Characterizing the dual roles of versican in angiogenesis and wound repair

by

Weining Yang

Supervised by Dr. Albert Yee

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Graduate Department of the Institute of Medical Science
University of Toronto

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University of Toronto

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ABSTRACT

While appearing to be disparate biological processes, wound healing and tumor progression utilize many similar molecular mechanisms. In particular, versican, a chondroitin sulfate proteoglycan has been shown to be upregulated in both of these contexts. We explored versican as both a coding and non-coding gene, given its versatile role in the extracellular matrix. Firstly, an ectopic expression plasmid containing the coding sequence of the versican V2 splice isoform was stably transfected into a primary human glioblastoma cell line. It was shown that the V2 isoform, expressed predominantly in the mature brain could play a pro-angiogenic role in malignant glioblastomas. These results were confirmed by in vitro cell-based assays as well as in a nude mouse xenograft model. Secondly, we examined the role of versican in cutaneous wound healing by overexpressing the 3′-untranslated region of the coding transcript in murine fibroblasts and a transgenic C57BL/6 mouse model. By binding to endogenous miRNAs which downregulate versican expression, expressing 3′-UTR regions or using similar miR sponging techniques can be used to assess genetic regulatory networks as well as observe any functional roles for non-coding regions of RNA. Understanding the coding and noncoding roles of versican plays will be valuable in understanding the multifaceted roles that this proteoglycan plays in the extracellular matrix.
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<tr>
<td>3'UTR</td>
<td>3'-untranslated region</td>
</tr>
<tr>
<td>ceRNA</td>
<td>competing endogenous RNA</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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Chapter 1

LITERATURE REVIEW

1.1 Introduction

Although initiated and propagated by different mechanisms, it has been hypothesized that certain physiologic steps in wound healing and tumor progression share similar genetic profiles, specifically in the inflammatory response and activation of the extracellular matrix (Riss et al. 2006; Grose 2004; Chang et al. 2004; Byun and Gardner 2013)). In 1863, Rudolph Virchow, widely considered as the father of modern pathology, noted that malignant transformation occurred more often in tissues which had been previously injured. He suspected that the origin of cancer was at sites of chronic inflammation (Balkwill and Mantovani 2001). He supported this initial hypothesis through anecdotal evidence.

Over a century later, Harold Dvorak, most widely known for the purification and characterization of Vascular Endothelial Growth Factor (VEGF), published a seminal paper in the New England Journal of Medicine, describing a similar hypothesis. In this paper, he suspected that tumors resembled ‘wounds that do not heal’ (Dvorak 1986). Dvorak studied tumor stroma and healing wounds and hypothesized that the differences were related to the continuous deposition of the initial fibrin clotting response observed in wounds. In tumors, the high levels of vascular permeability lead to a continuous fibrin leak which induces the formation of new blood vasculature. Given this, Dvorak hypothesized that the solid tumor matrix represented wound matrix that failed to progress normally. He also noted that extracellular
matrix components including hyaluronic acid, chondroitin, and dermatan sulphate proteoglycans, which were present in the wound matrix, were in fact elevated in solid tumors.

It was later demonstrated that healing wounds and tumor stroma demonstrate a common pattern of altered proteoglycan staining, in particular among chondroitin sulphate proteoglycans (CSPGs) (Yeo, Brown, and Dvorak 1991), corresponding with the altered cell proliferation, cell migration and collagen synthesis that these two biological processes involve. Since these initial manuscripts were published by Dvorak, a number of studies supporting the idea of tumors as wounds that do not heal, have been published (Riss et al. 2006; Chang et al. 2004; Yeo, Brown, and Dvorak 1991). Understanding these similarities, as well as differences, may be useful in the development of treatment strategies against malignant diseases associated with either neoplastic transformation or wound healing.

Proteoglycans are central components of basement membranes and are commonly associated with cell surfaces and the extracellular matrix. Specifically, CSPGs consist of a glycoprotein core and a chondroitin sulfate glycosaminoglycan (GAG) side chain. To this date, nine CSPGs have been identified – Aggrecan, Versican, Neurocan, NG2, CSPG5, SMC3, Brevican, CD44 and Phosphacan. As one of the matrix components that are upregulated in both wound healing and tumour progression, my thesis focused on the role of versican in both of these biological contexts. Specifically, the role of the versican V2 splice isoform in tumor angiogenesis was examined, as well as the role of the versican 3'-untranslated region in wound healing. Beyond the traditional role of the versican protein product, we hypothesize that the VCAN gene could also play a non-coding function as a sponge for microRNAs, endogenous gene regulators in the extracellular matrix. We examined this idea using a mouse model of cutaneous wound healing.
1.2 Versican

Proteoglycans are a major component of the extracellular matrix (ECM) and consist of a protein core, which is heavily glycosylated by covalently attached glycosaminoglycan (GAG) chains. Versican, a chondroitin sulfate proteoglycan (CSPG), is one of the main components of the extracellular matrix and is considered to be crucial to several key cellular processes involved in development and disease (Westling et al. 2004). Unlike other CSPGs which show tissue specific localization however, versican is distributed widely in a variety of tissues in the human body, suggesting a functional versatility (Bode-Lesniewska et al. 1996). Thus there is a differential temporal and spatial expression of versican, depending on cell type, tissue type as well as developmental or pathological timeframe.

There are four isoforms of versican, denoted as V0, V1, V2, and V3. All of the isoforms contain both N-terminal G1 and C-terminal G3 globular domains, separated by different central Chondroitin Sulfate regions (CS) (Sandy et al. 2001). The CS region is encoded by two exons encoding the CSα and CSβ domains. Through alternative splicing, both the CSα and CSβ domains are present in versican V0, but versican V3 has no CS domain. Versican V1 and V2 have only one of the CSβ and CSα domains, respectively.

1.2.1 Structure

Within the human genome, versican is encoded by the VCAN gene located on chromosome 5q 12–14. Human versican is encoded by 15 exons spanning over 90–100 kb (Naso, Zimmermann, and Iozzo 1994). Typically, the N-terminal globular domain (G1 domain) of versican contains an immunoglobulin-like motif and two proteoglycan tandem repeats which bind hyaluronan (HA). The C-terminal globular domain (G3 domain) contain two EGF-like
repeats, a complement regulatory protein-like repeat and a C-type lectin domain (Wight and Merrilees 2004). Analysis by reverse transcription polymerase chain reaction, cDNA sequencing and northern blot has demonstrated that the structural diversity of versican originates from alternative splicing processes. Designated V0, V1, V2 and V3, the respective sizes of the four versican isoforms are 370 kDa, 263 kDa, 180 kDa, and 74 kDa (Zimmermann et al. 1994; Naso, Zimmermann, and Iozzo 1994). Each isoform contains different lengths of glycosaminoglycan (GAG) binding regions with an accompanying variation in the number of attached GAG chains. Specifically, these isoforms differ by the presence or absence of two GAG attachment regions, GAG-α and GAG-β (Ito et al. 1995). The V0 isoform contains both GAG-α and GAG-β, while V1 contains GAG-β, V2 contains GAG-α, and V3 contains no GAG attachment domains (Kenagy, Plaas, and Wight 2006).

The GAG attachment regions carry 5 and 23 chondroitin-/dermatan-sulfate (CS/DS) chains depending on the versican isoform. The isoforms also have variable chondroitin 6-sulfate to chondroitin 4-sulfate ratios (Wight 2002). It has been recognized that the number, length, and molecular structure of GAG chains may also be affected by the G1 and G3 domains. In particular, the attachment of GAG chains has been reported to be inhibited by the versican G1 domain and promoted by the G3 domain (Wu et al. 2005).

Versican regulates intracellular processes including cell adhesion, proliferation, apoptosis, migration and invasion via the chondroitin and dermatan sulfate side chains. In addition, the versican G1 and G3 domains can interact with various intracellular or extracellular molecules (Lebaron 1996). To date, a wide range of molecules have been reported to interact with versican through either the G1 or G3 domains, or the GAG attachment region (Wu et al. 2005). It is known that the association of the versican G1 domain with HA is mediated by link
protein (LP). Both HA and LP protein have the ability to bind to the G1 domain of versican (Matsumoto et al. 2003). In addition to HA, versican has been shown to associate with tenascin-R (Aspberg, Binkert, and Ruoslahti 1995), fibulin-1 and -2 (Aspberg et al. 1999), fibrillin-1 (Isogai et al. 2002), fibronectin (Yamagata et al. 1986), P- and L-selectin (Kawashima et al. 2000), and various chemokines. Versican also binds to cell surface proteins including epidermal growth factor receptor (EGFR) (Wu et al. 2005), CD44 (Kawashima et al. 2000), and integrin β1 (Y. Wu et al. 2002). Recently, versican has been shown to act on macrophages through toll-like receptors, TLR2 and TLR6, leading to the production of inflammatory cytokines, and the promotion of tumor cell metastasis (Kim et al. 2009).

1.2.2 Regulation of versican expression

Versican is encoded by 15 exons encompassing over 90-100 kb of continuous DNA, with considerable conservation of both exon and intron sequences (Naso, Zimmermann, and Iozzo 1994). Versican expression is regulated by a promoter region harboring a TATA box located approximately 16 base pairs upstream of the transcription start site. Among its various regulatory mechanisms, there are potential binding sites for transcription factors, including TCF-4, AP1 and CCAAT enhancer protein (Domenzain-Reyna et al. 2009). The versican gene can also be bound by the tumor suppressor p53 at its first intron causing direct activation in a dose-dependent manner (Naso, Zimmermann, and Iozzo 1994). The versican gene is a reported target of several signaling pathways, including the Wnt pathway (Willert et al. 2002), GSK-3 beta pathway (Rahmani, Carthy, and McManus 2012), and PI3K pathway (Theocharis 2008).

Versican expression is known to be regulated by a number of cytokines including TGF-β, PDGF, IL-1α and IL-1β. TGF-β has been found to up-regulate synthesis of versican in glioma,
osteosarcoma and fibrosarcoma cells (Arslan et al. 2007; Nikitovic et al. 2006; Serra et al. 2005). Additionally, TGFβ1 is one of the major regulators of versican expression within the tumor stroma (Arslan et al. 2007; Cross et al. 2005). The induction of versican expression by TGF-β in benign prostatic hyperplasia (BPH) stromal cells is complemented with the negative effects of TGF-β on a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) -1, -5, -9, and -15. When coupled with increases in the TGF-β inhibitor TIMP-3, versican accumulates in the stromal cells of the prostate during BPH and prostate cancer (Cross et al. 2005). TGFβ2 has also been shown to increase versican expression in normal lung fibroblasts, fibrosarcoma and osteosarcoma cells (Berdiaki et al. 2008). With regard to other cytokines, platelet-derived growth factor (PDGF) treatment has been shown to increase versican expression in arterial smooth muscle cells (SMC) and human gingival fibroblasts (Haase et al. 1998). Furthermore, versican expression is up-regulated in lung fibroblasts treated with IL-1β (Tufvesson and Westergren-Thorsson 2000) and down-regulated in vascular SMCs treated with IL-1α (Lemire et al. 1999). The expression of versican can be also modified by Epidermal Growth Factor (EGF), insulin-like growth factor I and PDGF-BB in malignant mesothelioma cells (Syrokou et al. 1999). In addition, steroid hormones and gonadotrophins also modulate versican expression (Russell et al. 2003). Androgen receptors regulate the versican gene through an androgen response element located in the promoter region (Read et al. 2007).

There is increased evidence that the accumulation of the cleavage products of versican play an important role in cancer progression. The regulation of the G1 and G3 domains of versican by proteases is known to be important in regulating cancer cell motility as well as metastasis. A number of proteinase families are capable of cleaving versican to generate its proteolytic fragments. For example, matrix metalloproteinase (MMP)-1 (Perides et al. 1995), -2
(Passi et al. 1999), -3 (Perides et al., 1995), -7 (Halpert et al. 1996), and -9 (Passi et al., 1999) have been shown to degrade native, purified versican in vitro. ADAMTS-1 (Sandy et al. 2001), -4 (Sandy et al., 2001), -5 (Cross et al., 2005), and -9 (Enomoto et al. 2010) have all been reported to cleave either native versican or versican peptide substrates.

1.3 microRNAs

MicroRNAs are small non-coding RNAs of 18-24 nucleotides in length which bind through partial or perfect complementarity to their target microRNA recognition elements (MREs), located typically on the 3′-untranslated region (UTR) of target mRNAs. In this manner, they guide translational repression through either mRNA degradation or repression. Originally believed to be non-functional or ‘junk’ RNA, microRNAs are now understood to be highly conserved sequence-specific regulators of many developmental, physiological, and pathological processes.

1.3.1 Biogenesis

MicroRNAs are single-stranded RNAs of 18-24 nucleotides in length. They are transcribed from different genomic locations by RNA polymerase II, producing primary microRNA transcripts that contain one or more local hairpin structures. Most primary microRNA transcripts are polyadenylated and capped at the 5′-end: features of transcription by RNA polymerase II. Interestingly, microRNAs encoded by C19MC, the largest human microRNA cluster, are transcribed by RNA polymerase III (Borchert, Lanier, and Davidson 2006). MicroRNA genes are typically found in either independent non-coding regions or in the introns of protein-coding genes. Interestingly, there is some evidence that the expression of intron-encoded microRNAs may be associated with transcriptional regulation of their host gene
promoters. Some microRNAs are clustered in polycistronic transcripts, allowing coordinate expression of these microRNAs in a context-dependent manner. Typically, microRNA expression is under the regulation of transcription factors including c-Myc and p53, or their respective promoter sequences (Ozsolak et al. 2008). Interestingly, it has been shown that in some cases, microRNAs located in the same cluster can be independently regulated. For example, miRNA-433 and miRNA-127 arise from the same locus and overlap in a unidirectional manner. The 3’ coding region of the miRNA-433 gene harvests the promoter region of its neighbouring gene, miRNA-127. This allows independent regulation of the two neighbouring microRNAs, while allowing genetic information to be stored in a compact and efficient manner (Song and Wang 2008). Broadly speaking, most mature microRNAs are processed by the described canonical microRNA signaling pathway. Recently, microRNA specific exceptions have been reported, showing that these steps are not necessarily universal.

Following transcription, the primary RNA transcripts are then cropped by two RNase III-type enzymes, Drosha and Dicer (Kuehlbacher et al. 2007). In humans, these proteins are aided by the double-stranded RNA-binding domain containing proteins Pasha and TRBP (Kadener et al. 2009), corresponding with Drosha and Dicer, respectively. Following this, a precursor microRNA sequence, of approximately 70 nucleotides in length, is released into the nucleus. This precursor molecular, called the pre-microRNA is folded into a stem-loop structure which contains multiple bulges and mismatches. This pre-microRNA is exported into the cytoplasm in a Ran-GTP-dependent manner by Exportin-5 (Yi et al. 2003). Here, the pre-microRNA is further processed by Dicer, generating a duplex intermediate approximately 20 nucleotides in length. Following the thermodynamic asymmetry rule, a single strand of this microRNA duplex accumulates as the mature microRNA. This rule states that the 5’ region of the mature
microRNA lies at the end of the duplex with lower thermodynamic energy (Tomari and Zamore 2005). The mature microRNA is then assembled into effector complexes known as microRNA-containing ribonucleo-protein particles (miRNPs) which functionally resemble the RNA-induced silencing complex (RISC). While the components and functions of these complexes are not entirely understood, it is known that Argonaute (Ago) is highly conserved in both (Zisoulis et al. 2012). Our current understanding states that miRNPs contain a single stranded small RNA associated with an Ago protein.

Once the miRNP has been assembled, the microRNA sequence guides the complex to its target messenger RNA through Watson-Crick base pairing. In plants, microRNAs tend to bind to a single complementary site located in the 3'-untranslated regions of the target mRNA. This is in contrast with animal microRNAs, which have been found to bind to a number of partially complementary MREs in the 3'-UTRs (Liu et al. 2013). While microRNAs typically function by binding to the 3'-UTRs, it has been shown that MREs located in coding regions or 5'-UTR sequences are also functional (Lytle, Yario, and Steitz 2007). In animal microRNAs, complementarity is typically restricted to nucleotides 2-8 in the mature microRNA sequence, typically referred to as the seed sequence. An individual microRNA will induce transcript destruction if it has perfect or near-perfect complementarity to the target mRNA (Engels and Hutvagner 2006). In the presence of multiple, partially complimentary microRNAs however, mRNA levels are typically not strongly affected while protein accumulation is inhibited (Bartel 2009). Through partial binding with microRNA response elements (MREs) located primarily in the 3'-UTR, mature microRNAs post-transcriptionally repress gene expression through either transcript degradation or translational repression.
1.3.2 microRNA signaling pathway

Cleavage of mRNA bound by microRNAs is initiated by the RNA-induced silencing complex (RISC), which directly cleaves a single phosphodiester bond on the target transcript. The highly conserved core of RISC is composed of highly conserved Ago proteins which contain the signature PAZ and PIWI domains (Elkayam et al. 2012). Recent studies support a model wherein siRNAs and microRNAs are positioned between the PAZ and PIWI domains of Ago proteins, alongside the cleavage site of the target mRNA. It is likely that Ago proteins contain mRNA-cleaving activity resultant from the oligonucleotide-binding fold found in PA domains which anchor small RNAs. Notably, PIWI domains have been reported to contain domains resembling RNase H, which cleaves RNA in DNA-RNA hybrids. Furthermore, PIWI domains have also been reported to have a conserved binding pocket for the 5’-phosphates of small RNAs (Parker, Roe, and Barford 2005).

In bacteria, overexpressed human Ago2 complexed with siRNA has been shown to direct mRNA cleavage through the formation of a minimal RISC, demonstrating that human Ago2 is the only protein needed for this process (Rivas et al. 2005). This recombinant RISC was not stimulated by adenosine triphosphate, suggesting that other proteins were responsible for cleavage product release. The active site of this recombinant minimal RISC was shown to be a Asp-Asp-His motif. While all human Ago proteins can bind to microRNAs and siRNAs, only RISC complexes containing Ago2 can support mRNA cleavage. It has been shown that the loss of Ago2 reduces the expression and function of mature microRNAs, identifying Ago2 as a prime candidate gene for coordinating the regulation, biogenesis, and function of human microRNAs.
1.3.3 microRNA target prediction and validation

Hundreds of microRNAs have been identified by experimental or bioinformatics techniques in animals, plants and viruses, with the number of newly reported microRNAs continuing to increase. Despite this, the understanding of microRNA function remains preliminary because few microRNA targets have been identified and fully validated (Kuhn et al. 2008).

The identification of microRNA targets or microRNA recognition elements (MREs) is typically the first step in studying microRNA functioning. Given that the experimental methods used for identifying microRNAs are laborious and difficult to use in large-scale studies, bioinformatics approaches have been developed to predict microRNA binding targets in vertebrates. *In silico* methods however, have a high false positive rate and the results of these studies need to be experimentally verified. Typically, microRNA:MRE duplexes consist of a 5’ end seed region, central bulges or loops resulting from base-pairing mismatch, and a 3’-end tail region. Researchers typically emphasize the seed region in microRNA-mediated gene regulation. Recently however, the role of the central loops in microRNA:MRE duplexes have come into play and there is evidence showing that the size of this central loop may affect the efficiency of microRNA-mediated gene regulation (Ye et al. 2008).

Computational databases including miRanda, TargetScan, DIANA-microT and RNAhybrid are used to predict MREs in 3’-UTR sequences deposited in publicly available databases. Typically, complementarity based on Watson-Crick base-pairing, the conservation of target 3’-UTR sequences across different species, and the thermodynamics of the microRNA:MRE duplexes are used in these databases. The number of binding sites, the distance
between binding sites, the cost of disruption to existing mRNA 3'-UTR secondary structure and target-site accessibility are also used as factors in computational algorithms. Given these criteria however, there remains a high level of false positive predictions and a lack of target overlap between different algorithms. Given this, it is critical that results which are obtained in silico are validated using established experimental methods.

Currently, a number of techniques are used to experimentally validate microRNA targets. The most commonly used experiments include a luciferase reporter assay in combination with western blot to detect protein expression levels. Luciferase reporter constructs are designed such that the 3'-UTR of interest is cloned downstream of a luciferase reporter gene and co-transfected with a synthetic microRNA mimic (Nicolas 2011). If the mimic indeed binds to the 3'-UTR of interest, then luciferase reporter activity will be repressed and light output as assessed by luminometer or by micro-plate reader will be decreased. Conversely, if there is no binding of the microRNA mimic to the 3'-UTR, then light output will be unchanged relative to the control constructs. Typically a construct which contains no predicted MREs, and a construct which contains a mutation in the seed region of the MRE, are used as negative controls. Experimentally, both of these controls are expected to be unaffected by the microRNA mimic. An unrelated oligo that will not bind to the predicted MREs is also used to normalize the obtained luminometer values. While other high throughput techniques have been developed recently, the luciferase reporter assay is usually considered to be the gold standard for assessing the binding of individual microRNAs to their respective MREs (Martin et al. 2014).
1.4 Non-Coding RNAs

Non-coding RNAs (ncRNAs) are functional RNA molecules which are not translated into protein products. Collectively, non-coding RNAs are transcribed from regions of genomic DNA referred to as RNA genes (Knowling and Morris 2011). Non-coding RNAs encompass highly abundant and functionally active RNA molecules such as transfer RNAs (tRNAs), ribosomal RNAs (rRNA), microRNAs, siRNAs and long non-coding RNAs. The exact number of non-coding RNAs within the human genome is unknown, but has been estimated to range in the thousands. With the recent advances in sequencing and bioinformatics analyses, many of ncRNAs have been annotated but not yet functionally validated.

Many of the highly conserved and abundantly found ncRNAs are involved in protein translation. For example, ribosomes consist of mostly ribosomal RNA which catalyzes the translation of nucleotide sequences into protein. tRNAs, another non-coding RNA, act as an adapter between mRNAs and their corresponding nascent protein product (Björk et al. 1987). tRNAs are encoded by genes referred to as tDNAs, and the gene product is further processed by the ubiquitous ribonucleoprotein (RNP), RNase P, which generates mature 5'-ends of tRNAs by cleaving the 5'-ends of precursor tRNAs (Randau, Schröder, and Söll 2008).

Other highly conserved non-coding RNAs among eukaryotes comprise the spliceosome which removes intron sequences from nascent transcripts, forming mature mRNAs. The spliceosome comprises five small nuclear RNAs and a number of associated protein factors, collectively referred to as small nuclear ribonucleic particles (snRNPs). U1, U2, U4, U5, and U6 are the small nuclear RNAs which make up the major spliceosome in eukaryotes (Bindereif, Wolff, and Green 1990). Assembly of the spliceosome occurs on the precursor mRNA which contains sequence specific elements that are utilized during spliceosome assembly. These
elements include the 5’ end splice, the branch point sequence, the polypyrimidine tracts, and the 3’ end splice site. Polypyrimidine tracts recruit factors to the 3’-splice sites and possibly the branch point sequences, which contain a conserved adenosine for the first step of splicing (Coolidge, Seely, and Patton 1997).

Small non-coding RNAs are also involved in DNA replication. Y RNAs, which are components of the Ro60 RNP, fold into a conserved stem and are characterized by a bulged cytosine residue which is required for Ro binding (Christov et al. 2006). Y RNAs interact with chromatin and initiation proteins, including the origin recognition complex, which binds to origins of replication in an ATP-dependent manner. The subunits of this complex are encoded by the ORC1-6 genes (Ohta et al. 2003).

Another class of non-coding RNA includes long non-coding RNAs, which are non-protein coding transcripts greater than 200 nucleotides in length (Wierzbicki 2012). Large scale cDNA studies have estimated that approximately one-fifth of transcription in the human genome is associated with protein-coding genes, thus indicating that there are approximately four times more long non-coding RNA sequences than protein-coding RNA. In contrast with microRNAs and other small RNAs, long non-coding RNAs typically lack the strong conservation across diverse species. Well characterized long non-coding RNAs including Air and Xist are also poorly conserved, perhaps suggesting that non-coding RNAs may be subject to different selective pressures (Brockdorff 2002). For example, selection may conserve only small RNAs or short regions of long non-coding RNAs which are constrained by sequence or structure-specific interactions. The poor conservation of long non-coding RNAs may also be the result of rapid adaptive selection, suggesting that these RNAs may be more susceptible to evolutionary pressures than protein-coding genes. Indeed, conserved regions of the human genome that have
been subject to evolutionary change relative to the chimpanzee genome, correspond mostly with non-coding regions of the genome that are transcribed (Bird et al. 2007). Furthermore, there is evidence indicating that many sequences in the mammalian genome show poor conservation at the primary sequence level but are conserved at the secondary structure level (Torarinsson et al. 2006). This may indicate that even functionally validated long non-coding RNAs which are not subject to structural or functional constraints can evolve more quickly.

In addition to playing key roles in splicing, translation, and DNA replication, non-coding RNA molecules are known to affect the expression of thousands of genes in cis or in trans. microRNAs are now understood to be key regulators of many biological processes, including wound healing. Recent studies have shown that other cellular components, in particular non-coding transcripts and non-coding regions of coding transcripts, can regulate the function of mature microRNAs by acting as natural microRNA sponges which co-regulate and compete for shared microRNAs. These non-coding RNAs are thought to function as ‘competing endogenous RNAs’ (ceRNAs) which regulate microRNA functions, thus co-regulating the expression of other transcripts (Salmena et al. 2011; Tay, Rinn, and Pandolfi 2014). This ceRNA mechanism has been reported independently by various groups, most recently in the context of metastatic lung disease. The expression of HMGA2, a non-histone DNA architectural factor, has been associated with metastatic lung adenocarcinoma for which it contributes to cancer progression and metastasis. Kumar et al., showed that HMGA2 functioned both as a protein-coding gene and as a non-coding RNA by altering microRNA targeting of TGF-beta co-receptor 3 (Tgfr3), thus driving TGF-beta signaling in non-small cell lung cancer (Kumar et al. 2014; Tay, Rinn, and Pandolfi 2014). Analysis of patient gene-expression data further showed that HMGA2 and TGFBR3 were coordinately regulated. Similarly, in a report by Poliseno et al., a PTEN
pseudogene, PTENP1, was found to regulate PTEN cellular levels and suppress tumor cell growth by competing with endogenous microRNA, thus functioning as microRNA decoys (Poliseno et al. 2010).

1.5 The importance of 3'-Untranslated Regions

The 3’-untranslated region (UTR) refers to the section of messenger RNA that immediately follows the translation termination codon, and often contains regulatory regions which influence post-transcriptional gene expression. The median length of human 3’-UTRs is approximately 700 nucleotides, but can range from approximately 60 to 4000 nucleotides (Tanguay and Gallie 1996). The regulatory components within 3’-UTRs play a role in transcript polyadenylation, localization and degradation, giving the 3’-UTR a great variety of regulatory functions.

The length of 3’-UTRs also play a role, given that longer 3’-UTRs are typically associated with lower levels of gene expression. This correlation has been attributed to a higher probability of increased MREs present on longer 3’-UTR sequences. It has also been observed that rapidly proliferating cells express transcript isoforms with shorter 3’-UTR sequences, which can allow cells to avoid microRNA-mediated repression (Akman, Can, and Elif Erson-Bensan 2012). Indeed, rapidly proliferating cancer cells have been shown to express substantial levels of mRNA with shortened 3’-UTRs, usually resulting from alternative cleavage and polyadenylation (Mayr and Bartel 2009). These shortened mRNA isoforms exhibit increased transcript stability and resulted in a ten-fold increase in protein product accumulation, in part through the loss of canonical microRNA activity. In highly expressed neuronal genes, the average 3’-UTR length is
approximately 1300 nucleotides, nearly double the length of expressed non-neuronal genes (Sood et al. 2006).

In addition to length, nucleotide composition also affects the properties of 3'-UTRs. Specifically, UTRs that are GC-poor tend to have longer lengths than those which are GC-rich (Kircher, Bock, and Paulsen 2008). AU-rich elements, which can range in size from 50-150 nucleotides and are located in the 3'-UTRs can stabilize mRNA transcripts through multiple copies and arrangements of the consensus sequence AUUUA (Schaaf and Cidlowski 2002). Iron response elements, which produce a stem-loop structure within UTRs can cause either increased stability or degradation of the transcript, depending on the presence of specific proteins and on intracellular iron levels (Cmejla, Petrak, and Cmejlova 2006). Specifically, AU-rich element binding proteins (ARE-BPs) can promote mRNA stability or initiate translation, and is critically involved in cell growth and differentiation. ARE-BPs affect mRNAs encoding cytokines, transcription factors, receptors, membrane proteins, growth factors, and proto-oncogenes (Barreau, Paillard, and Osborne 2005).

Signals such as the nuclear polyadenylation signal, AAUAAA, located towards the end of the 3'-UTR also initiate the synthesis of the poly(A) tail (Lin et al. 2009; Sheets, Ogg, and Wickens 1990). While this is the most common polyadenylation signal, cytoplasmic polyadenylation can occur during development, regulating maternal mRNAs. These signals occur normally within 100 base pairs of the canonical nuclear polyadenylation signal, and generally have the structure UUUUUUUUAU (McGrew and Richter 1990). Cytoplasmic polyadenylation elements are bound by proteins that function in concert with other proteins to control translation (Pichon et al. 2012). Alternative polyadenylation, another mechanism involved in 3'-UTR mediated gene regulation, produces transcript isoforms which differ only in
their 3'-UTR sequence (Tian and Manley 2013). This is a powerful mechanism which allows complex organisms to express proteins in a spatiotemporal-dependent manner. Alternative polyadenylation is utilized by approximately half of the known human genes, and affects transcript stability, localization, and translational efficiency.

3'-UTRs play integral roles in gene expression through microRNA response elements, AU-rich elements, and polyadenylation signals. Given that MREs are typically the most prevalent motif within 3'-UTRs, the interaction between microRNAs and MREs allows for highly coordinated gene expression at different developmental and pathological time frames (Jia et al. 2013).

Increasingly, it is understood that dysregulation or alterations in 3'-UTR sequences can alter the expression of genes and may be linked with a disease phenotype. Given that 3'-UTR binding proteins and microRNAs affect the processing and exporting of mRNA, mutations in these regions may also affect other genes. For example, mutations in the termination codon of 3'-UTRs have been linked with epidermolysis bullosa simplex (Muller et al. 1999) and aniridia (Guo et al. 2013). Mutations in the polyadenylation signal of the 3'-UTR have been linked with hemoglobin H disease (Huang et al. 2013), immune disfunction, and polyendocrinopathy. Retrotransposon insertion of tandem repeat sequences into the 3'-UTR of fukutin has been linked to Fukuyama-type congenital muscular dystrophy (Taniguchi-Ikeda et al. 2011). CTG repeats within the 3'-UTR of dystrophia myotonica protein kinase has been shown to cause myotonic dystrophy type 1 (Amack, Reagan, and Mahadevan 2002). Given these recent advances in our understanding of the 3'-UTRs, it is highly likely that the 3'-UTRs are critical regions in tissue function and disease. Understanding the functions of regulatory elements within these regions will be critical in elucidating the molecular pathways involved in these processes.
1.6 Angiogenesis

Angiogenesis refers to the formation of new blood vessels from existing vasculature. Although blood vessels are beneficial for tissue growth and regeneration, they can fuel malignant disease and are exploited by tumor cells to promote malignant growth and metastasis. Indeed, angiogenesis is one pathologic hallmarks of glioblastoma, among the most lethal forms of brain cancer. In these cases, the dysregulated balance between pro- and anti-angiogenic signals leads to aberrant blood vessel formation and poor patient prognosis. The most developed treatment for glioblastoma is currently a monoclonal antibody to vascular endothelial growth factor (VEGF), bevacizumab. Bevacizumab has been shown to improve the progression-free survival in glioblastoma but has not been shown to provide an overall survival benefit in glioblastoma (Spezzer 2012).

As key effector molecules of the cell surface and pericellular environment, proteoglycans are known to perform multiple functions in tumor progression and angiogenesis (Lozzo and Sanderson 2011). In particular, the wide ranging functions of versican have been attributed to the central GAG-binding region of versican, and to the G1 and G3 terminal domains which collectively interact with a variety of extracellular matrix and cell surface structural components. While versican V0 and V1 are expressed in the central nervous system, it is known that versican V2 is expressed predominantly in the mature brain, where it acts as a potent inhibitor of neurite (Schmalfeldt et al. 2000; Schweigreiter et al. 2004). Given the expression pattern of versican V2 as well as the critical role of proteoglycans in angiogenesis, we sought to further understand the function of this splice isoform in glioblastoma.
Versican is one the important ECM components at the center of the angiogenesis-associated network (Ricciardelli et al. 2009). Upregulated expression of versican was found in pro-angiogenic arterial SMCs which contributed to the proliferation and migration of these cells (Evanko, Angello, and Wight 1999). The deposition of versican has been found to be linearly correlated with the number of microvessels in tumour stroma (Melrose, Ghosh, and Taylor 2001; Labropoulou et al. 2006). Indeed, versican accumulation in germ cell tumors is related to both metastatic potential and neovascularization (Labropoulou et al. 2006). Versican is actively processed during the early stage of vascular endothelial growth factor A (VEGF-A)-induced pathological angiogenesis in tumors (Fu et al. 2011). VEGF-A initiates vasculature formation from preexisting venules, in part, by inducing the expression of endothelial cell proteases such as ADAMTS-1 and MMP-15. These proteases act in concert to degrade venular basement membrane versican (Fu et al. 2011). In addition, increased expression of versican is often associated with elevated levels of HA in the vascular and perivascular elastic structures in malignant tumors (Koyama et al. 2007). It is believed that versican acts as a key player in HA-mediated angiogenesis by enhancing recruitment of host stromal cells (Koyama et al., 2007). A recent study has documented that versican can activate resident fibroblasts and endothelial cells in the tumor stroma through both TLR2 and its co-receptors TLR6 and CD14. This elicits the production of proinflammatory cytokines including interleukin-8, a proinflammatory CXC chemokine that potentiates neutrophil infiltration, angiogenesis and metastatic growth (Kim et al. 2009).

The versican G3 domain has been found to enhance angiogenesis both in vitro and in vivo (Zheng, Wen, et al., 2004). G3 domain expression enhanced endothelial cell adhesion, proliferation, and migration in vitro and blood vessel formation in nude mouse tumors (Zheng,
Wen, et al., 2004). G3-expressing cells and tumors formed by these cells express elevated levels of fibronectin and vascular endothelial growth factor (VEGF) (Zheng, Wen, et al. 2004). In the presence of versican, fibronectin and VEGF, endothelial cell adhesion, proliferation, and migration were found to be significantly enhanced. Removal of the complex containing these molecules reversed these processes (Zheng, Wen, et al. 2004). The above study indicated that the G3 domain directly binds to fibronectin and forms a complex together with VEGF (Zheng, Wen, et al. 2004). Potential effects on angiogenesis include enhancing vascular endothelial proliferation, migration, and vessel formation. The interactions between tumor cells, surrounding stromal components and neo-vascularization in breast cancer may include interactions among versican, VEGF and fibronectin (Yee et al. 2007).

1.7 Wound Healing

Wound healing is a complex process that involves an inflammatory response followed by subsequent changes in keratinocyte, fibroblast and endothelial cells which close the wounded area and regenerate skin tissue. After the initial insult, wounds fill with blood components leading to the exposure of plasma coagulation factors. This is followed by the deposition of a provisional fibrin-rich matrix. After several hours, epithelial migration restores epithelial integrity while leukocytes invade the fibrin matrix. After several days, this matrix becomes infiltrated with both capillary endothelium and fibroblasts that serve to produce a collagen-rich matrix. As the wound contracts, this provisional matrix is degraded and leaves a collagen-rich scar (Broughton, Janis, and Attinger 2006).

It is now understood that the physiological functions of skin as well as tissue remodeling are controlled by extracellular matrix (ECM) molecules such as versican and by intricate genetic
regulatory networks including microRNA. Exploiting endogenous microRNA feedback loops using versican 3'-UTR overexpression allows for a novel mechanism to study the functions of versican in wound healing as well as transcriptional co-regulation via the proposed ‘ceRNA’ mechanism.

In terms of post-translational regulation, microRNAs are also understood to play a critical role in wound healing. Understanding the mechanisms of microRNA functions in wound inflammation, angiogenesis, and remodelling may lead to the development of therapeutic strategies for management of chronic non-healing wounds.

As the largest organ of the body which protects against environmental hazards, most wounds occur in the skin. The physiological functions of skin are strictly controlled by the gene regulatory networks that involve numerous factors including regulation of gene expression by microRNAs. MicroRNAs play integral roles in the maintenance of skin physiology. In normal skin, miRNA-200 and miRNA-205 are highly expressed, both of which target Zinc finger E-box-binding homeobox 1 (ZEB1) and survival of motor neuron protein-interacting protein 1 (SIP1). Since SIP1, also known as ZEB2, is a transcriptional repressor of E-cadherin, repression of SIP1 by these two microRNAs is expected to regulate E-cadherin positively for maintaining epithelial stability (Gregory et al. 2008).

During the cutaneous wound healing process, expression of certain microRNAs is aberrantly regulated. miRNA-210 regulates keratinocyte proliferation and wound repair (Biswa et al. 2010). Under the hypoxic conditions of chronic wound repair, this microRNA is up-regulated. One of the targets for miRNA-210 is the transcription factor E2F3, which attenuates keratinocyte proliferation and wound closure (Gou et al. 2012). In mammalian skin wound healing, a scarring phenotype is always seen, and only fetal mammalian skin can heal without
scar formation. When comparing these types of skin, some microRNAs are found to be differentially expressed, including miRNA-29b, miRNA-29c, and miRNA-192 (van Rooij et al. 2008). It is known that TGF-β, Smads, and β-catenin are implicated in scar formation. Repression of these proteins by miRNA-29b and miRNA-29c may influence the formation of scars.

Psoriasis of the skin is an immune-mediated chronic inflammatory disease. Several microRNAs have been found to be up-regulated in psoriasis including miRNA-203, miRNA-146a, and miRNA-21, while miRNA-125b has been found to be down-regulated (Sonkoly et al. 2007). These two types of microRNAs may play roles in regulating the pathogenesis of psoriasis. Systemic sclerosis is a chronic disorder characterized by scarring in the skin and other organs, whereby normal extracellular molecules are progressively replaced by collagen. MiRNA-29a can repress expression of type I and type III collagens and is implicated in the formation of the sclerotic (Maurer et al. 2010; Wang et al. 2011). Since cell self-renewal, proliferation, differentiation, survival, migration, invasion, and morphogenesis are key to proper healing, understanding the microRNA regulatory networks of these processes will be key for further understanding wound healing. We showed that the a number of microRNAs could be regulated through its binding to the 3'UTR of versican in the process of wound repair, given the essential role of versican in the extracellular matrix.
Chapter 2

RATIONALE, AIMS AND HYPOTHESIS

Versican, a member of the CSPG family and a versatile member of the ECM, has been shown to be upregulated in both tumor progression and wound healing. Although seemingly disparate, these two biological processes have been demonstrated to utilize similar cellular components and molecular pathways (Riss et al. 2006; Grose 2004; Chang et al. 2004). Beyond the traditional role of the versican protein product, which we examined in tumor angiogenesis, we suspect that the VCAN gene also plays a non-coding function as a microRNA sponge. We examined this idea using a mouse model of cutaneous wound healing. Thus, using our two model systems, we hypothesize that versican plays both a coding and noncoding role in the extracellular matrix.

Project 1: Versican V2 in angiogenesis

If the versican V2 splice isoform plays a pro-angiogenic role in a cell and xenograft model of tumor angiogenesis, then CD-1 nude mice injected subcutaneously with V2-overexpressing U87 (glioblastoma) cells will show an increased level of vasculature relative to a control tumor. Similarly, versican V2 overexpression in these cells would elicit cellular phenotypes associated with tumor progression and angiogenesis. Specifically, I hypothesize that the overexpression of versican V2 through an ectopic expression construct will increase tumor angiogenesis as assessed by an in-vitro model of tube-like structure formation as well as by immunohistochemical quantification of tumor vasculature.
Specific Aim 1: To determine the effect of Versican V2 on tumor cell survival and proliferation as well as potential angiogenic effects in co-culture conditions

U87 glioblastoma cells stably transfected with either a V2 expression construct or mock control will be subject to cell proliferation assays, cell survival assays as well as cell cycle analysis. Formation of tube-like structures in a co-culture system with endothelial cells will also be examined. Cell lysates will be subject to western blot to examine protein expression in our cell models. An siRNA approach will be used in combination with these experiments to confirm the observed phenotypes in our cell-based model.

Specific Aim 2: To determine the effect of over expression of V2 on tumor angiogenesis in vivo

To observe the effects of versican V2 over expression in vivo, five-week-old C57Bl/6 strain nude mice were injected subcutaneously with V2- or vector-transfected U87 cells (5x10^6 cells per mouse). The mice were sacrificed for tumor harvest when tumor sizes of the tumors reached the limited size set by Sunnybrook Animal Committee. Organs were freshly excised and fixed in formalin overnight, immersed in 70% ethanol, embedded in paraffin, and sectioned by a microtome. Sections were examined by H&E staining as well as immunohistochemical analysis of versican expression and markers of blood vessel formation.

Project 2: Versican 3'-untranslated region in cutaneous wound healing

If the versican 3'-untranslated region (VUTR) acts as a competing endogenous RNA in a cell and murine model of wound healing, then transgenic mice subject to dermal wound healing experiments would show an increased rate of wound repair relative to control wildtype mice. Similarly, using a murine fibroblast model of the proliferative phase of wound healing would show an increased migratory capacity in cells stably transfected with the VUTR sequence.
hypothesized that the overexpression of the VUTR region through an ectopic expression construct would increase dermal wound repair as assessed by an established transgenic mouse model and fibroblast cell model.

Specific Aim 1: To determine the effect of Versican 3’UTR on wound healing in vivo

Transgenic mouse strains were previously developed by microinjection of versican 3’UTR fragment into fertilized zygotes of C57BL/6xCBA F2 mouse. Injected eggs were then implanted into oviduct of female mouse. Transgenic strains were maintained by backcrossing with C57BL/6xCBA F1 mouse. Positive transgenic mice were identified by genotyping using GAPDH as a control. By the age of 4 weeks, transgenic mice and control group were subject to skin biopsy with a punch (Miltex), creating a pair of full-thickness, excisional wound, which had diameters around 5 mm on the back of the mice. The wound sizes were measured everyday thereafter, and all mice were sacrificed at seventh day when some wounds were healed. Tissue samples were harvested for further analysis. The wound healing procedure was approved by the Animal Use Committee of Sunnybrook Research Institute. Tissue samples were analyzed using immunohistochemistry, western blotting and real-time PCR analysis.

Specific Aim 2: To determine the effect of Versican 3’UTR on fibroblast cell migration in vitro

To test the effects of versican 3’UTR on cell migration, we stably transfected NIH3T3 fibroblasts with the versican 3’UTR construct or a control vector. We confirmed expression of the VUTR construct by real-time PCR. VUTR-transfected cells were then examined for their effect using an in vitro wound healing assay. Small-interfering RNA (siRNA) sequences were designed to bind to the versican 3’UTR region. An unrelated oligo was used as a control. The effects of the siRNAs were tested. Western blot analysis was performed and the siRNA treated
cells were found to express decreased levels of versican. As well, expression of β-catenin was down-regulated by siRNA transfection. The siRNAs were then used for functional assays. In cell migration assay, we detected decreased migration activity in the VUTR cells transfected with the siRNA compared with the control oligo.

Specific Aim 3: To determine the potential regulatory role of miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-69 on versican 3’UTR mediated wound healing

In this study, twenty luciferase reporter constructs were generated. Five were made to confirm targeting of versican 3’UTR by miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-69. For each construct generated, one counterpart construct was made, which contained a mutation site to abolish the targeting by the microRNA. Five constructs were generated to confirm targeting of beta-catenin 3’-UTR by miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690. Similarly, five counterpart constructs were generated, which contained mutations to abolish targeting by the microRNAs. For all construct generation, PCR products were digested, purified and inserted into SacI/MluI digested pMir-Report vector. As a negative control, the vector was inserted with a non-related sequence, which was amplified from the coding sequence of the chicken versican G3 domain. No significant endogenous microRNA binding was expected to occur in this fragment. These constructs were utilized to perform a dual luciferase assay using a commercially available kit.
Chapter 3
VERSICAN V2 IN GLIOBLASTOMA ANGIogenesis

A version of this chapter is published in FEBS Letters (Yang W and Yee AJ. 587(2):185-192)
Chapter 3

VERSICAN V2 IN GLIOBLASTOMA ANGIogenesis

3.1 ABSTRACT

Versican is a proteoglycan expressed in the extracellular matrix, where it regulates a variety of cell activities and affects tumor development. With alternative splicing, there are four versican isoforms, denoted V0, V1, V2 and V3. The V2 isoform is highly expressed in the mature brain but its function in the mature brain has not yet been elucidated. Since brain tumors are among the most angiogenic of human tumors, we investigated whether or not the V2 isoform plays a role in angiogenesis and found that the glioblastoma cell line U87 stably transfected with V2 formed tumors containing extensive vasculature. Although the V2-expressing cells grew slowly, they survived well in serum-free medium. They also displayed high adhesive ability to endothelial cells and facilitated tube-like structure formation. Importantly, fibronectin was up-regulated by V2 and mediated V2 function. Thus, versican V2 could be a potential target for intervention of brain tumor angiogenesis.

3.2 INTRODUCTION

Glioblastomas are inherently life-threatening due to their invasive and infiltrative character. To achieve this, glioblastomas stimulate the formation of new blood vessels, a process known as angiogenesis, which are structurally and functionally abnormal. Glioblastomas are among the most angiogenic human tumors. High-grade glioblastomas are characterized by more extensive vascularization than low-grade glioblastomas. The degrees of neovascularization in high-grade glioblastomas are a histological indicator of malignancy and patient prognosis (Ohgaki and
Kleihues 2007). As a consequence of extensive angiogenesis, a hostile microenvironment results in low oxygen tension and high interstitial fluid pressure leading to a more malignant phenotype associated with increased morbidity and mortality. We hypothesize that malignant tumor cells are capable of controlling endothelial cell activity by modulating expression of extracellular matrix molecules in the tumor microenvironment.

Versican is a hyaluronan-binding protein that belongs to a class of the large aggregating chondroitin sulfate proteoglycans located primarily in the extracellular matrix (Jones, Margolis, and Tuszyński 2003). By alternative splicing of two exons encoding chondroitin sulfate attachment sequences (CSα and CSβ domains), there exist four versican isoforms. These four isoforms include the largest isoform (versican V0), which is not subject to alternative splicing and contains both CSα and CSβ domains; versican V1, the larger of the alternatively spliced forms, which has the β domain for chondroitin sulfate attachment (Arslan et al. 2007); versican V2, the smaller of the alternatively spliced forms, which contains the α domain; and versican V3, which contains neither of the CS sequences. Versican V0 has a core protein of 3650 amino acids (Zako et al. 1995). Versican V0 is synthesized by the prechondrogenetic mesenchymal cells and plays a role in cell migration (Shinomura et al. 1993; Arslan et al. 2007). Versican V1 is synthesized by embryo and endothelial cells, contributing to cell proliferation and tumor growth (Sheng et al. 2005; Ito et al. 1995). Versican V2 is expressed in mature tissues and can induce cell apoptosis and repress tumor growth (Ito et al. 1995). Versican V3 is expressed in various mouse and human tissues and can inhibit tumor growth (Shinomura et al. 1993; Serra et al. 2005; Hernández et al. 2011).

Versican is highly expressed in the early stages of development, but becomes downregulated after tissue maturation (Dutt et al. 2011). In the adult human central nervous
system, versican is expressed mainly in the white matter of the frontal lobe, cerebellum, brain stem, and spinal cord, in close association with astrocytes and oligodendrocytes (Xiang et al. 2006; Sheng et al. 2005; Zheng, Vais, et al. 2004). Interestingly, during wound repair or tumor formation, versican expression is again upregulated (Wu et al. 2004; Lemire et al. 1999; Lebaron 1996). Additionally, increases in versican expression are associated with tumor formation (Bensadoun et al. 1996). It is detected in the interstitial tissues at the invasive margins of tumors, and in the perivascular elastic tissues associated with tumor invasion (Lebaron 1996). Increased versican immunostaining has been detected during tumor blood vessel formation. It appears that versican V0 and V1 are the key isoform in the early stages of tissue development. Given that versican V2 is the major isoform expressed in the mature brain, we hypothesize that the V2 isoform plays a role in glioblastoma angiogenesis. Using in vitro and in vivo approaches, this study was designed to test the role of the versican V2 isoform in brain tumor angiogenesis.

3.3 MATERIALS AND METHODS

3.3.1 Cell proliferation assay

Glioblastoma cell line U87 cells stably transfected with versican V2 isoform or a control vector (1x10⁵ cells) were seeded onto 6-well tissue culture plates in 10% FBS/DMEM medium and maintained at 37°C overnight. Cells were harvested daily and cell number was determined by haematocytometer.

3.3.2 Cell cycle analysis

U87 cells stably transfected with V2 or the control vector were cultured overnight. The cells were then harvested, washed, and resuspended in 4°C PBS and incubated in ice-cold 70%
ethanol for 3 hours. The cells were then centrifuged at 1,500 rpm for 10 minutes and resuspended in a propidium iodide (PI) master mix (40 mg/ml PI and 100 mg/ml RNase in PBS) at 5x10^5 cells/ml and incubated at 37°C for 30 minutes before analysis with flow cytometry.

3.3.3 Capillary formation assay

Vector- or V2-transfected U87 cells were grown to 80% confluency and then harvested using 0.05% trypsin with 0.53 mM EDTA. The cells were recovered by centrifugation, washed three times with DMEM, and resuspended in this medium at 4x10^4 cells/ml. Cells were mixed with YPEN or EOMA endothelial cell culture and the mixture was inoculated in a Matrigel-coated 8-chamber culture slides. Formation of tube-like structures was examined by light microscopy and analyzed using ImageJ software.

3.3.4 Colony formation assay

1x10^3 cells were mixed with 0.3% low-melting agarose (Seaplaque, FMC) in DMEM supplemented with 10% fetal bovine serum. The mixture was plated on 0.66% agarose coated cell culture plates and culture medium was replaced twice a week. Four weeks after inoculation, colonies were analyzed by light microscopy.

3.3.5 Western blotting

Cell lysates prepared from V2- or vector-transfected cells were seeded on 6-well plates at 10^6 cells/well by lysing the cells with 100 μl lysis buffer containing protease inhibitors. Protein concentrations were measured using a commercially available protein assay kit (Bio-Rad). Lysates containing 50 μg protein were subject to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane followed by immunostaining with a primary antibody
against fibronectin at 4°C overnight. 24 hours later, the membrane was washed and incubated with HRP-conjugated goat-anti-mouse secondary antibody for 2 hours at room temperature followed by ECL detection. After detection of protein bands, the blot was re-probed with anti-β-actin antibody to confirm equal sample loading.

3.3.6 Tumorigenicity assays in nude mice and immunohistochemistry analysis

Five-week-old CD1 strain nude mice were injected subcutaneously with V2- or vector-transfected U87 cells (5x10⁶ cells per mouse). When the sizes of the tumors reached the limited size set by Sunnybrook Animal Committee, the mice were sacrificed for tumor harvest. Organs were freshly excised and fixed in formalin overnight, immersed in 70% ethanol, embedded in paraffin, and sectioned by a microtome (Leica RM2255). The sections were de-paraffinized with xylene and ethanol and then boiled in a pressure cooker. After washing with Tris-Buffered-Saline (TBS) containing 0.025% Triton X-100, the sections were blocked with 10% goat serum and incubated with primary antibody against CD34 or fibronectin in TBS containing 1% bovine serum albumin (BSA) overnight. The sections were washed and labeled with biotinylated secondary antibody, followed by avidin conjugated horse-radish peroxidase provided by the Vectastain ABC kit (Vector, PK-4000). The staining was developed by DAB kit (Vector, SK-4100). The slides were subsequently stained with Mayer’s Hematoxylin for counter staining followed by slide mounting.

3.3.7 Statistical analysis

The results (mean values ± SD) of all the experiments were subjected to statistical analysis by t-test. The level of significance was set at p<0.05, and p<0.01.
3.4 RESULTS

Versican V2 enhances angiogenesis

U87 glioblastoma cells stably transfected with a versican V2 expression construct or a control vector were subcutaneously injected into CD-1 nude mice. Six weeks following injection, the mice were sacrificed and tumors were excised. The tumors formed by the V2-transfected cells were visibly enriched in vasculature, in comparison with vector-transfected cells (Fig 1A). During retrieval of the tumors, we found that the V2 tumors were very adhesive to mouse tissues and a high level of vascularity was observed at the site of tumor growth. By hematoxylin and eosin staining of tumor sections, it was observed that a large number of red blood cells could be detected in the V2 tumors, but not in the control tumors (Fig 1B).

To examine blood vessel formation, we immuno-stained the tumor sections to detect expression of CD34, a surface endothelial marker of blood vessel formation. The number of blood vessels in each tumor section was counted in 6 randomly selected image fields and analyzed statistically. Careful examination of the structures of each blood vessel did not reveal obvious structural differences between the V2 and control tumors. Typical blood vessel structures from each of the tumors are shown (Fig 2). Significantly more vascular structures were detected in the V2 tumors than in the control tumors (Fig 3).
Figure 1: U87-V2 tumor xenografts

(A) U87 cells (5x10⁶) stably transfected versican V2 isoform or a control vector were injected subcutaneously into CD-1 nude mice. Tumors were removed from the mice 4 weeks after the injection. Tumors formed by the V2-transfected cells appeared darker than that formed by the control cells. Representative images of the excised tumors are shown. (B) Tumors formed by cells transfected with V2 isoform (V2 tumors) or a control vector were subjected to H&E staining. Extensive bleeding was detected in the V2 tumors, but not in the control tumors.
Figure 2: CD34 expression in tumor xenograft sections

Tumor sections of U87-V2 xenografts were subject to immunohistochemistry analysis and probed with anti-CD34 antibody to detect blood vessel formation. Typical images of blood vessels from each mouse are shown. n=5
Figure 3: Analysis of CD34 expression in U87-V2 xenografts
U87-V2 tumor sections were subjected to immunohistochemistry probed with anti-CD34 antibody to detect blood vessels. The number of blood vessels was counted in 6 randomly selected imaging fields and statistically analyzed. Expression of a V2 transgene promoted blood vessel formation as compared with the control tumors. Means of both groups were calculated, analyzed by student’s t-test and the differences were determined to be statistically significant. n=5, p<0.0001.
Versican V2 inhibits U87 proliferation and colony formation in soft agar

To test the effects of the versican V2 isoform in vitro, we initially determined proliferation rates of U87 cells stably transfected with the V2 or control vector. We found that cells transfected with V2 had a reduced proliferative capacity relative to the control cells (Fig 4). We thus conducted an analysis of cell cycle progression and observed that relative to the control cells, more V2-transfected cells were detected in the G1 phase, and less in the G2 or S phases (Fig 5). This result indicated that fewer V2 cells were pre-mitotic relative to control cells, providing evidence for the anti-proliferative function of versican V2 in glioblastoma cells.

We also tested whether or not expression of V2 affected colony formation in soft agar. The cells were cultured in a mixture of 0.3% low melting temperature agarose with serum-containing DMEM overnight. Culture medium was replaced twice per week. Four weeks after inoculation, colonies which had formed were photographed and analyzed by light microscopy. We found that the versican V2 harboring cancer cells formed a fewer number of colonies which were also found to be smaller in size (Fig 6).
Figure 4: Expression of versican V2 inhibits cell proliferation.
U87 cells stably transfected with versican V2 transgene or a control vector were seeded on tissue cultures plates containing 10% FBS for five days and quantified using hematocytometer. Expression of V2 was shown to reduce the proliferative capacity of U87 glioblastoma cells. **$p<0.01$ at Day 5. Error bars, SEM (n=3).
**Figure 5: U87-V2 cell cycle analysis**

Cell cycle analysis indicated that a greater proportion of U87-V2 cells were detected in the G1 phase, while fewer cells were detected in the G2 and S phases, relative to the control cells (n=3).
**Figure 6: U87-V2 colony formation.**
U87-V2 cells were subject to soft agar colony formation assay. U87 cells stably transfected with a V2 transgene formed fewer colonics and colonics of a smaller size.
Figure 7: The expression of versican V2 promotes formation of tube-like structures
V2- and vector-transfected U87 cells were co-cultured with YPENn cells and inoculated in Matrigel, followed by examination of the formation of tube-like structures. The YPEN cells formed extensive tube-like structures when co-cultured with V2 cells, but did so only minimally when co-cultured with vector-transfected U87 cells.
V2 expression facilitates endothelial-tumor cell interaction

Subcutaneous injection into nude mice revealed that the tumors formed by V2-expressing cells harbored significantly more blood vessels than those formed by control cells. We examined whether the V2-expressing cells and the control cells might display different capacities in interacting with endothelial cells, a major component of blood vessels. In an angiogenic assay, we tested whether V2-expressing U87 cells could facilitate the formation of tube-like structures in Matrigel. The V2- and vector-transfected cells were co-cultured with YPEN cells and cultured in Matrigel, followed by examination of tube-like structure formation. We found that YPEN cells formed complex tube-like structures when mixed with the V2 cells, which did not occur in culture with the control cells (Fig 7). Analysis of tube length and tube complexity was performed by ImageJ software. Both measures of tube formation were significantly increased when endothelial cells were cultured with V2-expressing tumor cells. These results indicated that the V2-expressing cells were better able to facilitate the production of tube-like structures through the recruitment of endothelial cells.

Effects of versican V2 mediated through up regulated fibronectin expression

We next analyzed levels of fibronectin, an adhesion molecule, by Western blot. Our results showed that the V2-expressing cells expressed much higher levels of fibronectin than the control cells (Fig 8). Our results suggested that the versican V2 isoform might up-regulate fibronectin expression and enhance interaction of tumor cells with endothelial cells, leading to increased angiogenesis. To test whether fibronectin played a role in mediating V2 functions, we used an siRNA approach to silence fibronectin expression (Fig 9). V2-expressing U87 cells were transiently transfected with an siRNA construct (siFN-32) specifically targeting fibronectin. The
cells transfected with the siRNA construct or a control vector were subject to tube-like structure formation assay described previously. The experiment indicated that U87 cells transfected with siRNA targeting fibronectin, and co-cultured with EOMA cells, displayed decreased and length and complexity of tube-like structures, as compared with cells transfected with the V2 construct (Fig 10). A similar result was obtained with YPEN cells. Silencing fibronectin expression led to decreased formation of tube-like structures. These results indicated that fibronectin played an important role in mediating versican V2 function associated with angiogenesis.
Figure 8: Western blot analysis of fibronectin expression in U87-V2 conditioned media and cell lysate

(A) Cell lysates prepared from V2- and vector-transfected cells or were subject to western blot analysis probed with anti-fibronectin antibody. The same membranes were also probed with anti-β-actin antibody which served as a loading control. Expression of the versican V2 construct increased fibronectin levels compared with the vector-transfected cells U87 cells. (B) U87-V2 cultured media was prepared and analyzed by western blot. Expression of the V2 construct increased levels of secreted fibronectin.
Figure 9: Western blot analysis of fibronectin levels in U87-V2 transfected with siRNAs targeting fibronectin mRNA. Cell lysates prepared from U87 cells transiently transfected with siRNA constructs targeting fibronectin were analyzed on Western blot probed with an anti-fibronectin antibody, confirming silencing of fibronectin. Four different siRNAs (denoted siFN-31-34) were utilized.
Figure 10: Silencing fibronectin using siRNA decreased the formation of tube-like structures in a Matrigel coculture system. Cells transfected with siFN-32 or a control vector were mixed with YPEN cells, followed by inoculation in Matrigel for tube formation assay. Transfection with siFN-32 decreased the complexity of the tube-like structures. Cells transfected with siFN-32 or a control vector were also mixed with EOMA cells for tube formation assay. Silencing of fibronectin decreased the formation of tube-like structures.
3.5 DISCUSSION AND CONCLUSIONS

Both versican and fibronectin are extracellular molecules which play important roles in enhancing cell adhesion. Although all versican isoforms contain the N-terminal G1 domain and a C-terminal G3 domain, the expression and functions of each isoform appear to be differentially regulated. Previous studies have demonstrated that the V1 isoform can enhance cell proliferation while the V2 isoform can inhibit cell growth (Sheng et al. 2005). The V1 isoform can also stimulate tumor growth (Paulus et al. 1996). However, the effect of V2 isoform in tumor development has not yet been elucidated. Since the V2 isoform has been shown to be the primary isoform expressed in the mature brain, reports on versican expression and function in the brain are likely referring to this isoform. In the brain, versican is mainly expressed in the submeningeal layers of the cortex, around the blood vessels, and in a layer of cerebellum where it is observed to co-localize with tenascin and hyaluronan, two adhesion molecules (Paulus et al. 1996). In brain tumors, versican is detected in the interstitial tissues at the invasive margins, and in the perivascular elastic tissues associated with tumor invasion (Lebaron 1996). Increased versican immunostaining is typically detected at the neovascularure within tumors. Elevated versican expression is also observed in the mesenchymal tissues between the invasive clumps of carcinoma cells at the margins of infiltrating ductal carcinoma. Collectively, it is highly likely that versican may play an important role in tumor development and blood vessel formation.

Because the versican V2 isoform is the predominant isoform in the mature brain, we investigated the role of V2 isoform in tumor angiogenesis. Our study demonstrated that the versican V2 isoform could promote tumor angiogenesis as assessed by a subcutaneous xenograft tumor model and in vitro assays of tumor angiogenesis.
We initially assessed the effect of stably transfecting the versican V2 transgene into human glioblastoma U87 cells. We found that relative to vector transfected cells, this particular splice isoform played an anti-proliferative effect on U87 cells, by arresting cells in the G1 phase, the first checkpoint in the mitotic cell cycle. We also found that the V2-transfected U87 cells had a reduced capacity to form colonies in soft agar, a common assay used to assess the level of malignant transformation a cell population has undergone. One characteristic of tumor cells is their ability to grow without the support of a solid substratum. These cells can thus be seeded onto growth suspensions or soft media, upon which they float freely. Thus, the V2 isoform appeared almost paradoxically, to play an anti-tumorigenic role, until we performed the subcutaneous tumor injections. When we examined these cells in such a tumor xenograft, we found that although tumor volume was unchanged between the V2-transfected and control cells, the former group displayed a dramatic difference in tumor characteristics. We found that tumors formed by the V2-transfected cells appeared highly vascularized when examined by dissection microscope and when examined using immunohistochemistry. By Hematoxylin and Eosin staining, we found a much higher level of red blood cells and blood vessel formation in the V2 xenograft sections, relative to the control cells. By immunohistochemistry, we found an increase in CD34, an endothelial cell surface marker. Thus, our in vivo data suggested that the versican V2 splice isoform could play a pro-angiogenic role in glioblastoma cells.

One possible explanation of this could be through increased levels of interactions between tumor cells and endothelial cells, facilitating the formation of tube-like structures. We investigated this using a co-culture with endothelial cells seeded on Matrigel, a commercially available mixture of basement membrane components. We found that when co-cultured with V2-transfected tumor cells, the endothelial cells produced more tube-like structures than in co-
cultures with vector transfected cells. This lent support to the hypothesis that the V2 splice
isoform could increase interaction between tumor and endothelial cells. While this assay is one
of the most widely used in studies of angiogenesis, results should be interpreted with caution
given the heterogeneity of cells within both the tumor and the surrounding microenvironment.
Furthermore, given that this assay is a 2D cell culture assay, results should be interpreted in
conjunction with other tests such as the Aortic Arch Assay, an *ex vivo* organ culture system.
Given that our *in vitro* work was done alongside an *in vivo* tumor xenograft, we believe that this
is strong evidence of a pro-angiogenic role for versican V2.

Up-regulation of fibronectin expression appears to be key in angiogenesis. Fibronectin is
an adhesion molecule that plays a critical role in angiogenesis (Nicosia, Bonanno, and Smith
1993). Since versican G3 domain has been shown to bind to fibronectin, the V2 isoform may
play a role in angiogenesis by up-regulating and binding to fibronectin through its G3 domain.
To confirm this, we demonstrated that silencing fibronectin expression by siRNA approach
abolished V2’s effect in enhancing tube-like structure formation.

Glioblastomas are one of the most prevalent and aggressive primary malignant brain
tumours (Jain et al. 2007). Following aggressive surgery, radiotherapy and chemotherapy
regimens, these tumours typically remain fatal and have low survival expectancy. Glioblastomas
are highly angiogenic, a phenomenon likely driven by the presence of VEGF. Tumor
vasculature, unlike normal physiological vasculature however, is structurally and functionally
abnormal and cannot provide normal levels of oxygen and nutrients. These blood vessels are
typically described to have a ‘tortuous’ morphology and contribute to a hostile tumor
microenvironment characterized by low oxygen tension and high interstitial fluid pressure.
While anti-VEGF therapies have been shown to be effective in preclinical trials, results have
been disappointing in the clinical realm. Thus, identifying new mechanisms and proteins involved in tumor angiogenesis may be key in developing novel therapeutics or biomarkers for this disease. Given its high expression levels in the mature brain, we identified the versican V2 isoform as an extracellular matrix CSPG which may be co-opted during malignant transformation to play a pro-angiogenic role in glioblastomas.

Our results demonstrated that the versican V2 isoform played a role in tumor angiogenesis by facilitating interactions between tumor and endothelial cells leading to the formation of tube-like structure, and up-regulating fibronectin expression. Regulating the versican V2 isoform and fibronectin may be potential targets for the intervention of tumorigenesis and angiogenesis in brain cancer.
Chapter 4

VERSICAN 3’-UNTRANSLATED REGION IN CUTANEOUS WOUND HEALING

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Chapter 4

VERSICAN 3’-UNTRANSLATED REGION IN CUTANEOUS WOUND HEALING

4.1 ABSTRACT

Versican is an extracellular chondroitin sulfate proteoglycan that provides structural and biochemical support to cells. This study was designed to explore the role of versican in the process of wound repair. To elevate levels of versican, we ectopically expressed versican 3’-untranslated region (3’UTR) as a competitive endogenous RNA to modulate expression of versican by regulating microRNA functions. Using a transgenic mouse model, we demonstrated that dermal wounds closed faster in transgenic mice expressing the versican 3’UTR than in wild type mice. We stably expressed versican 3’UTR in NIH3T3 fibroblasts and found that the 3’UTR-transfected cells showed a greater migratory capacity relative to control cells. We found that the 3’UTRs of versican and β-catenin shared common microRNAs including miRNA-185, miRNA-203*, miRNA-690, miRNA-680, and miRNA-434-3p. As a consequence, expression of versican and β-catenin was upregulated as compared with the controls by ectopic transfection of the versican 3’UTR, which was confirmed in vitro in the NIH3T3 fibroblasts transfected with versican 3’UTR and in vivo in the versican 3’UTR transgenic mice. Transfection with siRNAs targeting the versican 3’UTR abolished the 3’UTR’s effects on cell migration and invasion. Taken together, these results demonstrate that versican play important roles in wound repair and that versican mRNAs could compete with endogenous RNAs for regulating microRNA functions.
4.2 INTRODUCTION

Wound healing is a complex process that involves an inflammatory response followed by subsequent changes in keratinocyte, fibroblast and endothelial cells which close the wounded area and regenerate skin tissue (Sen and Roy 2008). Understanding the molecular mechanisms of wound healing is essential for developing therapeutic strategies to manage acute and chronic non-healing wounds. To elucidate the signaling pathways involved in wound healing, different model systems have been developed. It is now understood that the physiological functions of skin as well as tissue remodeling are strictly controlled by extracellular matrix (ECM) molecules such as versican and by intricate genetic regulatory networks including microRNA.

Versican is a hyaluronan-binding molecule that belongs to a family of the large aggregating chondroitin sulfate proteoglycans expressed in the ECM (Sheng et al. 2005; Wight 2002). It was originally cloned from human fibroblasts and chicken limb buds (Ito et al. 1995). It is expressed in many embryonic tissues and skin (Nadanaka et al. 2001). Versican expression is associated with tissue development (Henderson and Copp 1998). Versican is the principal chondroitin sulfate proteoglycan in blood vessels, as well as in the intima and adventitia of most arteries and veins (Lebaron 1996). In vitro studies have shown that versican modulates cell adhesion (Yang et al. 1999), aggregation (Zheng, Vais, et al. 2004), migration (Ang et al. 1999), and tissue angiogenesis (Zheng, Vais, et al. 2004). This study was designed to explore the roles of versican in the process of dermal wound repair. To elevate levels of versican, we ectopically expressed the versican 3'-untranslated region (3'UTR) as a competitive endogenous RNA to modulate expression of versican by regulating microRNA functions.
MicroRNAs are single-stranded RNAs of 18-24 nucleotides in length. They are transcribed from genomic DNA, producing primary RNA transcripts that contain one or more local hairpin structures. The primary RNA transcripts are then cropped by the RNase III-type enzyme Drosha, releasing microRNA precursors into the nucleus. The precursor microRNAs are then brought to cytoplasm by Exportin-5, and processed to produce mature microRNAs. Through partial binding with the 3’-untranslated region (UTR), mature microRNAs repress gene expression post-transcriptionally. By silencing mRNA expression, microRNAs have been shown to have a central roles in physiological and pathological conditions including cell proliferation, differentiation, migration, survival, invasion, angiogenesis, tumor progression, and metastasis. Recent studies have shown that other cellular components, in particular non-coding transcripts, can regulate the function of mature microRNAs. These non-coding RNAs can function as competing endogenous RNAs (ceRNAs) to regulate microRNA functions. This was first demonstrated by the ectopic expression of 3’UTRs which was shown to regulate the function of endogenous microRNAs (Jeyapalan et al. 2011; Lee et al. 2009). Similarly, ectopic expression of a pseudogene of the tumor suppressor PTEN, namely PTENP1, could increase PTEN expression and regulate cell proliferation by competing for PTEN-binding microRNAs (Jeggari, Marks, and Larsson 2012; Poliseno et al. 2010; Tay, Rinn, and Pandolfi 2014). Ectopic expression of long noncoding RNAs function in a similar way by competing for microRNA binding and regulating their functions (Cesana et al. 2011). In our recent study, we found that transgenic mice expressing our versican 3’UTR construct developed liver tumors by inhibiting the activities of miRNA-144, miRNA-133a, miRNA-431, and miRNA-199a*, and up-regulating expression of fibronectin, CD34, and versican (Fang et al. 2013).
In this study, we employed a versican 3’UTR stable cell line and transgenic mouse as model systems to evaluate the effects of the versican 3’UTR on the migratory phases of wound healing. We found that ectopic expression of the 3’UTR promoted wound healing by regulating a number of microRNAs that targeted versican but also shared target sites with the 3’UTR of β-catenin. As a consequence, expression of versican and β-catenin was enhanced while the activities of the bound microRNAs were inhibited, leading to a combinatorial effect on cell migration and wound healing.

4.3 MATERIALS AND METHODS

4.3.1 Cell Culture

Cell line used was NIH-3T3 (Mouse embryonic fibroblast cell line, ATCC® CRL-1658™). Cells were kept in exponential growth phase as adherent monolayers. Cells were cultured in a 37°C, 5% CO₂ humidified incubator until reaching 70-80% confluency in Dulbecco’s Modification of Eagle’s Medium (DMEM) supplemented with 10% bovine calf serum (Gibco) and 200µg/mL Hygromycin B (Multicell Technologies).

4.3.2 Generation of Expression Constructs

In this study, twenty luciferase reporter constructs were generated. Five were made to confirm targeting of versican 3’UTR by miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690, producing Luc-Ver\textsuperscript{185}, Luc-Ver\textsuperscript{203*}, Luc-Ver\textsuperscript{434-3p}, Luc-Ver\textsuperscript{680}, and Luc-Ver\textsuperscript{690}. For each construct generated, one counterpart construct was made, which contained a mutation site to abolish the targeting by the microRNA, producing Luc Ver\textsuperscript{185} mut, Luc Ver\textsuperscript{203*} mut, Luc-Ver\textsuperscript{434-3p-mut}, Luc-Ver\textsuperscript{680-mut}, and Luc-Ver\textsuperscript{690-mut}. Five constructs were generated to
confirm targeting of β-catenin 3’UTR by miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690, producing Luc-Cat185, Luc-Cat203*, Luc-Cat434-3p, Luc-Cat680, and Luc-Cat690. Similarly, five counterpart constructs were generated, which contained mutations to abolish targeting by the microRNAs, producing Luc-Cat185-mut, Luc-Cat203*-mut, Luc-Cat434-3p-mut, Luc-Cat680-mut, and Luc-Cat690-mut.

Each construct was generated by two primers as follows. Luc-Ver185 by huVUTR-Sacl (5’ gg gagctc tgtccctaaaatgggcaacacagtg) and huVUTR-R185-MluI (5’ ggg ccc aacgctt ggctttgct gtaa gttga), Luc-Ver203* by huVUTR-Sacl and huVUTR-R203-MluI (5’ cc aacgcctt gggc aaccc aaat gact gaa cgg), Luc-Ver434-3p by huVUTR-Sacl and huVUTR-R434-3p-MluI (5’ ggg ccc aacgctt aaaa cttt ctaa aaaa gca gcc), Luc-Ver680 by huVUTR-Sacl and huVUTR-R680-MluI (5’ ggg ccc aacgctt atca tttt ctaga gaa gcc aaaa ct), and Luc-Ver690 by huVUTR-Sacl and huVUTR-R690-MluI (5’ ggg ccc aacgctt gtttt ctagct gcc cgg).

To generate the mutations, each forward primer was combined with a mutation primer (-mut) as follows: huVUTR-R203*-MluI-mut (5’ cc aacgctt gggc aaccc aaat gact gaa cgg acc tcctca) to generate Luc-Ver203*-mut, huVUTR-R434-3p-MluI-mut (5’ ggg ccc aacgctt aaaa cttt ctaa aaaa gca gcc ctt ccaaa) to generate Luc-Ver434-3p*-mut, huVUTR-R690-MluI-mut (5’ ggg ccc aacgctt gtttt ctagct gcc cgg tttt gcc) to generate Luc-Ver690-mut, huVUTR-R680-MluI-mut (5’ ggg ccc aacgctt atca tttt ctaga gaa gcc aaaa ct cgt cc ata ggc tg) to generate Luc-Ver680-mut, and huVUTR-R185-MluI-mut (5’ ggg ccc aacgctt ggctttgct gtaa gttga ctttaaag) to generate Luc-Ver185-mut.

The β-catenin 3’UTR constructs were generated as follows. Luc-Cat185 was generated by mu-βcatenin-Sacl (5’ ggg ccc gagctc tccctaggtaagagcttat) and mu-βcatenin-R185-MluI (5’ ggg
ccc aacgcgt gttc aga cac ta ca gc tgt at aga); Luc-Cat<sup>203</sup>, by mu-βcatenin-R203*-SacI (5’ ggg ccc gagctc caagctgatctctctatgggaacag) and mu-βcatenin-R203*-MluI (5’ ggg ccc aacgcgt tta gtg ttct acacc atta ctc); Luc-Cat<sup>690</sup> by mu-βcatenin-R203*-SacI and mu-βcatenin-R690-MluI (5’ ggg ccc aacgcgt ca tttc ta tc tg tc tat tat tat aca). Two binding sites for miRNA-434-3p and miRNA-680 had a few nucleotides overlapped. Thus, one construct was generated to cover both binding sites by using mu-βcatenin-R203*-SacI and mu-βcatenin-R680-MluI (5’ ggg ccc aacgcgt gttt aatt ctg aacc atttc tataa). The names Luc-Cat<sup>434-3p</sup> and Luc-Cat<sup>680</sup> were used in the figure for easily following.

The mutation primers were mu-βcatenin-R185-MluI-mut (5’ ggg ccc aacgcgt gttc aga cac ta ca gc tgt at aga cttct aagg, to generate mutation site for miRNA-185), mu-βcatenin-R203*-MluI-mut (5’ ggg ccc aacgcgt tta gtg ttct acacc atta ctc ccaa ctt ag, to generate mutation site for miRNA-203*), mu-βcatenin-R690-MluI-mut (5’ ggg ccc aacgcgt ca tttc ta tc tg tc tat tat aca a tttc ctt at c, to generate mutation site for miRNA-690), and mu-βcatenin-R680-MluI-mut (5’ ggg ccc aacgcgt gttt aatt ctg ttgg atttc tataa ccg gta ctgt, to generate mutation site for miRNA-680 and miRNA-434-3p).

For all construct generation, PCR products were digested with restriction enzymes SacI and MluI. The fragments were purified and inserted into SacI/MluI digested pMir-Report vector. As a negative control, the vector was inserted with a non-related sequence, which was amplified from the coding sequence of the chicken versican G3 domain using 2 primers, chver10051SpeI (5’ gggccccacactgatggagccatgatatag) and chver10350SacI (5’ gggccccagctcgaatctacgctcaaacatct). No significant endogenous microRNA binding was expected to occur in this fragment. These PCR products were then inserted into a SpeI- and SacI-digested pMir-Report Luciferase plasmid.
4.3.3 Transgenic Mice

Transgenic mouse strains were developed by microinjection of versican 3’UTR fragment into fertilized zygotes of C57BL/6xCBA F₂ mouse. Injected eggs were then implanted into oviduct of female mouse. Transgenic strains were maintained by backcrossing with C57BL/6xCBA F₁ mouse. Positive transgenic mice were identified by genotyping using GAPDH as a control.

4.3.4 Wound Healing

By the age of 1 weeks, transgenic mice and control group were subject to skin biopsy with a punch (Miltex), creating a pair of full-thickness, excisional wound, which had diameters around 5 mm on the back of the mice. The wound sizes were measured everyday thereafter, and all mice were sacrificed at seventh day when some wounds were healed. Tissue samples were harvested for further analysis. The wound healing procedure was approved by the Animal Use Committee of Sunnybrook Research Institute.

4.3.5 Western Blotting

Cell lysates were collected from the cultures and subject to SDS-PAGE electrophoresis on 10% separating gel (for β-catenin) or 7% gel (for versican) with a 4% stacking gel. The buffer system was made up of 1x TG (Tris-glycine buffer, Amresco product) and 1% SDS. The proteins separated on SDS-PAGE were transferred onto nitrocellulose membranes (Bio-Rad) in 1x TG buffer containing 20% methanol. The membrane was then incubated in blocking buffer TBST (10 mM Tris-Cl, pH8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk powder (TBSTM) for 1 hour while being gently shaken at room temperature. The membranes
were incubated with primary antibodies at 4 °C overnight. Membranes were then washed in TBST and incubated with appropriate the HRP-conjugated secondary antibodies diluted in TBST for 2 hours at room temperature. The bound antibodies were then visualized with a Chemiluminescent HRP Antibody Detection Kit (HyGLO, Denville Scientific Inc.).

4.3.6 Immunohistochemistry

Wound tissues were freshly fixed in 10% neutral buffered formalin overnight and immersed in 70% ethanol. After dehydration, the tissues were embedded in paraffin, and sectioned by microtome (Leica RM2255). Sections of 4 μm thickness were placed on slides and deparaffinized in xylene twice for 5 minutes. Slides were rehydrated in 100% ethanol for 3 minutes, followed by two 5 minute washes in dH₂O. The slides were brought to a pressure cooker in 10 mM sodium citrate buffer (pH 6.0) and then maintained at a sub-boiling temperature for 10 min to unmask antigenic epitope. After cooling, endogenous peroxidase activity was inhibited by incubating the sections in 3% H₂O₂ solution in methanol at 4°C for 20 minutes. The sections were rinsed twice in TBS for 5 minutes. Non-specific reactions were blocked with 10% goat serum at room temperature for 30 minutes. The slides were then incubated with a primary antibody solution (diluted in TBS containing 10% goat serum and 1% BSA) in a humidified chamber at 4°C overnight, followed by three washes in TBS. The slides were then incubated with biotinylated secondary antibody diluted in TBST at 37°C for 45 min. ABC reagent (Vectastain Elite ABC kit, Vector Lab) was prepared according to the manufacturer’s instructions and added to the sections followed by incubation at 37°C for 45 min. After three 5 minute washes in TBS, DAB (Peroxidase Substrate Kit, Vector Lab) was added to each section and the staining was monitored closely. Slides were immersed in dH₂O followed by
counterstaining in Mayer’s Hematoxylin. After dehydration in 95% ethanol and xylene, the sections were mounted with coverslips.

4.3.7 Antibodies

This included antibodies against α-smooth muscle actin (Abcam, cat No. Ab5694), vimentin (Cell Signaling Technologies: 3932S), N-Cadherin (Santa Cruz: Sc1502), fibronectin, (Abcam. Ab6328), Type I collagen (Abcam. Ab6308), Versican (US Biological. L1305A), β-catenin (Cell Signaling Technologies: 8480S), CD34 (from Abcam: Ab8158), and Ki67 (BD Bioscience: 550609).

4.3.8 Luciferase Assay

NIH3T3 fibroblasts were cultured in 12-well tissue culture plates at a cell density of 1×10^5 cells per well in DMEM containing 10% FBS. The cultures were maintained at 37°C for 24 h till sub-confluence. The cultures were co-transfected with the versican or β-catenin luciferase reporter constructs or the mutant luciferase reporter construct together with the corresponding microRNA mimics (from GenePharma, Shanghai, China) using Lipofectamine 2000 in 500 μl serum-free DMEM medium. Each transfection mixture also contained 10 ng pRL-Renilla Luciferase Reporter Vector. The Renilla plasmid served as an internal control of transfection efficiency to normalize the value of the firefly luciferase activities. A negative control construct (G3R) contained a non-related sequence ligated to a luciferase reporter, which was also transfected with the microRNA mimics at the same ratio. All reactions were performed in triplicate. Six hours after the transfection, the medium in the cultures was changed to fresh DMEM containing 10% FBS, followed by an additional incubation of 48 hours. The cells were then harvested and luciferase activity was measured using a Dual-Luciferase® Reporter Assay
System (Promega, Nepean, ON, Canada). In brief, after removing growth media and rinsing with PBS, the cultures were lysed in 1x Passive Lysis Buffer (150 μl per well). The culture plates were gently shaken at room temperature for 15 min. The lysates were collected into Eppendorf tubes for centrifugation. 100 μl of luciferase assay Buffer II was dispensed into luminometer tubes. 20 μl of lysate supernatant was transferred to each tube and mixed. Firefly luciferase activity was measured using a luminometer (Berthold, Germany). 100 μl of Stop & Glo® Reagent was dispensed into each luminometer tube and Renilla luciferase activity was measured. Relative luciferase activity was compared between the luciferase reporter construct, mutant luciferase reporter construct and the negative control construct. Each experiment was performed in triplicate.

4.3.9 Statistical Analysis

All experiments were performed in triplicate (or more). The numerical data were subject to independent statistical analysis (unless otherwise specified). The results (mean values ± SD) of all experiments were analyzed by Student’s t-test. The levels of significance was set at p<0.05 (*) and p<0.01 (**).

4.4 RESULTS

Expression of versican 3’UTR increased dermal wound healing in transgenic mice

In this study, we utilized transgenic mice expressing the versican 3’UTR (VerUTR) as a model system and subject these mice to dermal wound healing tests. One week after wounding, we found that VerUTR transgenic mice healed significantly faster than the wild type mice. We found that the VerUTR transgenic mice showed enhanced wound healing as compared to the
wild type mice (Fig 11A). Examination of the unhealed wounds revealed reduced wound sizes in the VerUTR transgenic mice (Fig 11B). The wound healing assay was performed in large number of mice and the healing process was monitored every day. The assay confirmed that the wounds closed significantly faster than in the wildtype mice (Fig 12).

Wound tissue samples were subject to H&E staining. Histological examination revealed characteristics of increased wound repair in the VerUTR transgenic mice (Fig 13A). We also found less muscle tissues detected at the wound site of the wildtype mice, while larger amounts of muscle tissues were found in the VerUTR transgenic mice (Fig 13B). The wound surface of the wildtype mice showed thinner layer of blood clot and contained fewer lymphatic cells in the clot as compare with the VerUTR transgenic mice, which had much thicker layer of clot and greater number of lymphatic cells (Fig 14A). Examination of cell number revealed collagen fibrils in the wound sites of the wildtype mice than the VerUTR transgenic mice (Fig 14B). Immunohistochemical analysis of wound tissues revealed an increased level of Ki67 positive cells in the VerUTR transgenic mice than in the wild type (Fig 15A). Expression of CD34 was quantified using three randomly selected fields of view (Fig 15B).
Figure 11. Enhanced wound healing in VerUTR transgenic mice. VerUTR transgenic and wildtype mice were subject to wound healing tests. One week after the wounding, healed mice were counted. VerUTR showed higher rate of wounding healing. (A) The unwounded mice were subject to wound size measuring. The VerUTR transgenic mice showed enhanced wound healing as compared with the wildtype mice. (B) Picture taken from the seventh day showed that the VerUTR transgenic mice had enhanced wound healing compared with the wildtype mice.
Fig 12. Time course of wound healing in VerUTR transgenic mice. Wound repair was followed closely in the course of healing process. The wounds closed significantly faster in the VerUTR transgenic mice than in the wildtype mice.
**Fig 13. Analysis of wound closure.** (A) Wound tissue samples were subjected to H&E staining. Faster close-up of the wound was detected in the VerUTR transgenic mice (arrows). Typical photos are provided for 2 transgenic mice and 2 wildtype mice. Scale bar, 400 μm. (B) On day 7, smaller amounts of mussel tissues were detected in the wildtype mice, while large amounts of mussel tissues were found in the VerUTR transgenic mice (arrows). Scale bar, 100 μm.
Fig 14. Histological examination of wound sections. (A) The wound surface of the wildtype mice showed thinner layer of blood clot and contained fewer lymphatic cells than the VerUTR transgenic mice (arrows). Scale bar, 50 μm. (B) The wounds in the wildtype mice contained much fewer cells than the wounds in the VerUTR transgenic mice. Scale bar, 50 μm.
Fig 15. Enhanced expression of Ki67 in VerUTR transgenic mice.
(A) Wound tissue samples were subject to immunohistochemistry analysis. Expressions of Ki67 increased in the VerUTR transgenic mice (arrows). Scale bar, 50 μm. (B) The positive Ki67 cells were quantified. **p<0.05
Random sections were immunostained using an anti-CD34 antibody (Fig16A). It was found that VUTR transgenic mice displayed increased expression of CD34 (Fig16B). Since cytoskeletal proteins and extracellular matrix play important roles in wound repair, we analyzed expression of a number of these molecules associated with tissue growth and wound repair. We found that expression of α-smooth muscle actin, vimentin, N-Cadherin, fibronectin, and type I collagen was elevated in the VerUTR transgenic mice as compared with the wild type (Fig 17). Scanning of the photos with NIH ImageJ software showed significant higher levels of these proteins in VUTR mice than in the wildtype (Fig 17).

**Versican 3'UTR enhances NIH3T3 cell migration**

To test the effects of versican 3'UTR on cell activities, we stably transfected NIH3T3 fibroblasts with the versican 3'UTR construct or a control vector. We confirmed expression of the VerUTR construct by real-time PCR (Fig 18). VerUTR-transfected cells were then examined for their effect using an in vitro wound healing assay. We found that VerUTR-transfected cells showed increased migration into the wounded areas than the control cells (Fig 19). We also found that the VerUTR-transfected cells adhered to tissue culture plates slower than the vector control after cell inoculation (Fig 20). This appeared to be consistent with the previously observed increase in migratory capacity.

**Versican 3'UTR regulates microRNA functions**

Analysis with computational algorithms revealed many potential binding microRNAs on the versican 3'UTR. We hypothesized that overexpression of the versican 3'UTR would bind and arrest the function of these microRNAs, resulting in increased versican expression. In
Fig 16. Enhanced expression of CD34 in VerUTR transgenic mice. (A) Wound tissue samples were subject to immunohistochemistry analysis. Expressions of CD34 increased in the VerUTR transgenic mice (arrows). Scale bar, 50 μm. (B) The positive CD34 stained blood vessels were quantified. **p<0.05
Fig 17. Enhanced expression of α-SMA, vimentin, and N-Cadherin in VerUTR transgenic mice. Wound tissue samples were subjected to immunohistochemistry analysis. Expressions of alpha-smooth muscle actin (A), vimentin (B), and N-Cadherin (C) increased in the VerUTR transgenic mice. Scale bar, 50 μm. The increased levels of the proteins were quantified (right panels). **p<0.05
Fig 18. Enhanced expression of fibronectin and collagen I in VerUTR transgenic mice.
Wound tissue samples were subjected to immunohistochemistry analysis. Expressions of fibronectin (A) and type I collagen (B) increased in the VerUTR transgenic mice. Scale bar, 50 μm. The increased levels of the proteins were quantified (right panels). **p<0.01, *p<0.05
**Fig 19. NIH3T3-VerUTR cell migration.** (A) Total RNAs were isolated from NIH3T3 fibroblasts transfected with VerUTR or the control vector, followed by real-time PCR analysis of the expression of the versican 3'UTR. n=3, **p<0.01. (B) VerUTR- and vector-transfected cells (4×10^5) were seeded onto 6-well dishes in 10% FBS/DMEM medium and maintained at 37°C till 95% confluence. The monolayer cells were wounded and cultured in 10% FBS/DMEM medium with 5 μM mytomycin for migration assays. Cells were photographed every 24 hours. Expression of VerUTR promoted cell migration. **p<0.01. Error bars indicate SD (n=3). (C) Typical photos of cell migration.
**Fig 20. Expression of VerUTR decreased cell attachment.** (A) VerUTR- and vector-transfected cells were inoculated in tissue culture dishes overnight. Cell attachment was examined under a light microscope. The VerUTR cells exhibited decreased cell attachment. (B) The number of adhered cells was quantified (lower panel). \( n=5 \), ** \( p<0.01 \)
addition, these microRNAs could also bind to mRNAs associated with wound repair, resulting in up-regulation of the associated molecules. We listed all these microRNAs and checked their potential targets associated with wound repair. We found that five microRNAs, miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690, showed potential binding sites on versican 3’UTR (Fig 21). These microRNAs also showed potential binding sites on β-catenin (Fig 25).

We hypothesized that overexpression of the versican 3’UTR would bind miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690. As a consequence, the function of these five microRNAs normally expressed by the cells would be arrested. As a result, the endogenous mRNAs of versican and β-catenin were free of targeting by these microRNAs, resulting in increased expression of versican and β-catenin (Fig 22).

To confirm the direct targeting of miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690 on versican 3’UTR, a series of luciferase assays were performed with each microRNA. Luciferase expression constructs were generated, each containing a versican 3’UTR fragment harboring the target sites of these microRNAs, resulting in Luc-Ver\(^{185}\), Luc-Ver\(^{203*}\), Luc-Ver\(^{434-3p}\), Luc-Ver\(^{680}\), and Luc-Ver\(^{690}\). In addition, five mutant constructs were generated, in which each of the potential target site was mutated resulting in Luc-Ver\(^{185\text{-mut}}\), Luc-Ver\(^{203^*\text{-mut}}\), Luc-Ver\(^{434-3p\text{-mut}}\), Luc-Ver\(^{680\text{-mut}}\), and Luc-Ver\(^{690\text{-mut}}\). The mutated sequences are shown in red and the structures of the constructs are shown (Fig 23).

In the luciferase assays, all predicted micrRNAs were found to bind to the versican 3’UTR linked to the luciferase reporter construct resulting in repression of luciferase activities (Fig 24). When the microRNA binding sites were mutated, there was a restoration in luciferase
Fig 21. Generation of luciferase constructs harboring VerUTR. (A) Potential sites in versican 3’UTR targeted by miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690. (B) The 3’UTR fragments of versican were cloned into the luciferase reporter vector pMir-Report resulting in 5 constructs. Mutated sequences (bold and underlined) were generated in the seed regions to abolish binding of the corresponding microRNAs.
Fig 22. Versican 3’UTR interacts with multiple microRNAs. We hypothesized that overexpression of versican 3’UTR would bind endogenous miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690, and thus free versican and b-catenin mRNAs for protein translation. The dot lines represent versican 3’UTR expressed by the CMV promoter.
Fig 23. Sequences of luciferase constructs harboring VarUTR fragments.
Fig 24. Luciferase assays confirming VerUTR targeting. NIH3T3 fibroblasts were cotransfected with different microRNA mimics and the luciferase reporter constructs harbouring versican or mutant versican 3'UTR fragments. A non-related fragment of cDNA was used as a control. Luciferase activity assays indicated that all 5 microRNAs, miRNA-185 (A), miRNA-203* (B), miRNA-434-3p (C), miRNA-680 (D), and miRNA-690 (E) repressed luciferase activities when it harbored the corresponding versican 3'UTR compared with the control (ctrl), which were reversed when the potential microRNA target site was mutated. n=3, *p<0.05, **p<0.01.
activity.

To confirm the targeting of miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690 on β-catenin 3’UTR, five luciferase constructs, Luc-Cat\textsuperscript{185}, Luc-Cat\textsuperscript{203*}, Luc-Cat\textsuperscript{434-3p}, Luc-Cat\textsuperscript{680}, and Luc-Cat\textsuperscript{690}, were generated, each containing a fragment of β-catenin 3’UTR, which harbored the target sites of these microRNAs. Five mutant constructs, Luc-Cat\textsuperscript{185-mut}, Luc-Cat\textsuperscript{203*-mut}, Luc-Cat\textsuperscript{434-3p-mut}, Luc-Cat\textsuperscript{680-mut}, and Luc-Cat\textsuperscript{690-mut}, were generated, in which each of the target site was mutated (Fig 25, 26). Luciferase activity assays indicated that all predicted microRNAs bound to the β-catenin 3’UTR linked to the luciferase reporter construct, resulting in decreased luciferase activities (Fig 27). When the microRNA binding sites were mutated, normal luciferase activities were restored. We confirmed expression of the versican 3’UTR competing microRNAs, including miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690, in wildtype and transgenic mice as well as in mock- and versican 3’UTR-transfected NIH3T3 fibroblasts. Although they were found expressed at different levels, all of them could be detected in the mice and the cells (Fig 28).

**Confirmation of versican 3’UTR effects**

In this study, we hypothesized that expression of versican 3’UTR would compete with endogenous versican and β-catenin for microRNA binding. To test this hypothesis, we analyzed expression of both versican and β-catenin in the VerUTR transgenic and wild type mice, and subject both to wound healing assays. It was found that the levels of versican and β-catenin were higher in the VerUTR transgenic mice than in the wild type mice (Fig 29). Cell lysate was also prepared from cells transfected with VerUTR or the control vector and subject to Western blot analysis probed with an anti-versican antibody. Transfection with VerUTR was found to
promote versican expression (Fig 30). Similarly, when probed with anti-β-catenin antibody, VerUTR cell lysate also revealed higher levels of β-catenin than the control (Fig 31). To test the effect of versican and β-catenin on cell migration, we transfected NIH3T3 fibroblasts with siRNAs targeting endogenous versican and β-catenin and found that silencing versican (Fig 32A) and β-catenin (Fig 32A) decreased cell migration (Fig 32B).

Small-interfering RNA (siRNA) sequences were designed to bind to the versican 3’UTR region. An unrelated oligo was used as a control. The effects of the siRNA were tested. Western blot analysis was performed and the siRNA treated cells were found to express decreased levels of versican (Fig 9A, upper). As well, expression of β-catenin was down-regulated by siRNA transfection (Fig 9A, lower). The siRNAs were then used for functional assays. In cell migration assay, we detected decreased migration activity in the VerUTR cells transfected with the siRNA compared with the control oligo (Fig 33)
Fig 25. Generation of luciferase constructs harboring β-catenin 3’UTR. (A) Potential sites in β-catenin 3’UTR targeted by miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-69. (B) The 3’UTR fragments of β-catenin were cloned and mutations were generated as shown.
Fig 26. Sequences of luciferase constructs harboring fragments of β-catenin 3'UTR.
Fig 27. Luciferase assays confirming β-catenin targeting. NIIHT3 fibroblasts were co-transfected with different microRNA mimics and the luciferase reporter constructs harbouring the β-catenin 3’UTR or mutant 3’UTR fragments. Luciferase activity assays indicated that all 5 microRNAs, miRNA-185 (A), miRNA-203* (B), miRNA-434-3p (C), miRNA-680 (D), and miRNA-690 (E) repressed luciferase activities when it harbored the corresponding versican 3’UTR compared with the control (ctrl), which were reversed when the potential microRNA target site was mutated. n=3, *p<0.05, **p<0.01.
Fig 28. Expression of VerUTR binding microRNAs. Levels of microRNAs were measured in the VerUTR- and vector-transfected NIH3T3 fibroblasts and in the VerUTR transgenic and wildtype mice by real-time PCR.
Fig 29. Analysis of versican and β-catenin expression by immunohistochemistry. The wounded tissue sections were immunohistochemically stained with antibody against versican (A) and β-catenin (B). VUTR transgenic sections showed higher levels of these proteins than the control sections (upper). The intensity of staining was quantified using NIH ImageJ software (lower). Scale bar, 50 μm.
Fig 30. Silencing endogenous versican decreased cell migration. (A) Cell lysate prepared from cells transfected with VerUTR or the control vector was subject to Western blot analysis probed with antibody against versican. Transfection with VerUTR promoted versican expression. (B) NIH3T3 fibroblasts were transfected with three siRNAs targeting endogenous versican for cell migration assay. Silencing versican decreased cell migration. n=3, **p<0.01.
Fig 31. Silencing endogenous β-catenin decreased cell migration. (A) Cell lysate prepared from cells transfected with VerUTR or the control vector was subject to Western blot analysis probed with antibody against β-catenin. Transfection with VerUTR promoted β-catenin expression. (B) NIH3T3 fibroblasts were transfected with three siRNAs targeting endogenous β-catenin for cell migration assay. Silencing β-catenin decreased cell migration. n=3, **p<0.01.
Fig 32. Confirmation of versican 3’UTR mediated effects. (A) The NIH3T3 cells expressing VerUTR were transfected with siRNAs against versican 3’UTR. Cell lysates prepared from siRNA-treated cells were subject to western blot analysis. The levels of versican (upper) and β-catenin (lower) decreased as a result of siRNA targeting VerUTR. (B) NIH3T3 fibroblasts expressing VerUTR were transfected with siRNAs against versican 3’UTR. Cell migration assays were conducted and it was found to have a decreased rate of migration compared with the control. n=3, **p<0.01.
Fig 33. **siRNAs targeting VerUTR decreased cell migration.** NIH3T3 fibroblasts expressing VerUTR were transfected with siRNAs against versican 3’UTR or a control oligo. Cell migration assays were conducted and it was found that transfection with siRNAs targeting versican 3’UTR decreased cell migration compared with the control.
4.5 DISCUSSION AND CONCLUSIONS

Our studies showed that ectopic expression of the versican 3’UTR led to increased expression of endogenous versican. Modulating endogenous microRNA provides an ideal approach to study the functions of this large proteoglycan. This technique allows the study of the overexpression of versican with normal physiological functions. Interestingly, ectopic expression of the versican 3’UTR was also shown to enhance β-catenin expression. Since β-catenin has been found to regulate versican expression through promoter interactions (Rahmani et al. 2005), our experiments have characterized a novel feedback loop by which β-catenin and versican levels are continuously regulated. The 3’-untranslated region of the versican transcript, in addition to down-regulating protein expression through canonical microRNA functioning, also act to co-regulate transcripts which are targeted by the same pool of microRNA. Among others (Lee et al. 2010), this includes the mRNA of β-catenin. We demonstrated that both the versican 3’UTR and β-catenin 3’UTR shared common binding sites for miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690. It appeared that over-expression of the versican 3’UTR bound and inhibited the activity of these microRNAs, resulting in promotion of versican and β-catenin expression. Through various in vitro and in vivo functional assays, we found that fibroblast migration and dermal wound healing process were promoted. The physiological relevance of our study is strongly supported by the literature showing that up-regulation of versican and β-catenin plays important roles in cell migration and wound repair.

Versican was originally cloned from human fibroblasts and developing chicken limbs (Dours-Zimmermann and Zimmermann 1994). Versican is also expressed in many other tissues including embryos, the developing retina, skin, hair follicles, and arterial smooth muscles
(Yamagata et al. 1986; Yao et al. 1994; Zako et al. 1995). It has been reported that versican can associate with a number of ECM and cell surface molecules. With the interaction with these cell surface or extracellular molecules, versican can form a pericellular coat to support cell adhesion and migration (Ricciardelli et al. 2007). Due to the interaction of versican and its associated molecules with water, a hydrated gel can be formed to support cell migration and wound repair (Wight and Merrilees 2004).

The promotion of versican on wound repair was also supported by its direct roles in a variety of cellular activities. Ectopic expression of a recombinant mini-versican expression construct in NIH3T3 fibroblasts was found to enhance cell proliferation, while deletion of the EGF-like motifs from the versican G3 domain reduced this effect on cell growth. Overexpression of versican G1 domain construct is shown to reduce cell adhesion and increase cell proliferation (Yang et al. 1999). Versican G3 domain can bind to β1-integrin and mediates cell adhesion (Wu et al. 2002). When expressed in NIH3T3 cells, the V1 isoform of versican can enhance cell proliferation and protect cells from apoptosis (Sheng et al. 2005). In addition, Expression of the V1 isoform can induce mesenchymal-epithelial transition (MET) in NIH3T3 fibroblasts (Sheng et al. 2006). This was associated with morphological changes and dramatic alterations in both membrane and cytoskeleton architecture. Molecular analysis showed that V1 promoted a “switch” in cadherin expression from N- to E-cadherin, resulting in epithelial specific adhesion junction. Thus, increased expression of versican not only enhanced cell migration and wound repair, but also facilitated a variety of cell activity in fibroblasts associated with the wound healing process. In wound healing, fibroblasts play a crucial role in wound closure. Fibroblasts are responsible for most collagen and elastin synthesis, as well as organization of the ECM components.
β-Catenin is extensively expressed in tissues and serves diverse functions. Structurally, β-catenin is localized mainly to the adherent junctions, where it is bound to the cytoplasmic domain of E-cadherin and mediates cell-cell adhesion (Clevers 2006). While forming the complex with E-cadherin, β-catenin mediates the canonical WNT signaling pathway. After being activated, β-catenin translocates to the nucleus where it binds to the TCF/LEF family of transcription factors and triggers expression of the target genes. The signaling pathway of WNT/β-catenin complex plays roles in tissue injury and repair. In addition, β-catenin also mediates TGF-β1 signaling pathway in wound healing. TGF-β1 can substantially activate β-catenin signaling in primary fibroblast cultures (Cheon et al. 2004). TGF-β1-mediated fibroblast proliferation and migration are dependent on β-catenin expression, confirming the central roles of β-catenin in wound repair (Cheon et al. 2002). TGF-β1, a central regulator in wound healing is also known to enhance versican synthesis, thus playing a role in wound repair.

In mesenchymal stem cells, TGF-β1 activates β-catenin signaling pathway to control the self-renewal and osteogenic differentiation of mesenchymal stem cells (Jian et al. 2006). TGF-β1 can lead to the disassembly of the β-catenin/cadherin complex and facilitate cell migration (Nelson and Nusse 2004). In bone fracture repair, β-catenin plays an important role in the healing process (Chen et al. 2007). WNT/β-Catenin signaling has also been shown to enhance bone mass and density (Glass et al. 2005). In emphysema, decreased β-catenin signaling causes dysfunctional repair (Kneidinger et al. 2011). In animal and patients with acute lung injury, up-regulation of β-catenin was found to play roles in the healing processes (Adamson et al. 1999). Given its critical role in the healing process, inhibition of β-catenin signaling is understood to impair epithelial repair. However, mutation of AXIN2, in a component of the signaling pathway, stabilizes β-catenin and activates β-catenin/T-cell factor signaling, leading to the development of
colorectal cancer (Lammi et al. 2004). It is conceivable that up-regulation of β-catenin expression by ectopic transfection of versican 3’UTR would promote cell migration and tissue repair. Our study has raised a point for further consideration: whether transcripts that encode proteins with similar functions or proteins in the same signal pathway can bind to common microRNAs, forming a signaling network for gene regulation. This awaits further investigation.

To uncover the mechanism by which expression of versican 3’UTR promoted cell migration and wound healing, we screened for microRNAs that targeted versican as well as related molecules playing similar roles leading to these phenotypes. By using the computational algorithm FindTar (http://bio.sz.tsinghua.edu.cn/findtar), a long list of microRNAs was revealed. We then analyzed microRNAs that could potentially target molecules playing similar roles in cell migration and wound healing. Interestingly, β-catenin 3’UTR was found to share five common microRNAs with versican 3’UTR including miRNA-185, miRNA-203*, miRNA-434-3p, miRNA-680, and miRNA-690. While the functions of miRNA-434-3p, miRNA-680, and miRNA-690 have not been reported, miRNA-203 is understood to be a skin-specific microRNA (Yi et al. 2008). miRNA-203 can promote epidermal differentiation and restrict proliferative potential thus inducing cell-cycle exit. This result suggests that increased activity of miRNA-203 may in fact slow down tissue repair. Our results indicate that versican 3’UTR expression enhanced cell migration and wound repair. Since miRNA-203 is among the microRNAs binding to versican 3’UTR, arresting the functions of this skin-specific microRNA would predictably be associated with enhanced cell migration and wound repair. In addition, amongst the microRNAs involved in this study, miRNA-185 has attracted extensively attention. miRNA-185 can induce cell cycle arrest and suppress cell growth (Takahashi et al. 2009) It can also target c-Myc, RhoA and Cdc42 and inhibit cell proliferation (Liu et al. 2011; Liao and Lu 2011). Thus, it appears
that expression of the versican 3’UTR could enhance wound repair by repressing miRNA-185 activity.

Our study also raises the question of the role of the versican 3’untranslated region in fibrotic wound healing. In addition to the effects of β-catenin on TGF-β1 signaling and versican signaling, we characterize a novel feedback loop by which the versican 3’UTR up-regulate signaling proteins upstream of versican. This has implications in pathological fibrosis, which awaits further study. Indeed, versican, TGF-β1 and β-catenin signaling have been implicated in various levels of tissue fibrosis (Yang et al. 2012). Understanding the functions of whole transcripts will be invaluable in gene targeting and therapeutic development. Our work demonstrates the importance of RNA signaling networks in wound healing, both in vitro and in vivo.

In conclusion, we have demonstrated that ectopic expression of versican 3’UTR promoted cell migration and wound repair by arresting the activities of the binding microRNAs. This led to the increased expression of β-catenin. Increased expression of versican and β-catenin and repression of the binding microRNA activity facilitated cell migration and wound repair. Using this approach, it becomes possible to manipulate the function of any endogenous mature microRNAs.
Chapter 5

GENERAL DISCUSSION

While appearing to be disparate biological processes, wound healing and tumor progression utilize many similar molecular mechanisms. In particular, versican, a chondroitin sulfate proteoglycan has been shown to be upregulated in both of these contexts. We explored versican as both a coding and non-coding gene, given its versatile role in the extracellular matrix. This project served to investigate the potential roles of versican in two unique biological contexts. Firstly, we sought to investigate the potential role of versican V2 in glioblastoma by utilizing an overexpression construct in a cell-based model and tumor xenograft. To this end, we demonstrated that this particular splice isoform expressed in the mature brain could enhance angiogenesis and may play a key role in the poor prognosis of glioblastoma.

Secondly, a plasmid over-expression construct harboring the 3’-untranslated region of the proteoglycan versican was utilized to study the role of versican in cutaneous wound healing. We demonstrated that the overexpression of versican accelerated the rate of wound closure in our mouse model. More importantly, we validated the use of the 3’-untranslated region to increase the expression of otherwise difficult to study proteins, such as proteoglycans.

The methods utilized and results of these projects are relevant for further studies of proteoglycans and microRNAs, and will also be discussed in greater detail here.
5.1 Tumor progression and wound healing

In addition to the uncontrolled proliferation of cancer cells, the growth of solid tumors requires active participation by non-transformed cells such as fibroblasts, vascular endothelium, smooth muscle, and immune cells—components also utilized in physiological wound healing. Given that wound healing involves the formation of new tissue from existing quiescent tissue, it has been reasoned that tumors resemble wounds that do not heal (Dvorak 1986).

A study by Chang et al. examined the genetic response of serum treatment on fibroblasts isolated from five different tissues, as well as tumor samples obtained from these tissues (Chang et al. 2004)). Indeed we have demonstrated another mechanism by which the versican gene, VCAN, may function. In addition to playing a coding role, through the translation of different versican splice isoforms, this gene may in fact play a non-coding role through its various microRNA response elements. This additional layer of complexity may contribute to the multifaceted aspects of this proteoglycan plays in the extracellular matrix.

Examining both wound healing and tumor progression provides a better context by which to explore the continuum of roles that versican can play. While we have not definitely demonstrated that tumors act as wounds that do not heal, we have characterized one CSPG that has shown to be dramatically upregulated in both of these processes.

5.2 Technical Limitations

To evaluate whether the V2 splice isoform played a role in glioblastoma angiogenesis, we stably transfected an expression construct into the U87 glioblastoma cells, an immortalized cancer cell line.
One concern is that the use of an immortalized cell line may not accurately represent the diversity and heterogeneity that is observed within human tumors. The genetic and epigenetic heterogeneity that typically arises from genomic instability in cancer, leads to distinct morphological phenotypes. These can include variations in metabolism, proliferative capacity as well as metastatic potential. It is believed that this variation is observed both within an individual tumor, and between tumors. This poses significant challenges when designing treatments against cancer and when identifying clinically relevant biomarkers. Adding a further layer of complexity is the diversity that can occur in the tissue surrounding a solid tumor, the tumor microenvironment. These differences impose a range of selective pressures which can lead to a range of tumor sub-clones, even within the confined region of a solid tumor.

Given this complexity, producing clinically relevant model systems for studying cancer has remained a challenge for scientific research. While the use of immortalized cell lines is an accessible and well-established model for exploring cancer biology and testing the use of potential anti-cancer drugs, these cells do not properly capture the biological complexity that is observed in cancer. Furthermore, given the recent advances in personalized cancer therapy, immortalized cell lines are not an ideal model system and cannot meet the needs of these personalized therapies.

The use of primary cells isolated from patient tumors will be important in personalized cancer therapy. However the use of primary cells in research needs to be performed carefully, ideally with minimal cell passages which can impose unintended selective pressures. Challenges surrounding the use of primary cells can also include the variability in phenotype, which can result from the tremendous genetic diversity observed in patient cancer cells. Given that our research project was designed to study the function of one splice isoform of versican, the use of a
well-established model system was better suited to our research goal. Thus the use of the U87 cell line, a well-studied glioblastoma model system was selected, to reduce any confounding factors that may have been associated with the use of primary cells.

The use of a tumor xenograft as a model system is another factor to be considered when considering the results and their biological context. While the use of an ectopic xenograft model provides an accessible method for assessing tumorigenicity by palpation, this system may not accurately reflect the tumor microenvironment that leads to the development of tumor cells in patients. Ideally, a combination of both ectopic and orthotopic models of tumor xenografts should be used when studying genetically altered tumors.

Orthotopic models present a greater degree of complexity relative to xenograft models. Implanting tumor cells into their original tissues allows the growth of tumors which are comparable to their human counterparts and have similar incidences of tumor metastasis. Conversely, in classical ectopic models, injected tumors do not typically lead to metastasis. Currently, the National Cancer Institute recommends that in vitro molecules must demonstrate their efficacy in orthotopic xenograft tumor cell lines in nude mice, prior to pre-clinical and clinical development. In these instances, the tested substances are injected intra-peritonealy and tumor regression is analyzed by in vivo imaging or by sacrificing the animals. In general, orthotopic tumor models are considered to be more clinically relevant and are better predictive models of efficacy than standard subcutaneous models. In terms of assessing drug efficacy for tumor cells, orthotopic models provide a useful source of data if utilized correctly.

In this study, versican transgene was used to ectopically overexpress the V2 splice isoform in U87 glioblastoma cells. The use of a transgene is effective for producing high levels
of protein expression which can be useful for producing reliable model systems of disease. Given the localization of the V2 splice isoform in the mature brain, we looked to study the effects by overexpressing this coding sequence under a strong promoter. The use of this system can allow for a robust phenotype that may be observed earlier than in other model systems. Transgenic animal models however, utilize random integration to become incorporated into the host genome. Thus the expression patterns of the overexpressed protein may not be consistent with what is naturally observed.

Alternatively, a ‘knock-in’ model may be utilized to achieve more biologically relevant expression patterns and expression levels. These models utilize targeted knock-in gene vectors which integrate the desired gene into a specific locus using homologous recombination. Gene knock-in technology is often used to study the regulatory mechanisms behind protein expression, specifically the effects of putative promoter and enhancer regions. Most frequently, this technology is utilized to generate mutant mice, given the ability of embryonic stem cells to participate in the formation of germline cells when the mice are transplanted into an early embryo. The major hurdles associated with generating gene targeted mice are obtaining the correct homologous recombination event and obtaining successful germline transmission.

This technology can also be utilized to generate knock-in cell lines which can be enriched by incorporating a selectable marker into the target vector, and subsequently the desired genetic locus. From the obtained enriched set of cell lines, a molecular analysis of genomic DNA is performed to isolate the cells which have undergone the desired homologous recombination event. Given that our V2 research project was designed primarily to study the overexpression of this proteoglycan, a transgenic cell model was appropriate and could produce a robust and verifiable phenotype.
The different tools available in reverse genetic approaches have provided valuable insights into the functioning of versican. The use of a versican knockout model has previously been reported, using a targeting vector with the phosphoglycerate kinase promoter-driven neomycin resistance gene, inserted into exon 3 of the versican coding region. Backcrossing the heterozygotes with C57BL/6 mouse 16 times revealed that all of the homozygotes died within the early embryonic stage (E10.5). The homozygotes were small in size and had dilated hearts as compared to the wildtype mice. These mice displayed normal cardiac looping but had ventricular septal defects, suggesting that versican and proteoglycan aggregates are necessary in the atrioregular canal cushion and ventricular septa (Hatano, 2012). Thus, a versican knockout mouse model is unavailable given the associated defects in heart function and blood vessel formation.

To circumvent the embryonic lethality of a complete versican knockout mouse model, conditional knockout models have been utilized. The use of a site-specific Cre recombinase driven by the Prx1 was reported, generating Prx1-Cre/Vcan(flox/flox) mouse. These mice were fertile and viable, but histological analysis of newborn mice revealed hypertrophic chondrocytic nodules in the cartilage, joint tilting, and a slight temporal delay in chondrocyte differentiation. Immunohistochemistry of Prx-Cre/Vcan(+/+) revealed that these digits contained an accumulation of versican protein, concommitant with increased expression of TGF-beta. Conversely, the Prx1-Cre/Vcan(flox/flox) mice without versican expression displayed decreased incorporation of TGF-beta. These results implied that versican facilitates chondrogenesis and joint morphogenesis, through the localization of TGF-beta (Choocheep, 2010). The use of conditional knockout models can provide tremendous insight into versican knockdown beyond the developmental window where they are critically needed.
While the complete knockout of versican has shown to be embryonic lethal, strategies have been developed to knock out specific isoforms of versican. Importantly, the genetic knock out of versican V0/V2 has been previously reported. A targeting vector containing a floxed neomycin thymidine-kinase under the control of the Herpes Simplex virus thymidine kinase promoter was used to specifically target the versican V0/V2 isoforms. This was done by utilizing two 3.9 kb genomic arms corresponding to portions of exon VII, which codes for the GAG-alpha region, and adjacent intronic regions, into the targeting vector. Using this specifically targeted vector, an ER-retention signal and early translational stop codon was introduced into exon VII. This targeted vector was introduced into embryonic stem cells by electroporation and ES cells that had undergone the desired homologous recombination events were selected and identified by Southern Blot. These ES cells were then injected into C57BL/6 blastocysts and transplanted into a foster mother. The floxed neo-tk cassette was removed by crossing the V0/V2 nco-tk mice with mice expressing Crc under the control of a strong CMV promoter (María T Dours-Zimmermann et al. 2009).

This mouse model was used to explore the role of versican V0/V2 during post-natal development. Given the splice sites of the versican coding transcript, a concomitant elimination of the largest versican isoform (V0), along with versican V2 knockout, was not preventable. While the mice were viable and fertile, they displayed significant alterations to the formation of extracellular matrix surrounding the Nodes of Ranvier. The clustering of nodal sodium channels and the paranodal structures were not affected. The knockout of versican V2 was concomitant with a loss of tenascin-R and phosphacan from the perinodal matrix, strongly suggesting that versican V2 may organize the ECM in vivo, possibly through the control of a nodal receptor.
Chapter 6

FUTURE DIRECTIONS

6.1 Glycosaminoglycans as Inhibitors of Angiogenesis

Pharmacologic inhibition of angiogenesis is among the most well studied approaches to the treatment of solid tumors. Currently, there is active research into the use of heparin-derived substances to modulate angiogenesis that is dependent on cell-surface heparan sulfate proteoglycans (Koo et al. 2008). Heparin is a highly negatively charged sulfated glycosaminoglycan and is typically used as an injectable anticoagulant in humans or to produce an anticoagulant surface in medical devices. Heparin is stored in basophils and mast cells and released by secretory granules into circulation during tissue injury (Min and Paul 2008). In addition to its function as an anticoagulant, heparin may also act as a defensive mechanism against bacteria or other foreign particles at sites of injury.

For example, heparin sulfate proteoglycans have been shown to promote the growth and metastasis of myeloma and breast cancers which are prone to bone metastases. This effect is likely due to the cleavage product of syndecan-1 which favours the tumor microenvironment (Sanderson et al. 2004). In terms of mechanism, the anti-angiogenic effects of heparin-derived molecules are likely due a heparin dependent release of Tissue Factor Pathway Inhibitor which is known to modulate blood coagulation (Kemme et al. 2002).

The potential is great for developing heparin-like molecules for the treatment of cancer, as well as other diseases including Adult Respiratory Distress Syndrome (Hofstra et al. 2012) and Inflammatory Bowel Disease (Y.S. Ang et al. 2000). Importantly however, the anti-
coagulant effects of injectable heparin may represent an undesirable outcome in cancer treatments, leading to excessive bleeding in patients. Thus the development of heparin-derivatives with low anti-coagulant activity, which can act as competitive inhibitors of angiogenic factors such as fibroblast growth factor-2 or vascular endothelial growth factor could be an alternative approach.

Due to the diverse functions of glycosaminoglycans and proteoglycans, targeting and the use of mimetics should be studied with a number of considerations. Primarily, the context-dependence of proteoglycan expression and function should be examined in detail. For example, particular GAGs may inhibit cancer cell attachment and reduce instances of distant metastasis but may also promote the escape of tumor cells into circulation. Similarly, targeting proteoglycans which function in angiogenesis may have unintended effects if expression is stably reduced. Indeed, these approaches may best be used in combination with conventional treatment modalities providing a synergistic effect in cancer treatment. Given that there are currently no active clinical trials examining the degradation or ectopic expression of proteoglycans, this is an area of study that necessitates further pre-clinical and clinical research.

6.2 Targeting versican for therapeutic purposes

Given the diverse roles that versican has been shown to play in cancer initiation and progression, a number of approaches have been tested to target versican for therapeutic uses, with hope of producing clinically relevant treatment modalities.
6.2.1 Targeting versican in synthesis

Targeting versican synthesis is believed to be a potential measure in reducing the biological functions of this tumor promoting agent. The effects of growth factor and cytokine signaling such as PDGF, TGF-β, EGF, VEGF and IL-1β which promote versican expression, can be reversed by following treatment with various tyrosine kinase inhibitors (Shimizu-Hirota et al. 2001). Blocking these growth factors and cytokines with specific tyrosine kinase inhibitors has been effective in certain cases. The tyrosine kinase inhibitor genistein has been shown to block versican expression induced by growth factors in malignant mesothelioma cell lines (Syrokou et al. 1999). In vascular SMC, genistein inhibits PDGF-stimulated versican expression in a dose-dependent manner (Schönherr, Kinsella, and Wight 1997). In SMC, the tyrosine kinase inhibitor herbimycin A, the mitogen-activated protein kinase inhibitor PD98059, and the EGF receptor inhibitor AG1478 have been reported to reduce angiotensin II enhanced versican expression (Shimizu-Hirota et al., 2001). In addition to selective tyrosine kinase inhibitors, these growth factors can also be inhibited at the translational level by antisense oligonucleotides or blocked via monoclonal antibodies which inhibit the ligand-receptor interaction (Theocharis, 2008). However, there is no studies report whether these therapeutic approaches are effective in modulating in vivo versican expression.

Chronic inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease are characterized by airway remodeling with altered ECM deposition, especially versican (Bensadoun et al. 1996; Passi et al. 1999; R. Huang et al. 2006). Recent studies have revealed that alteration of versican deposition in asthmatic airways can be inhibited by a number or asthma drugs. Montelukast, a leukotriene receptor antagonist, inhibits versican expression in both bronchial and arterial SMCs (Potter-Perigo et al. 2004). Other asthma drugs such as
Budesonide and Formoterol can repress versican deposition in human lung fibroblasts and airway SMCs (Burgess et al. 2006). Further studies are needed to evaluate the effects of these drugs on expression of versican in malignancy.

Expression of versican can be modulated post-transcriptionally by a number of microRNAs such as miRNA-143, miRNA-138 and miRNA-199a-3p (Lee et al. 2009; Wang, Hu, and Zhou 2010; Morton et al. 2008). Theses microRNAs are considered to be tumor-suppressors, often downregulated in malignant tumors (Lee et al. 2009). MicroRNA-based therapeutic strategy for targeting versican can be introduced by short double-stranded synthetic RNA loaded into an RNA-induced silencing complex. Another strategy utilizes the expression of hairpin pre-microRNA in a viral vector system, which leads to increased expression of these tumor-suppressor microRNAs (Seto 2010).

6.2.2 Targeting versican in processing

With increased knowledge of the roles of versican proteolytic fragments in cancer progression, current studies highlight targeting versican processing as a novel strategy to prevent and control cancer cell invasion and metastasis. Antibodies against to the ADAMTS specific versican cleavage site inhibit glioma cell migration in a dose-dependent manner (Arslan et al. 2007). GM6001 (Galardin), a MMPs and ADAMTS proteases inhibitor, has been shown to inhibit cancer cell invasion and metastasis in several kinds of carcinoma (Almholt et al. 2008; Hao et al. 1999). Other protease inhibitors such as catechin gallate esters, present in natural sources including green tea, have been shown to selectively inhibit ADAMTS-1, -4 and -5 catabolism (Vankemmelbeke et al. 2003). Versican G3 fragments have been known to enhance cancer cell growth, invasion, metastasis, and chemical resistance via EGFR signaling (Du et al.
The selective EGF receptor inhibitor, AG1478 prevents G3 fragment enhanced cell growth, migration, invasion and chemical resistance in vitro (Du et al. 2010). Application of versican specific protease inhibitors or proteolytic fragments involved in cell signaling pathways can be further explored. Preventing versican catabolism and proteolytic fragment accumulation may provide novel therapeutic targets for cancer invasion and metastasis.

6.2.3 Targeting versican and its binding molecules

The unique and versatile structure of versican lends itself to multiple types of interactions through either protein–protein or protein–carbohydrate interactions. As mentioned previously, the versican G1 domain can interact with HA and CD44, forming a polarized pericellular sheath mediated around tumor cells, thus promoting their motility, invasion and metastasis (Matsumoto et al. 2003; Ricciardelli et al. 2007). It has been found that tumor cell formation of the pericellular matrix with HA and versican can be inhibited by treatment with HA oligomers (Evanko, Angello, and Wight 1999). HA oligomers can block the interaction between HA and versican, and are promising inhibitors of cancer dissemination (Ween, Oehler, and Ricciardelli 2011). Disruption of the HA-CD44 interaction with HA oligomers has been reported to significantly inhibit the growth of B16F16 melanoma cells (Zeng et al. 1998). Therefore the application of HA oligomers is an attractive agent for inhibiting the formation of versican-HA-CD44 complexes, providing valuable targets against cancer metastasis.

6.2.4 Targeting versican's chondroitin sulfate chains

Increasing evidence has revealed that versican CS plays an important role in cancer biology as it is involved in interactions with tumor cells and related molecules, such as growth factors and cytokines (Mikami and Kitagawa 2013). Thus in theory, the roles of CS in
promoting tumor growth, invasion and metastasis, could be through chemical modification of CS, or through CS with altered sulfation patterns. A recent study showed that modified CS injected directly into nude mice breast tumors reduced or abolished cancer cell growth without apparent toxic effects to adjacent tissue (Pumphrey et al. 2002). The further development of CS targeted anticancer drugs delivered using cationic liposomes may further represent a novel strategy to prevent local tumor growth and metastasis (Lee et al. 2002).

The multifaceted roles of versican in regulating cell behavior are critical in tumor development. Key processes such as tumorigenesis, angiogenesis and metastasis have all been shown to be mediated through versican. In addition to understanding the tissue and isoform specific functions of versican, it will be important to identify strategies for the therapeutic targeting of this proteoglycan. Understanding versican's wide array of regulatory mechanisms will provide a rational basis for further clinical development.

6.3 Translation of microRNA research

Since microRNAs were first brought to the forefront of biomedical research in 2002, there has been an explosion of interest and research in this field. MicroRNAs involved in physiological regulation as well as pathological conditions have been identified. Given this, there has been tremendous excitement as well as investment into the translation of microRNA research into the clinical realm.

6.3.1 MicroRNAs in therapy

Generally, a single microRNA is predicted to have hundreds of biological targets. Computational algorithms provide significant insights into annotating microRNAs and predicting
functional targets, but there are still a number of considerations to be made. Firstly, expression levels of microRNAs can vary between tissue and cell type, and between pathological timeframes. Secondly, the targeting capacity of microRNAs may be reduced depending on the state of the sequence harboring the MREs. For example, 3'-untranslated regions in cancer cells are known to become shortened over time, reducing the potential effects of microRNA binding. Similarly, point mutations in 3'-UTR sequences can have a similar effect. In fact, many single nucleotide polymorphisms in 3'-UTR sequences have been shown to be linked with diseases including diseases including human acute myeloid leukemia, alpha-thalassemia, neuroblastoma, and keratinopathy (Chatterjee and Pal 2009).

Given the large number of predicted targets for any microRNA, it is likely that only a fraction play a functional biological role. The idea of using microRNA in therapeutics is appealing, given that a single target is not inhibited as is the case for protein inhibitors or conventional RNAi strategies. In contrast, the use of microRNA in therapeutics could potentially lead to the modulation of entire gene programs that become deregulated. While this idea is highly appealing, significantly more research needs to go into the function of individual microRNAs, as well as microRNA delivery, stability and avoiding immune responses associated with gene therapy. Currently, there are a number of microRNA drugs in clinical trials, most notable an inhibitor of microRNA-122, a liver specific microRNA which acts as a key regulator of fatty acid metabolism (Girard et al. 2008). This locked nucleic acid based antisense oligonucleotide was tested in patients with chronic hepatitis C virus (HCV) genotype 1, and showed prolonged dose dependent reductions in HCV RNA levels without any evidence of viral resistance (Gehert et al. 2014).
In addition to microRNA inhibitors, the use of synthetic microRNAs as replacement therapy is also being tested. The principle is to restore levels of microRNAs to that of healthy surrounding tissues. Most notably, miRNA-34a is among the most well-documented tumor-suppressor microRNAs, being a transcriptional product of the tumor suppressor p53 (Hermeking 2010). The introduction of miRNA-34a has shown anti-tumor effects in a number of preclinical models. Unlike anti-miR drugs however, synthetic microRNAs need to be double-stranded for their proper processing. Thus this requires the use of a complex delivery vehicle which is similar to endogenous microRNA microvesicles or exosomes. Accordingly, advances such as nanoparticle based liposomes have been made and clinical trials are currently ongoing (Cheng and Saltzman 2012; Wang, Langer, and Farokhzad 2012; Kota et al. 2009).

6.3.2 MicroRNAs in disease diagnostics

Given the high stability of microRNAs in paraffin-embedded tissue samples and human plasma, the use of microRNAs as diagnostic tools has been highly attractive to researchers. Endogenous microRNAs are highly stable even when secreted into plasma circulation, given their small size. They resist nuclease degradation through enclosure in microvesicles or exosomes. An early report demonstrated that microRNA expression profiles could classify cancers by developmental origin (Lu et al. 2005). Using a bead-based microRNA profiling method, this paper also showed that microRNAs were generally downregulated by cancers (129 out of 217 microRNAs). This paper was the first to highlight the potential clinical application of microRNAs as diagnostic biomarkers. Since this time, a number of microRNAs have been implicated as potential diagnostic tools. For example, ER$^+$/PR$^-$HER2$^+$, ER$^-$/PR$^+$HER$^+$ and ER$^-$/PR$^-$HER2$^-$ breast cancer tumors exhibit distinct microRNA patterns with miRNA-205 being highly
expressed in triple negative breast cancers and miRNA-145 showing the reverse trend (Sempere et al. 2007). miRNA-15a/16-1 has been shown to act as a prognostic biomarker in chronic lymphocytic leukemia (CLL) and let-7a has shown promise as a marker for lung cancer (Hanlon, Rudin, and Harries 2009). MicroRNA expression profiles have also been used to distinguish different forms of heart disease, muscular disorders and neurodegenerative disease. In addition to serum and plasma, microRNAs have also been detected in urine samples with miRNA-126 and miRNA-182 being related to human urinary bladder cancer. Similarly, microRNAs have been detected in saliva samples, with miRNA-125a and miRNA-200a displaying lower expression levels in patients with oral squamous cell carcinoma (Park et al. 2009).

MicroRNA research has advanced significantly since their initial discovery twenty years ago. The use of microRNA inhibitors or synthetic microRNAs in therapy, as well as their use in diagnostics, is particularly exciting and may bring new treatment options within the next decade.

6.4 Future Projects

Having described several potential future pathways of study, there are specifically two interesting areas motivated by this thesis work which may be of promise in the near term. Given that the role of the versican 3'-untranslated region was explored only in physiological wound healing, it would be interesting to explore the role of this noncoding RNA region in pathological or fibrotic wound healing. Versican has been implicated in the fibroblast-myofibroblast transition, which is critical in wound healing, as well as pulmonary fibrosis (Hattori et al. 2011; Bensadoun et al. 1996; Roberts 2003). Myofibroblasts represent the contractile and proliferative phase of fibroblasts, which are normally quiescent and slow to replicate. During normal repair processes, myofibroblasts are lost through apoptosis, and remain as scar tissue. In pathological
fibrosis, these myofibroblasts persist and induce increased extracellular matrix synthesis and tissue contraction. Given this, much research has centred on the differentiation, proliferation, survival and ECM synthesis on myofibroblasts. While these studies focused primarily on the V0 and V1 coding isoforms, understanding whether there is a noncoding role of versican in chronic or pathologic wound healing, as in tumor biology (Tay, Rinn, and Pandolfi 2014; Fang et al. 2013), would provide valuable mechanistic insights. Furthermore, given the critical role of beta-catenin in fibroproliferative diseases (Bowler, O’Gorman, and Gan 2007), and the microRNA feedback loops between versican and β-catenin which we characterized, it appears plausible that versican may play a noncoding role not only in tumor progression, but also in fibrotic disease.
Chapter 7

CONCLUSIONS

Beyond the traditional role of the versican protein product, which we examined in tumor angiogenesis, we hypothesized that the VCAN gene could also play a non-coding function as a sponge for microRNAs, endogenous gene regulators. We examined this idea using a mouse model of cutaneous wound healing. Thus, using our two model systems, we studied versican as both a coding and noncoding gene in the extracellular matrix.

In this thesis, two different models were used to assess the function of versican in different biological contexts. Firstly, an ectopic expression plasmid containing the coding sequence of the versican V2 splice isoform was stably transfected into a primary human glioblastoma cell line. We have demonstrated that the V2 isoform, expressed predominantly in the mature brain plays an important pro-angiogenic role in malignant glioblastomas. These results were confirmed by in vitro cell-based assays as well as in a nude mouse xenograft model. Understanding the dual role of this splice isoform will help guide the identification of potential molecular targets or diagnostic biomarkers for potential clinical applications.

Secondly, we examined the role of versican in cutaneous wound healing by overexpressing the 3'-untranslated region of the coding transcript in murine fibroblasts and a transgenic C57BL/6 mouse model. Both model systems demonstrated that versican expression is upregulated using this strategy, increasing the migratory capacity of fibroblasts and increasing the rate of wound closure in vivo.
Thirdly, through innovative research approaches we have shown that expressing 3’-UTR regions or using similar miR sponging techniques can be used to assess genetic regulatory networks as well as characterize any functional roles for non-coding regions of RNA. Importantly, this research adds to a growing body of literature suggesting transcriptional co-regulation of gene transcripts through microRNA binding.

The observations reported here provide a conceptual framework for understanding the biology of versican and the regulation network of microRNAs. By first understanding mechanisms of physiology and disease, we can further effort in discovery by developing more effective diagnostic biomarkers and therapeutic options directed towards patient care. In particular, understanding versican as both a coding and noncoding gene, may represent another mechanism that allows this versatile proteoglycan to function in the extracellular matrix. An improved understanding of versican and its regulatory pathways may contribute to developing therapeutic modalities to inhibit versican function.
REFERENCES


a Requirement for Versican Proteolysis in Regulating Palatal Mesenchyme Proliferation.”


