and showed much greater degree of self-association than HisLC under the same conditions. However, the majority of molecular species were found at the appropriate molecular weight for a monomer, as observed in the following SDS gel for HisLC.
Figure 2.3 Purification of HisLC
HisLC transformed E.coli cells were lysed and the inclusion bodies were separated through centrifugation, washed, then dissolved in a solubilization buffer (L). Dissolved inclusion bodies were centrifuged once more and purified using a Ni$^{2+}$ charged chelating sepharose column. The column was washed in succession with column buffer, column buffer with 1M NaCl, column buffer with 1M NaCl, pH 6.0, and again with column buffer (FT, in this order). Proteins with non-specific interactions to the Ni$^{2+}$ column were pre-eluted by column buffer with 200mM imidazole (W). HisLC fusion protein was then eluted by a 200mM to 1M imidazole gradient (Eluted HisLC). Fractions are analyzed by SDS-PAGE. Predominant product was HisLC based on molecular weight confirmation by mass spectrometry.
2.3.3 **Biotinylated HisLC interacts with versican-like molecules in fibroblast cell lysate**

In order to determine any possible interactions between the lectin domain of versican and fibroblast cell surface, biotinylated HisLC construct was used as ligand in far western blotting with HFL1 cell lysate. To this end, HisLC construct was biotin-labeled using the EZ-Link NHS-PEO Solid phase biotinylation kits (Figure 2.4, ½ diluted in lane 5; and neat concentration in lane 6). Cell lysate of fibroblasts, grown in culture plates, contains high-molecular weight proteins that interact with biotinylated HisLC under non-reducing conditions (Figure 2.4, -DTT, lane 1, stacking gel). Biotinylated HisLC also interacts with a number of other molecules in fibroblast cell lysate, with varying molecular weights ranging from 30 kDa – 100 kDa. Three strongest protein bands that interact with biotinylated HisLC are of the same molecular weight as HisLC (29 kDa) and HisLC aggregates (Biotinylated HisLC +DTT, arrows). Note that there is no non-specific interaction between the secondary Avidin-HRP antibody and any of the proteins in the cell lysate (Figure 2.4, control), and that Avidin-HRP interacts only with the biotin group on biotinylated HisLC (Figure 2.4, lanes 5 and 6).
Figure 2.4 Versican G3 construct (HisLC) interacts with ligands in HFL1 cell lysate

Biotinylated HisLC construct interacts with a number of proteins in the HFL1 cell lysate (lanes 1-4, increasing dilutions from 0 to 1:5, 1:25, and 1:125 respectively), of similar molecular weight to multimers of HisLC (arrows, +DTT). Versican-like high molecular weight species (lanes 1 and 2, arrow) can be observed in HFL1 cell lysate under non-reducing (-DTT) conditions, in the stacking gel. Biotinylated HisLC (lanes 5 and 6, 1:100 and 1:20 dilutions respectively) was included in the same gel to illustrate the intensity of the signal and confirm specific interaction of biotinylated HisLC with Avidin-HRP antibody. Control represents PVDF membranes not treated with biotinylated HisLC prior to incubation with Avidin-HRP antibody.
The pattern of these high molecular weight species, which interact with HisLC in the cell lysate (Figure 2.4), is similar to what is generally observed with purified versican on western blot (Figure 2.2). As such, we examined the possibility that these high molecular weight species may in fact be versican. We employed a number of different methods, and assessed the protein content of fibroblast cell lysate (Figure 2.5, CL) with the versican-specific 2B1 antibody, HisLC antibody, and alcian blue stain which stains for glycosaminoglycan chains, in parallel to a biotinylated HisLC far-western blot. The high molecular weight molecule detected in biotinylated HisLC blot corresponded to the pattern of molecules detected by versican 2B1 antibody and by alcian blue stain. This observation supports an interaction between the biotinylated HisLC construct and versican species in the cell lysate. This experiment also confirmed that lower molecular weight fragments in fibroblast cell lysate, as detected by biotinylated HisLC, contain the C-terminal domain of versican as they are also recognized by HisLC antibody (Figure 2.5, arrows between 41.3-89 kDa).
Figure 2.5  Versican G3 construct (HisLC) interacts with versican and other fragments containing versican C-terminal domain in the fibroblast cell lysate

Biotinylated-HisLC (Biotin-HisLC) interacts with high molecular weight versican (arrow; 210 kDa and top of the gel) in fibroblast cell lysate (CL). Other entities (arrows; 41.3-89 kDa) are also recognized in the western blot by biotinylated HisLC. Western blot with anti-versican 2B1 antibody, which recognizes a site near the C-terminal domain, identifies high molecular weight versican aggregates (arrow; top of the gel), versican (arrow; 210 kDa), and lower molecular weight fragment containing the 2B1 epitope. HisLC antibody marks versican (arrow; 210 kDa) and other fragments containing the C-terminal domain of versican (marked by arrows). Alcian Blue stain of the cell lysate shows the presence of glycosaminoglycan-containing high molecular weight versican aggregates in the fibroblast cell lysate.
2.3.4 Versican intermolecular interactions modulated by its C-terminal domain

In the previous section, we showed that biotinylated HisLC interacted with fragments in the HFL1 fibroblast cell lysate that were the same size as the LC monomer and aggregates (Figure 2.4), and with fragments of versican that contained the C-terminal domain of versican (Figure 2.5). Based on these observations, intermolecular interactions of versican through its C-terminal domain were investigated further. Purified C-terminal construct of versican was loaded onto a polyacrylamide gel and transferred to a PVDF membrane after electrophoresis. The biotinylated versican construct was used as a ligand in far-western blotting experiments to examine intermolecular interactions. Our data showed that HisLC interacts with biotinylated HisLC construct, and confirmed our previous observation that HisLC monomer forms higher molecular weight aggregates (Figure 2.6), illustrating a specific intermolecular interaction between the G3 domains of versican molecules.
Figure 2.6 Biotinylated HisLC Interacts with HisLC monomer and multimers
Purified HisLC protein construct was subjected to SDS-PAGE and western blot with HisLC antibody. HisLC monomers and aggregates recognized by the antibody show a similar pattern to the proteins which interact with biotinylated HisLC in far-western blotting experiment.
In summary, the HisLC domain seemed to interact with itself and the full G3 domain of versican under non-reducing conditions, suggesting that versican may self-associate through disulfide bond formation between the LC domains at its C-terminus.

2.3.5 Biotinylated HisG3 interacts with proteins in fibroblast and macrophage cell membrane

To gain a better understanding of the interactions of C-terminal domain of versican with the cell surface of HFL1 fibroblasts and U937 macrophages, cell membrane proteins from both cell types were isolated from the cell lysate using the “Nitrogen Bomb” method. Biotinylated HisG3 (full C-terminal construct of versican) was used as a probe in far-western blotting experiment (Figure 2.7, Western Blot), under reducing (+DTT) and non-reducing (-DTT) conditions, to analyze the size of the cell membrane proteins that interact with it. Far fewer proteins in HFL1 fibroblast cell membrane interact with biotinylated HisG3 in comparison to the many protein bands that are detected in U937 macrophage cell membrane. There were a very large number of proteins in the cell membrane fraction that bound labeled HisG3, and we were unable to unravel the identity of HisG3 interacting proteins.
**Figure 2.7 Versican HisG3 Interacts with HFL1 and U937 Cell Membrane Ligands**

Cell membrane fractions from HFL1 and U937 macrophage cells were analysed with SDS PAGE under reducing (+DTT) and non-reducing (-DTT) conditions at two different concentrations. The interaction of cell membrane proteins with biotinylated HisLC was examined and the results are presented here (Western Blot). The two lanes under each cell type represent different concentrations of cell membrane proteins, with lane 2 containing half as much protein as lane 1. Total protein content was also visualized by silver staining method (silver stain).
2.3.6 HisG3-coated magnetic beads interact with each other and with the surface of fibroblasts

In order to identify cell surface molecules that interact with versican C-terminal domain, we used HisG3-coated magnetic beads. HisG3 versican constructs were covalently bound to carboxyl-terminated magnetic beads with about 90% efficiency. HisG3-bound magnetic beads were then used as bait to pull down any cell surface ligands that may exist in the pool of cell membrane proteins isolated from human lung fibroblasts and macrophages (Figure 2.8). The HisG3 coated magnetic bead chromatography led to the visualization of a number of proteins from the HFL1 fibroblasts and U937 macrophages cell membrane fraction. Our hypothesis was that any major G3-interacting protein would be pulled down by G3-coated beads and not by control (glycine-coated) beads. From the silver stained gels alone, no major cell surface ligands for G3 domain of versican could be detected in either cell type. The protein band pattern of the eluate from glycine-coated magnetic beads and that of HisG3-bound magnetic beads were visually identical. One caveat of using 1-dimensional gel electrophoresis to separate a complex mixture of proteins is that a single band on the gel may represent several different proteins indistinguishable by this method. However, within the limits of this technique, no major targets for protein sequencing were visualized.
Figure 2.8 HisG3 coated magnetic beads interact with HFL1 and U937 cell membrane

Cell membrane fraction isolated from total cell lysate (lane 1) was incubated with magnetic beads coated with either glycine (control, lanes 2-4) or HisG3 (lanes 5-7). The unbound proteins were collected in the supernatant (lanes 2 and 5) and the beads were washed with column buffer (lanes 3 and 6). The bound proteins were eluted with SDS containing sample buffer (lanes 4 and 7) and visualized by silver stain.
In order to determine if HisG3-bound magnetic beads could bind to cell surface of fibroblasts, we performed cell culture experiments through which the binding patterns of HisG3-bound, and control beads, could be observed with high resolution differential interference contrast (DIC) microscopy (Figure 2.9). Images taken of the fibroblast cell interaction with the magnetic beads showed that HisG3-coated beads interact with each other, and with the fibroblast cell surface. Control glycine-bound beads, on the contrary, did not aggregate and seemed to uniformly cover the glass slide on and around the cells. Cell microscopy of magnetic beads coated with HisG3 protein construct confirmed our previous results (Figure 2.6) that HisG3 self-associates and interacts with the cell surface of fibroblast cells. It is important to note that aggregate formation from beads individually coated with G3 shows that HisG3-HisG3 interactions form from HisG3 monomers, rather than as an artifact of aberrant folding of the HisG3 construct.
Figure 2.9 HisG3 coated magnetic beads interact with each other and HFL1 cells
HisG3 coated magnetic beads (top) and glycine coated magnetic beads (bottom) were incubated with HFL1 fibroblast cells for 15 minutes and viewed at 10X magnification. Almost all HisG3 coated magnetic beads are found in association with each other and localized on HFL1 cell surface. In comparison, glycine coated beads are dispersed throughout the cell culture plate.
2.3.7 Hyaluronidase treatment of cell cultured fibroblasts leads to the release of versican from fibroblast cell surface

HFL1 fibroblast cells, plated to 75% confluence in culture flask, were treated with hyaluronidase to determine whether degradation of hyaluronan would release cell surface versican. The intensity of fluorescent immunostain to versican, in association with HFL1 fibroblast cell surface, decreases with increase in the concentration of hyaluronidase (33.3 – 333.3 µg/ml) and with increasing time of incubation of cells with hyaluronidase (4 – 8 hours). Versican was released from the fibroblast pericellular matrix into the extracellular media, as observed by fluorescence microscopy using both the LeBaron antibody (Figure 2.10) and by western blotting with the 2B1 antibody (Figure 2.11). It is important to note that no fluorescent staining was observed for versican in the control groups, which were treated only with the secondary fluorescent antibody. Both LeBaron and 2B1 antibodies have been characterized in western blots as specific for versican, both in our and other laboratories. The release of versican was confirmed with western blot analysis using 2B1 antibody (Figure 2.11, arrow head). The silver stain of the proteins released into media also shows an increase in the concentration of a number of proteins including a species that corresponds to high molecular weight versican (Figure 2.11, arrow head). These immunofluorescence studies suggest that most versican is releasable from the cell surface by hyaluronidase treatment within 8 hours. These results strongly support the hypothesis that versican is held at fibroblast cell surface mainly through the interaction of its N-terminus with hyaluronan.
Figure 2.10 Versican is released from HFL1 cell surface on treatment with hyaluronidase
The morphology of human fetal lung fibroblasts was studied on culture slides with increasing concentration of hyaluronidase (0 - 333.3 µg/ml) at different time points (30 minutes, 1 hour, 4 hours, 8 hours). Cells were stained with Lebaron antibody (Versican, green) and Hoechst stain (nuclei, blue) and observed at 40X magnification with a Leica microscope after being fixed. Hyaluronidase degrades hyaluronan and releases versican from the pericellular matrix, observed as decreasing green fluorescent stain. Changes in the nuclear morphology are also observed.
All images were captured at 500ms exposure time, and bar = 25µm
Figure 2.11 Versican is released from HFL1 cell surface upon treatment with hyaluronidase
High molecular weight versican (lane 4, arrow) was released from the fibroblast cell matrices through degradation of hyaluronan by hyaluronidase. Increasing versican concentration is apparent with increasing concentration of hyaluronidase (0 - 333.3μg/ml from Lane 1-4), at 8 hours of incubation. 2B1 antibody was used in the preparation of the western blot.
2.3.8 Hyaluronidase treatment of fibroblasts induces changes in cell morphology

Fibroblast treatment with hyaluronidase lead to another observation, that as versican-hyaluronan complex is released from fibroblast cell surface, the morphology of nucleus is changed from a healthy dividing state into one marked by chromatin clumping and nucleus swelling, perhaps indicative of necrosis (Figure 2.12). This is particularly visible in the cells treated with higher concentration of hyaluronidase (333.3 µl/ml) and with higher incubation periods (8 hours). Versican association with the cell surface is essential for normal fibroblast cell morphology, and release of versican from the cell surface can force fibroblasts to conform to the lines of stress laid out by collagen fibers.
Figure 2.12 Fibroblast nuclear morphology is altered upon hyaluronidase treatment
Release of versican from fibroblast cell surface is dependant on the concentration of hyaluronidase and duration of incubation period. Nucleus morphology is clearly altered by the degradation of hyaluronan and release versican from the cell surface of fibroblasts as evident in the bottom right panel. This change is marked by chromatin clumping and nucleus swelling, perhaps indicative of necrosis. Bar = 25μm
2.4 Discussion

Increased versican expression is often observed in association with proliferating cells within remodeling tissue in lung and cardiovascular diseases and in cancer. In this study, we tested the hypothesis that versican interacts with the cell surface of fibroblasts and macrophages through its C-terminal domain. Our results have failed to identify any ligands on the macrophage cell surface, and have lead us to believe that versican is held near the fibroblast cell surface mainly through the interaction of its N-terminus with hyaluronan. These results are in concert with other research that suggests interactions of G1 domain of versican with HA results in the formation of a pericellular matrix that is required for the proliferation of arterial smooth muscle cells\(^7\).

We have shown that hyaluronidase treatment of cultured fibroblast cells leads to shedding of versican from the cell surface and alteration of fibroblast phenotype, *in vitro*. Similarly, other studies have shown that dissolution of the pericellular matrix by treatment of the cells with HA oligosaccharides inhibits SMC proliferation\(^7\). CD44, the main cell receptor for hyaluronan, may also interact with versican through its chondroitin-sulfate chains\(^48\). The binding of versican with CD44 may be mediated by the interaction of CS chains of versican with the carbohydrate-binding domain of these molecules\(^48\), and it has been suggested that versican interaction may complement or modulate CD44 mediated adhesion and migration. Nonetheless, hyaluronidase treatment seems to release most versican from the cell surface.

Our data are consistent with a model where versican can self-associate through G3 domain and can form large aggregates\(^45\). Our G3 constructs could also interact with versican through interactions with the C-terminal domain. It has been hypothesized that versican G3 motif is involved in the formation of intermolecular disulfide bonds that stabilize the matrix, and disruption of these interactions can affect cell adhesion and cell-matrix stability\(^45\). Our search for cell surface receptors that would bind versican
C-terminal domain, using G3 constructs, did not lead to identification of possible novel ligands.

Evidence in the literature for a direct interaction of versican with the cell surface relies upon vector-driven over-expression of versican constructs or deletion of segments within such constructs. For example, an engineered chimeric molecule named “mini-versican” has been shown to modestly stimulate NIH 3T3 cell proliferation, and it has been suggested that the EGF-like modules in the G3 domain are responsible for the observed enhanced cellular proliferation. Deletion of the G3 domain or the EGF-like repeats, they reported, eliminated the effect of over-expression or addition of versican products on cell proliferation. It is important to note, however, that the G3 construct used to show enhanced cellular proliferation contained an MRGS-His tag, while the G3 mutant which did not result in cell proliferation had the MRGS-His tag removed along with the EGF sequences. MRGS sequence is similar to the RGD (Arg-Gly-Asp) tri-peptide which is frequently found in proteins that interact with integrin cell adhesion receptors (Reviewed in63, 64). Possible interaction of MRGS with cell surface integrin receptors could explain the observed difference in cell behavior in the presence of G3 and G3-mutant constructs.

This group has also reported that addition of anti-sense against EGF receptor could block the effect of added versican, although versican has not been shown to bind or activate the EGF receptor. In contrast, studies of rat SMC that were retrovirally transduced to express versican V3, which lacks the GAG binding domains, showed decreased cell proliferation and migration and increased cell adhesion. It has been suggested that the effects of V3 on SMC indicate that over-expression of versican G3 domain constructs do not universally promote cell proliferation. Effects of V3 on cell behavior may be as a result of competition for binding to cell surface-associated endogenous versican (V0/V1) ligands, such as HA. This suggestion is supported by the observation that the formation of the HA-versican V0 and V1 pericellular matrix is inhibited in cells that express versican V3.

Taken together, the data in the literature suggest that versican has important functions at the cell surface that influences cell behavior, but more studies are needed to elucidate the mechanism of its action on the cells. Our data are consistent with versican-versican and versican-hyaluronan interactions at the cell surface found in other studies. Against a
background of versican-versican and versican-hyaluronan interactions, with these cell
types, we were unable to find evidence for other major receptor-ligand interactions.

The deposition of a transient granulation tissue and its subsequent maturation and
remodeling is the most clinically significant phase of wound healing. Myofibroblasts
proliferate in a matrix rich in fibronectin\textsuperscript{67}, tenascin, proteoglycans versican, biglycan,
and the glycosaminoglycan hyaluronan\textsuperscript{1,2}. Versican is believed to play a significant role
in this matrix through interactions with the cell surface and with its binding partners, and
thus has been the focus of much research in many different tissues.

Our findings support previous research, and suggest that versican is held at fibroblast cell
surface predominantly through its interactions with hyaluronan, and that formation of this
pericellular matrix is essential for the maintenance of fibroblast cell phenotype.
Homodimerization of G3 through formation of disulfide bonds may also be significant, as
it may contribute to cell adhesion and cell-matrix stability. In addition, lack of any direct
interactions between fibroblast cell surface proteins and versican C-terminal constructs
lead us to believe that versican may exert its effects on the cells either as a structural
molecule in the matrix through interacting with its binding partners, or through
biochemical properties of its glycosaminoglycan chains. In this context, it is interesting
that versican exists in four isoforms that differ principally in the number of covalently
bound GAG chains. This reflects different biological functions for these splice variants,
possibly through influencing charge densities in the extracellular matrix. The biological
significance of GAG density and the resulting charge density that may influence versican
interaction with various elements in the wound healing matrix is unknown, but worthy of
further investigation.
2.5 References


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3. FIBROBLAST CELL MORPHOLOGY IN 3-DIMENSIONAL COLLAGEN-VERSICAN-HYALURONAN MATRIX: A MODEL TO STUDY MYOFIBROBLAST CELL BEHAVIOR

3.1 Introduction

The wound healing process is classically divided into four phases which include homeostasis, inflammation, proliferation and remodeling (reviewed in1,2). Once inflammation is turned off, the process of epithelialization and matrix formation is initiated by proliferating fibroblasts. Fibroblasts migrate into the former clot and lay down a provisional matrix rich in proteoglycan versican, glycosaminoglycan hyaluronan, fibronectin, tenascin, and a number of other proteins. PDGF and activated TGF-β, along with other granulation tissue components, signal differentiation of fibroblasts into contractile myofibroblasts which are the main cell type responsible for wound closure. In the final remodeling phase of wound healing, fibroblasts express high levels of type-I collagen, and an organized collagenous matrix replaces proteoglycan and fibronectin. As the provisional matrix is degraded, myofibroblasts go into apoptosis and the anatomy and function of the tissue is restored. However, repeated insult and the ensuing inflammation result in prolonged myofibroblast proliferation and excessive deposition of granulation tissue at the site of injury. The accumulation of non-functional and excessive scar tissue, or fibrosis, is associated with many clinical problems such as keloid or hypertrophic scar formation in the skin, delayed nervous system regeneration, lung and liver dysfunction, and atherosclerosis3,4.

The tensile forces that develop within the wound matrix during repair are normally relieved through a combination of biosynthetic activity and wound contraction. Fibroblasts in the granulation tissue are responsible for both biosynthesis of the new connective tissue matrix, and contraction of the matrix4-6 by differentiating into myofibroblasts7,8 which contain actin stress fibers and α-smooth muscle actin9. Versican is the main proteoglycan whose expression is upregulated by fibroblasts and

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2 A version of this chapter will be submitted for publication. Pourmalek, S, and Roberts, C. Fibroblast cell morphology in 3-dimensional collagen-versican-hyaluronan matrix.
myofibroblasts at the site of injury. In the fibrotic lung, the early accumulation of versican occurs in association with proliferating and contractile myofibroblasts, prior to deposition of collagenous matrix in later stages of the disease\textsuperscript{10, 11}. In atherosclerotic lesions (reviewed in\textsuperscript{12}), versican is found in association with proliferating vascular smooth muscle cells\textsuperscript{13}. The spatial and temporal association of large splice-variants of versican, namely V0 and V1, with proliferating and contracting myofibroblasts (as defined by \(\alpha\)-SMA and collagen type-1 expression) has also been observed in all fibrotic lung diseases\textsuperscript{10}. Studies suggest that growth factors that regulate smooth muscle cell type proliferation and migration, such as TGF-\(\beta\)\textsuperscript{14} and PDGF\textsuperscript{15}, are also involved in upregulating versican\textsuperscript{15, 16} and hyaluronan\textsuperscript{17, 18} expression, and formation of versican-hyaluronan aggregates\textsuperscript{19}.

Versican is a member of hyalectan family of proteoglycans\textsuperscript{20, 21} which interact with hyaluronan through their highly homologous globular N-terminus\textsuperscript{22}. Glycosaminoglycan chains, bound to the core of the molecule\textsuperscript{23-25}, are responsible for many of the structural and functional properties of these proteoglycans. Two distinct glycosaminoglycan binding domains, GAG-\(\alpha\) and GAG-\(\beta\) are unique to versican and bind 10-30 chondroitin sulfate chains. Splicing of this region also gives rise to 4 different isoforms of versican that are differentially expressed in different tissues. Studies to date suggest that different versican splice variants namely: V0 (containing GAG-\(\alpha\) and GAG-\(\beta\)), V1 (containing GAG-\(\beta\)), V2 (GAG-\(\alpha\)), and V3\textsuperscript{26, 27} (devoid of both glycosaminoglycan binding regions), exhibit different functions in development and regeneration. In comparison, aggrecan which is found as a structural component in cartilage, contains the most GAG of approximately 100 chondroitin sulfate chains. C-terminal (G3) globular domain of lecticans, like the N-terminal domain, is also highly conserved. C-terminal domain of versican consists of two EGF repeats, a C-type lectin domain and complement regulatory protein (CRP)-like domain\textsuperscript{28}. The complex structure of versican has lead to the discovery of multiple functions for this molecule in different tissues\textsuperscript{29}, in development and disease\textsuperscript{30-36}.

As fibroblasts are normally embedded within a collagen-rich matrix \textit{in vivo}, the use of three dimensional collagen and fibrin matrices have become more popular as models best
resembling the granulation tissue matrix\textsuperscript{37}. Spatial signals in a 3D matrix can control cell morphology\textsuperscript{38} and gene expression, and research shows that transitioning between 2D and 3D matrices by mechanically flattening a 3D matrix\textsuperscript{39} or sandwiching cells between two 2D surfaces to mimic a 3D environment\textsuperscript{40} can induce such morphological changes, even though the same molecules and growth factors are present. Fibroblast cells allowed to interact with collagen matrices can penetrate into the substance of the matrix and become entangled with matrix fibrils\textsuperscript{41, 42}. Achieving tensional homeostasis has been suggested as the main reason\textsuperscript{43, 44} for the observed changes in signaling and migration pattern of the cells\textsuperscript{39, 45-47}, and the remodeling of the matrix\textsuperscript{5, 48-50} that follows. It is possible that in attached stiff collagen matrices, resistance of collagen fibrils to mechanical forces exerted by fibroblasts\textsuperscript{51} leads to increased intracellular tension, and differentiation of fibroblasts into $\alpha$-smooth muscle expressing myofibroblasts\textsuperscript{5}.

In this study, we tested the hypothesis that fibroblast cell morphology is altered in a versican-hyaluronan, three-dimensional collagenous matrix. We investigated the roles of versican and hyaluronan in relieving tension, and their effects on myofibroblast cell morphology and proliferation, in an \textit{in vitro} model of wound healing matrix. In order to investigate the influence of collagen gel matrix containing stimulating factors found in granulation tissue of healing wounds on the morphology of HFL1 human fetal lung fibroblast cells, we established a three-dimensional collagen-proteoglycan gel matrix system to study fibroblast cell behavior under 5 different conditions: buffer, hyaluronan, versican/hyaluronan, aggrecan/hyaluronan, and versican G3/hyaluronan, against a background of 3D collagen gel matrix. The experiment is illustrated in the schematic below (Figure 3.1).
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Figure 3.1 Illustration of 3D Collagen Experiment

In short, low passage fibroblast cells were seeded in collagen gels under each of the five different conditions. Gels were submerged in serum free media, as I found in preliminary experiments that fetal calf serum contains versican fragments. Fetal calf serum also contains growth factors such as PDGF and TGFβ-1 that can stimulate versican expression. Fluorescent staining and immunofluorescent staining was used to study changes in cell and nuclear morphology, actin expression, and α-smooth muscle actin expression. Our study shows that fibroblast cell morphology is affected by the presence
of granulation tissue components, versican and hyaluronan, and that versican is capable of inducing $\alpha$-smooth muscle actin expression and fibroblast differentiation into a myofibroblast phenotype.

3.2 Materials and Methods

Expression and Purification of Versican – Human fetal lung (HFL1) fibroblast cells American Type Culture Collection (Manassas, VA) were cultured in 75-cm$^2$ flasks (Sarstedt; Quebec, Canada) in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT) to 80% confluence. Cells from two confluent 75-cm$^2$ flasks were trypsinized and transferred to a 850-cm$^2$ tissue culture roller bottle (Becton Dickinson) with 200ml of DMEM and incubated at 37°C in a BELCO Biotechnology Roll-in incubator. Serum free conditioned medium from fibroblast cultures (CM) was collected and centrifuged at 1500 X g for 15 minutes to remove cellular debris. Then, Urea and salt concentrations in HFL1 conditioned media were adjusted to 7M Urea and 0.4M NaCl and loaded onto Q-Sepharose Fast Flow ion exchange resin (Amersham Biosciences, Piscataway, NJ) at approximately 1 litre CM per 5 mls resin. The column was washed with a 10 fold bed volume of 7M Urea, 0.4M NaCl, 0.1M NaOAc, pH 6.0 before elution with 7M Urea, 1.5M NaCl, 0.1M NaOAc, pH 6.0. Peak fractions were pooled and dialyzed against PBS (140mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, 1.8mM NaH$_2$PO$_4$, pH7.4) exhaustively, then flash frozen with liquid nitrogen and stored at -70°C. Fractions were monitored for versican content by alcian blue (Sigma, St. Louis, MO) staining of SDS-PAGE gels$^{52}$ and by Western blotting. Purified versican concentration was estimated using the dimethylmethylen blue (DMMB, Serva, Heidelberg) assay$^{53}$ to quantify sulfated glycosaminoglycan using known concentrations of chondroitin sulfate C as standards (Seikagaku). The concentration of versican was estimated based on an average of 1.5 mg total proteoglycan per 1 mg sulfated glycosaminoglycan detected with an estimated concentration of 1.12 mg/ml or approximately 1.12 $\mu$M versican.
Generation of Recombinant Constructs (HisL, HisLC, HisG3) – Before ligating the His-tagged C-terminal construct cDNA to the expression vector pGYMXC, the PCR product was amplified using the pPCR-Script strategy. Briefly, cDNA amplified from PCR was purified using a QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario). The DNA fragment was ligated into the pPCR-Script Cam SK(+) plasmid (Stratagene, CA, USA). The ligation mixture contained 1µl of 10X reaction buffer, 1µl of Srf I restriction enzyme (5U), 1µl of T4DNA ligase (4U), 0.5µl of 10mM rATP, 3µl of the HisLC insert (190ng), and 1µl of the cloning vector (10ng) for a 100:1 molar ratio of insert to cloning vector. The mixture was diluted to 10 µl with ddH2O, gently mixed, and incubated at room temperature for 1 hr before heating at 65°C for 10min. Two µl of the ligation mixture was used for heat shock transformation of supercompetent E. coli strain DH5α. The bacteria was transferred to 50µl of 2X YT media (1.0% w/v yeast extract, 1.6%w/v tryptone, 0.5% w/v NaCl, pH 7.5) and agitated at 275rpm for 30min at 37°C before plating on LB agar plates containing 30µg/ml chloramphenicol. Plates were incubated for 16hrs at 37°C. Colonies were selected and grown in 5ml of terrific broth media (1.2% w/v tryptone, 2.4% w/v yeast extract, 0.231% w/v KH2PO4, 1.254% w/v K2HPO4, 0.4% glycerol) with 30µg/ml chloramphenicol at 37°C for 16hrs at 275rpm. The plasmid was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario). The insert was removed from the CAM SK(+) plasmid by Hind III and NheI restriction enzyme digestion and purified by QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario). The insert was ligated into the expression vector pGYMX using the HindIII and Nhe I sites. The ligation reaction mixture contained 2µl of 10X reaction buffer, 1µl T4 DNA ligase, 2 µl 10mM ATP, 2µl of the pGYMX expression vector (15ng), a 1:1 weight ratio of insert to cloning vector and diluted to 20µl with ddH2O. This was heated at 60°C for 10min, placed on ice for 30min, and incubated at 16°C for 16hrs. Two µl of the ligation mixture was used for heat shock transformation of supercompetent E.coli strain DH5α following the above protocol before plating on LB agar plates containing 100µg/ml ampicillin. Colonies were selected and grown in 5ml of terrific broth as described above. DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario). The identity of the new HisLC pGYMX construct was confirmed by Hind III and Nhe I restriction enzyme digestion and by DNA sequencing.
performed by Nucleic Acids-Protein Service (NAPS) at the University of British Columbia.

**Transformation, Expression and Purification of Engineered Versican C-terminal Domain Constructs (His-L, His-LC, and His-G3)**– The pGYMX His-G3 expression vector was transformed into E.coli BL21(DE3) competent cells at a ratio of 1µl of cDNA into 50µl BL21 Gold E.coli cells. Briefly, the mixture was iced for about an hour before a 90 seconds heat shock at 42˚C. The mixture was iced for 2 minutes, and incubated at 37˚C for 30 min in shaker with 50µl of 2XYT media. The cells were then plated on Luria–Bertani (LB) agar plates containing 100µg/ml ampicillin. 5ml of superbroth (0.8% w/v yeast extract, 1.0%w/v tryptone, 0.5% w/v NaCl, 0.1% glycerol, pH 7.5) and incubated in 37˚C shaker for 24 hours. Next, another plate of agar was inoculated with a single colony and incubated at 37˚C for 16 hrs at 275rpm. Aliquots of this log phase seed culture were used to inoculate 3.5L of superbroth in a1:1000 (v/v) ratio with 100µg/ml ampicillin and incubated at 37˚C for 24hrs at 275rpm. Collected cells were then washed with 500ml of NET buffer (100mM NaCl, 1mM EDTA, 20mM Tris-HCl, pH 8.0) then lysed in 250ml of lysis buffer (50mM NaCl, 1mM EDTA, 20mM Na₂HPO₄, 1mg/ml lysozyme, 1mM PMSF, pH 8.0) for 2hrs at 37˚C and 275rpm and sonicated with 5 sec bursts. The inclusion bodies were washed with 500ml of NET buffer then dissolved in a solubilization buffer (8M Urea, 10mM Tris-HCl, 100mM Na₂HPO₄, pH 8.0) for 16hrs at 4˚C. Dissolved inclusion bodies were centrifuged at 20000rpm for 1hr and purified using a 30ml Ni²⁺ charged chelating sepharose column (Amersham Pharmacia) equilibrated in column buffer (8M Urea, 0.5M NaCl, 20mM Na₂HPO₄, pH 7.4). The column was washed in succession with 10-fold bed volume of column buffer, column buffer with 1M NaCl, column buffer with 1M NaCl, pH 6.0, and again with column buffer. Proteins with non-specific interactions to the Ni-chelate column were pre-eluted by a 10-fold bed volume of column buffer with 200mM imidazole. His-LC fusion protein was then eluted by a 200mM to 1M imidazole gradient over a 10-fold bed. Fractions were analyzed by SDS-PAGE and stored at –20˚C.

**Refolding of HisG3 Recombinant Protein** – Peak fractions obtained from the imidazole gradient were pooled and diluted 20-fold before dialysis in equal volume of
refolding buffer (18.2mM Na$_2$CO$_3$, 24mM NaHCO$_3$, 125mM NaCl, 1:10 ration of 3mM Cysteine/Cystine, pH 10.0) with aeration at room temperature. Refolding buffer was changed every 2 hrs for 8 hrs before exhaustive dialysis with refolding buffer minus the redox pair of Cysteine/Cystine for complete removal of urea. Because the pooled fractions were diluted 20 fold before refolding, a 10ml Ni-chelate column equilibrated in 18.2mM Na$_2$CO$_3$, 24mM NaHCO$_3$, 125mM NaCl, pH 10.0 was used to concentrate the diluted pool. His-LC was eluted with 18.2mM Na$_2$CO$_3$, 24mM NaHCO$_3$, 125mM NaCl, 500mM imidazole, pH 10.0. Peak fractions were pooled and dialyzed against excess volume of 18.2mM Na$_2$CO$_3$, 24mM NaHCO$_3$, 125mM NaCl, pH 10.0. The protein pool was aliquoted into 1ml fractions, flash frozen with liquid nitrogen, and stored at –70°C.

**Electrophoretic Techniques** – Samples in nonreducing 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 12.5% (separating) acrylamide. Stacking and separating gels were kept during staining and 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: 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 2.5% (w/v) bovine serum albumin, 20 mM Tris, 5 mM EDTA, 0.9%NaCl, and 0.3% (v/v) Tween 20. RP57 rabbit polyclonal antibody (British Biotech, 1mg/ml), raised against a peptide corresponding to the structural z binding S-loop in all MMP catalytic domain, was used to detect general MMP expression, 1:250 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-rabbit horseradish peroxidase-conjugate (Bio-Rad) secondary antibody was diluted to 1:2000. 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)
3D Collagen Cell Culture System – Human fetal lung fibroblasts (HFL1) 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) in cell culture flasks. Once cells were at 70% confluence, they were trypsinized, rinsed with serum-free media, and resuspended in PBS and kept on ice. Collagen solution was prepared following the manufacturer’s instructions for 3D Collagen Cell Culture System (Chemicon International, ECM675). Collagen solution was then mixed with either: PBS; PBS and 20µg/ml HA; 0.55mg/ml aggrecan and 20µg/ml HA; 0.50 mg/ml versican and 20µg/ml HA; or 0.53 mg/ml HisG3 and 20µg/ml HA. HFL cells, suspended in PBS in the previous step, were counted and mixed with this solution at a final concentration of 4x10^4 cells/ml. 20µl droplets of each solution were placed in the center of 8well chamber slides (Lab-Tek, Electron Microscopy Sciences, Hatfield, PA, USA) and incubated at 37ºC for up to 4 hours until the gels polymerized. Flipping plate upside down kept cells from attaching to the slide, ensuring their suspension in a 3D environment. Once gels polymerized, serum-free media was added to the cells and they were allowed to spread in the 37ºC incubator for either 12 hours, 24 hours, 48 hours, or 72 hours time points.

Cytochemistry – At the end of each time point, cells were fixed in 4% paraformaldehyde in PBS for 15 minutes in 37ºC incubator. In the first run, monoclonal antibody to α-smooth muscle actin (Clone 1A4, Sigma ImmunoChemicals, St. Louis, MO, USA) was used to establish the contractile physiology of the fibroblast cells, 1:400 dilution; α-mouse AlexaFluor-594, 1:2000. In the second run, Mitochondria were stained with 333nM MitoTracker Red CMXRos (Invitrogen M-7512, Life Technologies) for 20 minutes before being fixed, to follow apoptotic behavior. In live cells, MitoTracker dye accumulates in the Mitochondria and due to its reactivity with the thiol groups of peptides and proteins, it is retained within the mitochondria during the fixing and permeabilization process. As a result, cells with compromised mitochondria such as those going into apoptosis will exhibit less fluorescent mitochondria under the microscope. Fixed cells were permeabilized with 0.1% Triton X-100 and PBS for 1 minute and washed with PBS before being blocked by freshly prepared 1% bovine serum albumin (BSA) solution in
PBS for 5 minutes. Cells were then incubated in Alexafluor-488 Phalloidin (Molecular Probes, Eugene, Oregon, USA) at 1:1000 dilution in 1% BSA/PBS for 30 minutes. After being washed with PBS, cells were incubated in Hoechst stain (33342, Invitrogen) at 1:2000 dilution in 1% BSA/PBS for 10 minutes. Cells were then washed with PBS and stored with ProLong Gold antifade reagent (Invitrogen, Molecular Probes) on a covered slide.

Fluorescent Light Microscopy – Fixed and stained cells were viewed using a Leica CTR fluorescent light microscope. Images were captured at 40X magnification, in layers of 0.75µm thickness using the multi-channel z-stack capture option using Q-Imagine Retiga Exi mounted camera, on monochrome setting, and OpenLab software (4.0.2). Emission wavelengths were set to best view each stained organelle: actin filaments were viewed at 520nm; nuclei at 456nm (DAPI), and mitochondria at 594nm (Texas Red). Once images were captured, each image was deconvoluted using the Nearest Neighbor DCI function to remove unfocussed light. All layered images taken of the same coordinates but at different points along the z-axis (height) were merged to create one 3-dimentional image of cells spread in the collagen matrix. The appropriate color of the stain was then applied to the image, and images captured under different emission wavelength of different organelles were merged into one composite.

3.3 Results

Collagen solution was prepared following the manufacturer’s instructions for 3D Collagen Cell Culture (Chemicon International, ECM675) with a final collagen content of 80%. Based on the manufacturer’s fact sheet, ECM675 collagen solution is 99.9% pure native atelomeric avian collagen, with approximately 85% type I and 15% type III collagen protein. The Collagen solution was mixed with either: PBS; PBS and 20µg/ml HA; 0.55mg/ml aggrecan and 20µg/ml HA; 0.50 mg/ml versican and 20µg/ml HA; or 0.53 mg/ml HisG3 and 20µg/ml HA. HFL cells, suspended in PBS in the previous step, were counted and mixed with this solution at a final concentration of 4x10^4 cells/ml. 20µl droplets of each solution were placed in the center of 8-well chamber slides (Lab-Tek, Electron Microscopy Sciences, Hatfield, PA, USA) and incubated at 37ºC for up to
4 hours until the gels polymerized. The collagen gels containing fibroblast cells were subsequently submerged in serum-free media, and fixed at identified time points in preparation for staining and microscopy. The results presented here are representative of at least 3 different trials, with each trial containing at least three different replicas (excluding some of the replicas that did not polymerize properly, or were lost during the preparation process for fluorescence microscopy).

3.3.1 Physical properties and polymerization rate of collagen I matrix is influenced by versican and hyaluronan

According to manufacturer’s instructions, the polymerization of collagen should initiate after 60 minutes once gels are transferred to a 37°C incubator. Our observation was that while 60 minutes was enough for PBS and hyaluronan containing droplets to polymerize, longer incubation periods were necessary to achieve polymerization in versican and aggrecan containing gels (3-4 hours). These proteoglycans inhibited collagen self-assembly. We found that versican and aggrecan containing gels were especially prone to release from the slide and floating in the media during media changes and preparation for fluorescent microscopy, while PBS containing gels were tightly attached to the bottom of the plate. These results are not surprising as negatively charged hyaluronan, and the sulfated glycosaminoglycan chains of aggrecan and versican are known to attract and retain water molecules. This lengthened the polymerization period and appeared to give the matrix altered hydrodynamic properties.

3.3.2 Fibroblast cell morphology is affected by the composition of pericellular matrix

Fibroblast (HFL1) cells were grown in a three dimensional collagen matrix (Figure 3.2, 3.3) droplet containing either: phosphate buffered saline (PBS) as control; hyaluronan; versican and hyaluronan; or aggrecan and hyaluronan. Cells were incubated for 12, 24, 48 or 72 hours in serum free DMEM media, then fixed and stained with Alexafluor 488 Phalloidin (green, actin filaments), Hoechst stain (blue, DNA) and α-smooth muscle actin antibody (red), in preparation for cell microscopy.
Fibroblasts grown in 3D collagen matrix, with phosphate buffer saline added as control at 10% of total volume, spread out at a much slower rate (at 24h-48h) than cells in the presence of hyaluronan and versican (12h). In addition, these cells did not achieve a contractile myofibroblast morphology, with cellular projections into the matrix and cytoskeletal organization of actin fibers, even at longer incubation periods.

In contrast, fibroblast cells in collagen gels containing hyaluronan spread rapidly (12h), form long extensions into the matrix, and have a highly organized cytoskeleton, characteristic of migrating and proliferating fibroblasts. Fibroblast cells in the presence of hyaluronan alone, however, seemed to express lower amounts of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA, red immunostain) than those grown in the presence of versican and hyaluronan.

Fibroblast cells grown in versican/hyaluronan containing gels showed extended morphology and expressed high levels of \( \alpha \)-SMA (red immunostain), characteristics of myofibroblasts. After 48h of incubation, cells in the presence of versican showed apoptotic characteristics of cell and nucleus rounding, and compartmentalization of cell content.

Fibroblasts grown in the presence of aggrecan and hyaluronan showed the most retracted morphology from the start of incubation, and throughout the incubation period. Cells in the presence of aggrecan did not spread, and were round and detached from the collagen matrix. Only with longer incubation periods a slightly more extended morphology was observed.

Cells under all four conditions showed apoptotic characteristics of cell shrinkage and chromatin condensation at 72 hours incubation period. In these experiments, it appeared that fibroblasts grown in the presence of versican show apoptotic changes at a shorter incubation period (48 hours) than cells grown under the other three conditions. Nuclear fragmentation was also clearly visible in collagen matrices containing versican.
Figure 3.2 HFL-1 Cell Morphology in 3D Collagen Matrices Under 20X Magnification

The morphology of human fetal lung fibroblasts were examined in 3D collagen matrix droplets under five different conditions, with the matrix containing either: phosphate buffer as control (PBS); 0.5 μg/ml hyaluronan (HA); 25 μg/ml versican and 0.5 μg/ml hyaluronan (VC); or 25 μg/ml aggrecan and 0.5 μg/ml hyaluronan (AGG). The cells were incubated at 37°C in DMEM media without serum, and fixed at 12, 24, 48 or 72 hours. Cells were stained with Hoechst (nuclei, blue), Phalloidin (Actin, green) and an antibody to α-smooth muscle actin (red) for analysis of cell morphology. Yellow is observed in these merged images from the overlap of green and red stains. Bar = 50 μm.
Figure 3.3 HFL-1 Cell Morphology in 3D Collagen Matrices Under 63X Magnification

The morphology of human fetal lung fibroblasts were examined in 3D collagen matrix droplets under five different conditions, with the matrix containing either: phosphate buffer as control (PBS); 0.5μg/ml hyaluronan (HA); 25μg/ml versican and 0.5μg/ml hyaluronan (VC); or 25μg/ml aggrecan and 0.5μg/ml hyaluronan (AGG). The cells were incubated at 37°C in DMEM media without serum, and fixed at 12, 24, 48 or 72 hours. Cells were stained with Hoechst (nuclei, blue), Phalloidin (Actin, green) and an antibody to α-smooth muscle actin (red) for analysis of cell morphology. Yellow is observed in these merged images from the overlap of green and red stains. Bar = 15 μm.
3.3.3 Fibroblast cell apoptosis at 72 hours of incubation is independent of collagen matrix composition

Fibroblast (HFL1) cells were grown in a three dimensional collagen matrix droplet (Figure 3.4) containing either: phosphate buffer saline (PBS, control); hyaluronan; versican and hyaluronan; versican HisG3 construct and hyaluronan; or aggrecan and hyaluronan. Cells were incubated for 24, 48 or 72 hours in serum free DMEM media, then fixed and stained with Alexafluor 488 Phalloidin (green, actin filaments), Hoechst stain (blue, DNA) and Mitotracker dye CMX-Ros (red, mitochondrion), in preparation for cell microscopy.

Similar to the previous set of experiments (Figure 3.2, 3.3), cells in presence of PBS and aggrecan showed limited ability to spread out in the 3D collagen matrix, and were in a retracted state. However, cells in the presence of versican, hyaluronan, and G3 domain of versican spread out and form long extensions into their matrix. Cells in the presence of versican seemed to exhibit a more contractile phenotype and were less flattened compared to cells grown in the presence of hyaluronan or the G3 domain (24 hours). Fibroblasts in versican-rich matrix also contained higher number of active mitochondria spread throughout the cell cytoplasm (24h, 48h).

Fibroblasts grown under all conditions displayed apoptotic characteristics at 72 hours incubation. Cell shrinkage, chromatin condensation and fragmentation and formation of fragmented sacs containing cell content were visible in collagen matrices under all five conditions. Reduced fluorescence in the mitochondria, due to reduced uptake of MitoTraker Red dye, was also observed and could be interpreted as loss of mitochondrial membrane potential, a clear sign of the apoptotic state of a compromised cell.
Figure 3.4 Fibroblast cell apoptosis does not depend on the composition of 3D collagen matrices (40X Magnification)

The morphology of human fetal lung fibroblasts were examined in 3D collagen matrix droplets under five different conditions, with the matrix containing either: phosphate buffer as control (PBS); 0.5μg/ml hyaluronan (HA); 25μg/ml versican and 0.5μg/ml hyaluronan (VC); 25μg/ml HisG3 construct and 0.5μg/ml hyaluronan (G3); or 25μg/ml aggrecan and 0.5μg/ml hyaluronan (AGG). The cells were incubated at 37°C in DMEM media without serum, and fixed at 24 hours, 48 hours or 72 hours. Cells were stained with Hoechst (nuclei, blue), Phalloidin (Actin, green) and MitoTracker dye (Mitochondrion, red) for analysis of apoptotic behavior. Yellow is observed in these merged images from the overlap of green and red stains. Bar = 25 μm.
3.4 Discussion

In this study, we first assessed collagen matrix assembly in the presence of proteoglycan versican/aggrecan and glycosaminoglycan hyaluronan, through visual cues such as the rate of polymerization and resistance to separation from the slide. Our results point to versican and hyaluronan as factors in delayed collagen matrix assembly. We found that presence of versican and hyaluronan could delay collagen matrix polymerization and result in a weaker matrix, which is more prone to detachment from the slide and tearing during media changes. Indeed, it is well-known that proteoglycans and glycosaminoglycans of connective tissue matrix can influence fibrillogenesis and matrix architecture. Hyaluronan is an anionic polysaccharide that can bind to a large amount of water to form a viscous hydrated gel that gives connective tissue an ability to spread the pressure around and to resist compression loads\textsuperscript{57}. A recent study of the effects of HA on collagen fiber assembly showed that although the spontaneously packing of collagen monomers was not affected by HA after a longer incubation period (48 h), the diameter of collagen increased significantly at the initial incubating time and reached a constant diameter after 6 h of incubation. The collagen fibers were thinner in diameter upon addition of HA before 24 h incubation period (as compared with the pure collagen) and then increased to the same range of diameter after 48 h incubation\textsuperscript{57}. It is also possible that as an anionic polymer, HA could aggregate or adhere onto the positively charged regions of collagen and interfere with the interaction of collagen monomers and collagen fibrillogenesis. Moreover, an examination of collagen formation in reconstituted collagen matrices prepared from the PG/GAG components of interstitial mucosa which included hyaluronic acid and chondroitin sulfate, revealed that collagen fibrils had increased in density, but decreased in diameter and length, compared to those of matrices lacking the PG/GAG components\textsuperscript{58}. It was also reported that interstitial ECM polymerized in absence of the PG/GAG components appeared similar to matrices prepared with type I collagen alone\textsuperscript{58}. Increasing the concentrations of extracted PG/GAG components in the solution, however, resulted in a progressive increase in the nucleation of collagen fibril formation and a decrease in the rate of fibrillogenesis\textsuperscript{58}. In this regard, versican could be functioning to inhibit collagen accumulation in fibrosis.
Next, we tested the hypothesis that versican and hyaluronan alter the morphology of fibroblast cells in a three-dimensional collagenous matrix. We found that fibroblast cells grown in the presence of HA formed well organized actin stress fibers not observed in cells grown in collagen gels alone. In addition to stress fibers, cells grown in the presence of both versican and HA upregulate their α-SMA expression, and seem to spread long dendritic extensions into their matrix.

It is known that cells with prominent stress fibers are generally present during activated conditions such as wound repair and fibrosis. Studies of cells in attached stiff collagen matrices have suggested that resistance of collagen fibrils to mechanical forces exerted by fibroblasts leads to increased intracellular tension, and differentiation of fibroblasts into α-smooth muscle expressing myofibroblasts. Formation of stress fibers and large extensions also resembles the morphology of fibroblasts grown on a 2D culture plate, which are under continuous tensional stress from the surface of culture plates that resist deformation. Unlike the final product of cell contraction in 3D collagen matrices, which leads to a relaxed matrix, cells in 2D are continuously under pressure and never relax. When placed back into a 3D matrix, the cells from the culture plate re-acquire an elongated spindle-shaped phenotype. This activated myofibroblast phenotype is also observed with our fibroblast cell line which, when grown in planer culture dishes in the presence of serum, expresses large amounts of versican and proliferate until confluent. Based on all these observations, it seems that versican and hyaluronan are necessary components of the matrix in which activated myofibroblasts reside, as is observed with myofibroblasts in our 3D collagen matrix system.

By examining cell morphology in a matrix that contains hyaluronan and the G3 domain of versican, we also tested the hypothesis (discussed in Chapter 2) that versican exerts its effects on cells through its C-terminal EGF modules. It has been suggested that versican can stimulate proliferation in some cells through the interaction of its C-terminal EGF modules with the cell surface EGF receptors. We found that cells grown in the presence of G3 domain of versican and hyaluronan showed no difference in morphology to cells grown in the presence of hyaluronan alone. This observation lead us to believe that the whole versican molecule is necessary for versican to exert its influence on cells.
We also found that cells in the aggrecan-rich matrix have rounded retracted morphology and do not show any cellular extensions into the matrix. It is generally believed that the bulky glycosaminoglycan chains of chondroitin sulfate proteoglycans are anti-adhesive and that they sterically inhibit cell surface receptors, such as integrins, to interact with their ligands in the matrix, such as collagen. Considering one aggrecan molecule contains about 7-8 times more glycosaminoglycan chains than versican, the difference in the number of sugar chains bound to the core of the protein may contribute to the difference in cell morphology observed in versican- versus aggrecan-rich matrices. That the physical properties of the polyanionic glycosaminoglycan chains is the major determinant in the roles of these proteoglycans in the matrix, is further supported by the different distribution pattern for versican and aggrecan in vivo during development and disease. For example, the difference in versican and aggrecan localization during the development of central nervous system has been attributed to their partially complementary roles\textsuperscript{63}. In developing cartilage, versican is gradually replaced by aggrecan\textsuperscript{64} suggesting a role for versican as a temporary scaffold for the developing cartilage matrix\textsuperscript{65}. This signifies the role of versican and the contributions of its glycosaminoglycan chains on the physical properties of the matrix and the cellular response in wound healing matrix.

A number of mechanisms have been suggested for versican regulation of cellular proliferation\textsuperscript{66}. Versican can maintain the integrity of the ECM by interacting with hyaluronan\textsuperscript{22}. Further to maintaining the architecture and viscoelastic properties of tissues, HA modulates cell functions such as adhesion, migration and proliferation via interaction with specific cell surface receptors (such as CD44). Hyaluronan-CD44 signaling can lead to actin cytoskeleton reorganization\textsuperscript{67-69} and cell proliferation\textsuperscript{70} in tumor cells, and it has been suggested that the formation of versican-hyaluronan complex at the cell surface may facilitate the migration and proliferation of smooth muscle cells\textsuperscript{19,71}. We have also shown that the degradation of hyaluronan and release of versican from the pericellular matrix of fibroblast cells can lead to altered nuclear morphology and cell death (Chapter 2). Versican’s chondroitin sulfate chains can also interact with and localize a variety of growth factors\textsuperscript{72,73} and cytokines in the ECM, which can indirectly regulate cellular behavior\textsuperscript{74,75}. In this regard, proteoglycans are a matrix store of growth
factors and chemokines that can contribute to cell survival and proliferation.

Studies of self-assembling\textsuperscript{76} HA molecules have shown that HA, in association with aggregating CSPG, increases the viscosity of the matrix\textsuperscript{77, 78} and adds swelling pressure through the CS chains\textsuperscript{79, 80}. CSPG aggregation can potentially stiffen the HA network\textsuperscript{81} and influence cell behavior through mechanically coupled signaling\textsuperscript{82}. Other studies of fibroblast cell behavior in 3-dimentional (3D) collagen matrices, which resemble the \textit{in vivo} wound environment, provide convincing evidence that mechanical tension is a factor in transforming resting fibroblasts to contractile myofibroblasts that express $\alpha$-smooth muscle actin\textsuperscript{6, 8, 37}. Alpha-smooth muscle actin is increasingly seen as a “mechano-sensitive” protein that localizes to stress fibers in response to mechanical strain\textsuperscript{83}. In addition, versican’s ability to bind hyaluronan and form highly hydrated, supra-molecular aggregates\textsuperscript{84}, can contribute to swelling pressure and thus influence the mechanical properties of matrices such as blood vessel walls or remodeling tissue which are under pressure. This role for versican resembles the role of its family member, aggrecan, in resisting compressive forces put on cartilage. That is why we also compared the morphology of cells grown in aggrecan which, in comparison to versican, contains about 7-8 times more chondroitin sulfate chains. In the lungs, degradation of human airway smooth muscle-associated matrix is associated with decreased passive tension and alterations in smooth muscle contractility\textsuperscript{85}. This is consistent with the role of this matrix in modulating smooth muscle contractility by resisting compression. The large size (>1000 kDa) and hydration capability of versican, may also sterically hinder the interaction of integrins (large family of cell adhesion molecules) with their cell surface receptors\textsuperscript{86, 87}.

In our experiments with lung fibroblast cells grown in 3D collagen matrices, cells under all conditions showed apoptotic behavior at 48 hours of incubation. Most cells went into apoptosis at 72 hours of incubation. As these cells were grown in serum free media, the lack of growth factors and absence of other extracellular stimulants, pertinent to their survival, could have caused cells to go into apoptosis. We chose to use serum-free media in order to maintain the separation in matrix components under each condition. As fibroblasts are capable of expressing versican, and they do so particularly faster under the
influence of growth factors found in serum, starving cells from serum could maintain the integrity of matrix for longer periods. Also, we have found versican fragments in serum which could influence the results. Our experience with fibroblast cells grown on plastic culture plates has been that trypsinized fibroblast cells require fetal calf serum to establish a viable cell culture (data not shown). Once cells have attached to the bottom of culture plate and start proliferating, and once they reach a certain cell density, they can stay alive after serum starvation. In order to avoid this problem in the future, the media could be enriched with certain serum components, or cell morphology could be investigated in shorter time periods. More experiments are needed to confirm the effects of serum starvation on lung fibroblasts in our 3D collagen gel system.
3.5 References

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4. Versican Degradation by Macrophage Matrix Metalloproteinases MMP-7, MMP-12, and MMP-2

4.1 Introduction

Fibrosis, or abnormal wound healing, is marked by excessive deposition of provisional matrix by persisting myofibroblasts\(^1\). In many forms of human lung fibrosis, proliferating myofibroblasts alters the normal structure and function of the lung by crossing the basal membrane and depositing a provisional matrix in the alveolar spaces at the site of injury\(^2, 3\). We were first to identify versican, a large chondroitin sulfate proteoglycan, as an abundant yet transient component of this provisional matrix in many different types of fibrotic lung disease\(^4, 5\).

Versican is a member of hyalectan family of proteoglycans which interact with hyaluronan (HA) through their highly homologous globular N-terminus. Versican also interacts with a variety of molecules in the extracellular matrix (ECM) through its glycosaminoglycan (GAG) chains, bound to the core of the molecule. Versican’s globular C-terminal (G3) domain consists of two EGF repeats, a C-type lectin domain and complement regulatory protein (CRP)-like domain. The complex structure of versican\(^6\) has lead to the discovery of multiple functions for this molecule in different tissues, in development and disease.

Versican is distributed widely in adult human tissues\(^7-10\). In the fibrotic lungs, the early accumulation of versican occurs in association with proliferating myofibroblasts prior to deposition of collagenous matrix characteristic of the later stages of the disease\(^11, 12\). Versican may promote cellular proliferation through several mechanisms\(^13\). The N-terminal domain of versican is implicated in smooth muscle cell proliferation and migration through formation of a pericellular matrix, rich in versican and hyaluronan\(^14, 15\). Versican’s chondroitin sulfate chains interact with a variety of growth factors\(^16, 17\) and cytokines in the ECM, which can indirectly regulate cellular behavior\(^18, 19\). Versican

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\(^3\) A version of this chapter will be submitted for publication. Pourmalek, S, Overall, C, and Roberts, C. Versican degradation by macrophage matrix metalloproteinases MMP-7, MMP-12, and MMP-2.
C-terminal domain can also stabilize the matrix and cell-matrix interactions and influence cell morphology through disulfide bond formation with the G3 domain of other versican molecules \(^{20}\), and interaction with a number of other ligands found in the matrix of proliferating cells.

Due to the effects of versican on fibroblast cell proliferation and survival, and the role that versican synthesizing myofibroblasts play in wound healing, the significance of versican degradation and ECM remodeling in wound healing has been the subject of many studies. Matrix degrading metalloproteinases are a major group of matrix remodeling enzymes, implicated in the wound healing process\(^{21-24}\). The expression of several matrix metalloproteinases (MMPs), namely MMP-\(^7\)\(^{25}\)\(^{26}\) and MMP-12\(^{27}\) increases in pulmonary fibrotic diseases as a result of injury. In this study, we localize the expression of these enzymes to macrophages that accumulate at the site of versican-rich lesions in the later stages of pulmonary fibrotic diseases. We also present our original data on the sensitivity of versican to these macrophage metalloproteinase, and compare the cleavage pattern of versican by MMP-7, MMP-12 and MMP-2. The results of our study supports our hypothesis that macrophage metalloproteinases are involved in removal of the versican-rich fibroproliferative lesions by cleaving and degrading versican, removal of which is associated with fibroblast cell apoptosis.

### 4.2 Materials and Methods

**Patient Samples** – Lung tissues used in this study were obtained from the University of British Columbia St Paul's Hospital Pulmonary Research Laboratory tissue registry as previously described\(^4\). 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 (BOOP) or usual interstitial pneumonia (UIP). 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\(^4\). 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.
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\(^4\).

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\(^{28, 29}\) (Seikagaku, Tokyo, Japan), dilution 1:400; rabbit polyclonal anti-versican\(^30\) used as previously described\(^4, 5\), dilution 1:500; rhMMP-7 monoclonal antibody (MAB9071, R&D Systems, USA, 0.5mg/ml) dilution 1:50; hMMP-12 mouse monoclonal antibody (R&D systems, MN, USA), dilution 1:40 or 25µg/ml; MMP-2 antibody (a kind gift from Dr. Chris Overall; 79µg/ml), dilution 1:50. 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, CA, USA) and DAB (3,3'-diaminobenzidine) as substrate (Vector Laboratories, CA, USA) according to the manufacturers 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, OH, USA).

Expression and Purification of Versican – Human fetal lung (HFL1) fibroblast cells American Type Culture Collection (Manassas, VA) were cultured in 75-cm\(^2\) flasks (Sarstedt; Quebec, Canada) in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum
(FBS; HyClone, UT, USA) to 80% confluence. Cells from two confluent 75-cm\(^2\) flasks were trypsinized and transferred to a 850-cm\(^2\) tissue culture roller bottle (Becton Dickinson) with 200ml of DMEM and incubated at 37˚C in a BELCO Biotechnology Roll-in incubator. Serum free conditioned medium from fibroblast cultures (CM) was collected and centrifuged at 1500 \(\times\) g for 15 minutes to remove cellular debris. Then, Urea and salt concentrations in HFL1 conditioned media were adjusted to 7M Urea and 0.4M NaCl and loaded onto Q-Sepharose Fast Flow ion exchange resin (Amersham Biosciences, NJ, USA) at approximately 1 litre CM per 5 mls resin. The column was washed with a 10 fold bed volume of 7M Urea, 0.4M NaCl, 0.1M NaOAc, pH 6.0 before elution with 7M Urea, 1.5M NaCl, 0.1M NaOAc, pH 6.0. Peak fractions were pooled and dialyzed against PBS (140mM NaCl, 2.7mM KCl, 10mM Na\(_2\)HPO\(_4\), 1.8mM NaH\(_2\)PO\(_4\), pH7.4) exhaustively, then flash frozen with liquid nitrogen and stored at -70˚C. Fractions were monitored for versican content by alcian blue (Sigma, MO, USA) staining of SDS-PAGE gels\(^{31}\) and by Western blotting. Purified versican concentration was estimated using the dimethylmethylen blue (DMMB) assay (Serva, Heidelberg)\(^{32}\) to quantify sulfated glycosaminoglycan using known concentrations of chondroitin sulfate C as standards (Seikagaku, Japan). This is possible considering versican V0 and V1 are the primary mRNA splice variants expressed in proliferating HFL1 cultures. 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 \(\mu\)M versican.

Generation of Recombinant Constructs (HisL, HisLC, HisG3) – Before ligating the His-tagged C-terminal construct cDNA to the expression vector pGYMXC, the PCR product was amplified using the pPCR-Script strategy. Briefly, cDNA amplified from PCR was purified using a QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario). The DNA fragment was ligated into the pPCR-Script Cam SK(+) plasmid (Stratagene, CA, USA). The ligation mixture contained 1\(\mu\)l of 10X reaction buffer, 1\(\mu\)l of Srf I restriction enzyme (5U), 1\(\mu\)l of T4DNA ligase (4U), 0.5\(\mu\)l of 10mM rATP, 3\(\mu\)l of the HisLC insert (190ng), and 1\(\mu\)l of the cloning vector (10ng) for a 100:1 molar ratio of insert to cloning vector. The mixture was diluted to 10 \(\mu\)l with ddH\(_2\)O, gently mixed, and incubated at room temperature for 1 hr before heating at 65˚C for 10min. Two \(\mu\)l of the
ligation mixture was used for heat shock transformation of supercompetent E. coli strain DH5α. The bacteria was transferred to 50µl of 2X YT media (1.0% w/v yeast extract, 1.6% w/v tryptone, 0.5% w/v NaCl, pH 7.5) and agitated at 275rpm for 30min at 37°C before plating on LB agar plates containing 30µg/ml chloramphenicol. Plates were incubated for 16hrs at 37°C. Colonies were selected and grown in 5ml of terrific broth media (1.2% w/v tryptone, 2.4% w/v yeast extract, 0.231% w/v KH₂PO₄, 1.254% w/v K₂HPO₄, 0.4% glycerol) with 30µg/ml chloramphenicol at 37°C for 16hrs at 275rpm. The plasmid was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario). The insert was removed from the CAM SK(+) plasmid by Hind III and NheI restriction enzyme digestion and purified by QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario). The identity of the new HisLC pGYMX construct was confirmed by Hind III and Nhe I restriction enzyme digestion and by DNA sequencing performed by Nucleic Acids-Protein Service (NAPS) at the University of British Columbia.

Transformation, Expression and Purification of Engineered Versican C-terminal Domain Constructs (His-L, His-LC, and His-G3)– The pGYMX His-G3 expression vector was transformed into E.coli BL21(DE3) competent cells at a ratio of 1µl of cDNA into 50µl BL21 Gold E.coli cells. Briefly, the mixture was iced for about an hour before a 90 seconds heat shock at 42°C. The mixture was iced for 2 minutes, and incubated at 37°C for 30 min in shaker with 50µl of 2XYT media. The cells were then plated on Luria–Bertani (LB) agar plates containing 100µg/ml ampicillin. 5ml of superbroth (0.8% w/v yeast extract, 1.0% w/v tryptone, 0.5% w/v NaCl, 0.1% glycerol, pH 7.5) and incubated in 37°C shaker for 24 hours. Next, another plate of agar was inoculated with a single colony and incubated at 37°C for 16 hrs at 275rpm. Aliquots of this log phase seed culture were used to innoculate 3.5L of superbroth in a1:1000 (v/v) ratio with 100µg/ml ampicillin and incubated at 37°C for 24hrs at 275rpm. Collected cells were washed with 500ml of NET buffer (100mM NaCl, 1mM EDTA, 20mM Tris-HCl, pH 8.0) then lysed in 250ml of lysis buffer (50mM NaCl, 1mM EDTA, 20mM Na₂HPO₄, 1mg/ml lysozyme, 1mM PMSF, pH 8.0) for 2hrs at 37°C and 275rpm and sonicated with 5 sec bursts. The inclusion bodies were washed with 500ml of NET buffer then dissolved in a solubilization buffer (8M Urea, 10mM Tris-HCl, 100mM Na₂HPO₄, pH 8.0) for 16hrs at
4˚C. Dissolved inclusion bodies were centrifuged at 20000rpm for 1hr and purified using a 30ml Ni²⁺-charged chelating sepharose column (Amersham Pharmacia) equilibrated in column buffer (8M Urea, 0.5M NaCl, 20mM Na₂HPO₄, pH 7.4). The column was washed in succession with 10-fold bed volume of column buffer, column buffer with 1M NaCl, column buffer with 1M NaCl, pH 6.0, and again with column buffer. Proteins with non-specific interactions to the Ni-chelate column were pre-eluted by a 10-fold bed volume of column buffer with 200mM imidazole. His-LC fusion protein was then eluted by a 200mM to 1M imidazole gradient over a 10-fold bed. Fractions were analyzed by SDS-PAGE and stored at –20˚C.

**Refolding of HisG3 Recombinant Protein** – Peak fractions obtained from the imidazole gradient were pooled and diluted 20-fold before dialysis in equal volume of refolding buffer (18.2mM Na₂CO₃, 24mM NaHCO₃, 125mM NaCl, 1:10 ration of 3mM Cysteine/Cystine, pH 10.0) with aeration at room temperature. Refolding buffer was changed every 2 hrs for 8 hrs before exhaustive dialysis with refolding buffer minus the redox pair of Cysteine/Cystine for complete removal of urea. Because the pooled fractions were diluted 20 fold before refolding, a 10ml Ni-chelate column equilibrated in 18.2mM Na₂CO₃, 24mM NaHCO₃, 125mM NaCl, pH 10.0 was used to concentrate the diluted pool. His-LC was eluted with 18.2mM Na₂CO₃, 24mM NaHCO₃, 125mM NaCl, 500mM imidazole, pH 10.0. Peak fractions were pooled and dialyzed against excess volume of 18.2mM Na₂CO₃, 24mM NaHCO₃, 125mM NaCl, pH 10.0. The protein pool was aliquoted into 1ml fractions, flash frozen with liquid nitrogen, and stored at –70˚C.

**Electrophoretic Techniques** – Samples in nonreducing 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 12.5% (separating) acrylamide. Stacking and separating gels were kept during staining and 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: 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 2.5% (w/v) bovine serum albumin, 20 mM Tris, 5 mM EDTA, 0.9%NaCl, and 0.3% (v/v) Tween 20. Anti-versican 2B1 (Seikagaku Corporation, Tokyo, Japan), 1:500 dilution, was used for detection of Versican at an epitope near the C-terminal domain; and anti-G3 antibody (LC2, in-house antibody), 1:10,000 dilution, for detection of the C-terminal domain of Versican. 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 to 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, USA) or captured using the ChemiGenius-2 bio-imaging system and Gene Snap software (Perkin Elmer, Woodbridge, ON, Canada).

Versican and HisG3 Degradation with MMPs – Versican and HisG3 were incubated with selected MMPs (MMP-2 (kindly provided by Dr. Chris Overall, Faculty of Dentistry, University of British Columbia), MMP-7 (USB Corporation, Cleveland, Ohio), and MMP-12 (provided by Dr. Richard Dean, University of British Columbia) at equal substrate:enzyme ratio (1:0, 1:2.5, 1:5, 1:10, 1:20, 1:40, 1:80) diluted in the respective buffers for 24 hours at 37°C. Detection was by silver staining and Western blotting with appropriate versican antibodies. Enzyme digestion was stopped by the addition of EDTA for a final concentration of 10mM. HisG3, purified in our laboratories, was incubated with expressed and purified human MMP-7 at substrate:enzyme ratio of 1:20, at 37°C for 24h.

Sequencing of HisG3 fragment cleaved by MMP-7 – MMP-7 digested HisG3 fragments were first concentrated by Microcon YM-3 concentrators (Millipore, Ontario, Canada). Concentrated samples were then analyzed by 15% SDS-PAGE and transferred to PVDF membrane. The membrane was stained with 40% methanol, 1% Acetic Acid, and 0.1% R-250 for 15min, destained 3 times in 50% methanol, for 5min periods. The highly stained band at around 26kDa was sequenced by N-terminal sequencing at NAPS center at UBC (Vancouver, Canada).
4.3 Results

4.3.1 Macrophages surround versican-rich lesions 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 4.1). Staining of the matrix and the cells involved in this type of pulmonary fibrosis revealed fibroblast cell proliferation in areas of intensive versican staining (Fig 4.1, A). Alcian blue stain, typically used to stain glycosaminoglycans, also stained the intraluminal buds signaling the presence of a matrix rich in versican decorated with chondroitin sulfate chains (Fig 4.1, B and C). 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. Another pathological characteristic of BOOP was the accumulation of macrophage cells around the fibroproliferative lesions in the late stages of the disease (Fig 4.1, D).

4.3.2 Macrophages surrounding versican-rich lesions express high levels of MMPs

Macrophages surround versican-rich lesions in later stages of human pulmonary fibrosis and their accumulation is concomitant with versican degradation. An immunohistochemical examination of tissue from pulmonary fibrotic lung showed increased expression of several MMPs by macrophages around versican-rich lesions. Population of macrophage cells surrounding versican-rich lesions were higher in Fibrotic lung samples (Fig 4.1, F H J L) than Normal lung tissue (Fig 4.1, E G I K). The levels of MMP-7, MMP-12 and MMP-2 expression were also higher in Fibrotic lung compared to Normal lungs or the control with no antibody staining against matrix metalloproteinases (Fig 4.1, L). Strongest staining of these metalloproteinases is observed in association with the macrophage cells, which suggests a role in the changes that take place in the matrix subsequent to the arrival of macrophages.
Figure 4.1 MMP expressing macrophages surround fibroproliferative lesions in human IPF
Figures A-D examine the molecular environment of fibroproliferative lesions in the lung. Versican was stained with LeBaron antibody (A-red), glycosaminoglycans stained with alcian blue (B C-blue), α-smooth muscle actin immunostained with an antibody (C-red), collagen stained with Picosirius Red (B-red), macrophages identified with CD68 immunostaining (D-Purple). MMP expression by macrophages that surround versican-rich lesions were examined in figures E-L. Immunostaining of MMP-7 (E F), MMP-2 (G H) and MMP-12 (I J) in normal lung (center panels, E G I K) and fibrotic lung (right panels, F H J L) respectively, reveal increased MMP expression (brown) by macrophages in fibrotic lung. The controls (K L) with no primary antibody treatment for MMPs showed no brown staining associated with macrophage cells. Bar = 100 µm.
4.3.3 Versican degradation assays by macrophage metalloproteinases

The macrophage matrix metalloproteinases (MMPs), MMP-7 (matrilysin), MMP-12 (macrophage metalloelastase), and MMP-2 (gelatinase), were chosen as candidate macrophage enzymes that were localized to the resolving phase of wound healing in the lung. Considering versican slowly disappears in the final stages of wound healing and this phase is concurrent with macrophage co-localization around versican-rich lesions in a rat BOOP model of the lung (data not shown), sensitivity of versican to these enzymes were examined by silver staining and detected by western blotting. Versican was expressed by Human Lung Fibroblast (HFL1) cell culture, and purified using a Nickel column. The purified Versican was incubated with each of the chosen MMPs at 37°C at a range of ratios for 24h. We also examined the degradation pattern of the C-terminal construct of versican (G3 domain). Histidine tagged G3 was expressed by transfected E-coli cells and purified, and used in degradation assays with MMPs. 2B1 mouse monoclonal antibody, which recognizes an epitope in the C-terminal domain of versican\textsuperscript{29} was used in all cases.

4.3.4 MMP-7 degrades versican at multiple sites

The MMP-7 degradation of versican resulted in a drastic change in intact versican concentration with increase in enzyme concentration as detected by silver stain and western blot (Figure 4.2A and 4.2B respectively). High molecular weight intact versican molecules were trapped at the top of the stacking gel (Figure 4.2, Lane 1). With the introduction of a small amount of MMP-7 enzyme (1:80 enzyme : substrate), the large fragmented versican moved further down the stacking gel (Figure 4.2, Lane 2). As the concentration of MMP-7 enzyme increased, fragments of different sizes appeared sequentially, from larger than 120 kDa to about 20 kDa (Figure 4.2, Lanes 2-7). The degradation products were better observed in the western blot, recognized by the 2B1 mouse monoclonal antibody, than the silver-stained gel. However, both methods clearly illustrated the ability of MMP-7 enzyme to cleave versican at multiple sites.
Figure 4.2 Versican degradation pattern by MMP-7 enzyme
Versican (lane 1) purified from the media of human lung fibroblast cells are visibly degraded with increasing concentrations (1:80, 1:40, 1:20, 1:10, 1:5, 1:2.5) of enzyme to substrate using active MMP-7 enzyme (lanes 2-7 respectively). Note the appearance of higher molecular weight fragments in lane 2 and 3, and their subsequent degradation in lanes 4-7. (A)Western blot analysis using 2B1 versican antibody, and (B)Silver stained gel.
4.3.5 Recombinant human MMP-12 degrades C-terminal domain of versican

Our data showed, for the first time, the susceptibility of 2B1 epitope of versican (near the C-terminal domain) to degradation by macrophage metalloelastase (Figure 4.3A). From the western blot analysis of the degradation pattern, a rapid disappearance of versican antibody (2B1) signal was observed. From the silver stain, we inferred that high molecular weight versican is not prone to cleavage by the enzyme. Even though low molecular weight fragments appeared as the enzyme concentration increased (Figure 4.3B), there seemed to be little effect on the concentration of high molecular weight versican trapped at the top of the stacking gel.
Figure 4.3 Versican degradation pattern by MMP-12 enzyme
Versican (lane 1) purified from the media of human lung fibroblast cells are visibly degraded with increasing concentrations (1:80, 1:40, 1:20, 1:10, 1:5, 1:2.5) of enzyme to substrate using active MMP-12 enzyme (lanes 2-7 respectively). (A) Western blot analysis using 2B1 versican antibody, and (B) Silver staining. Note that the epitope near the C-terminal domain of versican that is detected by the 2B1 antibody degrades efficiently by MMP-12. The degradation pattern of versican as analyzed by the silver stain shows slower degradation of versican molecule as a whole. Low molecular weight fragments accumulate through lanes 5-7.
4.3.6 MMP-2 degradation of versican is limited

Our western blot and silver stain analysis (Figure 4.4A and 4.4B respectively) of versican degradation by recombinant MMP-2 showed a pattern that might be expected from selective degradation. There seemed to be limited cleavage of versican into smaller fragments that appeared with the lowest concentration of enzyme (Lane 2) when compared to the intact versican (Lane 1). Interestingly, as the enzyme concentration increased, the degradation pattern did not change. The same pattern was observed in silver stain (40kDa – stacking gel) and western blot (as a smear in the stacking gel) from lane 2-7.
Figure 4.4 Versican degradation pattern by MMP-2 enzyme
Versican (lane 1) purified from the media of human lung fibroblast cells are visibly degraded with increasing concentrations (1:80, 1:40, 1:20, 1:10, 1:5, 1:2.5) of enzyme to substrate using active MMP-2 enzyme (lanes 2-7 respectively). (A) Western blot analysis using 2B1 versican antibody, and (B) Silver staining. Note that both the western blot and silver stain analysis show limited and selective degradation of versican by MMP-2 enzyme.
4.3.7 Versican C-terminal (G3 domain) is cleaved by MMP-7

From the western blot degradation pattern of intact versican by MMP-7 (Figure 4.2 A), the presence of several degraded protein bands (lanes 4 – 7; 20 – 40 kDa) suggest that C-terminal domain of versican may be prone to proteolytic degradation by this enzyme. The degradation pattern of HisG3 construct of versican with MMP-7 on the PVDF membrane immunoblotted with anti-G3 antibody is shown (Figure 4.5). A small degraded fragment, approximately 26kDa in size, appeared with the introduction of smallest amount of enzyme (Figure 4.5, lanes 2-5). The cleavage site of this fragment was determined by N-terminal sequencing. The product was sequenced to the histidine 3204 – leucine 3205 site in the lectin domain of the C-terminal of versican. Our reported cleavage site is comparable to the cleavage sites reported for other substrates of this enzyme, with either leucine or isoleucine amino acids at the P1’ site\textsuperscript{34}. HisG3 monomers (~30kDa), dimers (~60kDa) and aggregates (at the top of the gel) started to disappear with higher concentrations of MMP-7 (Figure 4.5, lanes 4 and 5).
Figure 4.5 HisG3 is degraded by rhMMP-7 enzyme

HisG3 (lane 1) is visibly degraded by hMMP-7 with increasing concentration (1:40, 1:20, 1:10, 1:5) of enzyme to substrate (lanes 2-5, respectively). The band that appears towards the bottom of the gel with estimated molecular weight of 26 kDa was identified, by N-terminal sequencing, as the cleavage product at histidine 3204 - leucine 3205.
4.4 Discussion

The basic science of wound healing and fibrosis are covered in a number of recent reviews\textsuperscript{35-37}. The highly regulated process of normal wound healing is initiated at the site of injury by blood clotting and inflammation, followed by fibroblast migration and proliferation in a provisional matrix. The final stage of wound healing is characterized by matrix resolution and remodeling into a collagenous matrix, and by myofibroblast apoptosis. In the pathological process of fibrosis, excessive matrix deposition by proliferating myofibroblasts in the remodeling process leads to scarring and abnormal organ function.

The balance between ECM deposition and degradation is integral to a normal wound healing process. Versican is abundant in fibroproliferative lesions in the fibrotic lung, and is associated with proliferating myofibroblasts in the early stages of remodeling\textsuperscript{4, 5}. Versican promotion of cell proliferation in other matrices is well established\textsuperscript{13-15, 38}. Histochemical studies in our laboratory have shown that versican is a transient component of the provisional matrix\textsuperscript{4, 5}, and it is known that versican is absent from the collagenous matrix at later stages of the disease course\textsuperscript{39, 40}. The mechanism of removal of versican from the healing matrix, however, is unknown.

Immunohistochemical data presented in this study show that macrophages, identified by anti-CD-68 antibody staining, surround versican-rich fibroproliferative cores and even penetrate some of these lesions in the fibrotic lung (Figure 4.1). Our data also shows that expression of MMP-2, MMP-7, and MMP-12 is upregulated in the macrophages that accumulate at the site of lesions in the later stages of pulmonary fibrotic diseases (Figure 4.1), suggesting that a substrate for these proteinases lies within these lesions.

Matrix metalloproteinases are key regulators of all stages of wound healing including inflammation, re-epithelialization, and matrix remodeling\textsuperscript{41-43}, as it is the case in the lungs. It is shown that expression of MMP-7 and MMP-12 increases in asbestos-induced lung injury in mice and it is suggested that these metalloproteinases promote fibrosis through effects on inflammation\textsuperscript{26}. Also, matrilysin expression increases in airway epithelial cells and alveolar type II cells in cystic fibrosis and facilitates
re-epithelialization\textsuperscript{24}. We are the first, however, to show an increased expression of MMP-7, MMP-12, and MMP-2 in tissue repair. We also make a strong case for metalloproteinase involvement in proteolytic cleavage and degradation of versican (Figures 4.2-4.5) in the remodeling matrix.

It is known that matrilysin is constitutively expressed in the epithelium of peribronchial glands and conducting airways in normal lung. Up-regulation of matrilysin after injury, however, suggests a role for this metalloproteinase in injury-mediated responses of the lung. Matrilysin (MMP-7) expression increased in pulmonary fibrosis \textsuperscript{25}. Matrilysin knockout-mice were dramatically protected from pulmonary fibrosis in response to intratracheal bleomycin \textsuperscript{25}. Other studies suggest that versican may be a substrate for this enzyme. It has been shown that versican is present at the site of MMP-7 expression by lipid laden macrophages in atherosclerotic tissues\textsuperscript{44}. Versican has also been detected in the same areas as MMP-7 in actinic damage\textsuperscript{45}. Our biochemical data shows that versican is a substrate for macrophage metalloproteinases, MMP-7 (Figure 4.2), MMP-2 (Figure 4.3), and MMP-12 (Figure 4.4), and that the versican degradation pattern by each of these three metalloproteinases is unique. MMP-7 seems to cleave intact versican at multiple sites as seen in both the immunoblot (Figure 4.2A) and silver stained gel (Figure 4.2B). MMP-7 is known to be an effective enzyme in degrading the extracellular matrix, and due to lack of a hemopexin domain characteristic of other MMPs, it has multiple cleavage site activities. This seemingly indiscriminate degradation pattern points to a possible role for MMP-7 in clearing versican from the provisional matrix.

MMP-12 is upregulated in the airways of rats with allergic bronchial asthma, and is mainly expressed in airway epithelia and alveolar macrophages \textsuperscript{27}. Degradation of high molecular weight versican with MMP-12 resembles its degradation pattern by MMP-7 when only the immunoblots are compared (Figure 4.3A). A closer look at the silver stained gels (Figure 4.3B), however, leads one to postulate that MMP-12 activity is more focused on the G3-domain of versican. Considering the immunoblots are prepared by an antibody that recognizes a site near the C-terminal domain of versican\textsuperscript{29}, and that the large intact versican is still apparent at the top of the silverstained gel, it is possible that MMP-12 cleaves the 2B1 epitope near the G3 domain which not only reduces the
molecular weight of the intact versican slightly, as seen in the silver-stained gel, but also appears to completely degrade versican, as detected by the western blot.

Versican degradation pattern by MMP-2 is quite distinguishable from that of MMP-7 and MMP-12. Limited and what seems like selective cleavage of versican by MMP-2 is observed both by the immunoblot (Figure 4.4A) and the silver stained gel (Figure 4.4B). It is important to note that active site titration of MMP-2 with TIMP-2 has shown this enzyme to be fully active at the time of incubation with substrate (data not shown). MMP-7 and MMP-12 were already in their active form and did not require activation. Thus, the same number of active enzyme per substrate was used in all cases. The presence of high molecular weight intact versican at top of the stacking gel, and appearance of few low molecular weight bands with increasing enzyme concentration may point to a different role played by MMP-2, as compared to MMP-7 and MMP-12. MMP-2 may cleave versican to produce molecules that regulate cell behavior or to destabilize the matrix, though this hypothesis needs to be studied further.

From the results of versican degradation by macrophage enzymes MMP-7 and MMP-12, it is clear that versican C-terminal domain is susceptible to degradation by macrophage MMPs investigated in this study. The activity of MMP-7 on the C-terminal domain of versican was further investigated by N-terminal sequencing of one of the fragments produced from cleavage of HisG3 construct. The product corresponded to the histidine 3204-leucine 3205 site in the lectin domain of the C-terminal of versican, and the sequence was comparable to other cleavage sites reported for this enzyme with either leucine or isoleucine amino acids at the P1’ site. The significance of this specific cleavage is discussed below.

Versican degradation could result in destabilization of a hyaluronan- and versican-rich pericellular matrix, the structural integrity of which is required for proliferation of myofibroblast cells. Degradation of versican may also lead to release of growth factors bound to its chondroitin sulfate chains and perhaps leave them more vulnerable to cleavage by the pool of MMPs present at the site of repair. The degradation of G3 domain of versican by MMP-7 and MMP-12 is also physiologically significant
considering its roles in stabilizing cell-matrix interactions\textsuperscript{20} and modulating cell proliferation\textsuperscript{13,46}.

Our results are supported by other studies that point to the colocalization of MMPs with versican, a substrate for the enzymes, in other matrices. For example, the amount of immunoreactivity for versican, identified as a substrate for MMP-7, was increased in sun-damaged skin, and versican was detected in the same areas as MMP-7\textsuperscript{45}. In atherosclerotic lesions, matrilysin expressed by lipid-laden macrophages localized to areas of versican deposition and could cleave proteoglycan versican, \textit{in vitro}, possibly disrupting the structural integrity of the plaques\textsuperscript{44}. Versican isolated from rabbit lung is cleaved by purified MMP-2 in a limited fashion\textsuperscript{47}, comparable to the data that we have presented here using versican expressed by human lung fibroblasts. Together, these results make a strong case for the involvement of MMP-2, MMP-7, and MMP-12 in the breakdown of versican in later stages of wound healing process.

It has been suggested that reduced growth factor expression and increased matrix turnover could be responsible for myofibroblast cell apoptosis\textsuperscript{48}. It has been shown that MMP activity enhances myofibroblast apoptosis in lungs\textsuperscript{49}. We have uncovered the degradation pattern of versican by three matrix metalloproteinases expressed by macrophages surrounding the fibrotic lesions in human lung diseases. Although the association between cell apoptosis and degradation of versican is circumstantial, and does not imply causality, better understanding of the molecular mechanism of matrix resolution in pathological conditions such as fibrosis can lead to the development of new therapies for the treatment of these diseases.
4.5 References

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5. CONCLUSIONS AND FUTURE DIRECTIONS

This study centers around wound healing and fibrosis in the lungs. Replacement of normal lung architecture with collagenous matrix causes decreased lung air space volume and obstruction of gas exchange, which can result in considerable loss of lung function and ultimately respiratory failure and death. Persistence and magnitude of initial injury, multiple modes of tissue injury and slower than normal clot resolution are all possible causes that may lead to pulmonary fibrosis instead of normal wound healing. Whatever the cause, however, heavy proteoglycan deposition in association with proliferating myofibroblasts is central to persistence of active lesions in most fibrotic lung diseases.

My thesis is largely focused on the role of versican in the transient granulation tissue and subsequent matrix maturation and remodeling. Studies in our laboratory have shown a clear association between versican and proliferating and migrating contractile myofibroblasts in fibroproliferative lesions of fibrosis in the lungs. We now know that deposition of large amounts of versican and hyaluronan in association with active fibroproliferative lesions at the early remodeling stages is a constant feature of all of major fibrotic lung diseases. Macrophages accumulate around versican-rich fibroproliferative lesions in the later stages of wound healing, and have been implicated in the resolution of these lesions.

The main hypotheses tested in this thesis were:

1. Versican interacts with ligands on the surfaces of fibroblasts and macrophages (Chapter 2);
2. The interaction of versican with the cell surface promotes fibroblast cell proliferation (Chapter 3); and
3. Versican is removed from the versican-rich fibroproliferative lesions by the action of macrophage matrix metalloproteinases (Chapter 4).

The use of 3D collagen matrix, containing a versican-hyaluronan mixture, as an in vitro model of wound healing was also tested (Chapter 3).
5.1 Summary and Significance of Results

5.1.1 Generation of Versican C-terminal Constructs to Study Versican Interactions with the Cell Surface

In order to identify macrophage and fibroblast versican-binding cell surface ligands (Chapter 1), we expressed and purified C-terminal constructs of versican from transformed *E.coli* cells. Use of versican constructs simplifies the design of experiments and interpretation of data compared to the full-length proteoglycan. The negatively charged glycosaminoglycan chains prevent versican from penetrating SDS-gels, make it difficult to transfer onto a membrane for use in western blotting experiments, and render N-terminal sequencing and mass spectrometry ineffective. An added benefit to using constructs is that the interaction of versican’s ligands can be narrowed down to specific domains of the protein.

We used biotinylated constructs of the C-terminal domain of versican as baits in far-western blotting experiments with cell membrane fraction of fibroblasts and macrophages. We identified versican and versican fragments as the main ligands for HisG3 construct. Magnetic beads coated with versican's C-terminal construct and incubated with cultured fibroblast cells aggregated together and localized mainly to the surfaces of fibroblast cells, possibly through interacting with cell-surface versican C-terminal domain. Next, we examined whether versican is held at the fibroblast cell surface mainly through its interaction with cell surface hyaluronan. As hyaluronan was gradually degraded from the fibroblast cell surface with hyaluronidase, versican was released until almost no versican staining was observed at the cell surface. We also observed that the release of hyaluronan/versican complex from the cell surface resulted in morphological changes in the nuclei, and fibroblast cell death.

These results are consistent with versican-versican\(^1\) and versican-hyaluronan\(^2-5\) interactions at the cell surface found in other studies. Against a background of versican-versican and versican-hyaluronan interactions, with these cell types, we were unable to find evidence for other major receptor-ligand interactions. A shortcoming of using protein domain constructs in determining ligand-receptor interactions is the possibility that the construct is not properly folded into its natural tertiary structure. This
may be due to the absence of sequences in the full length protein that may assist in folding of the construct. In addition, new sequences which would be hidden in the full length protein may be revealed and may result in abnormal ligand-receptor interactions. Our findings, however, support previous research, and suggest that versican is held at fibroblast cell surface predominantly through its interactions with hyaluronan, and that formation of this pericellular matrix is essential for the maintenance of fibroblast cell phenotype.

5.1.2 Three-Dimensional Proteoglycan-Glycosaminoglycan-Collagen Gel Matrix as a Model of Wound Healing Matrix

Three dimensional (3D) collagen or fibrin matrices, containing cultured fibroblasts, have become popular as in vitro models of wound contraction. Lack of any direct interactions between fibroblast cell surface proteins and versican C-terminal constructs lead us to believe that versican may exert its effects on the cells either as a structural molecule in the matrix (through its interaction with hyaluronan and other matrix proteins), or based on the biochemical properties of its glycosaminoglycan chains. We investigated the biological significance of versican and its glycosaminoglycan chains on fibroblast cells in a three dimensional collagen matrix which contained hyaluronan and either versican or aggrecan.

We found that fibroblast cells grown in the presence of HA formed well-organized actin stress fibers and focal adhesions not observed in cells grown in collagen gels alone. In addition to stress fibers, cells grown in the presence of both versican and HA upregulate their α-SMA expression, and seem to have spread dendritic extensions in addition to prominent stress fibers and focal adhesions. It is known that cells with prominent stress fibers and focal adhesions are only present during activated conditions such as wound repair and fibrosis. We also found that cells in the aggrecan-rich matrix have rounded retracted morphology and do not show any cellular extensions into the matrix. Considering one aggrecan molecule contains about 7-8 times more glycosaminoglycan chains than versican, the difference in the number of polysaccharide chains bound to the core of the protein may contribute to the difference in cell morphology observed in versican- versus aggrecan-rich matrices. Other studies of
fibroblast cell behavior in fibronectin-collagen matrices confirm that the composition of the matrix is critical for regulating cell phenotype.

Several mechanisms have been suggested for the differentiation of fibroblasts to myofibroblasts. Combined effects of growth factors, such as TGF-β1 and PDGF, and mechanical tension, in particular, have been the focus of multiple reviews in recent years. Although it is unclear how mechanical stress and TGF-β1 signaling converge to promote increased α-SMA expression and myofibroblast differentiation, one possibility may be the expression of matrix proteins downstream of both growth factor and mechanical tension stimulation, namely versican and hyaluronan.

Based on our results, it seems that versican and hyaluronan are necessary components of the matrix in which activated myofibroblasts reside, as is observed with myofibroblasts in our 3D collagen matrix system. However, it is important to note that fibroblasts are not a homogeneous population and phenotypically diverse populations of fibroblasts can differ in growth rate and cytoskeletal arrangement. Also, migrating fibroblasts can change collagen concentrations and remodel the matrix throughout the experiment, which may explain the apoptotic behavior of our fibroblasts after 3 days of incubation.

5.1.3 Matrix Metalloproteinase Degradation of Versican

Once the process of wound contraction by differentiated myofibroblasts is completed, contractile myofibroblasts disappear from the scar through the process of apoptosis. Although it is well established that regression of granulation tissue occurs by apoptosis, factors and mechanisms that lead to myofibroblast apoptosis are not well understood. As myofibroblast apoptosis occurs concomitantly with granulation tissue degradation in vivo; and as granulation tissue proteoglycans, such as versican are essential for myofibroblast cell proliferation, the role of proteoglycan degradation in myofibroblast apoptosis were investigated.

Immunohistochemical data presented in this study show that macrophages, identified by anti-CD-68 antibody staining, surround versican-rich fibroproliferative cores and even penetrate some of these lesions in the fibrotic lung (Figure 4.1). Our data also shows that expression of MMP-2, MMP-7, and MMP-12 is upregulated in the macrophages that
accumulate at the site of lesions in the later stages of pulmonary fibrotic diseases (Figure 4.1), suggesting that a substrate for these proteinases lies within these lesions. Other studies also point to the colocalization of MMP-7 with versican in atherosclerotic lesions\textsuperscript{26} and sun-damaged skin\textsuperscript{27}.

MMP-7 seems to cleave intact versican at multiple sites, and this extensive degradation pattern points to a possible role for MMP-7 in clearing versican from the provisional matrix. Degradation of high molecular weight versican with MMP-12 resembles its degradation pattern by MMP-7, however, it seems MMP-12 activity is more focused on the G3-domain of versican. Versican degradation pattern by MMP-2 is quite distinguishable from that of MMP-7 and MMP-12. Limited cleavage of versican by MMP-2 was observed, although same number of active enzyme per substrate was used in all cases. This difference in degradation pattern may point to a different role played by MMP-2, as compared to MMP-7 and MMP-12. Limited proteolysis of versican by MMP-2 yields large fragments that might be expected to influence cell behavior or to destabilize the matrix, though this hypothesis needs to be studied further.

Versican degradation could result in destabilization of a hyaluronan- and versican-rich pericellular matrix, structural integrity of which is required for proliferation of myofibroblast cells\textsuperscript{2, 28-30}. Degradation of versican may also lead to release of growth factors bound to its chondroitin sulfate chains and perhaps leave them more vulnerable to cleavage by the pool of MMPs present at the site of repair. The degradation of G3 domain of versican by MMP-7 and MMP-12 is also physiologically significant considering its roles in stabilizing cell-matrix interactions\textsuperscript{1} and modulating cell proliferation\textsuperscript{31, 32}. It has been suggested that reduced growth factor expression and increased matrix turnover could be responsible for myofibroblast cell apoptosis\textsuperscript{33}. It has been shown that MMP activity enhances myofibroblast apoptosis in lungs\textsuperscript{34}. MMP substrate specificity and compartmentalization studies also support our versican degradation results. It has been suggested that MMP-7 is a more potent proteoglycanase than MMP-3 or MMP-9\textsuperscript{26}, and macrophage metalloelastase (MMP-12) is the most elastolytic enzyme of the MMP family\textsuperscript{35} capable of efficiently degrading fibronectin and chondroitin sulfate chains of proteoglycans\textsuperscript{36}. Also, MMP-2 binds to $\alpha_5\beta_3$ integrin\textsuperscript{37}.\textsuperscript{37}
whose expression is increased in human lung fibroblasts by TGF-β1, and MMP-7 binds to cell surface proteoglycans.

The following model is proposed:

i. In an effort to relieve tensile forces of the wound and in response to PDGF and TGF-β1, fibroblast cells up-regulate the expression of versican and hyaluronan among other granulation tissue components.

ii. Formation of versican-hyaluronan complexes at the cell surface increases local pressure on the cells and leads to myofibroblast contraction of the matrix.

iii. Versican provides a suitable environment for myofibroblast contraction, a necessary step in relief of external tension and return of the provisional matrix to a normal state.

iv. Macrophages which surround the versican-rich lesions in the final stage of wound healing release matrix metalloproteinases, such as MMP-7 and MMP-12, which degrade versican.

v. Fibroblast cells may ‘sense’ the drop in pressure in the matrix and go into apoptosis concomitant with versican removal from the matrix.

vi. This leaves a collagenous matrix which resembles the normal tissue architecture.

The following diagram is a schematic representation of our new hypothesis which incorporates our new findings into the current understanding of the mechanism of wound healing. Studies of the 3D models suggest that mechanical tension in anchored matrices leads to intracellular tension and formation of stress fibers as the fibroblast cells differentiate first into proto-myofibroblasts with organized stress fibers and then into myofibroblasts with α-SMA decorated stress fibers. Once the gels are contracted or released from their support (free-floating gels), cells go into a quiescent state and the process of apoptosis begins. In vivo, this drop in mechanical tension could be due to the degradation of versican by macrophage matrix metalloproteinases. The stiffness of the provisional matrix of wounds at different stages of wound healing has estimated through a number of different studies (reviewed in ). Tension in newly synthesized granulation
tissue is comparable with that of the newly polymerized collagen gels at 10-100 Pa\textsuperscript{44}. Gradual increase in tension induces the formation of stress fibers\textsuperscript{20,45} which for fibroblasts grown on soft two-dimensional culture substrates occurs at 3,000-6,000 Pa\textsuperscript{46,47}. The expression of α-SMA in stress fibers occurs at around 20,000 Pa as demonstrated for contractile wound granulation tissue and for myofibroblasts cultured on elastic substrates\textsuperscript{48}. Matrix stiffness of greater than 50,000 Pa\textsuperscript{48} has been measured for mature granulation tissue and other fibrotic tissues\textsuperscript{48}. 
Figure 5.1 Schematic of Wound Healing

- Resting Fibroblasts in Relaxed Matrix
- Apoptotic Myofibroblast in Collagenous Matrix
  - Versican is Degraded by MMPs
- Contractile Myofibroblast with high levels of intracellular stress fibers (green) and α-SMA (red)
  - [Merge: yellow]
- Proto-myofibroblast with low levels of intracellular stress fibers (green) and α-SMA (red)
- Matrix Pressure
  - 10-100 Pa
  - 3000-6000 Pa
  - 15,000-30,000 Pa

Key:
- Macrophage
- PDGF
- TGF-β
- Matrix Metalloproteinases (MMP)
- Fiber Under Remodeling
- Organized Collagen Fiber
- Hyaluronan (HA)
- Versican

- ↑ Collagen
- ↓ Versican
- ↓ HA
- ↑ TGF-β
- ↑ PDGF
- ↑ HA
- ↑ MMP-7
- ↑ MMP-12
- ↑ MMP-2
- ↓ Versican
- Matrix Pressure

- Slow Decrease in Pressure

5.2 Future Studies

Future studies in our laboratories could involve our newly established *in vitro* model of wound healing in which fibroblasts are grown in a 3-dimensional collagen-versican-hyaluronan matrix. Cell morphology could be videotaped on a live stream to evade the effects that fixing and permeabilizing cells could have on cell morphology, and also to observe cell contraction, migration, and proliferation. The stiffness of the matrix could also be measured at different time points. As experiments in this study were performed in serum free media and in the absence of any growth factors, the effects of different growth factors and serum could be studied on cells embedded in collagen matrix. In terms of investigating protein expression patterns by cells under different conditions, the changes in gene expression can be evaluated by comparing the mRNA content of cells grown in the presence or absence of versican. Genechip arrays for gene expression analysis are provided by a number of companies, including Affymetrix, which provides whole genome arrays, and SuperArray Biocsience, which produces focused microarrays. Another method for evaluating changes in gene expression is “stable isotope labeling with amino acids in cell culture” (SILAC)\(^\text{49}\). This method relies on the incorporation of amino acids that have stable isotopic nuclei, such as deuterium \(^2\text{H},\ 13\text{C},\ 15\text{N}\). The genes that show significant altered expression will then be evaluated for their physiological relevance to fibroblast and macrophage cell morphology in wound healing.
5.3 References


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