SPRR3 REGULATION AND FUNCTION IN THE ATHEROSCLEROTIC MICROENVIRONMENT

By

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To my parents

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I have often said that just as it takes a village to raise a child, it likewise took a huge community to produce this scientist. This is my tribute to that community.

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ABBREVIATIONS

Δ ..... Deletion
AIE ..... Arterial Intima Enriched
Akt ..... v-akt murine thymoma viral oncogene homolog 1
Ang II ..... Angiotensin II
BEC ..... Biliary Epithelial Cells
CABG ..... Coronary Artery Bypass Graft
cAMP ..... Cyclic Adenosine Monophosphate
cDNA ..... Complementary DNA
CE ..... Cornified Envelope
CRP ..... C-Reactive Protein
CS ..... Cyclic Strain
CT ..... Computed Tomography
DAPI ..... 4',6-diamidino-2-phenylindole
DNA ..... Deoxyribonucleic Acid
EC ..... Endothelial Cell
ECM ..... Extracellular Matrix
EMT ..... Epithelial to Mesenchymal Transition
Erk ..... extracellular signal-regulated kinases
FIH ..... Fibrointimal Hyperplasia
FITC ..... Fluorescein isothiocyanate
GSK3 ..... Glycogen Synthase Kinase
HDL ..... High Density Lipoprotein
HRP ..... Horseradish Peroxidase
IL-6 ..... Interleukin-6
ILK ..... Integrin Linked Kinase
IP ..... Immunoprecipitation
kDa ..... kiloDalton
LDL ..... Low Density Lipoprotein
MAPK ..... Mitogen-Activated Protein Kinase
MCP-1 ..... Monocyte Chemoattractant Protein-1
MDC ..... Methyldansylcadaverine
MMP ..... Matrix metalloproteinases
NADPH ..... Nicotinamide adenine dinucleotide phosphate
NF-κB ..... Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NO ..... Nitric Oxide
NOS ..... Nitric Oxide Synthase
PAR-1 ..... Protease Activated Receptor-1
PCR ..... Polymerase Chain Reaction
PDGF ..... Platelet Derived Growth Factor
PDI ..... Protein Disulfide Isomerase
PI3K ..... Phosphoinositide 3-kinases
PKAc ..... cAMP-dependent Protein Kinase
PKC ..... Protein Kinase C
PLL ..... Poly-L-Lysine

qRT-PCR ..... Quantitative Reverse Transcriptase PCR
RGD ..... Arginine-Glycine-Aspartic Acid Polypeptide
RNA ..... Ribonucleic Acid
SDS ..... Sodium Dodecyl Sulfate
SH3 ..... Src Homology Domain 3
SMA ..... Smooth Muscle Alpha-Actin
SM-MHC ..... Smooth Muscle Myosin Heavy Chain
SPRR ..... Small Proline Rich Protein
Tag ..... derived from the H-2K^{b}-tsA58 mouse
TGase ..... Transglutaminase
TGFβ ..... Transforming Growth Factor Beta
TNFα ..... Tumor Necrosis Factor Alpha
VDUP ..... Vitamin-D Upregulated Protein
VEGF ..... Vascular Endothelial Growth Factor
VSMC ..... Vascular Smooth Muscle Cell
vWF ..... vonWillebrand Factor

The Following Are Amino Acid Abbreviations Used in the Text

K ..... Lysine
P ..... Proline
Q ..... Glutamine
RGD ..... Arginine-Glycine-Aspartic Acid Polypeptide
X ..... Aliphatic Amino Acid

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CHAPTER I

INTRODUCTION

Biomechanics play a vital role in vascular biology, affecting the cells of arteries and veins under both physiological and pathological conditions. While many studies have shown that the *development* of atherosclerosis is particularly associated with biomechanical stress, the effect of stress on the cells in an *established* atherosclerotic plaque is less well studied. This document aims to define the role of biomechanics in the vasculature, particularly in vascular smooth muscle cells (VSMCs) of large arteries, in normal and atherosclerotic conditions. This will include a discussion of mechanosensing, mechanotransduction, and the physical adaptations by cells to biomechanical stress. Finally, SPRR3, a mechanically sensitive protein which is localized to atheromas, will be used as an example of VSMC adaptation to stress.

Normal vessel biomechanics

There are two primary stresses experienced by normal large arteries: shear stress and cyclic strain.[1] By contrast, veins experience only very low levels of either stress.[2] Shear stress is the frictional force experienced by the endothelium as blood flows through the lumen. Shear can be laminar, as it is in areas of uninterrupted flow, or turbulent, which occurs at curves or branch points. While endothelial cells (ECs) are the primary sensors of shear stress, some

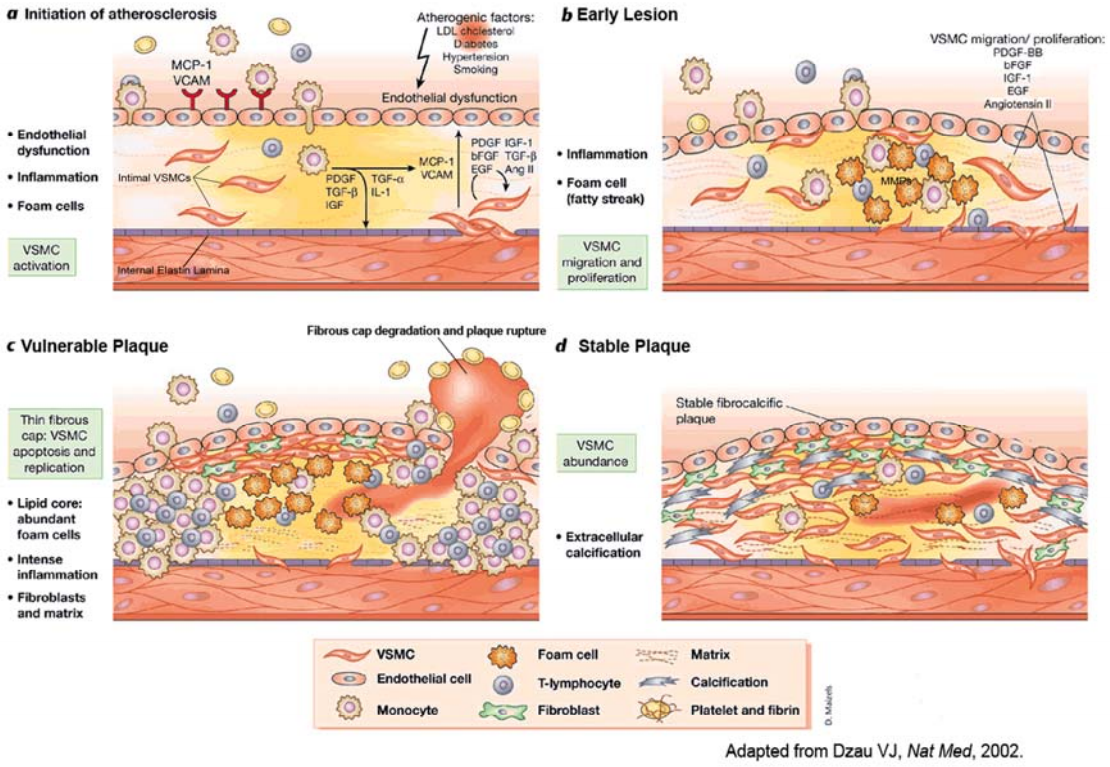
stress may be relayed to the VSMCs by transmural transmission through the extracellular matrix (ECM).[3] Cyclic strain is the tangential force on the arterial wall during systole. Unlike shear stress, cyclic strain primarily affects both ECs and VSMCs, and to a lesser degree, adventitial fibroblasts.[2] The effects of cyclic strain on VSMCs has been the subject of much study in the recent decades, and many methods have been developed for exposing cells and tissues to cyclic strain both *in vitro* and *in vivo* (an in depth discussion of the topic can be found in a review by Brown).[4] While cyclic strain is a major determinant of normal VSMC physiology, for example, by regulating proliferation and apoptosis, it also plays a pivotal role in various pathologies. These include hypertension, vein-graft intimal hyperplasia and failure, restenosis, and atherosclerosis.[5]

Plaque biomechanics

Atherosclerosis is a disease of large arteries characterized by the accumulation of lipid, ECM, and cells within the arterial intima.[6] As the underlying cause of most myocardial infarctions and strokes, atherosclerosis is ultimately responsible for up to 50% of all deaths in the United States, resulting in over $400 billion dollars in health care fees in the U.S. annually.[5, 6] In light of this toll on society, it is of great importance to understand atherosclerosis in order to improve detection and treatment of the disease. Much of the existing research in the field has focused on the role of lipids and inflammation in atherosclerosis. However, this manuscript will address the role of biomechanics in atherosclerotic disease development and progression.

Atherosclerosis occurs in large and medium-sized arteries and develops over time, starting with fatty streaks, progressing to intermediate lesions, and at the worst stage of the disease, advanced and complicated lesions (Figure 1-1).[7-9] Brief descriptions of each of these lesion types in human vasculature, as laid out by the American Heart Association Committee on Vascular Lesions of the Council on Arteriosclerosis, are as follows. Normal arterial intima consists of that part of the arterial wall from the endothelium down to the luminal edge of the media, as demarked by the internal elastic lamina. Thus, the intima comprises the endothelium, ECM (primarily collagens and elastin), and occasional VSMCs. Thick regions of the intima can be found throughout arteries (either focal or diffuse), and denote adaptive responses to physiological changes in hemodynamics.[7] The earliest pathological lesions are fatty streaks, or lipid deposits within macrophage foam cells in the intima. These may be so small as to be only microscopically detectable, whereas later lesions are generally visible to the naked eye upon autopsy.[8] As the lesion progresses to a true atheroma and foam cells continue to accumulate, VSMCs migrate into and proliferate within the intima, thereby increasing plaque size, secreting collagen to generate a fibrous cap over the plaque. Moreover, VSMCs and foam cells secrete matrix metalloproteinases (MMPs) which break down and remodel ECM molecules such as collagen and elastin.[9] An advanced atheroma contains all these components and usually a necrotic core, calcifications, and in the most advanced state, fissures and hemorrhages (Figure 1-1c).[10] VSMCs in this context undergo a number of processes which are pivotal in the process of atherosclerotic

Figure 1-1



Adapted from Dzau VJ, *Nat Med*, 2002.

Figure 1-1

Atherosclerotic progression is a complex process involving many cell types and ECM. The earliest events in atheroma formation are endothelial dysfunction and lipid accumulation in the arterial intima leading to macrophage infiltration and foam cell formation (a). As VSMC migrate and proliferate within the intima, an early lesion forms (b). If the plaque is relatively VSMC-poor (due to apoptosis), especially with a lipid-rich necrotic core and thin fibrous cap, the plaque is vulnerable to fissure and rupture (c). However, if VSMCs are abundant within the lesion and actively secrete ECM to generate a thick fibrous cap, then the plaque will remain relatively stable and is unlikely to cause a clinically-recognizable event (d).

Table 2-1: Primers used for qRT-PCR

	T_m	Product Size	Primer Sequence
18S Forward	60° C	100 bp	5'-CGCCGCTAGAGGTGAAATTCT-3'
Reverse			5'-CGAACCTCCGACTTTCGTTCT-3'
MMP-2 Forward	54° C	51 bp	5'-ATCGCTCAGATCCGTGGTG-3'
Reverse			5'-CCAAATGAACCGGTCCTTGA-3'
Elastin Forward	54° C	144 bp	5'-TTCCCCGCAGTTACCTTTCC-3'
Reverse			5'-AACCACCGCACCTGCAGA-3'
Sciellin Forward	52° C	145 bp	5'-CCTGAAAACACCTGACACCA-3'
Reverse			5'-TCCTGGCATTCACTTTAGCA-3'
Periplakin Forward	58° C	127 bp	5'-ACTGGAGTGACCGCAACCTC-3'
Reverse			5'-TGAAATTCTCATACTGGCGCC-3'
SPRR3 Forward	52° C	96 bp	5'-ATGTCCTTCAACGGTCACTCC-3'
Reverse			5'-CTCTTCGGTTGGTGGTCTAC-3'
Plakoglobin Forward	60° C	70 bp	5'-CAACCAGGAGAGCAAGCTGAT-3'
Reverse			5'-GTTACGCATGATCTGCACGAG-3'
Galectin 7 Forward	60° C	52 bp	5'-CACGGTGCTGAGAATTCGC-3'
Reverse			5'-ATGGAACCTGCTGGCATTG-3'
Envoplakin Forward	60° C	65 bp	5'-CTTCCTGAACCTGTGTATCTGCC-3'
Reverse			5'-GGAACCGGCGGTAGTCCT-3'

development. These include VSMC migration, proliferation, and switching from a contractile to a synthetic state.[11] All of these changes in vessel wall content result in an atheroma-specific microenvironment which is significantly different than the adjacent vasculature.

VSMCs in atherosclerosis

VSMCs accumulation is a hallmark of moderate to advanced atherosclerotic plaques. Within the plaque, VSMCs act in diverse manners, primarily by modulating proliferation, inflammation, ECM modulation, and contraction (Figure 1-2).[12] VSMC proliferation and hypertrophy within the plaque is largely mediated by platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ) which are both sensed and synthesized by the VSMCs.[4, 13] Early in plaque development, VSMCs secrete inflammatory mediators, such as monocyte chemoattractant protein 1 (MCP-1), interleukins, and tumor necrosis factor-α. These, along with surface expression of adhesion molecules such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, promote macrophage infiltration and accumulation.[12] It has been shown that VSMCs within an atherosclerotic plaque undergo phenotypic switching, moving from a more contractile to synthetic state.[13] As such, VSMCs along with other local cells, such as macrophage foam cells, modify the local atherosclerotic milieu by synthesizing ECM molecules, primarily collagens I and III as well as MMPs which degrade and remodel the ECM.[14] This remodeling is critical in determining the stability of the plaque; increased MMP activity is

Figure 1-2

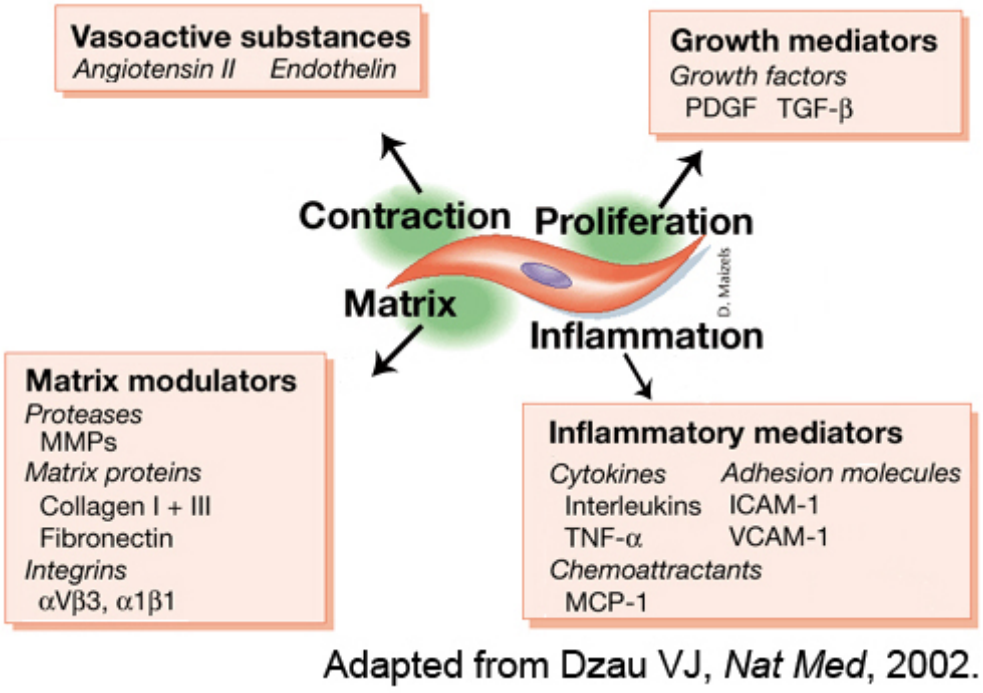


Figure 1-2

The VSMC plays a central role in atherosclerotic progression. VSMCs in atheromas function in four capacities. Contraction occurs in response to vasoactive substances such as angiotensin II and endothelin. VSMCs proliferate and hypertrophy by stimulation with PDGF and TGFβ. They also interact with the extracellular space by making and remodeling ECM and by binding it through integrins αvβ3 and α1β1. Finally, there is a significant release of inflammatory molecules into the local milieu.

thought to undermine plaque stability, thereby increasing the risk of rupture.[15, 16] Although they are in a primarily synthetic state, VSMCs within atheromas are still capable of responding to contractile stimuli, such as endothelin-1 and angiotensin II, which are present in the atherosclerotic plaque.[17-19] Thus, the VSMC is a key player in atherosclerosis development and progression. Gaining further insight into these cells, particularly as they respond to a lesser-studied stimulant, biomechanical stress, will aid in the study and treatment of atherosclerosis.

Altered stress/strain at branch points

It has long been known that biomechanical forces promote atherosclerosis, specifically at curves, branch points, and bifurcations.[20] Thus, atheromas most often arise in the branching coronary and carotid arteries, in the abdominal aorta at the branches for the abdominal arteries, and around the iliac bifurcation.[21] The effect of shear stress on ECs has long been related to the development of atherosclerosis. At regions where the blood flow is significantly disrupted and low, turbulent shear stress alters cellular behavior, leading to endothelial dysfunction. Aspects of this state include a generalized inflammatory state of ECs, which gives rise to generation of reactive oxygen species, altered surface markers, and increased lipid clearance into the intima.[22] Furthermore, though it is less-well understood, the cyclic strain at these points is also significantly affected, with the direction and magnitude of stretch being different from areas of the arteries which are not atheroma-prone. Hence, these altered

biomechanics that impact plaque initiation also impact VSMCs within an established plaque.[11]

Effect on VSMCs

The effect of shear stress on VSMCs has been investigated, though not to the same extent as on ECs. In addition to experiencing "by stander" shear stress via transmural transmission, certain surgical procedures denude the endothelium, directly exposing VSMCs to shear stress.[1, 22, 23] Studies on the effect of shear stress in VSMCs have considered the mechanotransduction of shear and resulting physical changes in the cells. For example, twenty-four hours of *in vitro* shear stress suppresses VSMC proliferation. The same report demonstrated a 2- and 5-fold upregulation of TGFβ and tissue-type plasminogen activator, respectively.[23] Further studies suggest the decreased proliferation may be due to the suppression of PDGF receptor expression and Src phosphorylation by non-uniform shear stress.[24] However, in another non-laminar shear stress model in which stress exposure was prolonged (10 days), VSMCs demonstrated 75% increased proliferation in an Erk1/2-dependent manner.[26]

Altered biomechanics within the plaque

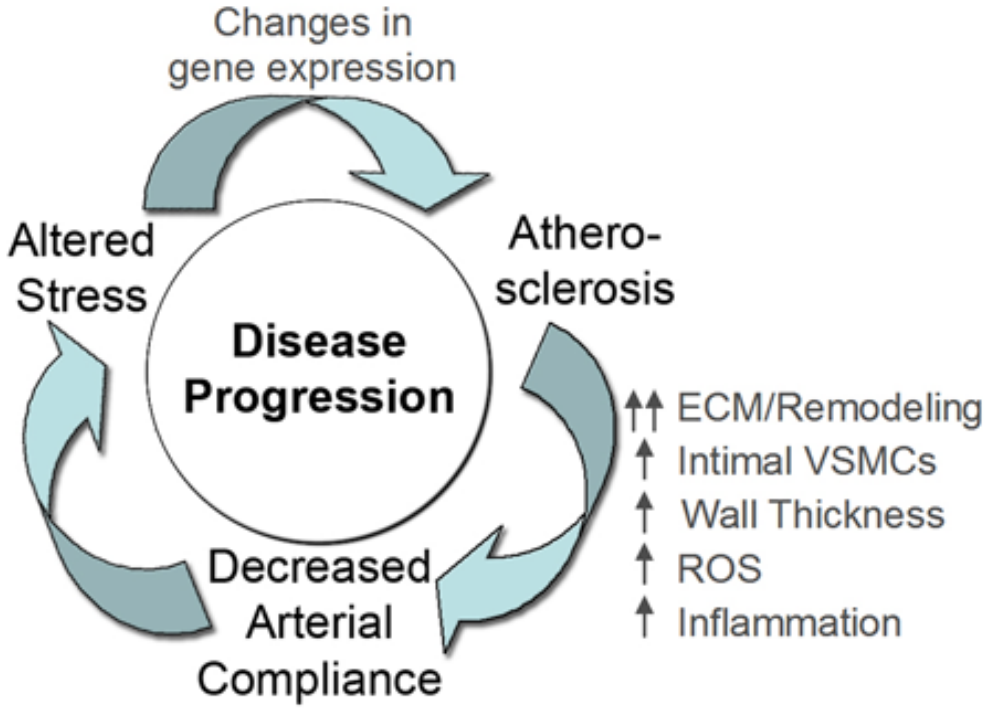
An established atherosclerotic plaque itself is subject to alterations in biomechanical stress and experiences distinct stresses compared to those felt by healthy vessels. Recent studies have shown that biomechanical stress caused by altered flow not only leads to atherosclerosis, but *atherosclerosis itself alters*

local biomechanics (Figure 1-3).[27-29] Recently, sensitive technological innovations have become available, such as intravascular ultrasound, which revealed that even early atherosclerotic lesions significantly affect vessel compliance.[27, 30] On the cellular level, there are many factors in the development of atherosclerosis which can affect vessel stiffness. For example, as the disease progresses VSMCs proliferate and hypertrophy and inflammatory infiltrates (macrophages and lymphocytes) further alter local cellular density. ECM synthesis and remodeling by macrophages and VSMCs further promotes local stiffening. Excess free cholesterol in the plaque can be taken up into the plasma membranes of the resident cells, promoting rigidity of the individual cells.[21, 22] As the plaque progresses, the interior can necrose and calcify, further altering vessel compliance.[5, 12] These changes in vessel compliance necessarily have an effect on the cells resident in the vessel, such as VSMCs. However, little is understood about the resulting molecular changes of VSMCs as they adapt to plaque biomechanics and their impact on plaque stability.

Fibrous cap

One of the vessel-stiffening events evident in mature plaques is the generation of a fibrous cap which plays an important role in plaque stability. The fibrous cap is a structure made up of VSMCs and collagen, which separates the lumen from the atheroma and stabilizes the plaque against rupture.[23, 24] A stable plaque is defined by a thick fibrous cap, extensive VSMC content, few macrophages, and a minimal necrotic core. On the other hand, an unstable, rupture-prone, or “vulnerable” plaque is defined by a thin fibrous cap, profound

Figure 1-3



Adapted from Y.S. Chatzizisis and G.D. Giannoglou, *Med Hyp*, 69

Figure 1-3

Interplay between biomechanics and atherosclerosis. Altered shear stress at branch point and curves is a well-known initiating step in atherosclerosis. The atherosclerotic plaque, in turn, promotes increased arterial compliance and distendability as lipid, cells, and ECM accumulate in the vessel wall. As the vessel wall stiffens, it alters blood flow and changes the local hemodynamics. Altered stress then further promotes atherosclerosis through mechanosensing resulting in changes in gene expression, thereby promoting increased plaque development.

macrophage infiltration, low VSMC content, and a large lipid- and calcium-filled necrotic core.[25] In the absence of a thick, stiff, protective top layer, the unstable plaque is prone to fracturing under the continuous shear and cyclic stresses.[29, 34]

Thus, biomechanical alterations in the vasculature, particularly in and around atherosclerotic plaques, are of significant biological and clinical interest. Of particular interest is how VSMCs, particularly those within the atherosclerotic microenvironment, sense and adapt to mechanical stress by altering gene regulation and cellular behavior. Therefore, the remainder of this document will focus on the mechanisms by which VSMCs sense stress and respond to it.

Mechanosensors—Integrins

Many proteins have been implicated in mechanosensing by VSMCs, including membrane oxidase NADH/NADPH, stretch-activated ion channels, G-protein coupled receptors, integrins, and others.[36-39] In the context of the atherosclerotic plaque, VSMCs are surrounded by ECM, primarily type I collagen, and to a lesser extent elastin, vitronectin, and fibronectin.[7] Cells relate to and sense this environment through integrin-ECM interactions, thus making this interface an important focus of study.[5] The integrins are a large family of heterodimeric cell adhesion receptors which primarily bind extracellular matrix. There are 18 α and 8 β subunits which heterodimerize into 24 unique integrins.[26] The majority of the work with mechanotransduction in VSMCs has implicated β_1, β_3, and various corresponding α subunits as sensors for mechanical stress that transduce signals through numerous pathways.[41-45] Integrins are ideal cellular

sensors of biomechanical stress, as they link the ECM to the cell cytoskeleton through transmembrane domains and can thereby transmit signals intracellularly. When they are not bound to ECM, integrins reside in an inert, or resting, state in which the ECM-binding sites are hidden. However, when triggered by various signals, both subunits undergo a conformational change to expose the binding sites and can thus bind to the ECM.[27] Classical integrin signaling begins by binding of a single heterodimer to its ligand which triggers clustering of other integrin heterodimers and their binding to the same ligand.[28] This leads to recruitment of various signaling and structural proteins to the intracellular end of the integrins, forming focal adhesions.[29] From focal adhesions, signals are transmitted within the cell, regulating various processes including gene regulation, cytoskeletal arrangement, and proliferation.[30] Integrin activation can occur not simply through the binding to specific ECM molecules, but also by sensing tension in the ECM.[31] Furthermore, integrins, ECM components, the cytoskeleton, and the nucleus are all connected such that a mechanical stress at any of these sites can be transmitted to the others.[51] The integrins of particular interest with respect to mechanosensing within atheromas, are $\alpha 1\beta 1$, $\alpha 7\beta 1$, and $\alpha 8\beta 1$, though many others have been implicated in VSMC biology.[32] Thus, extracellular stress and tension can be transmitted through the ECM via integrins to promote intracellular signaling events and alter gene regulation, as well as transferring the forces to the cytoskeleton.

The interaction between ECM and integrins can be modulated by cyclic strain which alters ECM production and remodeling. For example, human

VSMCs under prolonged cyclic strain (24-96h) were shown to upregulate collagen and fibronectin. This was further shown to be the result of synthesis and release of TGFβ by the cells in response to stretch; incubation with TGFβ-blocking antibodies was sufficient to abrogate collagen synthesis in response to stretch.[33] In another report, type I collagen and fibronectin synthesis was upregulated in VSMCs following 12-48h cyclic strain, with a maximal expression at 12h. Fibronectin regulation was shown to be dependent on signaling through the angiotensin type II receptor.[55] Furthermore, cells exposed to 24h cyclic strain upregulated expression of type I collagen through Akt activation.[34] Static stretch of VSMCs has been shown to significantly regulate elastin transcription as well.[35] Matrix remodeling genes are also affected by stretch. Asanuma *et al*, showed that human VSMCs exposed to up to 72h static or cyclic strain modulate MMP-2 and -9 synthesis and secretion.[36] In these cases, the expression of ECM and matrix-regulating proteins is important for vessel remodeling and adaptation to biomechanical stress.

Integrin activation can occur not only by binding their substrate, but also by mechanical activation via substrate tension.[40, 58] For example, it was shown that distinct signaling occurred downstream of the β_1 integrin when activated by binding to an RGD peptide substrate versus activation from mechanically pulling on it through the same substrate-receptor interaction[37]. Specifically, cAMP levels increased, by applying stress through the integrin.[37] In another report, anti-integrin antibody-coated magnetic beads were allowed to adhere to cells, thereby binding to and clustering integrins. While this binding did produce an increase in

total cellular protein phosphorylation compared to untreated cells, when a magnetic field was used to apply stress through the integrins, an increase in total protein phosphorylation was observed.[38] Furthermore, a similar study demonstrated that using optical tweezers to apply stress through integrins generated cytoskeletal stiffening around the focal adhesion.[39] These studies serve to distinguish the distinct responses of integrins to chemical and mechanical input.

There are many known pathways by which integrins transduce biomechanical stress into a chemical signal within the cell. Plating cells on different substrates has differing effects, depending on which integrin heterodimers are affected. It has been speculated, though not conclusively shown, that the α subunit is the actual mechanosensor, which then prompts the β subunit to undergo activation through an intracellular signal.[40] Reusch, et al, has shown that VSMCs differentially activated Erk1/2 and JNK when exposed to cyclic strain on either laminin or pronectin (an RGD-containing peptide) matrices, each of which engage different integrins.[41] Integrin αvβ3 has been shown to be required for the mechanosensitive activation of PI3K/Akt in rat VSMCs exposed to cyclic strain for 15min.[63] VSMCs that were stressed by direct application of force to various integrins through coated magnetic beads showed activation of Erk1/2 specifically through β3, but not α2 or β1 integrin subunits.[45] However, another study showed the opposite; applying direct biomechanical stress through either integrins β1 or α2 produced a global increase in tyrosine phosphorylation, specifically an increase in Erk1/2 phosphorylation.[38] The discrepancy between

these studies may be due to different methodology for applying stress. A similar study demonstrated an increase in cAMP levels, as well as phosphorylation of CREB and nuclear PKA-c, following mechanical stress application to the RGD-binding integrins.[37] Thus, a variety of downstream signaling events have been linked to mechanical stress on integrins. The pathway of action varies by integrin/ligand interactions, duration of stress, and mechanism of strain, nevertheless, it is clear that integrins play a significant role as mechanosensors. Notably, while integrins have been the most studied of the ECM-receptors, they are not the only ones which serve as mechanotransducers. The elastin-laminin receptor was demonstrated to transduce mechanical stimuli into cultured VSMCs.[64, 65]

Physical Changes Resulting from Biomechanical Stress

As an atherosclerotic plaque develops and forms as a result of previously described changes (ECM expression and remodeling, cellular infiltration and accumulation, etc), the region in and around the atheroma becomes increasingly different from the surrounding vasculature. These changes affect vessel compliance and result in a unique environment in which cells within the plaque experience stress differently than the neighboring tissue.[42] This distinctive milieu produces a distinct set of responses in VSMCs, including altered cytoskeletal arrangement, changes in VSMC proliferation, apoptosis, and phenotype, but most of these changes are determined by altered gene expression. VSMCs modulate an array of genes in atherosclerosis, but only in the presence of the

unique plaque microenvironment. For example, our lab and others have demonstrated expression of the integrin subunit α1 on VSMCs to be largely restricted to atheromas, though expression is rarely observed in VSMCs of normal vasculature.[43, 44] This is the result of the influence of the atheroma microenvironment, which is rich in various cytokines such as TGFβ, PDGF, and MCP-1 that modulate VSMC gene expression and behavior.[45, 46] Moreover, in the presence of this microenvironment, biomechanical stress will differentially affect the VSMCs; for example, as the $\alpha 1\beta 1$ integrin is present only within the atheroma, only there will it transduce cyclic strain down a specific pathway to regulate specific genes.[43] Thus, the response of VSMCs to biomechanical stress is ideally studied within the context of a specific setting such as the atherosclerotic microenvironment, and should be considered as a caveat when applying *in vitro* studies of biomechanics and VSMCs to the molecular understanding of atherosclerosis.

Gene Transcription

The primary manner in which physical changes arise following biomechanical stress is through modulation of gene transcription. For example, many groups have shown that cyclic strain activates α-actinin expression, thereby promoting VSMC differentiation.[73, 74] Other studies have demonstrated upregulation of a variety of genes in response to cyclic strain. For instance, studies have shown that chronic stretch increases ECM production, as well as integrin expression on VSMCs.[43, 44, 53] VSMCs exposed to cyclic strain for 24h have been shown to

upregulate transcripts of MCP-1. This effect is partially blocked by inhibition of PKC and tyrosine phosphorylation.[47] Human aortic VSMCs exposed to 20% cyclic strain demonstrated a 2-fold increase in protease-activated receptor-1 (PAR-1) in an NADPH oxidase-dependent manner, and was synergized in the presence of fibroblast growth factor. Paradoxically, *shear* stress downregulated the expression of PAR-1.[48] Exposure to 10% cyclic strain for 4h produced a 3.3-fold increase in expression of VEGF from VSMCs.[49] Another group has shown that expression and shedding of syndecan-1, -2, and -4, cell surface heparin sulfate proteoglycans which act as cell-surface receptors were increased with cyclic, but not static strain, in human aortic VSMCs.[78, 79] Furthermore, the proliferation-suppressing protein iex-1 is induced is in VSMCs exposed to 24h cyclic strain, downstream of NF-κB activation by mechanical strain.[80] Using magnetic twisting cytometry, D'Addario, *et al.* showed a force-dependent regulation of Filamin A through the β1 integrin.[50] These and other changes in gene expression have a variety of effects on VSMCs, including changes in the cytoskeleton, apoptosis, proliferation, and phenotypic state. Cyclic strain also affects gene expression in the atheroma in cells other than VSMCs. Low-levels of cyclic strain (0%-3%) on cultured monocyte/macrophages induced expression of class A scavenger receptor by up to 3.5-fold. This was consistent with the finding that many macrophages in atherosclerotic lesions of ApoE-deficient mice express the class A scavenger receptor, and this effect is augmented by inducing hypertension, and thus increasing physiological cyclic strain.[51] This further demonstrates the importance replicating the atheromatous milieu *in vitro* to

recapitulate physiology; not only do VSMCs respond to the stress, but other cells, particularly macrophages which would not otherwise be exposed to cyclic strain, are also affected by events specific to the atheroma.

Cytoskeleton

In the artery wall, ECs align along the direction of blood flow. However, VSMCs are arranged in a helix around the artery and are orientated 50°-70° relative to the axis of cyclic strain. A well-documented *in vitro* response of VSMCs to cyclic strain is the remodeling of the cytoskeleton such that the cells orient themselves perpendicular to the direction of stretch.[66, 67] This is believed to be due to rearrangement of the cytoskeleton, particularly actin stress fibers.[52] There are many possible molecular mediators of this effect. Standely*, et al.,* showed a mechanism of alignment which was partly dependent on nitric oxide (NO). NO synthase (NOS) inhibition in VSMCs exposed to 48h cyclic strain blocked cellular alignment perpendicular to the direction of stretch. Interestingly, neither supplementing NOS activity nor NO presence had any affect on VSMC alignment, suggesting that the mechanism may diverge from the more commonly recognized pathways of NOS action.[69] This is supported by another report in which VSMC alignment was shown to be dependent on reactive oxygen species and p38 MAPK, but not on PI3K, JNK, Erk1/2, or ion channels.[53]

Cytoskeletal proteins themselves can respond to cyclic strain. Rat aortic VSMCs exposed to acute 15% cyclic strain demonstrate translocation of zyxin from focal adhesions to the nucleus, where it affected expression of

mechanosensitive genes. However, this was a transient effect; following long-term strain (6h), the majority of zyxin returned to the cytoplasm.[71] While not a cytoskeletal element, the transcription factor Egr-1 relocated within the cell in response to stress. Egr-1 was not only increased in neonatal rat VSMCs exposed to cyclic strain, but was also shown to translocate to the nucleus in response to the strain.[72]

Apoptosis

VSMC apoptosis has various effects on the atherosclerotic plaque, and can modulate plaque stability, calcification, and inflammation.[15] Generally, cyclic strain increases VSMC apoptosis. Many groups have studied the mechanisms by which this occurs, with the goal of eventually reversing the effect. For example, the laboratory of Qingbo Xu has performed many studies on the signaling resulting from cyclic strain on VSMCs, particularly as it results in VSMC apoptosis. They have shown that when exposed to cyclic strain venous VSMCs undergo apoptosis through the β1 integrin in a p53- and p38-dependent manner.[2,44, 82] Another group has shown that 10% stretch in VSMCs plated on type I collagen induced cell cycle arrest compared to static controls, through p21 activation, along with a decrease in Rb phosphorylation.[54] Additionally, 25%, but not 7% cyclic stretch of porcine VSMCs induced a three-fold increase in apoptosis through JNK and p38 compared to static controls. This was possibly due to activation of the TNFα receptor 1.[85] The phenotype of VSMCs can also affect their apoptotic response to cyclic strain. Relative to VSMCs in a

proliferative, de-differentiated state, quiescent, differentiated cells exposed to 10% cyclic strain underwent up to 25% more apoptosis.[55] Finally, another group has shown that calpain expression partially attenuates strain-induced apoptosis, such that in the absence of calpain or with a calpain inhibitor, apoptosis increased significantly.[56] Thus, this could be considered as a target for affecting VSMC apoptosis. In general, changing the rate of apoptosis of VSMCs in an atheroma could be a potential method for modulating plaque stability.

Proliferation

VSMC proliferation is a key step in atherogenesis, and a plaque with high VSMC content is generally more stable and therefore less likely to initiate a clinical event. Several reports suggest that cyclic strain promotes proliferation in VSMCs.[74, 88] However, some work suggests that cyclic strain inhibits VSMC proliferation.[57] As with apoptosis, VSMC phenotype also affects the proliferative response to cyclic strain. Tock, *et al.,* showed that VSMCs isolated from fetal rats demonstrated more proliferation in response to cyclic strain (~2.5-fold increase over no strain controls) compared to VSMCs from adult rats, which showed no significant proliferation.[58] Interestingly, in cultured venous VSMCs, mechanical stretch has been shown to upregulate insulin-like growth factor-1 and its receptor, which, in turn, enhanced VSMC proliferation through PI3K. This revealed a novel pathway underlying the neointima formation observed when veins are exposed to arterial biomechanical stress in coronary artery bypass grafts.[59] Interestingly, cyclic strain in rat VSMCs has been shown to block

proliferation by inhibiting notch signaling.[91] Finally, it should be noted that mechanotransduction through integrins have a potent ability to effect VSMC proliferation; VSMC proliferation is promoted by cyclic strain in a mechanism which is dependent upon plating on specific substrates and binding through specific integrins. Wilson, *et al*., showed that the increased proliferation of rat VSMCs in response to cyclic strain could be partially abrogated by plating the cells on collagen rather than fibronectin or vitronectin (though plating on collagen did not completely abolish the proliferative phenotype), by blocking binding to fibronectin, or by blocking the fibronectin-binding integrins $\alpha v\beta 3$.[60] Venous VSMCs from mice cultured on collagen increase proliferation in a PI3K-dependent manner by about 2-fold when exposed to cyclic strain for 48h.[59] However, when rat VSMCs were cultured up to 10 days following cyclic strain, they demonstrated a decrease in proliferation.[91]

Phenotype

VSMC phenotype (either synthetic or contractile) is known to be mediated by cyclic strain. Albinsson *et al*., stretched mouse portal veins *ex vivo* and showed that those tissues under strain turned on transcription of several genes associated with the contractile phenotype. This was been shown to be dependent on increased actin polymerization with strain, which was related to signaling through Rho.[93] Expression of smooth muscle α-actin in rat aortic VSMCs is enhanced by cyclic strain by 2-3-fold. This was shown to be dependent up on signaling through JNK and p38.[58] Interestingly, another group

using a different stress model demonstrated that VSMCs degrade smooth muscle α-actin when exposed to high arterial-like strain, but to a lesser degree in low venous-like pressure. However, they also implicated a role for p38 in this process.[61] Finally, another group demonstrated a requirement for p38 in stress-mediated phenotype modulation, showing that a strain-induced increase in a-actinin could be ablated using a p38 inhibitor. Furthermore, they also showed that the increase in α-actinin and other markers of VSMC differentiation could be altered by changing strain frequency, such that the expression was highest at 1Hz, but dropped at 2Hz.[62]

The study of VSMCs has revealed a great deal of information into the role of biomechanical stress, particularly cyclic strain, in normal smooth muscle cell biology. It also opens a window into understanding the role of VSMCs in the pathogenesis of mechano-sensitive diseases such as atherosclerosis.

SPRR3

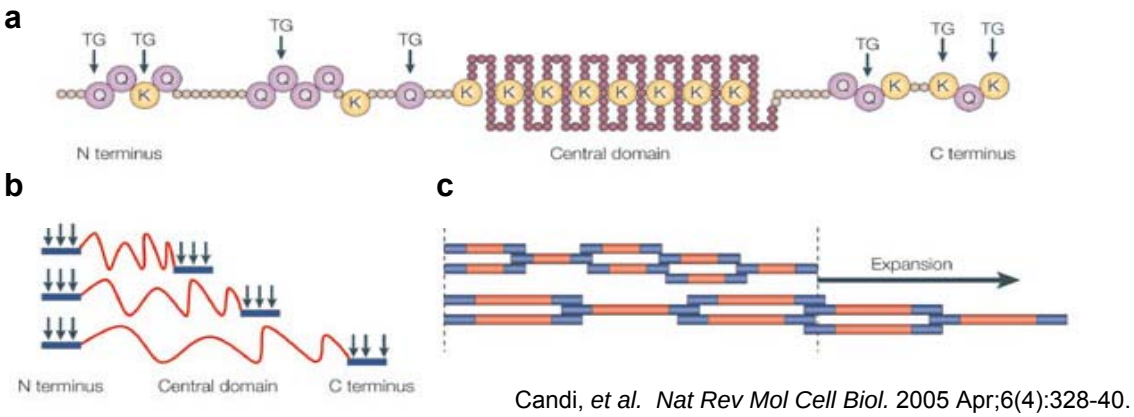
Recent work from our laboratory has identified SPRR3 as a mechanically-responsive protein. The study of this protein has revealed that it is an exemplary model of many of the mechanisms described above.

SPRR3 is a member of the family of Small Proline-Rich Repeat proteins, consisting of members that all possess glutamine- and lysine-rich head and tail domains and a proline-rich core. The flexible core domain is believed to impart to tissues an increased ability to stretch while the head and tail domains provide anchorage to other proteins. For example, SPRRs makes up 18% of the total

protein in the highly flexible mouse forestomach epithelium, but only 8% in the less elastic epidermis.[95, 96] Although SPRR proteins have been identified in VSMCs, cardiomyocytes, lungs, and uterine epithelia, the SPRRs have primarily been studied in the context of their association with the cornified envelope (CE) in stratified epithelium.[97-100] The CE is a 10-15nm thick proteinaceous structure located just inside the plasma membrane of stratified epithelial cells, such as terminally differentiated keratinocytes in the skin. The CE is a barrier against the chemical and mechanical assaults frequently experienced by the tissues in which it is found, such as the epidermis and esophageal epithelium.[101, 102] The architecture of the SPRRs is believed to play a central role in the function of the CE, as the N- and C-terminal domains are covalently bound by transglutaminases (TGases) through ε-(γ-glutamyl)lysine isopeptide bonds to other SPRRs and large structural CE members, such as loricrin and involucrin.[63] It is believed that the central core of SPRR3 has virtually no secondary structure, a vital characteristic in its function as a flexible cross-bridge between its binding partners.[103, 104] As illustrated in Figure 1-4, the central domain of SPRR3 allows the protein to expand significantly upon force application at either end. However, the function of members of the SPRR family outside the context of the CE is very poorly understood.

Our laboratory recently identified SPRR3 in the vasculature by using microarray analysis to compare aorta versus vena cava, in which a group of genes was recognized as being highly expressed in large arteries, but in low or undetectable levels in large veins.[64] These genes, one of which was SPRR3,

Figure 1-4



Candi, *et al. Nat Rev Mol Cell Biol.* 2005 Apr;6(4):328-40.

Figure 1-4

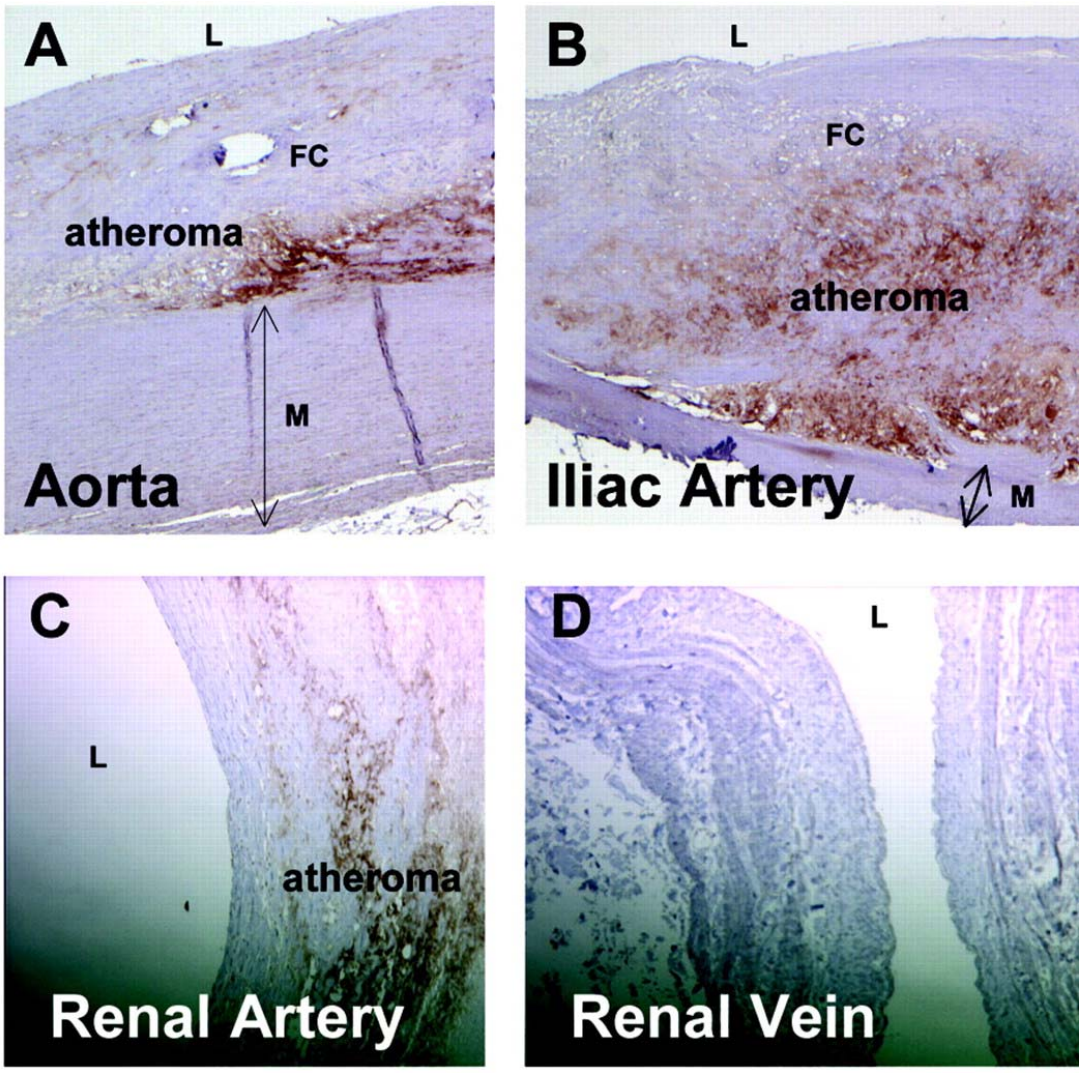
Members of the SPRR family of proteins share a unique architecture, with TGase substrate domains at the N- and C-termini and a highly flexible central domain (a). As stress is applied to either end of the protein, the proline-rich central domain can expand (b). When an SPRR protein is covalently bound to another protein at the N- and/or C-termini, the SPRR can act as an expansion unit, allowing the protein complex to extend when pulled upon by biomechanical stress (c).

had not previously been studied in the vasculature, though they were all known components of the CE. Interestingly, immunohistochemistry revealed that SPRR3 expression was restricted to VSMCs in areas of atherosclerosis in human arteries, but not in healthy vasculature (Figure 1-5).[64]

The fact that SPRR3 is known to play a role in adaptation to biomechanical stress in stratified epithelium, and that our lab found SPRR3 expression in the vasculature limited to regions of altered stress (atherosclerosis), lead us to hypothesize that SPRR3 in VSMCs is regulated by biomechanical stress. Furthermore, recent work from the laboratory of Dr. Kenneth Chien implicated another member of the SPRR family, SPRR1a, as being turned on as an adaptive response to biomechanical stress. Pradervand, *et al.*, showed that in cardiac tissue SPRR1a is induced in response to ischemic and biomechanical stress, and pointed to a possible cardioprotective role of SPRR1a.[99]

In light of these data, we decided to pursue a mechanical role for SPRR3 expression in VSMCs. As will be discussed in more detail in a later chapter, we have established that SPRR3 is regulated by cyclic strain in VSMCs. Moreover, we showed that this regulation is dependent upon mechanosensing by integrin $\alpha 1\beta 1$ through binding type I collagen. This finding explains why SPRR3 expression is restricted to VSMCs within atheromas and is not found in healthy arteries; $\alpha 1\beta 1$ expression is limited to the atherosclerotic plaque and is not found in other VSMCs.[43] This study is one of few reports showing distinct gene regulation downstream of mechanical stress on integrins.

Figure 1-5:



Young, PP, *et al., Circulation*, 2005

Figure 1-5

SPRR3 expression in the vasculature is limited to atheromas. Immunoreactivity to SPRR3 is detected extensively in atheromatous regions of various arteries, including the aorta (a), iliac artery (b), and renal artery (c). However, expression of SPRR3 is not detected in venous tissue, as it is relatively resistant to atherosclerotic development.

The study of the effects of biomechanics on the vasculature, particularly in cases of vascular pathology, is an area still ripe for exploration. The molecular changes arising from biomechanics on VSMCs within an established atheroma remain largely unknown, particularly as the local microenvironment of the plaque changes throughout plaque development and progression. A better understanding of the VSMC response to stress within the atherosclerotic milieu will promote the development of better detection and treatment options for atherosclerosis.

CHAPTER II

BIOMECHANICAL STRESS INDUCES NOVEL ARTERIAL INTIMA-ENRICHED GENES: IMPLICATIONS FOR VASCULAR ADAPATION TO STRESS[1]

Introduction

Blood flowing through the vasculature exerts a significant force on the cells of the arteries resulting in biomechanical stress: cyclic and static strain is produced as blood pressure pushes against the vessel wall in systole and diastole, respectively, and shear stress as the blood flows through the lumen [1, 65]. However, veins exist in a low pressure environment and are less subject to such forces [2, 66]. When veins are arterialized and subjected to a high-stress environment, such as when the saphenous vein is used as a coronary artery bypass graft (CABG), the primary response is extensive fibrointimal hyperplasia (FIH) followed by fibrosis and atherosclerosis that may eventually lead to complete graft failure [67, 68]. Within one year of surgery, 10-15% of grafts are occluded; within 10 years 70% of grafts exhibit some FIH, and over half of those contain lesions large enough to cause occlusion [69, 70]. Vascular smooth muscle cells (VSMCs) are a major constituent of the vascular wall and are key cells in neointimal lesions in grafted veins and atherosclerosis [71].

[1] This chapter has been adapted from the paper: *Biomechanical Stress Induces Novel Arterial Intima-Enriched Genes: Implications for Vascular Adapation to Stress*. Pyle AL, Li B, Maupin AB, Guzman RJ, Crimmins DL, Olsen S, Atkinson JB, Young PP: Cardiovascular Pathology, *In Press*

We previously identified a group of functionally related genes that we call the arterial intima-enriched (AIE) genes based on expression that is limited to the intimal VSMCs of large arteries, but not veins [64, 72]. The AIE genes include sciellin, periplakin, Small Proline-Rich Protein 3 (SPRR3), galectin 7, and plakoglobin. These gene products have been previously characterized in stratified epithelia where they contribute to the ability of the tissues to withstand chemical and biomechanical stresses. Specifically, these proteins are components of the cornified envelope (CE), a 10-15 nm thick highly cross-linked structure just inside the plasma membrane of stratified epithelial cells such as keratinocytes [73]. Of particular interest to this study are sciellin, periplakin, and SPRR3, each of which has a different function in the CE. Sciellin contains a core of conserved repeats and a unique proline-rich N-terminal domain that are proposed to serve a structural role. Furthermore, it possesses a C-terminal LIM domain that is believed to be a protein-interaction domain, possibly bestowing a scaffolding function on sciellin [74-76]. Periplakin, a member of the plakin family of proteins, is enriched in desmosomes where it acts as a mediator of intermediate filament assembly [77, 78]. Small proline-rich protein 3, or SPRR3, is cross-linked by transglutamination to other CE members, thereby potentially serving as a flexible linker of structural proteins in the CE. SPRR3 is hypothesized to have a highly flexible core of proline-rich repeats, the most flexible of the SPRR family, that confers elasticity on the CE [79, 80].

Because arteries are under considerably greater cyclic strain than veins, we hypothesized that at least a subset of the AIE genes are regulated by cyclic

strain in the arteries [81]. We examined their distribution and expression by immunofluorescence in post mortem samples of arterialized saphenous CABGs and mRNA transcript levels in an *in vitro* model system of cyclic strain. The results of this study provide the novel finding that VSMCs up-regulate the transcripts of the structural proteins sciellin, periplakin, and SPRR3 in response to prolonged biomechanical stress. These findings shed new light on the response of VSMCs to biomechanical stress.

Methods

Cell Culture and Biomechanical Stress:

We isolated VSMCs from excess human aortic tissue from heart explants. Briefly, the vessels were cleaned of all connective tissue and cut longitudinally to expose the lumen, which then was scraped to remove the endothelium. The vessel wall was peeled apart to expose VSMCs on both surfaces. These were then cut into 1 cm square pieces and placed smooth muscle-side down in SmGM2 (Clonetics) and held in place by wire mesh. Outgrowths of cells derived from the explants were expanded. This resulted in a mixed population of cells from both the media and intima. After one passage, co-immunocytochemistry was performed with both anti-smooth muscle alpha actin (1:1000, Sigma), anti-smooth muscle myosin heavy chain (1:250, Abcam), and anti-vonWillebrand factor (1:200, Dako). Cells were only used for experiments when cultures were 95%-100% smooth muscle alpha actin and smooth muscle myosin heavy chain

positive and vonWillebrand Factor negative. Cultures were maintained in SmGM2 and used between passages 4-9.

Cells were exposed to cyclic strain as follows. Cells were plated on collagen I coated Flex I elastomer-bottom plates or Flex II solid plates, as control, (Flexcell, Int.) and exposed to cyclic strain with 15-20% elongation for 12, 24, 48 and 72 hours (60 cycles/min) [47, 82]. One hour prior to starting strain, media was changed to 10% FBS/DMEM with antibiotics. Following strain application, cells were washed twice with PBS and harvested in Trizol for RNA extraction, or fixed for immunofluorescence. Cyclic strain experiments were performed a minimum of three times with cells from two independent cell isolations.

Reverse Transcription and Semi-Quantitative RT-PCR:

RNA from VSMCs was isolated with Trizol (Invitrogen) following the manufacturer's instructions and quality confirmed by an $A_{260/280}$ ratio >1.9. Semi-quantitative PCR analysis was performed as previously described [64, 83]. Briefly, 1ug RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). The cDNA was then used for semi-quantitative PCR using SYBR-green iQ PCR supermix and run in an iCycler Real-Time PCR thermal cycler (Bio-Rad). Primer sequences and conditions for use can be found in Table 2-1. All transcript levels were set relative to the level of 18S RNA present in each sample. The results are presented as fold change from non-stressed samples. Results are shown from three independent experiments, each of which was analyzed in triplicate.

Immunofluorescence:

Sections from formalin-fixed, paraffin-embedded human saphenous vein-derived CABG grafts and control non-arterialized saphenous veins were stained as described [64]. The duration of the bypass grafts used in our studies was 12-72 months post-implantation and were obtained from both male and female patients. These grafts were from individuals who received bypass grafts for ischemic heart disease. Based on our analysis of graft morphology, grafts that demonstrated significant intimal hyperplasia and/or atherosclerosis were used for further study. Human aortic VSMCs grown on elastomer membranes were fixed for 20 min at room temperature with 4% paraformaldehyde, followed by permeabilization with 0.4% Triton in PBS. Subsequently, the membranes were cut out of the dishes and cut into smaller wedges so that each membrane could be stained for several different proteins. Each membrane wedge was blocked for 1 hr with 10% goat serum and primary antibody applied in 3% goat serum overnight at 4^{o}C. The following primary antibodies were used: polyclonal SPRR3 (1:400, Alexis Biochemicals), polyclonal sciellin (1:400, the kind gift of Dr. Howard Baden, Department of Dermatology, Cutaneous Biology Research Center, Harvard Medical School, Massachusetts General Hospital, Boston, MA[84]), polyclonal periplakin (1:100, generated against R1729-G1754. The antibody was affinity purified and recognized a single band of 195 kDa from human skin protein lysate by western blot [85, 86]), monoclonal plakoglobin (1:200, Sigma), polyclonal galectin 7 (1:400, Bethyl Laboratories), monoclonal vimentin (1:100, Dako), monoclonal

Golgin 97 (1:100, Molecular Probes), monoclonal Prohibitin (1:100, Calbiochem), and monoclonal protein disulfide isomerase (PDI) (1:100, Affinity BioReagents). The slides/membranes were rinsed twice with PBS and incubated in either goat anti-rabbit-Cy3, goat anti-mouse-alexa 488 (1:200, Molecular Probes); selected membranes were co-stained with FITC- or rhodamine-labeled phalloidin (1:40, Molecular Probes). Finally, the samples were counterstained with DAPI and mounted as described [64, 87]. As a negative control, parallel staining was carried out with secondary antibody alone. Slides were viewed under a Zeiss Axioplan microscope (Carl Zeiss MicroImaging), and analyzed with MetaMorph Imaging system (Molecular Devices). The staining and co-localization was confirmed by fluorescent confocal microscopy using a Zeiss upright LSM510 confocal microscope and the images were analyzed using LSM Image Browser.

Statistical Analysis:

CABG data was analyzed by chi squared analysis and the stress data using a one-way ANOVA with Newman-Keuls Comparison analysis. Error bars represent ± standard deviation. $p<0.05$ was considered statistically significant.

Results

Sciellin and Periplakin are Accumulated in Coronary Artery Bypass Grafts

Saphenous veins are commonly used to bypass occluded coronary arteries [68]. Upon introduction into the arterial circulation, the saphenous vein graft can experience up to 10-fold increase in biomechanical forces and therefore

serves as an ideal *in vivo* model to test the hypothesis that the AIE genes are regulated by biomechanical stress [81]. Cadaveric paraffin-embedded saphenous vein CABGs were analyzed by indirect immunofluorescence with antibodies against sciellin, periplakin, SPRR3, plakoglobin, and galectin 7. The arterialized explants all contained extensive fibrointimal hyperplasia with varying degrees of thrombosis and plaque formation while the non-arterialized saphenous veins displayed only minimal intimal hyperplasia (Figure 2-1a). In the absence of neointima formation, non-arterialized veins were not recognized by any of the antibodies (b-f, right panels, Table 2-2, n=4 non-arterialized veins). However, when arterialized veins were examined for the AIE proteins, greater than 80% and 50% were positive for sciellin and periplakin respectively (Table 2-2, n=17 (sciellin) and n=26 (periplakin) and Figure 2-1b,c, left panels). However, consistent with our previous observations, SPRR3 expression was restricted to atherosclerotic plaques within grafts (Figure 2-1d) [64]. Furthermore, while plakoglobin and galectin 7 stained positive in some arterialized veins (but were negative in non-arterialized saphenous veins), the number of positive grafts was not statistically significant (Table 2-2, n=29 (plakoglobin and galectin 7), Figure 2-1e,f). These findings indicate that sciellin and periplakin protein accumulated *in vivo* in intimal VSMCs following arterialization. Additionally, our previously described finding of atheroma-associated SPRR3 expression in arteries has been extended to atheromas present in arterialized saphenous vein grafts [64].

Figure 2-1

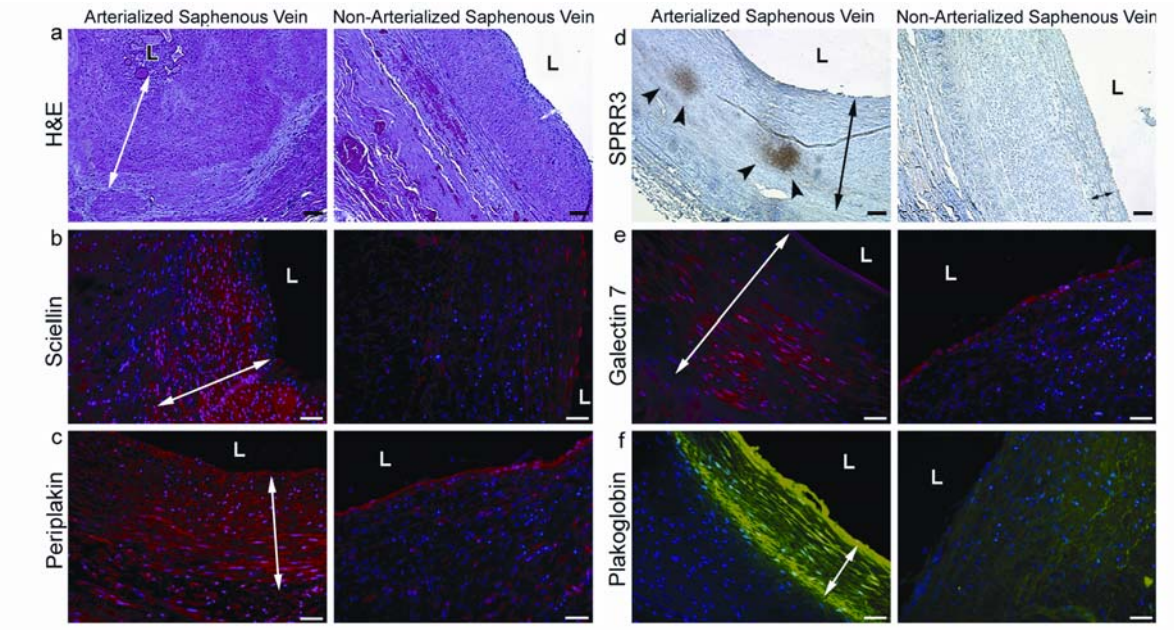


Figure 2-1
AIE proteins are accumulated in arterialized veins.
Cadaveric saphenous vein CABGs and non-arterialized saphenous veins were examined for expression of sciellin (b), periplakin (c), SPRR3 (d,arrowheads), galectin 7 (e), and plakoglobin (f) by indirect immunofluorescence. Extensive FIH is seen in the arterialized veins (arrows), but to a lesser extent in non-arterialized veins, as seen in H&E stained sections (a). L indicates lumen. Scale bar is 80 μm (a, d) and 40 μm (b, c, e, f).

Table 2-2: Quantitation of CABG staining.
Control vein is non-arterialized saphenous vein; CABG is saphenous vein coronary artery bypass graft. Each CABG counted per group represents a unique graft from different individuals.

	Control Vein	CABG	p-Value
Sciellin	0/4 (0%)	14/17 (82.4%)	<0.0002
Periplakin	0/4 (0%)	14/26 (53.8%)	<0.05
Galectin 7	0/4 (0%)	5/29 (17.2%)	>0.3
Plakoglobin	0/4 (0%)	2/29 (6.9%)	>0.3

Sciellin, Periplakin, and SPRR3 are upregulated by cyclic strain in vitro

Because increased biomechanical stress during arterialization is an important stimulus for intimal hyperplasia formation, we hypothesized that expression of the AIE genes was upregulated in response to biomechanical stress [71, 81, 88]. To test this, VSMCs were isolated from aorta samples from two individuals. The cells were then exposed to cyclic strain by applying a computer-controlled vacuum to elastomer-bottomed plates (with 15-20% stretch). Previous studies have measured the distention of human arteries at 18% elongation, and therefore a similar level of cyclic stretch was applied to the cells *in vitro* [89, 90]. This was followed with analysis by qRT-PCR of the mRNA transcript levels of two control genes, MMP-2 and elastin, which have been shown to be upregulated in VSMCs by biomechanical stress, and the six AIE genes [35, 36]. The VSMCs were responsive to cyclic strain at 48 h as demonstrated by the upregulation of MMP-2 and elastin transcripts by 2.09±0.366-fold ($p=0.035$, $n=4$) and 8.88±0.028-fold ($p=0.00162$, $n=4$), respectively (Figure 2-2a). Analysis of the AIE genes revealed upregulation of sciellin, periplakin, and SPRR3 at 48 and 72 h of cyclic strain (Figure 2-2b,c,d). Sciellin transcripts increased by 2.47±0.926-fold ($p\leq0.05$, $n=4$) and 4.67±0.421-fold ($p\leq0.05$, $n=3$), periplakin by 2.77±1.30-fold ($p\leq0.05$, $n=4$) and 1.56±0.028-fold, and SPRR3 by 2.1±0.18-fold ($p\leq0.05$, $n=3$) and 4.95±0.029-fold ($p\leq0.05$, $n=3$) after cyclic strain for 48 and 72h, respectively. However, the data were not statistically significant for plakoglobin, envoplakin, and galectin 7 transcripts (Figure 2-2e,f,g). These data suggest that sciellin, periplakin, and

Figure 2-2

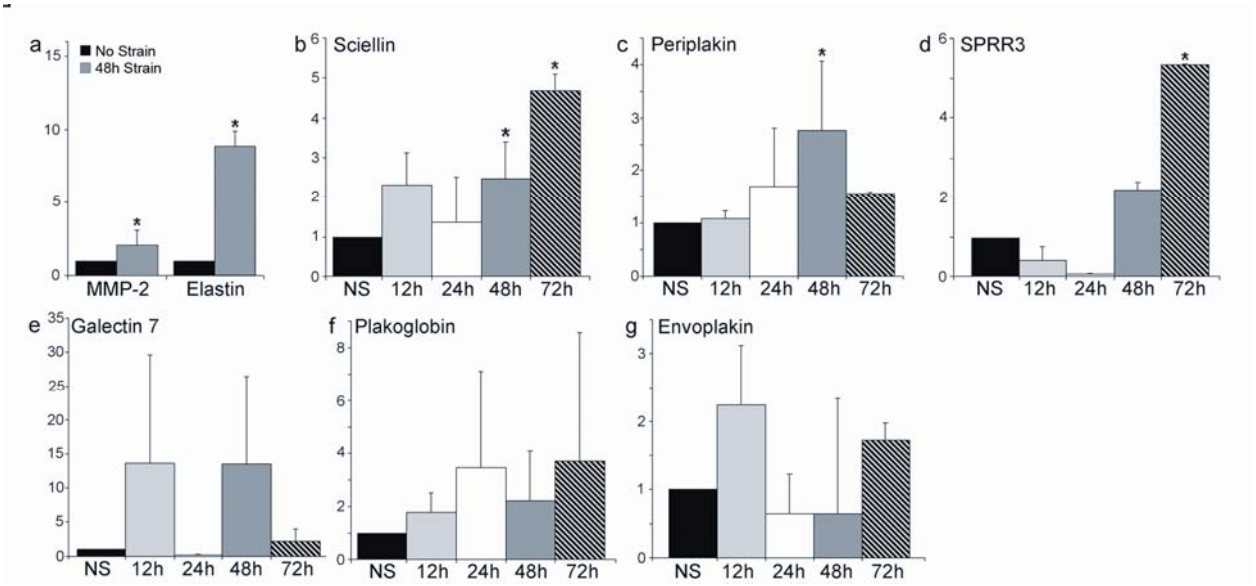


Figure 2-2
AIE gene expression regulation by biomechanical stress.
Real time RT-PCR revealed changes in transcript levels. (a) Fold change of MMP-2 and elastin after 48h. Sciellin transcripts increase significantly after prolonged cyclic strain of 48h and 72h (b), as do periplakin and SPRR3 (c,d). No statistically significant increase was seen for galectin 7, plakoglobin, or envoplakin (e, f, g). *p≤0.05; NS: No Cyclic Strain.

SPRR3 are all transcriptionally responsive to prolonged exposure to cyclic strain in human VSMCs.

Arterial Intima Enriched proteins had unique expression patterns in VSMCs.

Previous studies have shown that the location of a protein in VSMCs can be modulated in response to biomechanical stress [91-94]. Therefore, subcellular localization of sciellin, periplakin, SPRR3, galectin 7, and plakoglobin was studied by indirect immunofluorescence in VSMCs with or without 72h of cyclic strain. Galectin 7 and plakoglobin were only faintly detectable and did not change cellular localization with cyclic strain when compared to unstrained controls (data not shown). Sciellin, however, displayed a diffuse cytoplasmic and peri-nuclear expression pattern (Figure 2-3a) that did not change with stress. Sciellin failed to co-localize with other cellular elements, such as filamentous actin (Figure 2-3b), the intermediate filament vimentin, tubulin, the golgi apparatus (as detected by an antibody against golgin 97), or the endoplasmic reticulum (as detected by an antibody against PDI) (data not shown). Therefore, sciellin distribution did not correlate with any major structural elements within the cell. A previous study demonstrated that periplakin associates with vimentin in keratinocytes, establishing its role as a structural protein [77, 95]. Not surprisingly, periplakin co-localized with vimentin in VSMCs, but no change was noted with the addition of cyclic strain (Figure 2-3d). Periplakin did not co-localize with actin or tubulin (data not shown). Finally, SPRR3 was detected in a peri-nuclear region in cells without strain, whereas it was more widely distributed through the

Figure 2-3

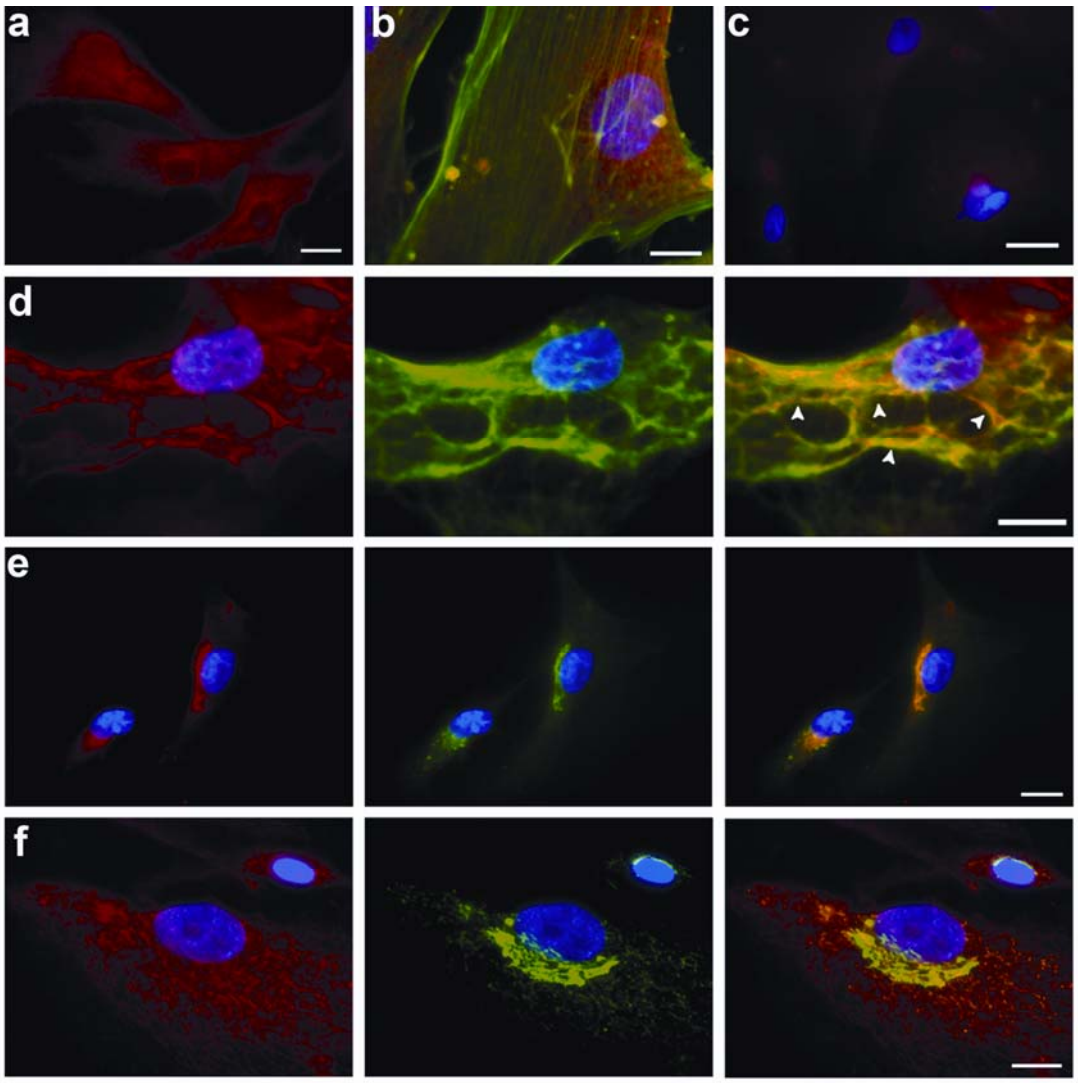


Figure 2-3
Localization of AIE gene products in VSMCs by indirect immunofluorescence. Sciellin (red) in a diffuse cytoplasmic and peri-nuclear pattern (a), does not co-localize with actin (FITC-phalloidin, green) (b). Secondary-only control (c). Periplakin (red) co-localized with vimentin (green) (co-localization—arrowheads) (d). SPRR3 (red) co-localizes with golgi marker (green) in the absence of cyclic strain (e) but is found in a cytoplasmic network after 72h stress (f). Nuclei are stained blue by DAPI. Scale bar is 20μm.

cytoplasm in a reticular pattern after 72h of cyclic strain. In unstrained cells, SPRR3 co-localized with the golgi apparatus (Figure 2-3e). However, after strain, the cytoplasmic distribution of SPRR3 did not co-localize with our selected golgi-, endoplasmic reticulum- or mitochondria-marker proteins. It also failed to co-localize with F-actin, vimentin, or tubulin (Figure 2-3f, data not shown). For all of these experiments, the secondary antibody-only negative control was negative (Figure 2-3c).

Discussion

The differences in the molecular profiles of adult arteries and veins are not well known, despite the established functional, morphological, and pathological differences [96]. We previously identified a group of genes that are expressed in arterial intimal smooth muscle cells, the arterial intimal enriched (AIE) genes [64]. This study demonstrated that expression of a subset of the AIE genes, specifically sciellin and periplakin, was increased in arterialized human veins (CABGs), and their transcripts, as well as that of SPRR3, which has an expression pattern limited to atherosclerotic plaques, were upregulated by cyclic strain.

The manner by which the AIE genes are regulated by cyclic strain remains unclear. The majority of studies that have examined signaling molecules in VSMCs in response to stress have focused on early time points (5min to 24h), whereas the effects on sciellin, periplakin, and SPRR3 were detected after 48h cyclic strain [81, 82, 92]. Our finding that the expression of these genes was

increased following prolonged exposure to cyclic strain (48 to 72h) may provide better insight into the mechanisms of VSMC adaptation *in vivo* to long-term mechanical stress [88, 97]. Furthermore, in light of the fact that the structural effect of cyclic strain in VSMCs is not completely understood, it is of particular interest that previous work in keratinocytes that has defined sciellin, periplakin, and SPRR3 as structural proteins is of interest [35, 91]. Modulation of other structural proteins by cyclic strain in VSMCs has been previously shown. Regulation of extracellular matrix proteins such as tenascin C and elastin by cyclic strain has been demonstrated [35, 91]. Zyxin, a focal adhesion protein, has been shown to translocate from focal adhesions to the nucleus where it is required for expression of certain mechanosenitive genes [91]. Thus, this study further develops the notion that cyclic strain regulates cellular function through the regulation of structural proteins, specifically sciellin, periplakin, and SPRR3. These alterations may have important implications during vascular adaptation to arterialization.

The function of the AIE genes in VSMCs remains unclear, but studies of AIE genes in stratified epithelia, where they serve as structural molecules, may yet shed light on their function in the vasculature. Sciellin was initially characterized in a screen of antigens generated from human keratinocyte CE fragments as a highly insoluble CE precursor and transglutaminase substrate [98]. Subsequently, sciellin was shown to contain a proline-rich N-terminal domain, a central 16 repeat glutamine- and lysine-rich motif—the transglutaminase substrate—and a C-terminal LIM domain [74, 76]. LIM domains, which are protein-

interaction motifs, have been demonstrated in other proteins associated with the cytoskeleton and biomechanical stress in VSMCs [91, 99]. Notably, there was no phenotype observed in sciellin knockout mice[76]. A yeast two-hybrid screen revealed that sciellin in keratinocytes interacts with vitamin D-upregulated protein (VDUP), an endogenous inhibitor of thioredoxin [84, 100]. Interestingly, VDUP expression has been shown to be regulated by biomechanical stress in cardiomyocytes and by oxidative stress in VSMCs, resulting in altered thioredoxin activity [100-102]. The relationship between VDUP and sciellin as well as VDUP and biomechanical stress implicates a possible role for sciellin and VDUP together in our system.

Periplakin has also been identified as a CE precursor. It is found in desmosomal plaques and interdesmosomal networks where it has been shown to associate with keratins and vimentin, and has been hypothesized to act as a scaffold for intermediate filament assembly [77]. Thus, our finding that periplakin co-localizes with vimentin in VSMCs is consistent with the data obtained from studies in keratinocytes. While the periplakin knockout has no obvious phenotype, deletions of other desmosomal proteins in mice, such as plakoglobin and desmoplakin, are lethal *in utero* with major defects in heart muscle resulting from an inability to function under biomechanical stress [78, 103, 104].

SPRR3 is a member of the small proline rich protein family that all contain glutamine- and lysine- rich head and tail domains and a proline rich core. In SPRR3, the head and tail domains are substrates for transglutamination, while the proline-rich central domain is highly flexible. This structure has been

correlated to a function as a cross-bridging protein, such that SPRR3 is transglutaminated on either end to provide mechanical resistance, while the flexible central domain confers elasticity to the protein complex, and thus the tissue [80]. Therefore, we expected to find SPRR3 associated with structural cellular elements, such as the cytoskeleton, but were surprised to see its association with the golgi apparatus. Thus, the role of SPRR3 in isolated VSMCs remains a mystery. Interestingly, another member of the SPRR family has recently been implicated in cardiovascular physiology. Pradervand, *et al*., showed that SPRR1a is induced in cardiac tissue in response to ischemic and biomechanical stress and pointed to a possible cardioprotective role [105].

This work holds implications for a variety of vascular pathologies. For example, the role of the AIE proteins in adaptation to biomechanical stress could be related to disease pathogenesis. Data in Chapter IV will demonstrate that SPRR3 can regulate VSMC proliferation, which could underlie VSMC proliferation in an atheroma and thus disease progression. Sciellin and/or periplakin could affect cellular proliferation and survival in a similar manner to promote vein graft neo-intima formation, perhaps by affecting cellular stability in the face of biomechanical stress. Future work could elucidate the means of function for the AIE genes in VSMCs, as well as establishing a pathway for their regulation by cyclic strain. The following two chapters will show the work to establish a detailed mechanism for SPRR3 regulation by cyclic strain, as well as work trying to determine the function of SPRR3 in VSMCs.

CHAPTER III

Regulation of the atheroma-enriched protein, SPRR3, in vascular smooth muscle cells through cyclic strain is dependent on integrin α1β1/collagen interaction[2]

As shown in Chapter II, we have demonstrated that the AIE genes sciellin, periplakin, and SPRR3 are all regulated by biomechanical stress, though the exact mechanism by which the regulation occurs remains unclear. Here we illustrate the means by which SPRR3 regulation occurs through cyclic strain.

Introduction

Atherosclerosis is known to arise in regions of the vasculature subjected to altered hemodynamic stress.[20] Recent studies have shown that biomechanical stress caused by altered flow not only leads to atherosclerosis, but atherosclerosis itself alters local biomechanics.[106, 107] New, more sensitive technological innovations, such as intravascular ultrasound, have revealed that even early atherosclerotic lesions significantly affect vessel compliance.[106, 108] Studies have considered changes in VSMCs gene expression in other vascular pathologies,[48, 109] however little is known about how locally altered biomechanics affect VSMCs within the context of atheromas.

[2] This chapter has been adapted from the paper: *Regulation of the atheroma-enriched protein, SPRR3, in vascular smooth muscle cells through cyclic strain is dependent on integrin alpha1beta1/collagen interaction.* Pyle AL, Atkinson JB, Pozzi A, Reese J, Eckes B, Davidson JM, Crimmins DL, Young PP: Am J Pathol 2008, 173:1577-1588

In a previous study we showed that the protein SPRR3 is highly expressed in advanced atheromas of human arteries.[64] SPRR3 is a member of the family of Small Proline-Rich Repeat proteins, consisting of members that all possess glutamine- and lysine-rich head and tail domains and a proline-rich core.[80] The flexible core domain is believed to impart to cells an increased ability to stretch while the head and tail domains are anchored to other proteins[80, 110]. Many members of the SPRR family of proteins serve as constituents of the cornified envelope, which is an insoluble protein complex formed under the plasma membrane in the uppermost layers of stratified squamous epithelium.[98 75 111] The cornified envelope plays a major role in the mechanical and barrier properties of these tissues.[79] A recent study identified other SPRR members as stress-inducible, cardioprotective proteins.[105] Both SPRR1a and 2a/b were identified as downstream targets of gp130 signaling that are strongly induced in cardiomyocytes in response to biomechanical stress.[105] Ectopic overexpression of SPRR1a protected cardiomyocytes from ischemic injury both *in vivo* and *in vitro.*[105]

Many proteins have been implicated in mechanosensing in VSMCs, especially integrins.[65, 112-114] Integrins are transmembrane adhesion receptors that primarily bind extracellular matrix (ECM).[115] They function in a non-covalently bound heterodimer composed of an α and a β subunit[26]. Studies have shown that chronic stretch and hypertension increase ECM production, as well as integrin expression on VSMCs.[35, 116, 117] Current concepts suggest that mechanical signals are transmitted from integrin-ECM binding sites to the

cytoskeleton and hence to other transduction molecules in the cytoplasm and nucleus.[118]

The recognized biomechanical disruptions around atherosclerotic plaques and the putative role of SPRR family members in epidermal and possibly cardiac biomechanics led us to test the hypothesis that SPRR3 gene expression was regulated by mechanical stress in VSMCs. This would represent a novel component of the molecular adaptation of VSMC to biomechanical alterations within atheromas. We investigated the mechanism of transcriptional regulation of SPRR3 by cyclic strain (CS) and implicated signal transduction via integrin α1β1 and collagen in SPRR3 gene regulation in VSMCs.

Methods

Materials:

Antibodies: anti-hSPRR3 (clone 4a; Alexis Biochemicals, San Diego, CA), anti-vWF (Dako, Glostrup, Denmark), anti-mouse integrin α1 subunit (clone Ha 31/8; BD Pharmingen, San Diego, CA), anti-mouse integrin α2 subunit (clone Ha1/29; BD Pharmingen), anti-β-actin (clone AC-15; Sigma-Aldrich, St. Louis, MO), anti-smooth muscle myosin heavy chain (clone 1G12; Abcam, Cambridge, MA), and anti-smooth muscle α-actin (α-SMA; clone 1A4, Sigma-Aldrich). We generated and affinity purified a polyclonal Armenian hamster anti-mouse SPRR3 against the peptide spanning amino acids V45-P56 of mSPRR3. By immunoblot, this antibody recognized a 30 kDa band from mouse esophagus lysate as well as COS-7 cells overexpressing SPRR3 (Image Clone ID:

4288753). This band was competed away by preincubation with the immunizing peptide (Figure 3-2a).

Animals:

All animals and procedures were carried out in accordance with the Vanderbilt Institutional Animal Care and Use Committee. C57Bl/6 (wildtype, WT), H-2K^{b}-tsA58[119] (Tag; colony maintained by J.R. at VUMC), and α1-integrin[120] and α2-integrin[121] subunit null mice were sacrificed between 2-3 weeks of age by cervical dislocation and their thoracic aortas were harvested for VSMC isolation. ApoE$^{-/-}$ and syngeneic Bl/6 WT control mice (gift of Dr. A. Hasty,[122] Vanderbilt University) were maintained on regular chow diet and sacrificed at 6 months of age for histologic evaluation of proximal aorta lesions.

Cell Culture:

Protocols involving human tissues were reviewed by the Vanderbilt Institutional Review Board. Human (h)VSMCs were isolated from excess aortic tissue from heart explants (in generous collaboration with Dr. Davis Drinkwater and Mr. Paul Chang). VSMCs were isolated from n=4 donors. The vessels were cleaned of adventitia, cut longitudinally, and the lumen was scraped to remove the endothelium. Segments from the media were cut into 0.5 cm^2 and cultured in SmGM2 media (Lonza, Basel, Switzerland) until outgrowth of cells. A similar procedure was used to obtain VSMCs from mice. Murine cells were maintained in 15% FBS/DMEM/Penicillin/Streptomycin with or without 200pM TGF-β.[123] Each independent isolation of VSMCs was assessed by immunofluorescence with anti-smooth muscle α-actin (α-SMA, 1:1000), anti-SM myosin heavy chain

(SM-MHC, 1:250), and anti-vWF (1:200). CS experiments were performed with cells that were 95%-100% α–SMA and SM-MHC positive and vWF negative. VSMCs were maintained at 37°C in 5% CO_2 and used between passages 4-9, except immortal Tag-VSMCs, which were culture expanded at 33°C in the presence of 10ng/ml interferon-γ and subcultivated for 10 days (5 passages) in regular media at 37°C prior to use.[124] VSMCs were treated with 75 μg/ml of LDL or mildly oxidized LDL in medium containing 1% FBS (kind gift of W. G. Jerome) for 48 or 72 hours.[125]

Biomechanical Stress Application:

VSMCs were exposed to CS by plating cells at $1x10^4$ cells/well on Flex I elastomer-bottom dishes or control solid Flex II plates (Flexcell Int, Hillsborough, NC) and allowed to adhere overnight. Plates were commercially prepared with type I collagen or pronectin F coating. Uncoated plates were treated with 100μg/ml poly-L-lysine for 3hr at 37°C. In some experiments, VSMCs were preincubated 1h on ice with 2μg antibody/$1x10^4$ cells. VSMCs were exposed to CS with 15-20% elongation for 24 to 72 hours at 1Hz (60 cycles/min) via application of a vacuum (15-20 kPa) by a computer-controlled mechanical strain unit (Flexercell 2000, Flexcell Int). All experiments shown were performed a minimum of three times.

VSMCs were exposed to a constant level of shear stress using a cone-plate viscometer. Cone angle and rotational velocity were selected to produce a steady shear stress at either 5 or 10 dynes for 12 and 24hr (1 dyne = 100mN). Following strain application cells were washed with PBS and harvested in Trizol

for RNA extraction, in 8M urea buffer for protein, or fixed for immunofluorescence.

Reverse Transcription and Semi-Quantitative (q) RT-PCR:

RNA from VSMCs was isolated with Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and quality was confirmed by an $A_{260/280}$ ratio ≥1.9, as well as clear bands of 28 and 18S rRNA by gel electrophoresis. Following isolation, RNA was treated with RNase-free DNase to eliminate contaminating DNA and processed with the RNeasy RNA cleanup kit (Qiagen, Valencia, CA). cDNA was generated using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) from 1µg RNA. The cDNA was then used for real-time PCR using SYBR-green iQ PCR supermix and run in an iCycler Real-Time PCR thermal cycler (Bio-Rad). Primer sequences are shown in Table 1. The average cycle threshold from triplicate reactions was used to quantify the relative amount mRNA present based on a standard curve. The differences between samples were determined based on the comparative Ct model [83]. Data was compiled by calculating ΔΔCt relative to the level of 18S RNA present in each sample. A standard curve composed of serial dilutions of a positive control was run with each reaction. A single amplification product was confirmed by the presence of a single band of the correct size by agarose gel electrophoresis, as well as by a single peak in a melt curve analysis of the PCR products. Furthermore, samples were only analyzed when the no-template control reaction was negative. The results are presented as average of fold change from non-stressed samples from

multiple, independent experiments; PCR analyses from each experiment performed in triplicate.

Immunofluorescence/Immunohistochemistry:

Human tissue was obtained from autopsies (post mortem time ranged from 2-12 hours) from males and females whose cause of death was not vascular related. Regions were from both proximal and distal large arteries (n=38) and large veins (n=8). Of these, n=12 of the human aortas contained primarily early atherosclerotic lesions (fatty streaks and intermediate lesions) and n=4 aortas that were histologically normal were analyzed by H&E and SPRR3 immunohistochemistry. Histologic analyses were performed by a cardiovascular pathologist (Dr. J. Atkinson). Sections from formalin-fixed, paraffin-embedded human arteries were stained as described.[64] Briefly, the slides were deparaffinized and subjected to antigen retrieval in citrate buffer. The primary antibodies recognized human SPRR3 (1:400) and α-SMA(1:1000). Frozen sections of mouse proximal aortas fixed with cold acetone were stained using antibodies against mouse SPRR3 (3E9.1; 1:250), α1-integrin subunit (1:100), α2-integrin subunit (1:200), and/or α-SMA (1:2000) followed by appropriate fluorescent-conjugated secondary antibodies. Human VSMCs grown on elastomer membranes were fixed for 20 min at room temperature with cold acetone, followed by permeabilization with 0.4% Triton in PBS. Subsequently, the membranes were cut out of the dishes. Each membrane was blocked for 1 hr with 10% goat serum and primary antibodies for SPRR3 (1:400), and SM-MHC (1:20) were applied in 3% goat serum overnight at 4^{o}C and incubated with

the appropriate secondary antibodies the following day. Slides were viewed under a Zeiss Axioplan microscope (Carl Zeiss MicroImaging, Thornwood, NY), and analyzed with MetaMorph Imaging system (Molecular Devices, Sunnyvale, CA). The staining and co-localization was confirmed by fluorescent confocal microscopy using a Zeiss upright LSM510 confocal microscope and the images were analyzed using LSM Image Browser (Zeiss).

Immunoblotting:

VSMC lysates were harvested by scraping in urea buffer (8M Urea, 75mM Tris pH 8.0, 2% SDS, 5% β-mercaptoethanol, protease inhibitors) and centrifuged at 10,000xg for 10min at 4^{o}C and the debris pellet discarded. Immunoblotting was carried out as described elsewhere.[126] Primary antibodies used were anti-mouse SPRR3 (3E9.1, 1:1000) and anti-β-actin (1:5000) with appropriate HRP-conjugated secondary antibodies.

Statistical Analysis:

Statistical analyses were performed using GraphPad Prism5. All data are shown as ± standard error of the mean. When comparing two samples, a Mann-Whitney U test was used and for experiments with multiple groups, a non-parametric Repeated Measures ANOVA with Bonferroni correction was used. p-values ≤ 0.05 were considered significant.

Results

SPRR3 localizes to VSMCs in atherosclerotic lesions. SPRR3 was detected even in early atherosclerosis within subendothelial intimal cells (Figure 3-1a,c). We detected specific staining in five out of six fatty streaks and all six

Figure 3-1

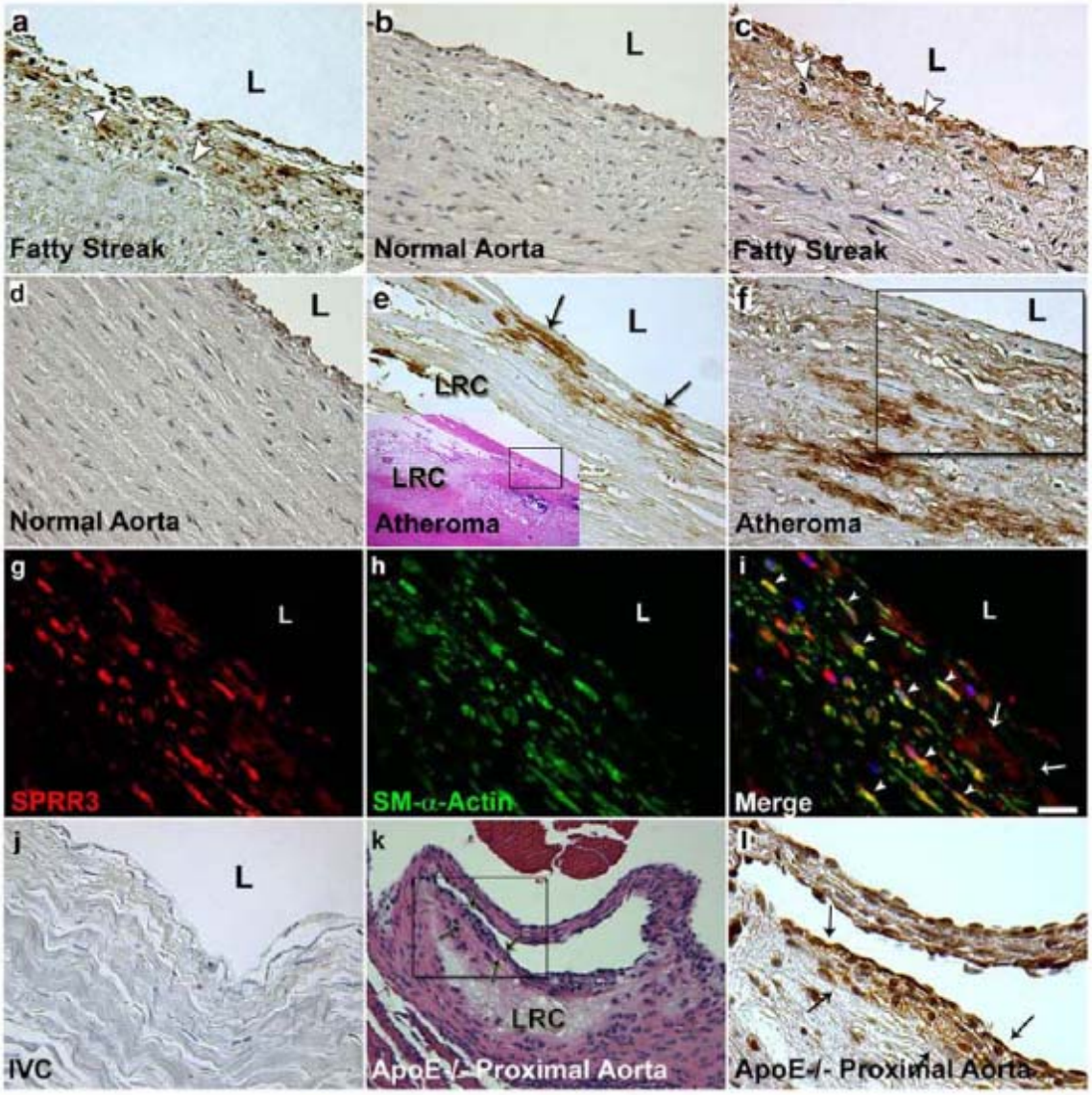

a
L
Fatty Streak
b
L
Normal Aorta
c
L
Fatty Streak
d
L
Normal Aorta
e
L
LRC
LRC
Atheroma
f
L
Atheroma
g
L
SPRR3
h
L
SM-α-Actin
i
L
Merge
j
L
IVC
k
LRC
ApoE-/- Proximal Aorta
l
ApoE-/- Proximal Aorta

Figure 3-1

SPRR3 is enriched in VSMCs in human and mouse atheromatous lesions. Representative sections from paraffin-embedded tissues of human arteries obtained from different individuals containing fatty streaks show SPRR3-positive staining (brown) within the affected intima (a, c). Corresponding regions with normal histology from the same vessel (b,d, respectively) are negative. Arrowheads designate foam cells. SPRR3-stained human aortas containing advanced atherosclerotic lesions (e-f) show extensive SPRR3 immunopositive staining within the fibrous cap (e) and dispersed through the lesion (f). An H&E of the lesion in (e) (inset) is shown with a box denoting the region magnified in (e). The tissue is fractured due to calcification within the atheroma. No SPRR3-staining detected within the vessel wall of histologically normal human inferior vena cava, nor was it found in the medial VSMCs beneath any of the lesions (j, data not shown). The boxed area in (f) corresponds to the region of the vessel examined by indirect immunofluorescence for co-staining of SPRR3 (g, red) and α-SMA (h, green). (i) Overlay in which co-localization is denoted with arrowheads. Note the presence of extracellular SPRR3 which does not co-localize with DAPI (i, arrows). Representative H&E stained proximal aortic lesion from $ApoE^{-/-}$ (k) and its serial section (l) stained for SPRR3. Immunopositive area coincided with the VSMC-rich fibrous cap (indicated by arrows; (l) is the magnification of the boxed area in (k)). Vessel lumen (L), Lipid-Rich Core (LRC). Scale bar = 20μm, 10x objective (a-f, j-k), 40x objective (g-i, l).

Figure 3-2

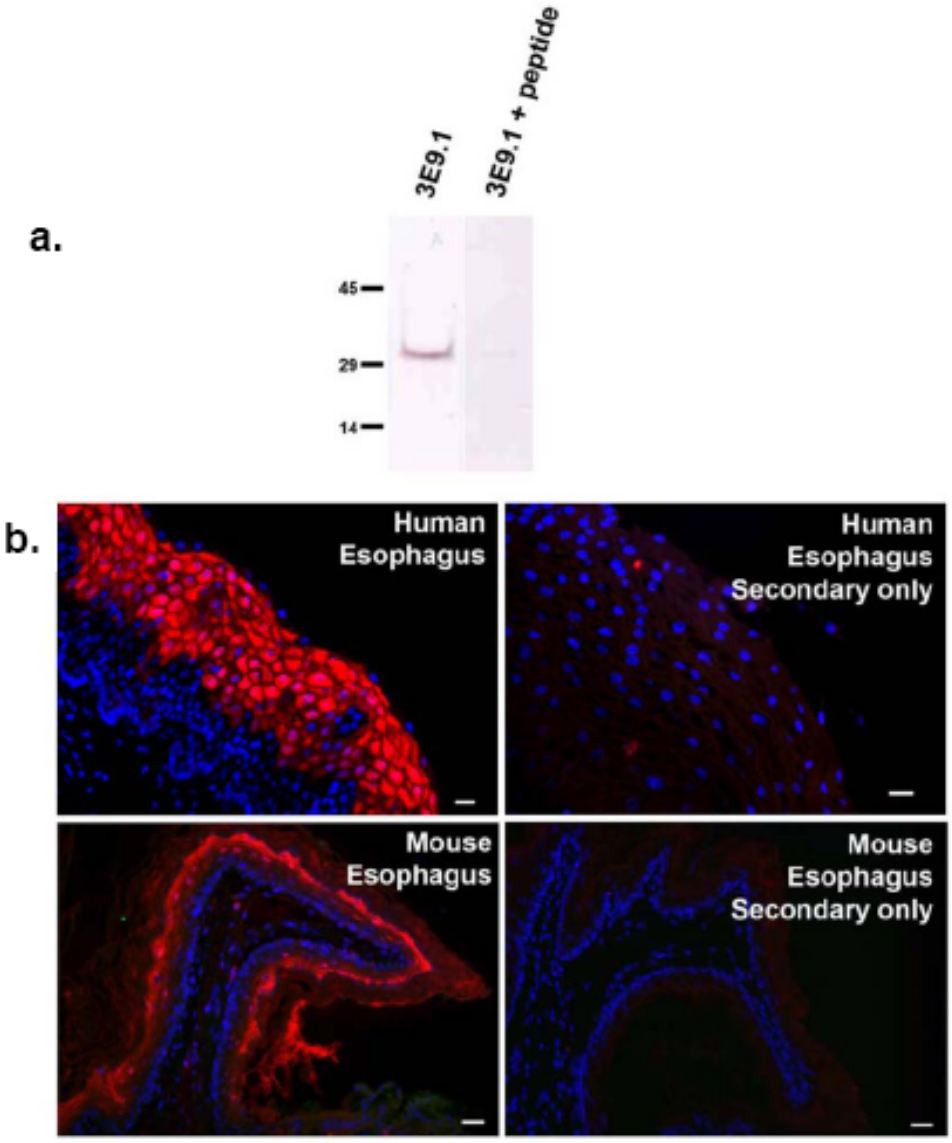


Figure 3-2

Validation of SPRR3 antibodies. (a) 3E9.1 recognized a 30kDa band in Cos7 cells overexpressing SPRR3. This band was competed away by preincubation with the immunizing peptide. (b) Positive (Esophagus) and negative (Secondary Antibody Only) controls for immunostaining both human and mouse tissue for SPRR3.

intermediate lesions examined, but not in normal regions within the same vessel (Figure 3-1a-d). Extensive SPRR3 immunoreactive areas were identified in all advanced atheromas (Figure 3-1e-f). No specific staining was observed in normal veins (Figure 3-1j). Immunofluorescence was used to co-localize expression of SPRR3 and α-SMA, a marker for VSMCs, within a human arterial atherosclerotic lesion.[127] Numerous areas of co-localization indicated that a large number of VSMCs within plaques expressed SPRR3 (Figure 3-1g-i, arrowheads). There was also some evidence of extracellular deposition of SPRR3, which may be the result of protein secretion or cell death (Figure 3-1g-i, arrows). SPRR3 staining was also evident in lesions within the smooth muscle cell-rich fibrous caps of the proximal aortas of Apo $E^{-/-}$ mice (Figure 3-1k,l). Human and murine esophagus were stained in parallel as positive control[128] and secondary antibodies alone were used as negative controls (Figure 3-2b*).*

SPRR3 gene expression is regulated by cyclic strain in VSMCs but not by lipids or shear stress. Independent preparations of primary hVSMCs were each assessed by immunofluorescence for expression of SM α-actin and SM-MHC (Figure 3-3a) to confirm the VSMC phenotype in >90% of the cultured cells prior to use in experiments. Furthermore, we demonstrated co-distribution in hVSMCs of SPRR3 with SM-MHC (Figure 3-3b), supporting the role of SPRR3 in biomechanics.[129, 130] To determine if SPRR3 expression was influenced by biomechanical stress when grown on native type I collagen, we measured SPRR3 mRNA levels by qRT-PCR in response to cyclic strain (CS) and shear

Figure 3-3

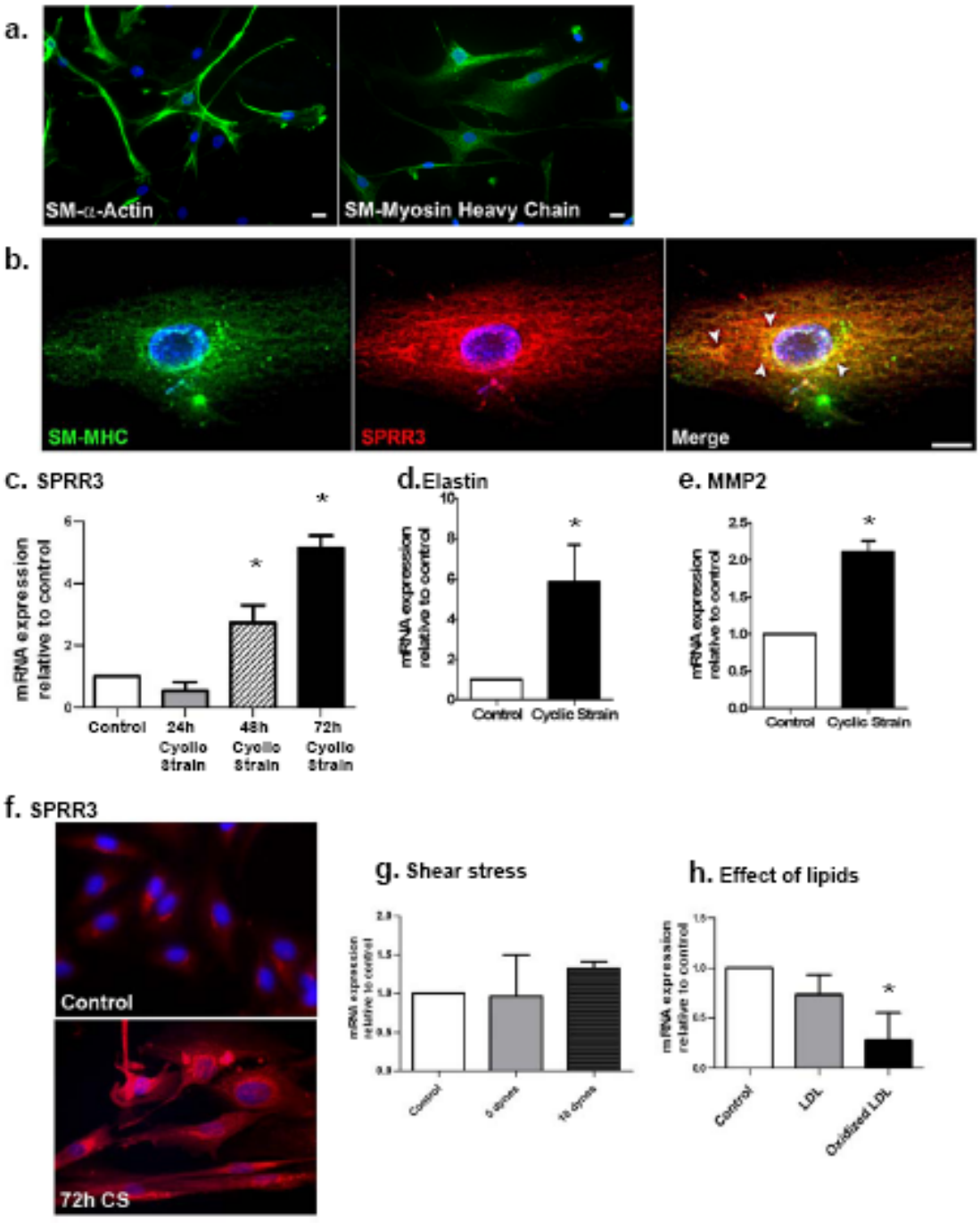

a.
SM-α-Actin
SM-Myosin Heavy Chain
b.
SM-MHC
SPRR3
Merge
c. SPRR3
mRNA expression relative to control
Control
24h Cyclic Strain
48h Cyclic Strain
72h Cyclic Strain
d. Elastin
mRNA expression relative to control
Control
Cyclic Strain
e. MMP2
mRNA expression relative to control
Control
Cyclic Strain
f. SPRR3
Control
72h CS
g. Shear stress
Control
5 dynes
15 dynes
h. Effect of lipids
mRNA expression relative to control
Control
LDL
Oxidized LDL

Figure 3-3

SPRR3 is regulated by CS in human VSMCs. (a) Primary hVSMC cultures expressed both α-SMA and SM-MHC. (b) SM-MHC (green) and SPRR3 (red) co-distributed (yellow, arrowheads). mRNA levels of SPRR3 (c), elastin (d, 48h), MMP2 (e, 48h) after exposure to CS relative to unstrained controls (n=4 independent CS experiments). Immunofluorescence of representative strained and unstrained cells from the same experiment demonstrated clearly increased cellular SPRR3 protein expression following 72h CS (f). Shear stress (24h) had no effect on SPRR3 transcript levels at either 5 or 10 dynes (g) ($n \geq 3$ independent primary VSMC preps and CS experiments). Culturing hVSMCs with lipids (h, only 48 h data shown) did not induce SPRR3 transcripts over control (n=4 independent experiments in triplicate). Scale bar = 20μm, 20x objective (a), 40x objective (b). *$p \leq 0.05$

stress. Collagen I was chosen as it is a predominant matrix protein in the arterial wall.[131] Following CS application for 24-72h, SPRR3 transcript levels were increased by 2.1±1.1- and 4.95±0.7-fold after 48 and 72h, respectively, of continuous strain as compared to unstrained cells (Figure 3-3c). As a positive control, we assessed concomitant upregulation of the previously studied stress-sensitive transcripts, elastin and MMP-2 (Figure 3-3d-e).[35, 36] Elastin and MMP-2 transcripts were increased by 5.85+1.8- and 2.11+0.4-fold, respectively, over unstrained control VSMC after 48 hours of CS. By immunofluorescence, SPRR3 protein levels were shown to increase following 72h CS (Figure 3-3f). As VSMCs are indirectly affected by shear forces[89, 132-134], we also investigated whether SPRR3 was transcriptionally regulated by shear stress in culture. Human VSMCs were exposed to shear stress at either an arterial level of 10 dynes or a venous level of 5 dynes for 12 or 24h (Figure 3-3g shows data for only 24 hour).[132, 133] There were no significant changes in transcript levels of SPRR3 following application of shear stress. Since VSMCs within atheromas are exposed to a lipid-rich environment, we investigated if SPRR3 expression was influenced by lipids. Levels of SPRR3 were significantly reduced after exposure to oxidized LDL (Figure 3-3h), while the addition of either oxidized or unoxidized LDL for 48h or 72h (only 48h data shown, Figure 3-3h) hours did not increase SPRR3 transcript levels in VSMCs.

Due to the limited availability of primary VSMCs from human aortic explants, it was of interest to determine if our results could be recapitulated using both mouse primary wildtype VSMCs (WT-VSMC) as well as VSMC lines

generated from the transgenic H-2K^b-tsA58 mouse (Tag-VSMC). This mouse possesses a heat-labile T-antigen (Tag) expressed behind the mouse major histocompatibility complex H-2K^b, which is widely expressed and is further inducible by interferon-γ. Therefore, cells isolated from these animals can be conditionally immortalized by growth at a permissive temperature of 33^{o}C and in the presence of interferon-γ, but can be returned to normal primary culture-like conditions when grown at 37^{o}C.[119] As primary cells must be used within 9 passages, conditionally immortal Tag-VSMCs enabled us to circumvent the difficulty of obtaining sufficient numbers for extended analysis. We confirmed that both WT-VSMCs and Tag-VSMCs grown at 37^{o}C expressed a-SMA and SM-MHC (Figure 3-5). Both WT and Tag-VSMCs were subjected to CS for 72h and both demonstrated statistically significant upregulation of SPRR3 mRNA transcript levels between ~2-2.5-fold, respectively (Figure 3-4a, c). Interestingly, both types of murine VSMCs showed a less robust CS-associated regulation than human VSMCs under parallel conditions. To determine if SPRR3 protein levels were regulated by CS, cell homogenates from cyclically strained (72h) and non-strained WT-VSMC were analyzed by immunoblot. SPRR3 expression was detected in WT-VSMCs after 72h CS, but not in unstrained, control samples. Expression in mouse esophagus was expectedly very high (Figure 3-4b).[128] Using human and mouse primary VSMCs as well as mouse Tag-VSMCs, we demonstrated consistent upregulation of SPRR3 transcripts and protein by CS.

Figure 3-4

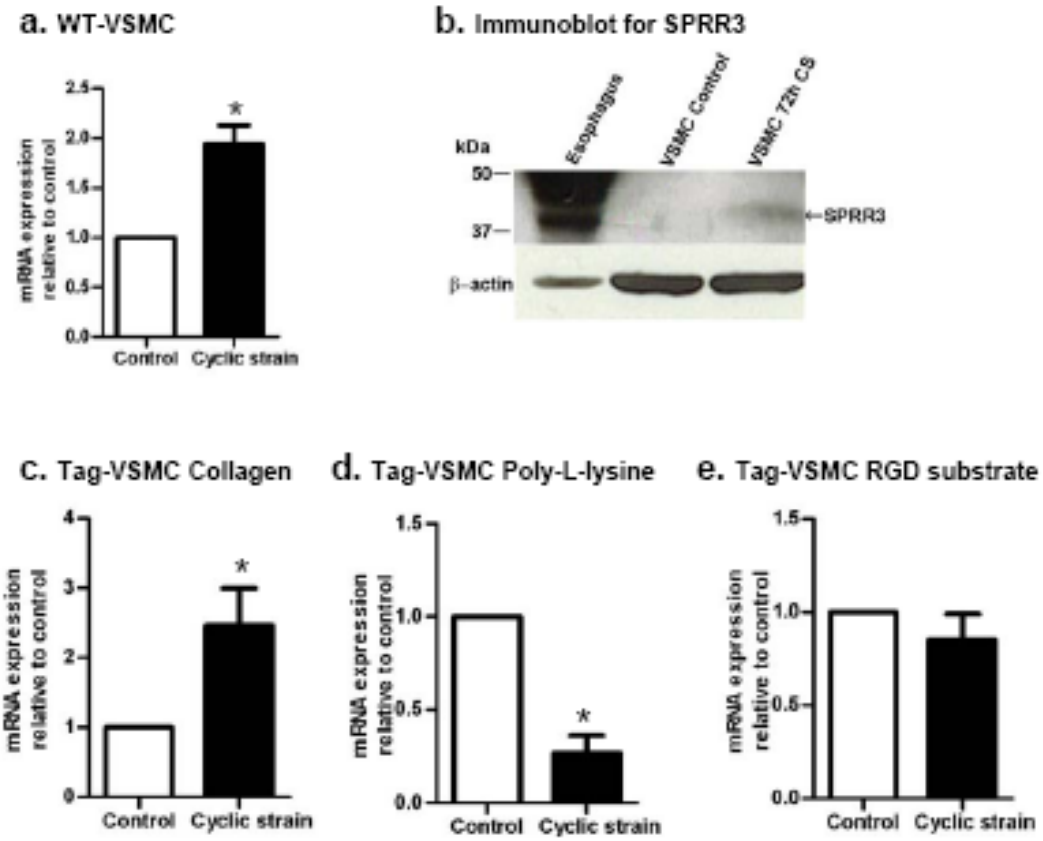


Figure 3-4

SPRR3 upregulation in murine VSMCs requires collagen. SPRR3 transcripts (a) and protein (b) upregulated by 72 h CS in WT-VSMCs ($n \geq 3$ independently isolated VSMCs; n=4 independent CS experiments). Similar fold increase in SPRR3 transcripts observed in Tag-VSMCs cultured on type I collagen (n=5) (c), but not on poly-L-lysine (n=3, d), or pronectin F (n=3, e, RGD substrate). *$p \leq 0.05$.

Figure 3-5

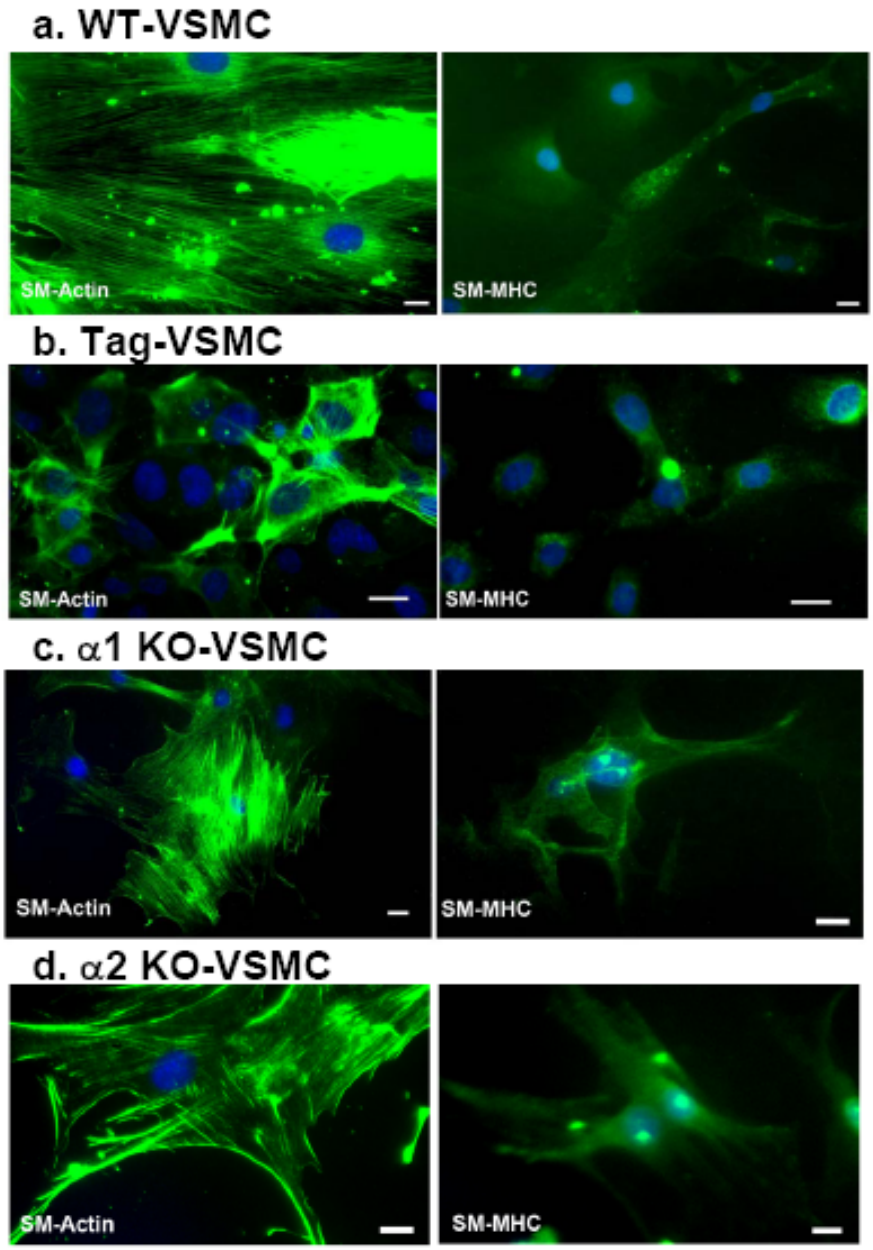


Figure 3-5
VSMCs from mice expressed SM α-Actin and SM-myosin heavy chain. Representative VSMCs from WT C57 Bl/6 mice (a, WT-VSMC), H-2K^{b}-tsA58 mouse (b, Tag-VSMC), α1-integrin knockout (c, α1 KO-VSMC), and α2-integrin knockout mouse (d, α2 KO-VSMC) all stained positive for α-SMA (left-hand panels) and SM-MHC (right-hand panels). Each independently isolated primary VSMC prep was evaluated once. Tag-VSMCs were periodically confirmed for expression. 20x magnification.

SPRR3 mechanoregulation is dependent on binding to collagen. The mechanisms by which mechanical signals are recognized by VSMCs and translated into molecular responses are not completely understood.[135] Intimal VSMCs within atheromas are exposed to an environment rich in ECM, particularly (type I) collagen.[9] Collagen and other ECM components bind cell surface integrins, which serve to connect the ECM to the actin cytoskeleton of cells.[136] Moreover, changes in traction force initiate intracellular signal transduction cascades via "outside in" integrin signaling.[136] We investigated whether regulation of SPRR3 by mechanical forces was modulated by specific extracellular matrices.

To determine the specificity of SPRR3 regulation for type I collagen, we exposed VSMCs to CS on various substrates. Tag-VSMCs were plated onto poly-L-lysine (PLL) (which allows non-integrin mediated adhesion) and pronectin F (which contains multiple RGD repeat peptides from human fibronectin) and subjected to CS conditons.[58, 137] Growth and gross morphology of the VSMCs were not markedly altered on these substrates. SPRR3 transcript levels were not increased in VSMCs after 72h of CS when cultured on either PLL or on pronectin F (Figure 3-4d-e). In fact, the PLL substrate resulted in a significant relative decrease (>2-fold) in SPRR3 transcript level with 72h of CS. These data indicated that SPRR3 regulation required VSMC contact with (type I) collagen.

Integrin $\alpha 1\beta 1$ is necessary for mechanosensitive upregulation of SPRR3. Based on the above data, we chose to investigate the role of the major collagen binding

integrins, $\alpha 1\beta 1$ and $\alpha 2\beta 1$[26], in SPRR3 regulation using function blocking antibodies to each of the integrin $\alpha 1$ or $\alpha 2$ subunits. Tag-VSMCs were preincubated with function blocking monoclonal antibodies against α1 or α2 integrin subunits prior to plating on type I collagen and application of 72h of CS. Incubation with α1 integrin subunit blocking antibodies abrogated the CS-mediated increase in SPRR3 expression (Figure 3-6a). By contrast, preincubation of VSMCs with $\alpha 2$ integrin subunit blocking antibodies induced a modest (1.5$\pm$.4-fold), but statistically insignificant upregulation of SPRR3 transcripts. There was a statistically significant difference in fold-change of SPRR3 transcripts between cells exposed to antibodies against $\alpha 1$ versus $\alpha 2$ (Figure 3-6a). To further strengthen these findings we utilized genetic models of integrin $\alpha 1$ or $\alpha 2$ subunit deletion. Primary VSMCs were isolated from aortas of mice lacking integrin $\alpha 1\beta 1$[120] or $\alpha 2\beta 1$.[121] VSMC cells derived from both integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ knockout mice were confirmed to express α-SMA and SM-MHC (Figure 3-5). KO-VSMC primary cells were plated on type I collagen, subjected to 72h CS, and assessed for changes in SPRR3 levels as compared to control cells without strain. As expected, the VSMCs from mice lacking integrin $\alpha 1\beta 1$ failed to upreguate SPRR3 whereas those from integrin $\alpha 2\beta 1$-/- animals showed a 2.3$\pm$0.6-fold ($p<0.05$) increase in SPRR3 transcript and a corresponding increase in protein similar to WT-VSMCs (Figure 3-6b,c). These data help explain why SPRR3 expression is limited to atherosclerotic regions of the vasculature; SPRR3 regulation will not occur in the absence of integrin $\alpha 1\beta 1$. It is of note that WT-VSMCs and Tag-VSMCs demonstrated enhanced SPRR3

Figure 3-6

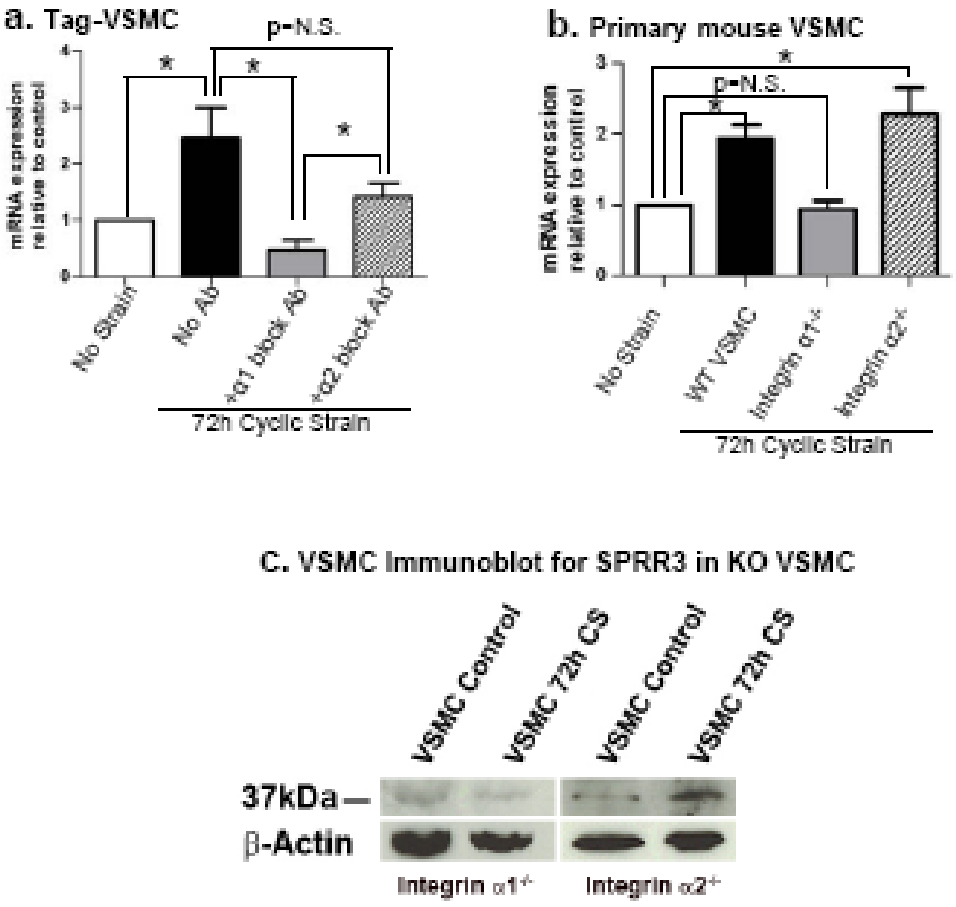


Figure 3-6

Transcriptional regulation by CS requires integrin α1, but not α2. (a) Relative fold change in SPRR3 mRNA transcripts in Tag-VSMCs exposed to CS following pretreatment with function blocking antibodies (n=4). (b) SPRR3 transcript levels were measured in primary VSMCs from WT or mice lacking designated integrin subunits after 72h CS (n=4 CS experiments from single isolation of VSMCs). (c) SPRR3 protein levels were increased in primary VSMCs from integrin α2β1-/- mice following 72h CS, but not in integrin α2β1-/- mice, as determined by immunoblot. *p≤0.05, NS: not significant.

regulation when the cells had been cultured for 48h prior to stress in 200pM TGF-β (Figure 3-7b). This observation was likely due to the upregulation of the integrin α1 subunit when VSMCs were cultured in 200pM TGF-β (Figure 3-7c); Furthermore, it is known that VSMCs in prolonged culture downregulate integrin $\alpha 1\beta 1$[44]. Consistent with this, we did not observe SPRR3 regulation with cyclic strain using cells in prolonged culture (over 9 passages) (Figure 3-7a). It should be noted, however, that there are numerous other cytokines and signaling factors which are known to affect integrin expression and these, as well as or in addition to TGFβ, may be influencing $\alpha 1\beta 1$ integrin expression in atheromas.[138]

To confirm the relevance of integrin α1β1 in this system, we assessed whether α1β1 integrins were expressed in VSMCs of atheromas. Proximal aortas from ApoE$^{-/-}$ mice containing atherosclerotic lesions were co-stained with antibodies against α1 or α2 integrin subunits and anti-SM α-actin antibodies. Expression of integrin α1 subunit was co-localized with the VSMC marker (Figure 3-8a-d), whereas no discernible integrin α2 staining was observed in this mouse atherosclerosis model.

Discussion

This study was designed to investigate the physiologic basis for atheroma-enriched expression of the SPRR3 protein demonstrated in both human and murine vasculature. Our results indicated that prolonged (>48 hr) CS of 20% resting length produced upregulation of SPRR3 RNA and protein in VSMCs. By contrast, exposure of VSMCs to varying amounts of shear stress or lipids failed

Figure 3-7

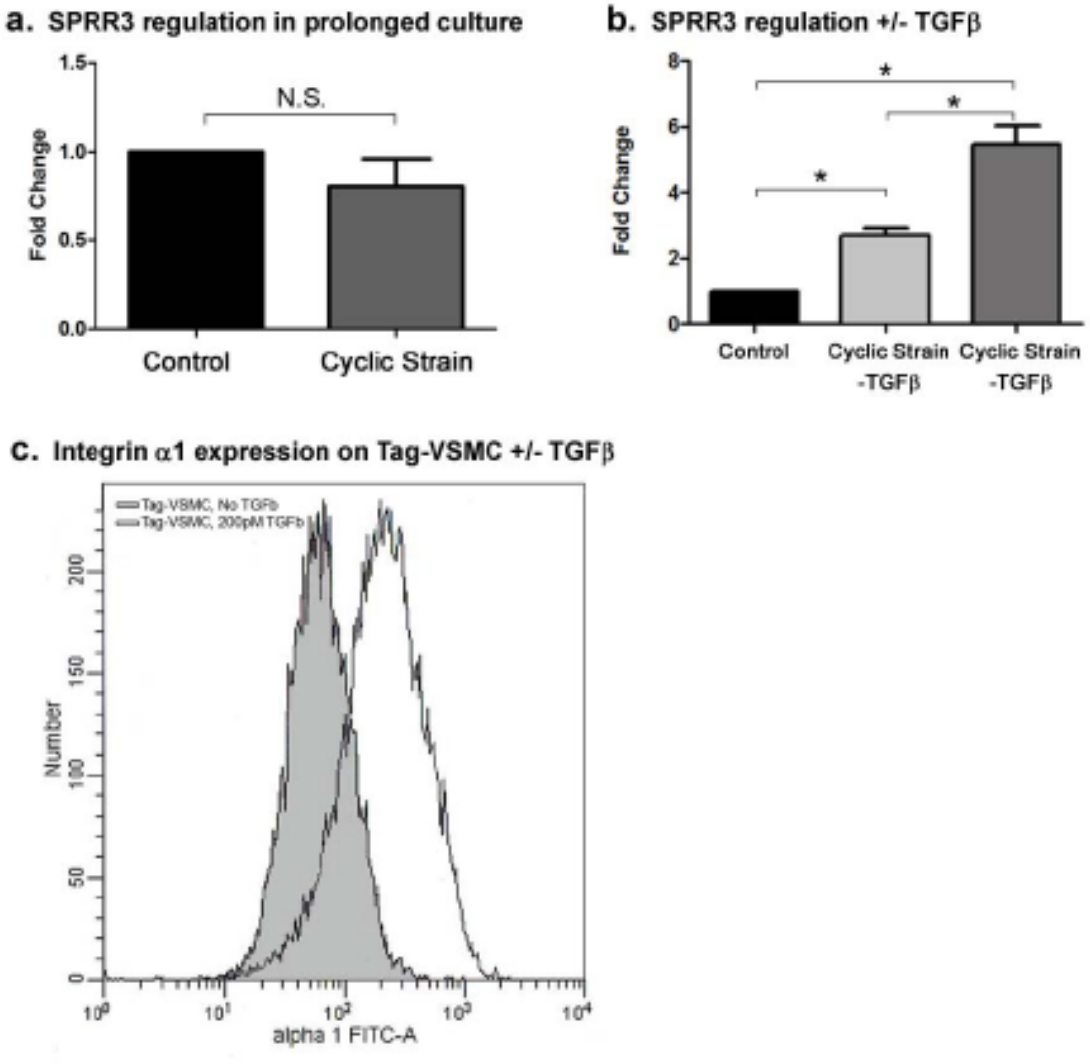


Figure 3-7

Altered SPRR3 regulation. (a) WT-VSMCs maintained over passage 9 did not regulate SPRR3 transcripts following 72h CS. (b) WT-VSMCs exposed to CS in the presence of 200pM TGF-β showed enhanced expression of SPRR3 relative to those maintained in media lacking TGF-β. (c) Surface expression of the α1 integrin subunit detected by flow cytometry was increased on Tag-VSMCs when cultured in 200pM TGF-β. n=4 *p≤0.05 statistically significant; NS: Not significant.

Figure 3-8

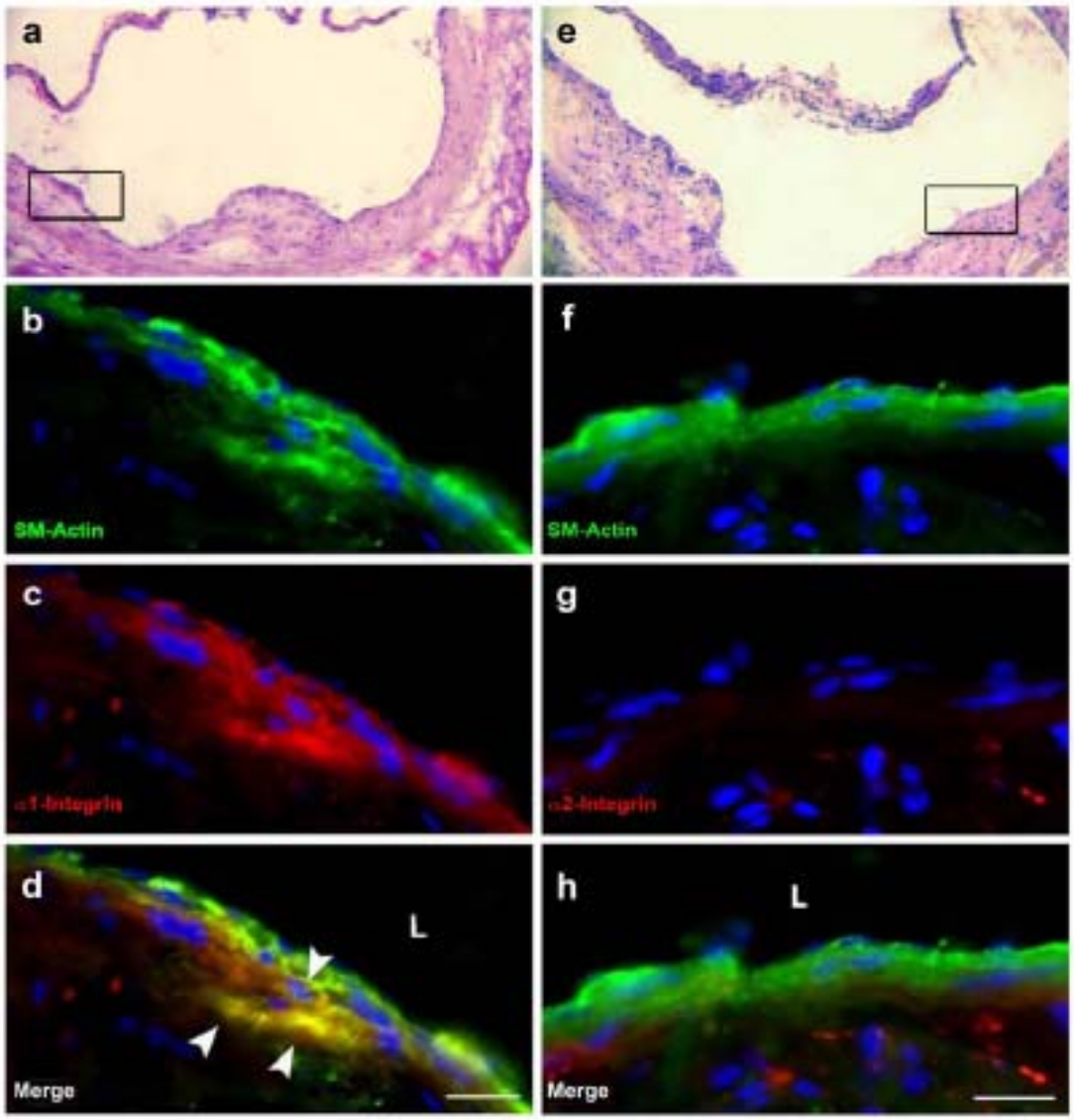


Figure 3-8

Integrin α1, but not α2, is expressed in VSMCs in atheromas. Indirect immunofluorescence of representative proximal aorta lesion from ApoE$^{-/-}$ mice (H&E, a,e) showed co-expression of α-SMA (b, f, green) and integrin α1 (c, red). (d) Merge, co-localization (yellow) marked by arrowheads. Integrin α2 expression was not detected, (g) Scale bar = 20μm, 40x magnification. L indicates vessel lumen.

to increase SPRR3 transcript levels (Figure 3-3). To our knowledge this is the first atheroma-specific protein whose expression is biomechanically regulated. It is of some interest that SPRR3 co-distributed with SM-myosin heavy chain in hVSMCs (Figure 3-3b). SM-MHC has previously been shown to be both biomechanically regulated as well as biomechanically active and hence this finding supports a role for SPRR3 in biomechanics.[129, 130] Because integrins are hypothesized to serve as prime cellular mechano-sensors, as they link the interior and exterior of the cell[116, 139], we sought to elucidate the effect of ECM and integrins in mechanoregulation of SPRR3 transcription. By exposing VSMCs to CS on various substrates, we demonstrated that SPRR3 regulation by CS required type I collagen, whereas cells grown on PLL or pronectin F failed to regulate SPRR3 with CS. Since type I collagen constitutes >70% of all collagen in atheromatous plaques, it represents the primary ECM circumscribing VSMCs within lesions.[131] Given that the CS effect required a collagenous substrate, we evaluated the role of the major collagen binding receptors, α1β1 and α2β1, in SPRR3 gene regulation using both an immunoblocking approach and a genetic deletion model. Taken together, the data suggest that the α1β1 collagen-binding integrin is required for mechanoregulation of SPRR3. Consistent with published studies, we confirmed integrin α1 subunit expression in VSMCs within murine lesions[140]. Expression of the integrin α2 subunit was not evident in murine lesions as is consistent with previous findings.[44, 141]

Since VSMCs are exposed to mechanical forces such as CS in healthy arteries as well as in atheromas, our findings do not fully explain why SPRR3

expression was restricted to plaques. One possibility for this phenomenon relates to the microenvironment of the plaque itself. VSMCs as well as macrophage foam cells are known to take up lipid in atheromas, so we investigated if lipid loading could upregulated SPRR3 transcript.[142] LDL and ox-LDL failed to enhance SPRR3 expression, although ox-LDL loading resulted in decreased SPRR3 transcription. While it is unclear why this may have occurred, it is known that lipid-loading VSMCs induces a variety of signaling pathways, as well as inducing an even more synthetic cellular phenotype.[143-145] Therefore, cells with an altered phenotype, in the absence of biomechanical stress, may downregulate SPRR3. However, this raises the issue of VSMC phenotype (e.g., contractile/differentiated vs. synthetic) as a variable in this system. VSMCs in the context of the atherosclerotic plaque are widely considered to reside in the synthetic state.[145, 146] Because this study investigates the regulation of SPRR3 in the context of the atherosclerotic plaque, it is optimal to use cells *in vitro* under culture conditions which promote a synthetic phenotype. SM-MHC is a marker of a differentiated/contractile VSMC. As seen in Figure 3-3 and 3-5, the VSMCs used in this study express low levels of this protein, suggesting that they are in a relatively synthetic state. Another potential explanation may be that VSMCs within atheromas upregulate α1β1 expression. We have observed *in vitro* downregulation of the integrin α1 subunit in some VSMCs after prolonged culture; these cells fail to demonstrate SPRR3 regulation (Figure 3-7a).[44] It is known that lesions locally express high levels of TGFβ, which is known to upregulate collagen and surface integrin expression[32, 147, 148]. We confirmed this

finding by demonstrating that TGFβ increased expression of α1β1 in cultured VSMCs. Accordingly, VSMCs upregulated SPRR3 in response to CS more robustly when cultured in the presence of 200pM TGFβ (Figure 3-7b,c). We also demonstrated highly localized expression of α1β1 on VSMCs constituting murine proximal aorta lesions. Hence, one hypothesis is that the atheroma microenvironment (inflammation, altered local compliance, etc.), perhaps through increased TGFβ, altered VSMC integrin expression and subsequent ECM/integrin signaling. However, other cytokines also present in this microenvironment may also be responsible for the integrin regulation.[138] Moreover, to further examine if biomechanical stress alone could upregulate SPRR3, we examined aortas isolated from mice subjected to continuous infusion of angiotensin II (Ang II) for two months, which induces systemic hypertension associated with increased medial thickness as compared to saline controls (samples obtained from Dr. Nancy J. Brown, Vanderbilt University).[149] We failed to detect either α1β1 or SPRR3 expression in arterial wall VSMCs in treated or control aortas (Figure 3-9). Together, the data suggest that biomechanical stimulation alone *in vivo* or *in vitro* was not sufficient for upregulation of SPRR3 transcript and protein expression in the absence of VSMC α1β1 integrin expression. A model of this proposed mechanism is shown in Figure 3-10. Nevertheless, the atherosclerotic plaque is a complex tissue with multiple variables that may affect the transcription of SPRR3. While many excellent studies have highlighted integrin/matrix interactions in regulating the physiology and gene expression in VSMCs, to our knowledge our study is among the first to

Figure 3-9

a.

	Vehicle	Ang II
Aortic Media, μm	49.4 ± 10.9	66.0 ± 29.3
Aortic Adventitia, μm	20.8 ± 7.43	48.4 ± 23.6

Data provided with permission from Dr. Nancy Brown, Vanderbilt University

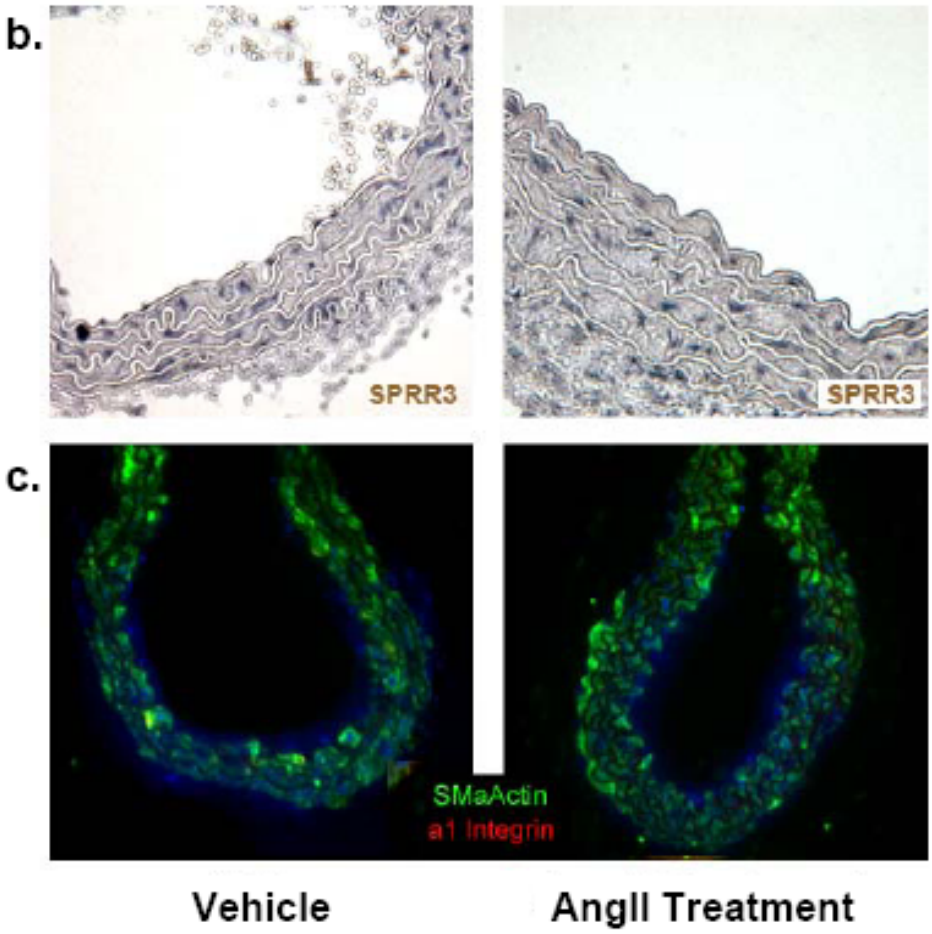


Figure 3-9

SPRR3 regulation in hypertension. (a) The effect of AngII-infusion on mouse aortic morphology. Note the increased medial thickness in the AngII-treated animals (n=6, vehicle control, n=7, AngII infused). (b) Aortas from AngII-infused and vehicle-control animals were subjected to immunostaining for SPRR3, but were all negative for SPRR3 expression. (c) Serial sections to those used to immunostain for SPRR3 were subjected to indirect immunofluorescent analysis for α-SMA (green) and anti-integrin α1 subunit (red). No α1 expression was detected on VSMCs.

Figure 3-10

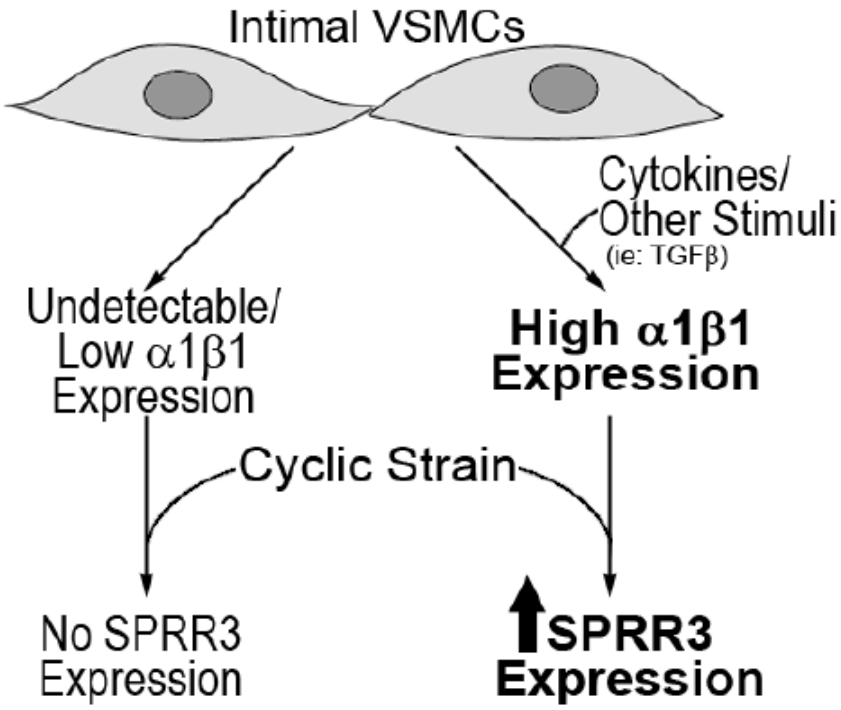


Figure 3-10
Model of SPRR3 regulation in VSMCs.

delineate a direct relationship between transcription of a mechanosensitive target gene and specific integrin/ECM signaling.

The function of SPRR3 in VSMCs within atheromas remains unclear. The architecture of SPRR3, which is transglutaminated via its N- and C-terminal domains to other structural proteins, including other SPRRs, is believed to play a central role in the barrier and stress function of the cornified envelope.[80] The central core of SPRR3 is considered to have virtually no secondary structure, a vital characteristic in its function as a flexible cross-bridge between its binding partners.[150, 151] SPRR3 is a substrate for transglutaminase (TGase) types I and III, which are expressed in murine vessels.[64, 150, 151] Interestingly, other studies have linked increased TGase activity to VSMCs and sites of atherosclerosis[152, 153], although no reports are available on the relationship between SPRR3 (or any substrates) and TGase gene family in VSMCs. It is tempting to speculate that SPRR3 may serve as a crosslinking substrate for TGase to stabilize the cytoarchitecture of VSMCs, although the relationship between SPRR3 and transglutaminases in the vasculature remains unexamined. Our data showing that SPRR3 is exclusively enriched in VSMCs within atheromas in response to mechanical stress implies that it may play a role in altered biomechanical compliance of the smooth muscle cell within an atheromatous lesion. Efforts in our lab are currently underway to better understand the role of SPRR3 within VSMCs using gene deletion models and identifying its crosslinked partners.

It has been reported that early atherosclerotic lesions (undetectable by angiography but evident by intravascular ultrasound and postmortem

histopathology) result in significantly reduced aortic compliance in a hyperlipidemic rabbit model.[106] These data have been confirmed by other studies by multiple groups.[154-156] One mechanism by which even an early atheroma may alter local biomechanics is from the direct effect of cholesterol on VSMC membrane fluidity. Alternatively, alterations of the physical properties of the ECM can be transduced to VSMCs.[24, 157, 158] While much data support that atherosclerosis itself alters vessel compliance and, thereby, biomechanical properties, the molecular changes within VSMCs in response to altered local biomechanics are poorly understood. This paper elucidates the mechanism by which SPRR3 is regulated by mechanotransduction in VSMCs within the context of the microenvironment of the atherosclerotic plaque.

While a mechanism for SPRR3 regulation has been established, the function of the protein in VSMCs remains to be determined. The following chapter will show data to test the hypothesis that SPRR3 functions by binding through transglutamination to other proteins.

CHAPTER IV

FUNCTION OF SPRR3 IN VSMCS

Introduction

VSMCs within the context of the atherosclerotic microenvironment are distinct from the smooth muscle cells in the adjacent normal tissue, as there are a distinct set of responses of VSMCs as they adapt to the atherosclerotic milieu.[159] For example, VSMCs within an atheroma are more proliferative, migratory, and synthetic.[46] Furthermore, as discussed in Chapter I, one distinction between normal and atherosclerotic tissue is decreased vascular compliance in the atheroma. Work in the field of biomechanics has shown that as extracellular forces increase, either directly (pushing or pulling on the cell itself) or indirectly (tension from stiffening matrix/decreased compliance), numerous changes arise within the cell. Stiffening in the ECM and generation of mechanical force leads to construction of new adhesion complexes and cytoskeletal rearrangement. Together, these responses to force result in a stiffer cellular architecture.[160, 161] Cellular stiffening is further increased by rearrangement and activation of cytoskeletal crosslinking proteins.[160-163] These and other responses may help the cell adapt to the compliance changes in the surrounding microenvironment.

We propose that SPRR3 acts in the atherosclerotic VSMC as an adaptive response to the altered compliance. Specifically, we hypothesize that within

high-compliance atherosclerotic tissue SPRR3 is bound by transglutamination at its N- and C- termini to the high tension cytoskeletal elements, where it acts as a crosslinker and serves to dissipate stress applied to the cell (see Figure 4-1). This model is consistent with previous work on SPRR3 which implicated it as a flexible crosslinker of structural cornified envelope proteins.[75, 80] Previous studies of SPRR3 localized the protein to the cellular perimeter in the cornified envelope of stratified epithelial cells. However, in VSMCs SPRR3 is localized within a cytoplasmic network when the cells are exposed to cyclic strain.[164] This distribution suggests that SPRR3 functions in a unique role in the vasculature, specifically in vascular adaptation to altered vessel compliance in atherosclerosis.

Methods

Cell culture:

VSMCs were isolated from the aortas of H-2K^{b}-tsA58^{119} transgenic mice (Tag-VSMCs). The vessels were cleaned of adventitia, cut longitudinally, and the lumen was scraped to remove the endothelium. Segments from the media were cut into 1mm^2 pieces and cultured in 15% FBS/DMEM/Penicillin/Streptomycin until outgrowth of cells. VSMCs were assessed by immunofluorescence with anti-smooth muscle α-actin (α-SMA, 1:1000), anti-SM myosin heavy chain (SM-MHC, 1:250), and anti-vWF (1:200). Experiments were only performed with cultures that were 95%-100% α–SMA and SM-MHC positive and vWF negative. Tag-VSMCs were culture expanded at

Figure 4-1

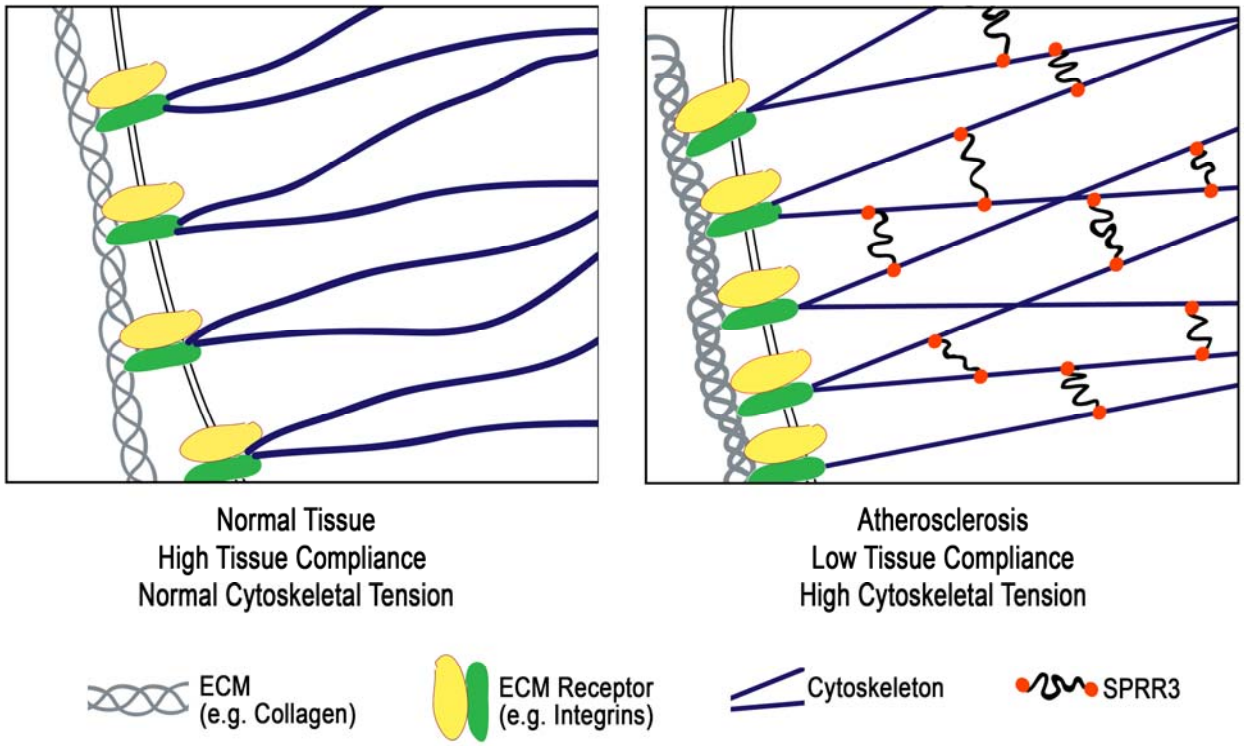


Figure 4-1

Hypothesized function of SPRR3. Under normal conditions, large arteries remain soft and highly compliant with low ECM content and the cytoskeleton of VSMCs within the arterial wall is under relatively low tension. Thus, the artery can distend and retract during systole and diastole, respectively. However due to ECM remodeling, calcification, and increased cellular content within the atherosclerotic milieu, the vessel wall becomes decreasingly compliant. As the microenvironment stiffens, cytoskeletal tension builds within the intimal VSMCs. We hypothesize that SPRR3 is covalently bound through transglutamination to cytoskeletal elements and yields flexibility to the otherwise stiff cytoskeleton.

33°C in the presence of 10ng/mL interferon-γ (this permits transformed-like phenotype) and subcultivated for 10 days (5 passages) in regular media at 37°C (this reverts the cells to a primary-like phenotype) prior to use.[124]

For retroviral transduction, wild type (WT) or mutant SPRR3 cDNA was subcloned into the LZRS-MS-IRES-GFP retroviral expression vector (a kind gift of Alyssa Weaver, Vanderbilt University) and transfected into Phoenix 293 packaging cells (maintained in 10% heat-inactivated FBS in DMEM). Phoenix cell transfection, viral harvest, and target cell transduction were performed as previously described.[165] Forty-eight hours following transduction, the transduced Tag-VSMCs were assessed for GFP expression and sorted by flow cytometry to enrich for GFP-positive cells. Transduced Tag-VSMCs will be referred for the remainder of the text as follows:

Tag-VSMC+GFP (GFP-only, empty vector control)

Tag-VSMC+WT (wtSPRR3-IRES-GFP construct)

Tag-VSMC+ ΔX (mutant SPRR3-IRES-GFP construct)

Mutational cloning:

Specific deletions of the wild-type SPRR3 cDNA were generated using QuikChange Mutagenesis as described elsewhere.[166] The mutant constructs are detailed in Figure 4-2.

Migration Assay:

Tag-VSMC migration was assessed using a modified Boyden-chamber assay as previously described.[167] Briefly, 70% confluent Tag-VSMCs with various transducts were subjected to serum-starvation overnight in 1%

Figure 4-2

Figure 4-2
Graphic illustration of various SPRR3 WT and mutant constructs. TGase substrate domains are highlighted in red in the amino acid sequence (a) and in the graphic depictions of the protein (b).

FBS/DMEM. Cells were harvested, and $4x10^3$ cells were added to the upper chamber of the apparatus with 15% FBS/DMEM in the lower chamber, and incubated for 5hr. Filters were fixed for 20min in 4% paraformaldehyde and stained with crystal violet. The number of cells migrated to the lower chamber were counted in each of eight high power fields per filter. Migration was assessed in triplicate for each cell type.

Proliferation Assay:

Tag-VSMC with various transducts were assessed for BrdU incorporation as well as Ki-67-staining nuclei to determine proliferative indexes. For BrdU incorporation, cells were plated at 500 and 1,000 cells/well of a 96-well plate and grown in 15%FBS/DMEM. For Ki67 staining, cells were plated at 10% confluence on glass coverslips coated with PBS, fibronectin (20μg/mL), collagen I (0.1mg/mL), gelatin (0.2%). When the cells reached approximately 90% confluence, the coverslips were fixed and stained as described elsewhere.[168]

Collagen Gel Contraction:

Tag-VSMCs at 60% confluence were harvested with trypsin and $2.5x10^5$ cells/mL (final concentration) were dispersed into collagen gels (1.25mg/mL final collagen concentration) and 0.3mL were plated in each well of a 24-well plate. After the gels solidified at 37°C for 30min, they were manually detached from the sides of the wells with a 30-gauge needle and 1mL media was added to maintain the cultures. The plates were scanned at defined time points and the area of the gels in pixels was calculated with MetaMorph Imaging system (Molecular

Devices, Sunnyvale, CA). Contraction was assessed in triplicate for each cell type.

Immunoblot and Immunoprecipitation:

Tag-VSMCs were grown to 90% confluence and harvested by scrapping in RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.05% SDS, protease inhibitors). Protein concentration was determined by a BCA protein assay (Thermo Fisher). For immunoprecipitation, cell lysates were pre-cleared against protein A beads and immunoprecipitated with protein A beads cross-linked (in 0.2M TEA pH 8.2) to a SPRR3 antibody. Samples were separated on SDS-PAGE gels. Immunoblotting was carried out as described elsewhere.[36] Primary antibodies used were anti-hSPRR3 (clone 4a, 1:1000), anti-smooth muscle α-actin (1:5000) and anti-β-actin (1:5000) with appropriate HRP-conjugated secondary antibodies.

Results

Generation of Tag-VSMC lines overexpression wtSPRR3 and deletion mutants.

To better understand the function of SPRR3, we generated VSMCs which constitutively expressed the human SPRR3 gene using a retroviral transduction system in the mouse-derived Tag-VSMCs. This allowed very specific detection of the overexpressed SPRR3 using an antibody which expressly recognized human SPRR3. There is no endogenous mouse SPRR3 detected in the Tag-VSMCs (consistent with findings in the previous chapter that SPRR3 expression was stress-dependent, see Figure 3-4b), but the human protein was robustly expressed following retroviral transduction (Figure 4-3a). Immunoblot for SPRR3

Figure 4-3

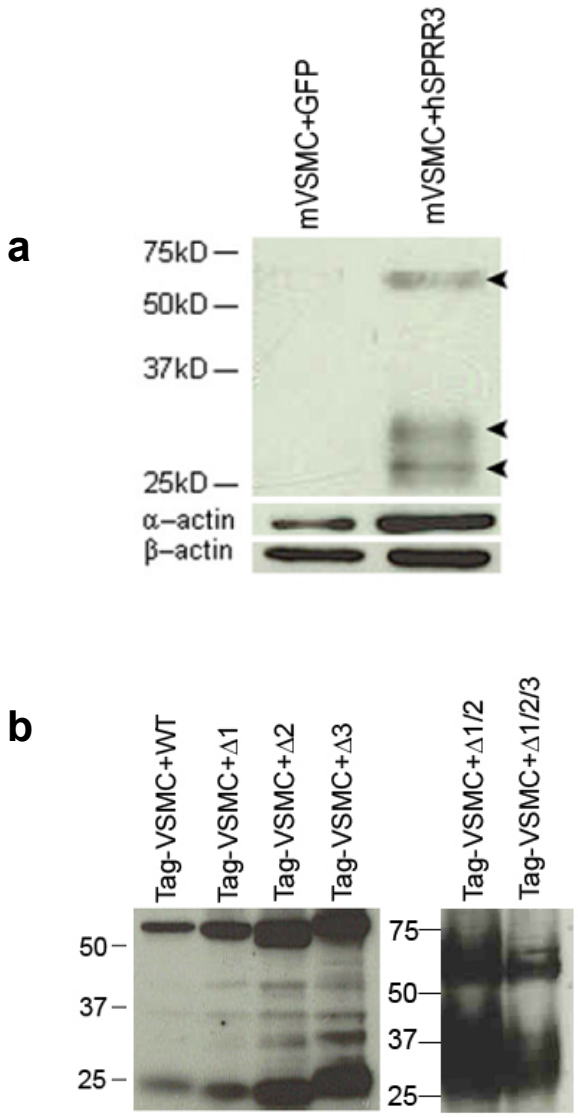


Figure 4-3
wtSPRR3 overexpression in Tag-VSMCs. Human SPRR3 expression was driven in Tag-VSMCs, and detected by immunoblot using an antibody specific for human SPRR3. Three bands were observed with SPRR3 overexpression: 25kDa, 30kDa, and 60kDa (a). Mutant human SPRR3 constructs with various TGase domain deletions were also detected by immunoblot (b). Molecular weight markers given in kilodaltons (kDa).

showed three prominent bands, one at approximately 25kDa, which represents monomeric SPRR3, one band at 30kDa, and another at 60kDa. This is consistent with the notion that SPRR3 is transglutaminated to itself and/or other proteins.[63] Transglutamination is a covalent linkage of two proteins through a ε-(γ-glutamyl)lysine isopeptide bond which is not readily disrupted by reducing conditions.[150] Furthermore, as SPRR3 has been shown to be transglutaminated at amino acids Q5-Q8, Q17-Q22, and K159-K161, we generated deletion mutants of each of these putative transglutaminase (TGase) domains.[63] The same pattern of bands was observed for the WT and mutant constructs. The two higher molecular weight bands were believed to be protein complexes containing SPRR3 covalently bound to an unknown protein through the action of TGases. However, as these bands remain in all the mutant constructs, it is unlikely that the bands arise from transglutamination. Alternately, other TGase domain sites may exist on SPRR3 which were not identified by *in vitro* TGase assays.[63] In order to determine the identity of the higher molecular weight bands detected by immunoblot, we performed immunoprecipiations (IP) with the same antibody used for immunoblot. Multiple attempts were made to IP a sample which could be identified by proteomic analysis. However, none were successful and the nature of the higher molecular weight bands remains unknown (representative experiment shown in Figure 4-4).

Figure 4-4

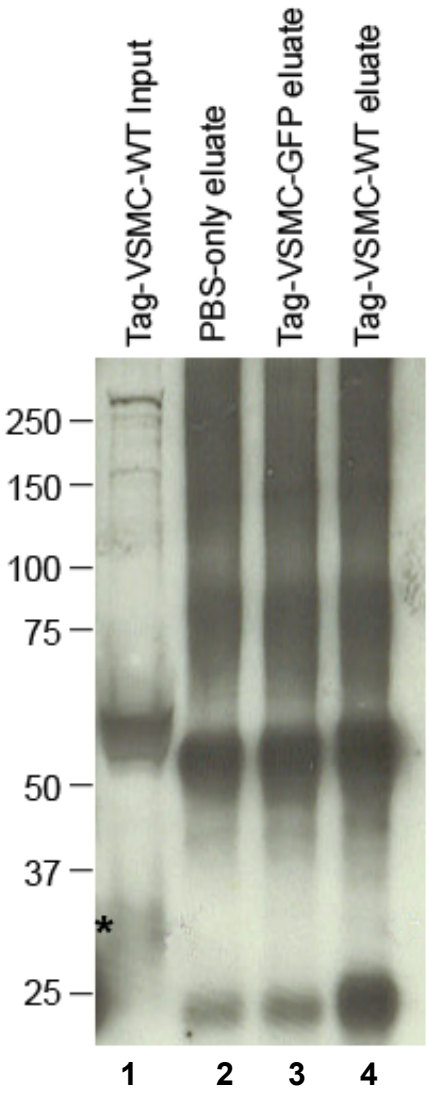


Figure 4-4

IP of SPRR3 brought down neither SPRR3 nor any binding partners. This image is from a single representative experiment. A small amount of input lysate was run in lane 1 for comparison (* monomeric SPRR3 in control lane). No unique bands were detected from the IP of lysates from cells overexpressing SPRR3 compared to PBS-only input control or lysate from GFP-only expressing cells. Molecular weight markers given in kDa.

Overexpression of wtSPRR3 affected proliferation, migration, and contraction.

Since the proliferative activity of VSMCs is altered in atherosclerosis, we sought to determine the rate of proliferation of the Tag-VSMC+GFP and Tag-VSMC+WT cells using two assays. A BrdU-incorporation assay in which the cells were plated directly onto the plastic tissue culture dish overnight followed by a 24h pulse of BrdU. This revealed decreased proliferation of the Tag-VSMC+WT (Figure 4-5a). Furthermore the same cells were plated at a very low concentration (around 10% confluence) on glass coverslips coated with various ECM components for 5-7 days and growth was assessed by Ki67-positive nuclei. In this case the Tag-VSMC+WT cells proliferated significantly faster than control when plated on PBS-coated coverslips. However, those cells grown on coverslips coated with fibronectin, gelatin, or collagen showed equal proliferation between Tag-VSMC+wtSPRR3 and control cells (Figure 4-5b). There are several possible causes for the inconsistency between the different proliferation assays. Firstly, the substrates are different in all cases: tissue culture plastic, glass, or ECM-coated glass. The interaction of each of these substrates with the cells is undoubtedly different and therefore may be responsible for the different proliferative indexes.[60] Secondly, the cells were grown at different densities in the two assays. Cell were grown very sparsely on coverslips for a prolonged period of time, whereas in the BrdU-incorporation assay the cells were at a relatively high density for a short time. The molecular events underlying these

Figure 4-5

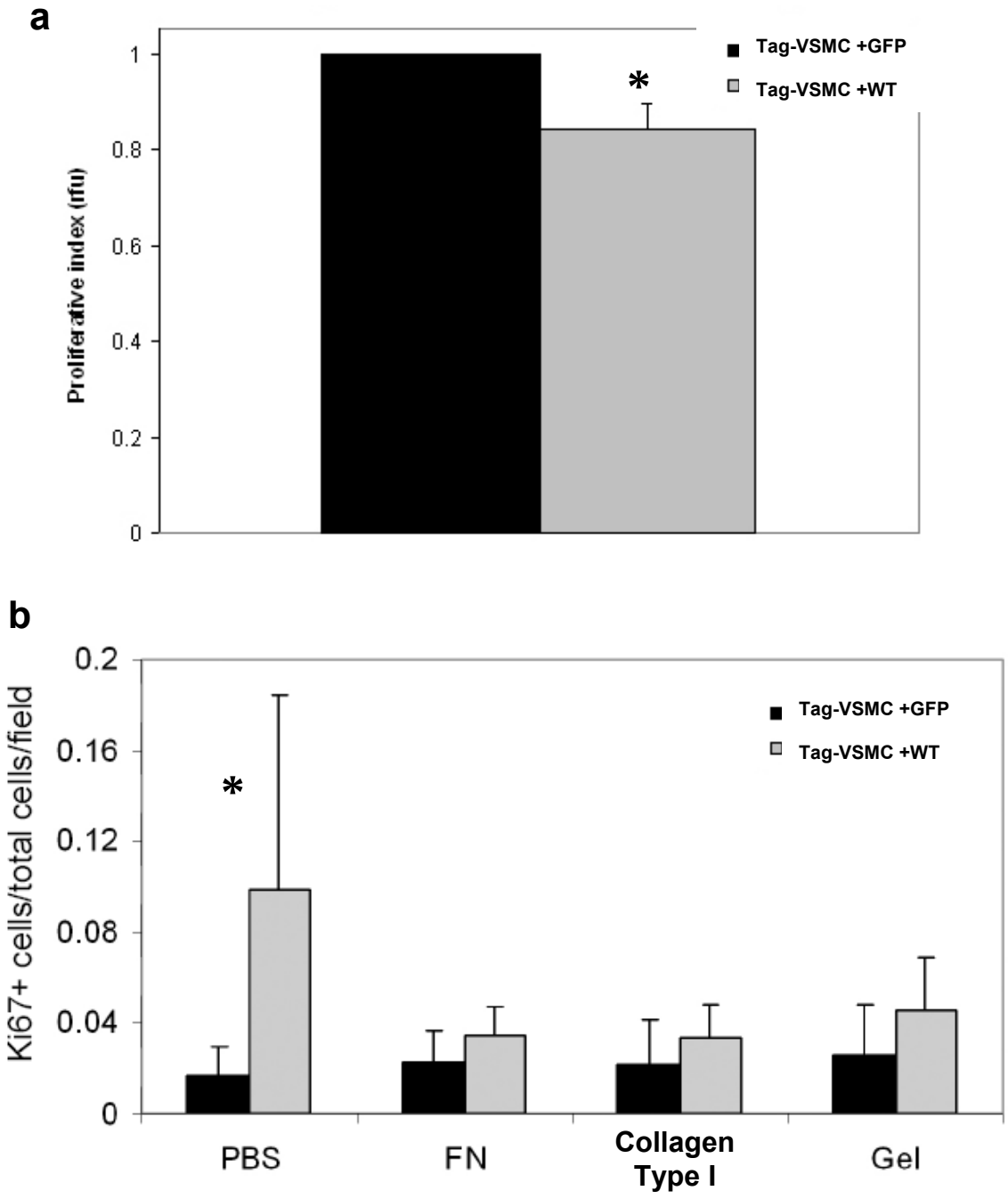


Figure 4-5

SPRR3 overexpression alters Tag-VSMC proliferation. BrdU-retention after 24hr reveals a decrease in proliferation when cells overexpress SPRR3 (a). However, when plated at a low concentration and grown for several days on coated glass coverslips, the opposite effect is observed, that overexpression of SPRR3 promotes proliferation (b). *p≤0.05

divergent results could provide some insight as to the function of SPRR3 in the future.

Since VSMC migration is a key step in atherosclerotic plaque progression, the cells were also assessed for their ability to migrate. As shown in Figure 4-6a, Tag-VSMC+WT migrated about 1.7+/-0.003-fold ($p=1.4x10^5$) more efficiently than control cells. The fact that SPRR3 expression promotes migration suggests that SPRR3 may play a role in the important step of VSMC migration from the media to intima in atherosclerosis progression.

Another characteristic of VSMCs within an atherosclerotic plaque is decreased contraction as the cells de-differentiate into a synthetic phenotype. Therefore, we determined the contractile capacity of the cells expressing SPRR3 using a collagen gel contraction assay. This revealed that Tag-VSMC+WT demonstrated a diminished ability to contract a collagen gel over time (Figure 4-6b, $p \leq 0.002$). These data showing increased migration and decreased contraction with SPRR3 expression were consistent with known behavior of VSMCs within an atheroma.

TGase inhibition

To test the hypothesis that transglutamination of SPRR3 is required for its function in VSMCs, we employed the TGase inhibitors, methyldansylcadaverine (MDC) and cystamine. These compounds function by competitive inhibition and active-site blocking of TGases, respectively.[169] Surprisingly, the higher molecular weight bands observed by immunoblot were not abrogated by 24h treatment with either TGase inhibitor (Figure 4-7). These findings suggest that SPRR3 existed

Figure 4-6

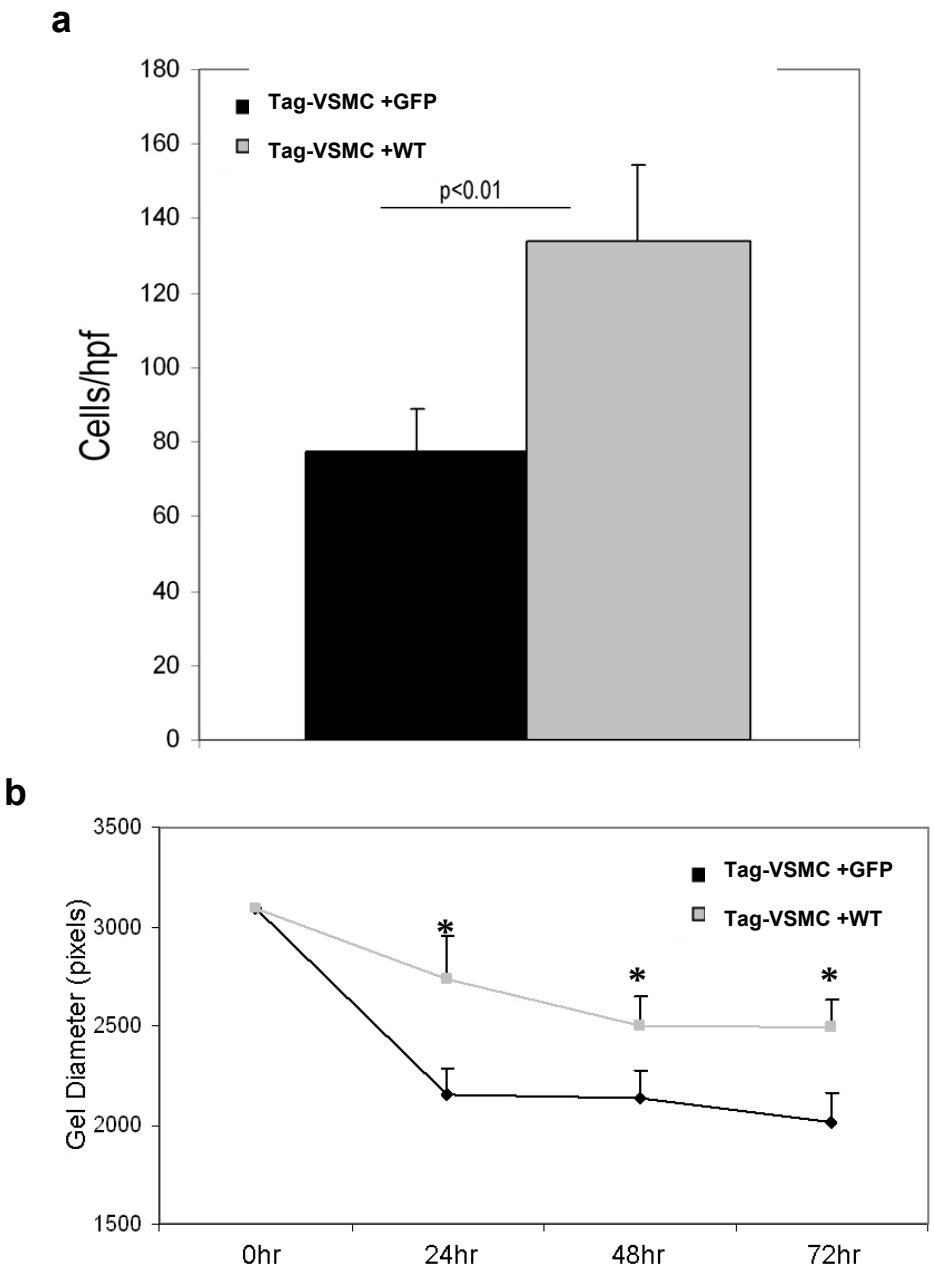


Figure 4-6

SPRR3 overexpression decreases Tag-VSMC contraction of collagen gels. Overexpression of SPRR3 in Tag-VSMCs produces a 1.73-fold increase in migration compared to GFP-only controls (a). The Tag-VSMC+WT cells, however, are less contractile than control cells, as evidenced by a relative inability to contract a collagen gel (b). *p≤0.002

only in a monomeric form and that the higher molecular weight bands are non-specific artifacts associated with SPRR3 overexpression. Alternately, because TGase-generated covalent bonds are non-reversible, it is possible that the higher molecular weight protein complexes were generated prior to TGase inhibition and are still observed due to a slow protein turn-over.

Although we could not detect changes in the stead-state population of SPRR3 with TGase inhibition, we sought to assay these treatments on active cellular processes. To test the effect of TGase inhibition on SPRR3 function, Tag-VSMCs+/-SPRR3 were pretreated for 24h with TGase inhibitors and then subjected to migration analysis. As seen in Figure 4-8, use of either TGase inhibitor reversed the effect of overexpressing SPRR3 in a dose-dependent manner. These data were seemingly in conflict with the immunoblot which suggested that transglutamination does not affect SPRR3. It is possible that the results observed using the TGase inhibitors were due non-specific effects; MDC and cystamine are known to produce off-target results on cultured cells.[170, 171] This may also explain the increased migration in control cells with inhibitor treatment.

SPRR3 mutants

As an alternate approach to TGase inhibition, the SPRR3 TGase-substrate domain mutants were employed to more specifically determine the effect of transglutamination on SPRR3 function. A number of possible protein-protein binding interactions can be envisioned based on these three TGase-substrate domains. As shown in Figure 4-10, SPRR3 could be bound to other

Figure 4-7

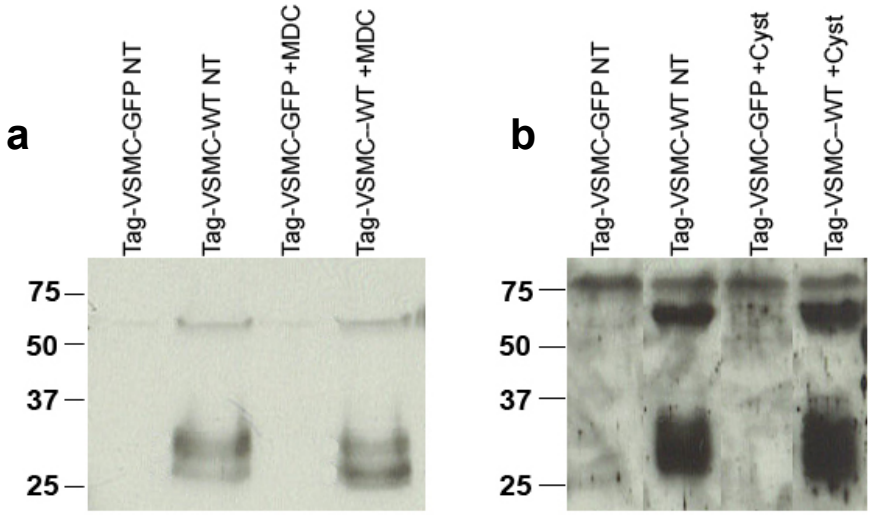


Figure 4-7

TGase inhibition does not affect the banding pattern of SPRR3 as detected by immunoblot. Tag-VSMCs +/- SPRR3 were grown in the presence of TGase inhibitors, MDC (a) or cystamine (b) for 24h. Molecular weight markers given in kDa.

Figure 4-8

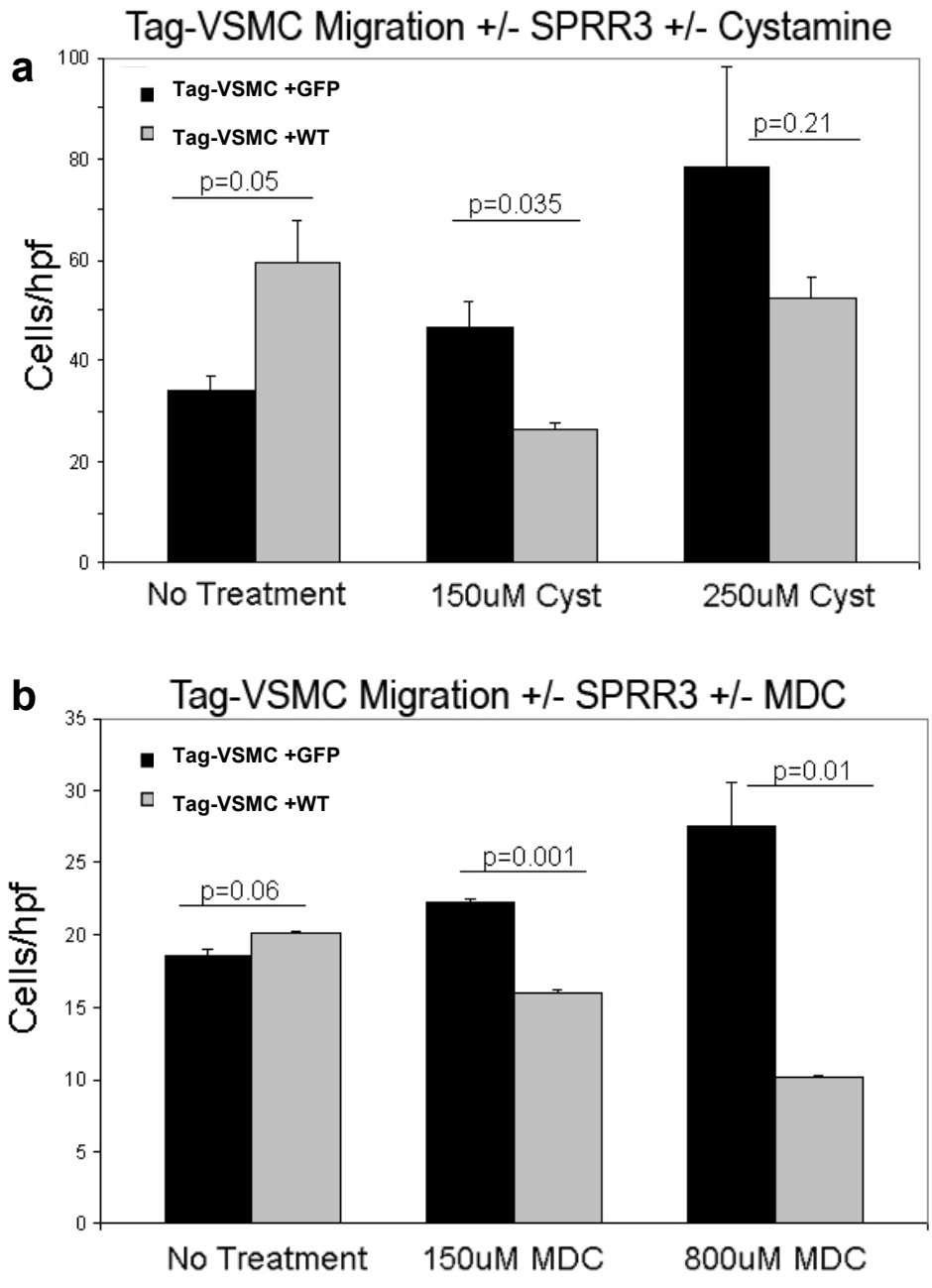


Figure 4-8
TGase inhibition decreases Tag-VSMC+WT migration. Pretreatment with either TGase inhibitor (cystamine (a) or MDC (b) decreases Tag-VSMC+WT migration while Tag-VSMC+GFP migration increases, both in a concentration-dependent manner.

SPRR3 molecules through any of those domains, as well as to other proteins, thereby producing the multiple bands observed by immunoblot for SPRR3. However, consistent with the TGase inhibition immunoblot, when these mutant constructs were expressed in Tag-VSMCs, the same banding pattern as wtSPRR3 was observed (Figure 4-3b). If any of the deleted domains are normally covalently bound to any other protein, the banding patterns should shift in either size or intensity with some of the mutant constructs. Therefore, these data suggested again that transglutamination of SPRR3 does not occur in this system with VSMCs.

Migratory and contractile capabilities of the cells with mutant SPRR3 constructs were assessed in comparison to GFP and wtSPRR3 transduced cells. As shown in Figure 4-9, only cells containing constructs with the Δ1 mutation (Δ1, Δ1/2, Δ1/2/3) demonstrated behavior similar to control cells, whereas the other mutants were phenotypically similar to those expressing wtSPRR3. Cells carrying a construct with a single deletion at site 1 alone also contracted and migrated like control cells (data not shown). Furthermore, in the contraction assay, cells containing mutants Δ2 and Δ3 are unable to contract the collagen gel. The divergent results between the migration and contraction assays (migration suggests that Δ2 and Δ3 are non-functional, while the contraction assay shows an effect of these regions opposite of the Δ1 region) indicate a possible role for the amino acids in regions 2 and 3 in contraction only, but not in migration. However, the role of SPRR3 in both migration and contraction is ablated in the Δ1 mutant, indicates a requirement for amino acids 5-8 in SPRR3

Figure 4-9

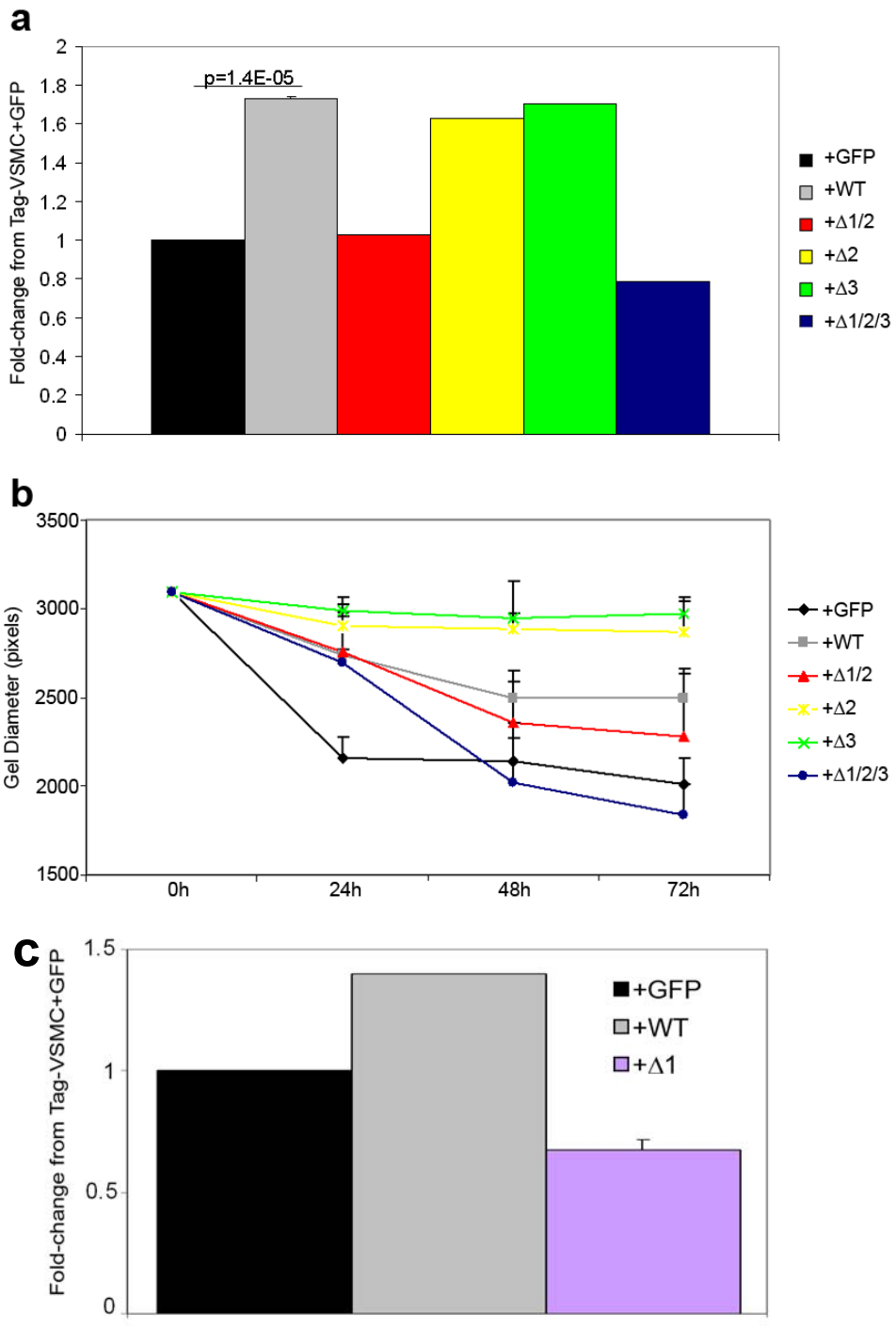


Figure 4-9

SPRR3 mutant constructs affect VSMC migration and contraction. Tag-VSMCs overexpressing mutant SPRR3 constructs with site 1 deleted (Δ1, Δ1/2, and Δ1/2/3) migrate (a,c) and contract (b) at levels similar to Tag-VSMC+GFP.

Figure 4-10

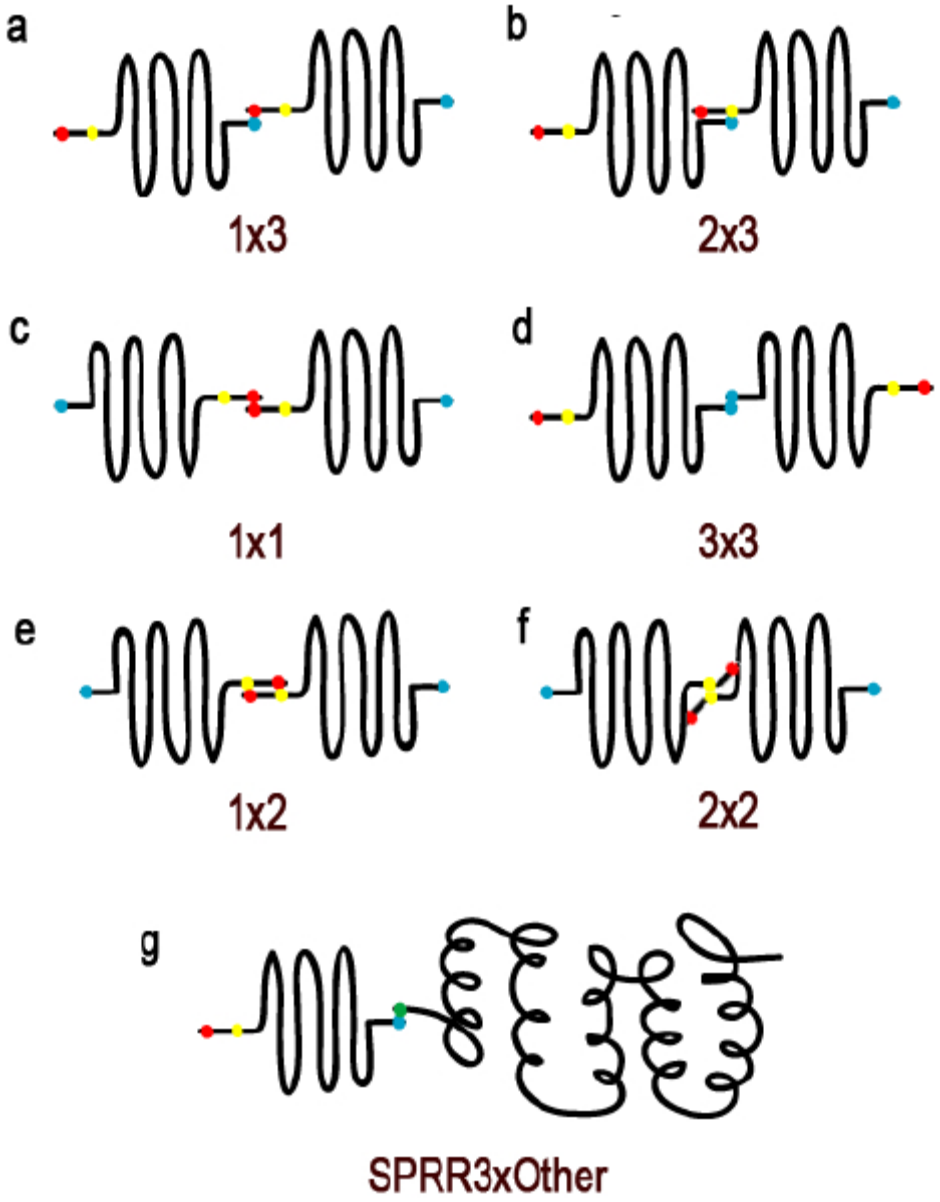


Figure 4-10
Possible arrangements of SPRR3 binding to other SPRR3 molecules (homodimer) or other proteins. Theoretically, SPRR3 could be bound to other SPRR3 molecules and other proteins through binding at the three TGase domains, site 1 (red), 2 (yellow), and 3 (blue). SPRR3 molecules could pair with each other through any combination of these domains, as shown (a-f) or with another TGase domain-containing protein (g).

(those deleted in the Δ1 mutant). If the phenotype were the result of transglutamination of two SPRR3 molecules binding to each other through site 1, then two molecules of SPRR3 would bind to each other as shown in Figure 4-10c, and would result in a 50kDa SPRR3 homodimer. However, this cannot be the case, as the 50kDa band observed by immunoblot remains in the Δ1 mutant cells (Figure 4-3b). Therefore, the effect mediated through amino acids 5-8 of SPRR3 are not likely the result of transglutamination. Unfortunately, this experiment did not definitively prove that SPRR3 was not transglutaminated. A more compelling experiment to demonstrate the transglutamination state of SPRR3 would be to utilize proteomic analysis of the various SPRR3 constructs isolated from Tag-VSMCs. We have made multiple attempts to do so, but have been unable to successfully purify and analyze SPRR3 from that system. Furthermore, preliminary data discussed in the next chapter, as well as recent work from another group suggests that SPRRs may function independently of transglutamination.[172] Therefore, we feel that the hypothesis that SPRR3 functions in VSMCs via transglutamination was incorrect.

Discussion

The hypothesis driving the work shown in this chapter was that SPRR3 functions as a flexible crosslinker in the cytoskeleton of VSMCs via transglutamination. However, the data present above indicated that the function of SPRR3 is independent of transglutamination of the protein. When wtSPRR3 is expressed in Tag-VSMCs, immunoblot analysis for SPRR3 revealed three

distinct, specific bands: one at approximately 25kDa (the predicted size of SPRR3) and two of higher molecular weights, suggesting that SPRR3 was covalently bound to some other molecule, presumably through transglutamination. Moreover, wtSPRR3 expression also lead to decreased proliferation and contraction and increased migration. Treatment of the SPRR3 overexpressing cells with TGase inhibitors yielded a dose-dependent decrease in Tag-VSMC+WT migration. However, the control cells also underwent a dose-dependent *increase* in migration and the banding pattern by immunoblot remained unchanged with TGase inhibitor treatment. These data produced an unclear picture of the role of transglutamination in the function of SPRR3. To more directly address the issue of SPRR3 transglutamination, specific deletion mutants of the TGase-substrate domains of SPRR3 were overexpressed in Tag-VSMCs. Migration and contraction of cells containing SPRR3 with the Δ1 site missing reverted to control levels, thereby indicating a dependence on the residues within the Δ1 site for SPRR3 function. However, this does not appear to be a TGase-dependent function, as the higher molecular weight moieties observed by immunoblot were not affected in the Δ1 mutant constructs. The function of SPRR3 in VSMCs remains uncertain, though it is evident that the region containing amino acids 5-8 is required for its function. Further mutational analysis will be required to determine which of these residues, either alone or in combination.

While these three assays mimic effects observed in VSMCs of atheromas, it should be noted that these are not all believed to act through the same

pathways. VSMC migration, contraction, and proliferation have all been shown to be affected by fibroblast growth factor, AngII, NADPH oxidase, PPARα, PKC, Ras, cAMP, PI3K, Akt, NF-κB, MAP-kinases, p38, JNK, and p16.[173-177] Given this broad array of pathways, we were surprised to see SPRR3 having an effect on proliferation, migration, and contraction. It is unlikely that SPRR3 is acting at a common point and affecting VSMCs in the same manner for each assay. This is supported by the fact that mutants Δ2 and Δ3 behave like WT SPRR3 in the migration assay, whereas cells expressing these constructs are completely unable to contract a collagen gel, unlike WT SPRR3. This suggests that different regions of SPRR3 may be required for different VSMC functions. Future work will continue to pursue the effects of amino acids 5-8 in SPRR3 function.

Recent work has implicated a non-structural role for another SPRR family member, SPRR2a, in biliary epithelial cells.[172] Based on this work and emerging preliminary data from our laboratory, it seems likely that SPRR3 may function in VSMCs to modulate intracellular signaling cascades, although the precise mechanism is yet to be elucidated. Nevertheless, it is clear that SPRR3 expression affects VSMC proliferation, migration, and contraction. Each of these processes likely plays a role in how cells within an atheroma adapt and react to the altered local microenvironment.

CHAPTER V

DISCUSSION AND FUTURE WORK

The studies presented in this document demonstrate our work on the regulation and function of SPRR3. We have shown that SPRR3 expression is regulated in VSMCs of atheromas by cyclic strain as the cells sense the microenvironment via $\alpha 1\beta 1$ integrin. We hypothesized that SPRR3 functions within VSMCs by transglutamination to structural proteins. However, the work presented in chapter IV lead us to conclude that SPRR3 likely functions through a different mechanism. Future work on SPRR3 will investigate an alternative function for SPRR3. Our lab has also generated a mouse deficient in SPRR3 which will be studied in order to gain more insight into SPRR3 function. These studies, particularly those in chapters II and III, serve as a reminder of the importance of the cellular microenvironment, a concept which has broad applications to cell biology. Finally, the novel discovery of SPRR3 as a specific marker of atherosclerosis makes it a prime candidate for development as a biomarker of atherosclerosis.

SPRR3 in intracellular signaling

A recent paper has suggested a function for an SPRR family member, SPRR2A, as a non-structural, signaling-related protein.[172] SPRR2A overexpression in biliary epithelial cells (BECs) decreases proliferation, increases

migration, and promotes a degree of epithelial to mesenchymal transition (EMT). Furthermore, SPRR2A was shown to be protective in response to BEC oxidative injury, which is consistent with another publication in which SPRR1a was shown to be cardioprotective following ischemic injury.[105, 172] Moreover, rather than acting in a structural manner, SPRR2A contains multiple sequences within its proline-rich region which act as SH3 ligand domains, and interacts with several SH3-containing proteins *in vitro*, including Src, Yes, and Abl. The authors suggest that the sequence PXPPXP (where P is proline and X is usually an aliphatic residue) is required for this function.[172] While many PXPPXP regions exist in SPRR3, they reside in the central domain and were therefore not directly affected by any of our mutant constructs. However, other putative functional domains in SPRR3 have been predicted by computational analysis.[178] Specifically, there is a class IV WW ligand domain (similar in function to SH3 ligand domain) at aa8-13, a region which is disrupted in the ∆1SPRR3 mutant.[179] The WW domain functions in a range of protein-protein interactions, particularly in linking signaling between the membrane and the cytoskeleton.[179] Currently, known WW domain-containing proteins include Pin 1, Nedd4, and WW-2.[179] While there is no known connection between SPRR3 and any of these proteins, the role of the WW-ligand interaction in relating signals from the cell membrane to the cytoskeleton suggests a possible function for SPRR3 as a signaling molecule. Future studies will address the biological role of the WW ligand domain using mutagenesis to determine which amino acids in the ∆1 region are required for SPRR3 function.

We hypothesize that SPRR3 regulates Akt phosphorylation, thereby promoting cell survival. This is supported by the fact that overexpression of wtSPRR3 results in an increase in Akt phosphorylation, an effect which is abrogated in the Δ1SPRR3 mutant (Figure 5-1). This was through PI3K activity, as evidenced by the lack of phosphorylation of Akt following incubation with the PI3K inhibitor, LY294002. Akt phosphorylation by phosphatidylinositol 3-kinase (PI3K) is primarily associated with promoting cell survival by evading apoptosis and it is possible that SPRR3 overexpression may lead to enhanced cell survival.[180] Cells overexpressing either SPRR1A or SPRR2A have better survival, though not necessarily in an Akt-dependent manner.[105, 172] Furthermore, SPRR2a and 2b is upregulated in bronchial epithelium following an allergic challenge and inflammation and SPRR1a has been shown to be expressed as an adaptive response to ischemic stress in cardiomyocytes as well as in neurons following peripheral axonal damage,.[105, 181, 182] Therefore, future work on SPRR3 will determine if overexpression of SPRR3 enhances cell survival following various challenges. Specifically, we will expose VSMCs overexpressing WT SPRR3 to ROS (in the form of H_2O_2 added to the media), hypoxia, and low serum conditions and asses the rate of cell death by apoptosis. Based on the increased phosphorylation of Akt by SPRR3, we expect that those cells overexpressing SPRR3 would demonstrate improved survival in the face of such insults. Blocking Akt, however, should abrogate such an effect.

While it is well known to affect cell survival, Akt has many other functions including cell growth, proliferation, transcription, and nutrient metabolism.[183] If

Figure 5-1

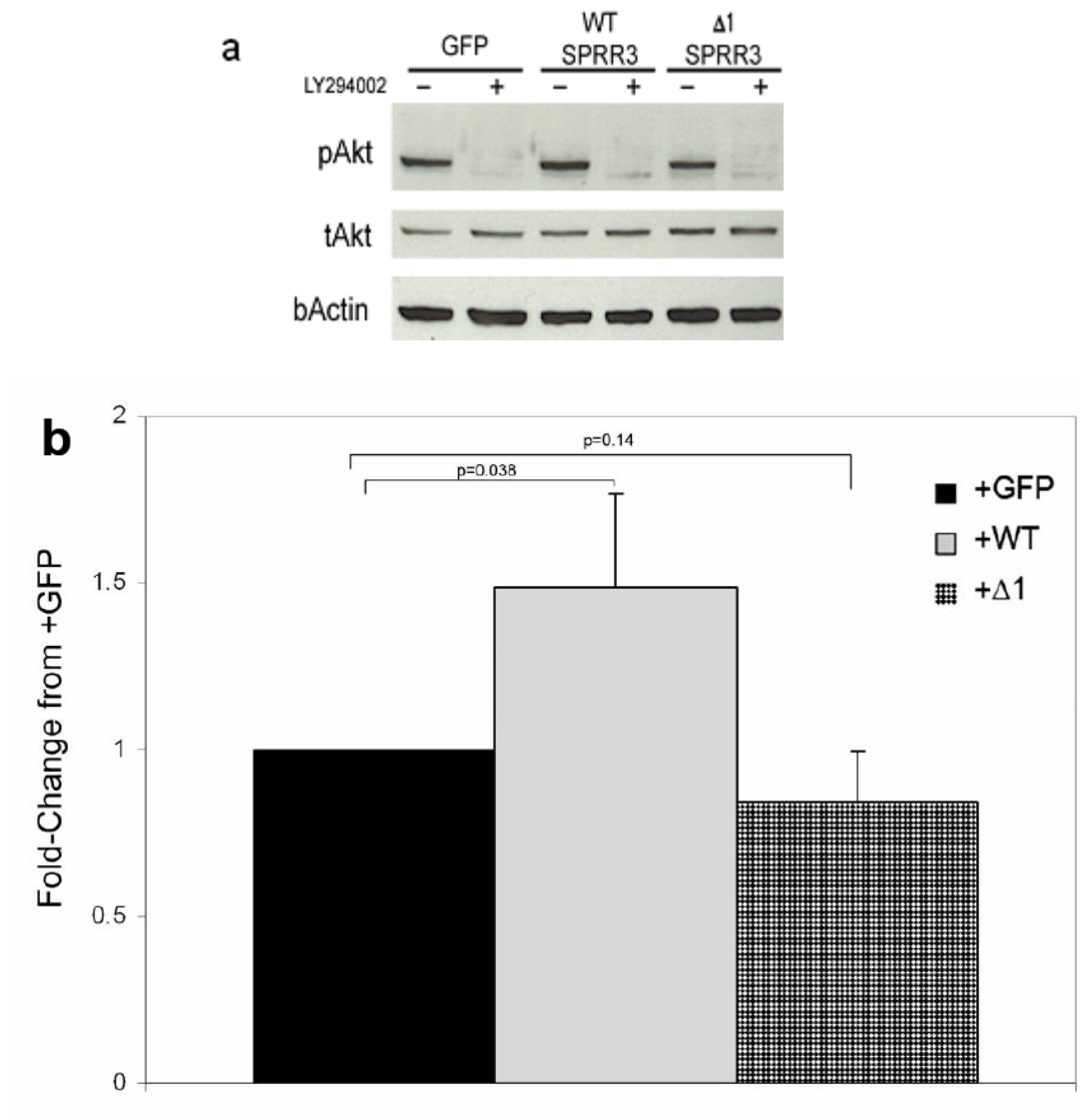


Figure 5-1

SPRR3 overexpression increases basal Akt phosphorylation. (a) A representative immunoblot showing phosphorylation of Akt (pAkt) at serine 437 (top panel), as well total Akt and βActin. Levels of pAkt were determined relative to total Akt and βActin. Overexpression of wtSPRR3 resulted in a modest increase in pAkt, but this increase was abolished in the Δ1 SPRR3 mutant. Preincubation with PI3K-inhibitor, LY294002, blocked Akt phosphorylation. (b) Quantitation of Akt phosphorylation as assessed by immunoblot (n=4).

SPRR3 is not shown to mediate cell survival through Akt as described above, we may look at these other phenotypes. Moreover, while still controversial, data exists showing Akt phosphorylation can occur through integrin-linked kinase (ILK), which interacts with the cytoplasmic tails of β1 and β3 integrins.[184] In particular, studies have shown that in VSMCs, ILK is an important mediator of cellular behavior by activating Akt.[184] It remains unclear, however, if ILK directly phosphorylates Akt or if it acts through PI3K (our data supports a role for PI3K, as its inhibition abrogates the effect of SPRR3 overexpression).[185, 186] Nevertheless, this supports a role for Akt signaling downstream of integrin activation.[180]

In addition to our finding that SPRR3 overexpression affects Akt phosphorylation, we have also observed an effect on type I collagen synthesis. Two-dimensional difference gel electrophoresis (2-D DIGE) of protein lysates from VSMC +WT SPRR3 and GFP-control cells showed a significant up-regulation of type I collagen (Figure 5-2a). This finding was confirmed by qRT-PCR and also showed that collagen Ia1 transcripts, which are increased in cells overexpressing WT SPRR3, are not up-regulated in cells expressing the D1 mutant. This suggests that the amino acids 5-8 are required for this function as well (Figure 5-2b). Especially in light of the requirement for amino acids 5-8 for both increased Akt phosphorylation and collagen synthesis, we hypothesized that the collagen synthesis may be dependent upon Akt. In very preliminary data, we have shown that increased collagen synthesis by SPRR3 is ablated by the PI3K inhibitor, LY294002 (Figure 5-3). While still very preliminary, these data suggest

Figure 5-2

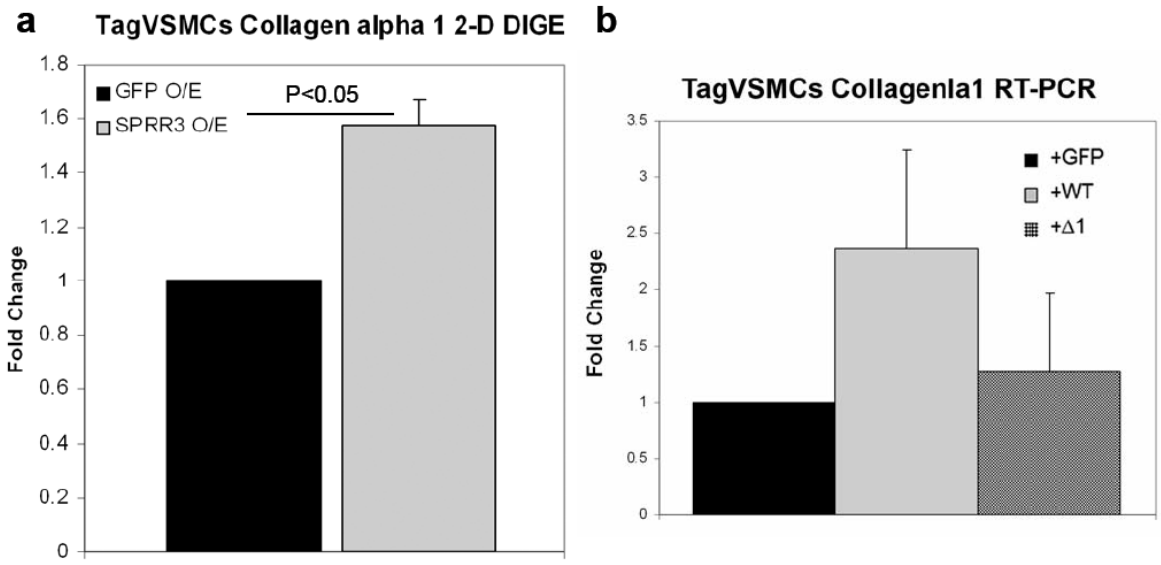


Figure 5-2

SPRR3 overexpression increases type I collagen expression. VSMCs overexpressing WT SPRR3 were found to have increased type I collagen protein by 2-D DIGE analysis (a) as well as increased collagen I a1 transcript synthesis by qRT-PCR (b). However, cells expressing the Δ1 SPRR3, did not show an increase in collagen I a1 transcription (b).

Figure 5-3

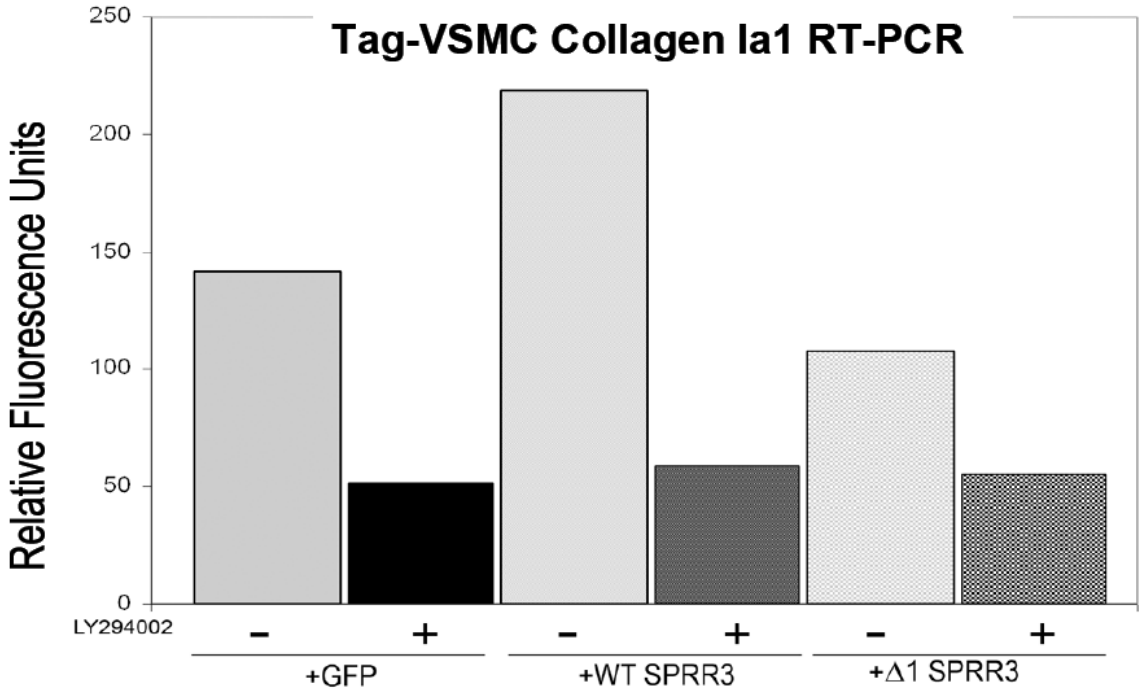


Figure 5-3
SPRR3-dependent collagen up-regulation is Akt-dependent. WT SPRR3-overexpressing VSMCs show increased collagen Ia1 transcript levels, but not in the presence of the PI3K inhibitor, LY294002.

that SPRR3 modulates type I collagen synthesis in an Akt-dependent manner (see Figure 5-4).

Given its known function as a structural element, it is also conceivable that SPRR3 may act as a scaffolding protein, by bringing other signaling proteins into close proximity (Figure 5-4). In this way SPRR3 may affect intracellular signaling. Given the known structure and function of SPRR3, it is unlikely that it would act in this capacity, as there are no other studied protein-interaction domains. However, the proteomic analysis which identified the WW-binding domain also revealed several other putative protein-interaction domain sequences. Most of these were within the highly flexible central domain of the protein and are therefore unlikely to be functional. This remains an area open for future study.

The proposition that SPRR3 modulates intracellular signaling pathways is truly a new paradigm for the manner in which the protein is thought to function. In recent years, many reports have emerged in which various SPRR family members were discovered in tissues other than stratified epithelium.[64, 105, 172, 181, 182, 187-191] Since these tissues are not known to have a cornified epithelium in which the SPRR proteins could function, it follows that these proteins must perform another role. It is our hope that this work will be among the first to show a new mechanism of action for SPRR proteins, specifically that they can participate in signaling pathways, particularly the cell survival pathway via Akt.

Figure 5-4

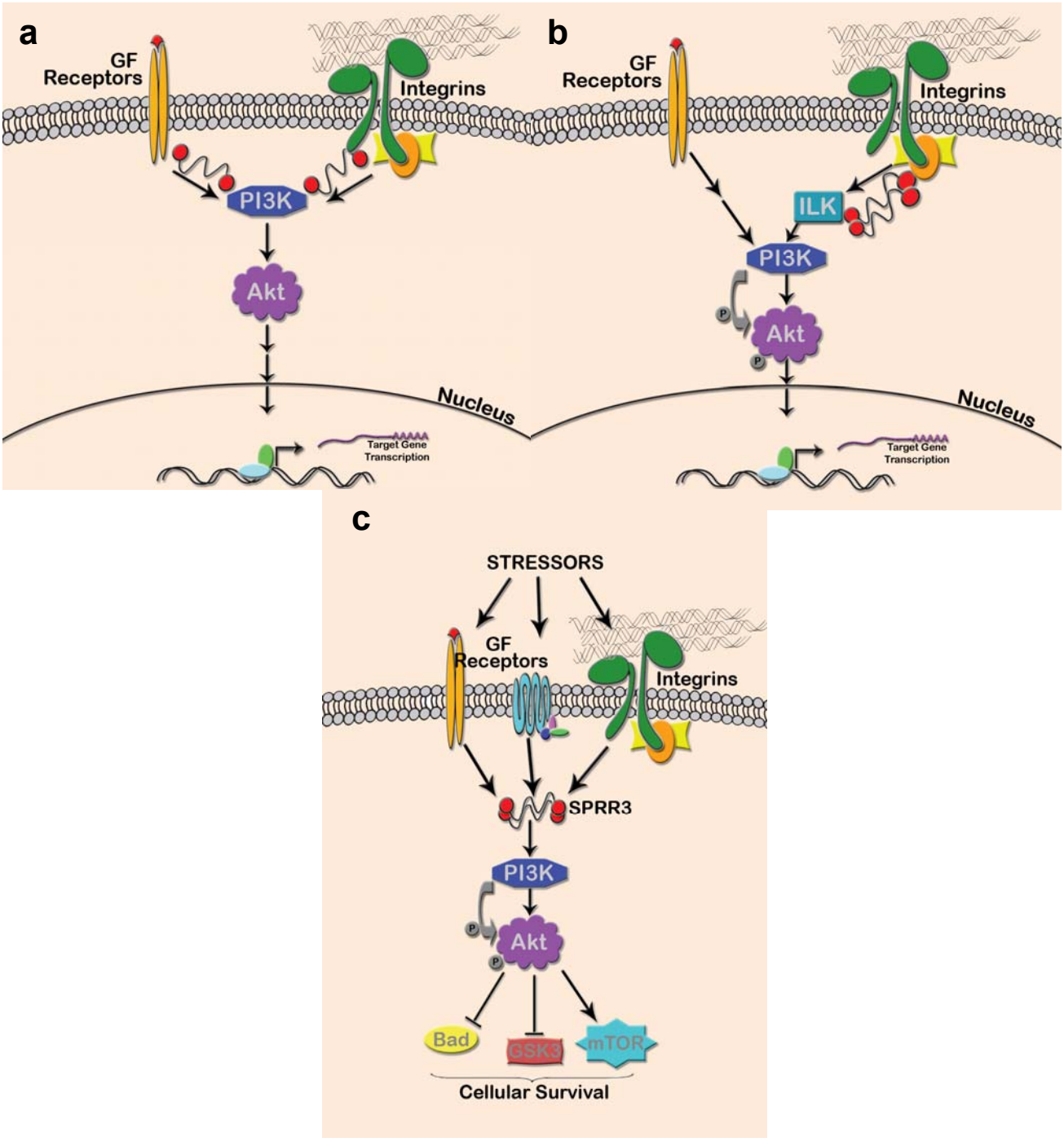

a
GF Receptors
Integrins
PI3K
Akt
Nucleus
Target Gene Transcription
b
GF Receptors
Integrins
ILK
PI3K
P
Akt
P
Nucleus
Target Gene Transcription
c
STRESSORS
GF Receptors
Integrins
SPRR3
PI3K
P
Akt
P
Bad
GSK3
mTOR
Cellular Survival

Figure 5-4

Possible models of SPRR3 function. We have established a role for SPRR3 in modulating Akt phosphorylation (a-c). Given its known function as a structural molecule in other systems, it is possible that SPRR3 may act as a scaffold, brining various intracellular proteins into proximity, such as integrins or other cell-surface receptors, and signaling molecules such as PI3K (a). Furthermore, based on the amino acid sequence, SPRR3 may function to modify other proteins, thereby acting as a signaling molecule. As shown in b, it is conceivable that SPRR3 may modulate Akt activity by enhancing ILK function downstream of integrins. Finally, in light of studies showing other SPRR family members to be turned on in tissue-stress conditions, SPRR3 may function to integrate various stress signals (be it mechanical or chemical) through different pathways. Akt could be activated through SPRR3 to activate pathways for increased cell survival, such as mTOR and GSK3 (c).

SPRR3 knockout mouse

In order to gain further understanding into the function of SPRR3, our lab generated a mouse deficient in SPRR3. The mice were aged to over 14 months with no evident phenotype. Since SPRR3 is minimally expressed in VSMCs *in vivo* in the absence of atherosclerosis, and because mice do not naturally develop atherosclerosis, it was not surprising that no vascular phenotype was observed. In order to determine what effect the absence of SPRR3 will have in atherosclerosis, we plan to cross the SPRR3 knockout mouse with the atherosclerosis-prone ApoE knockout mouse. Based on the data that SPRR3 overexpression in VSMCs leads to increased migration and decreased contraction (which are characteristic of VSMCs in a developing plaque), we hypothesize that absence of SPRR3 will decrease atherosclerotic progression in the ApoE null mice.

Other work from our lab has implicated SPRR3 as biomechanically responsive in cardiac tissue, though no cardiac phenotype was evident in the knock-out animals. To further investigate the role of SPRR3 in the heart, we subjected SPRR3-null and litter-mate control animals to a pressure-overload model of cardiac hypertrophy by transverse aortic constriction. Surprisingly, the $SPRR3^{-/-}$ mice demonstrated improved cardiac function (as determined by percentage of fractional shortening) and less fibrosis (Figure 5-5). Future work will further continue to dissect the mechanism by which SPRR3 mediates cardiac fibrosis.

Figure 5-5

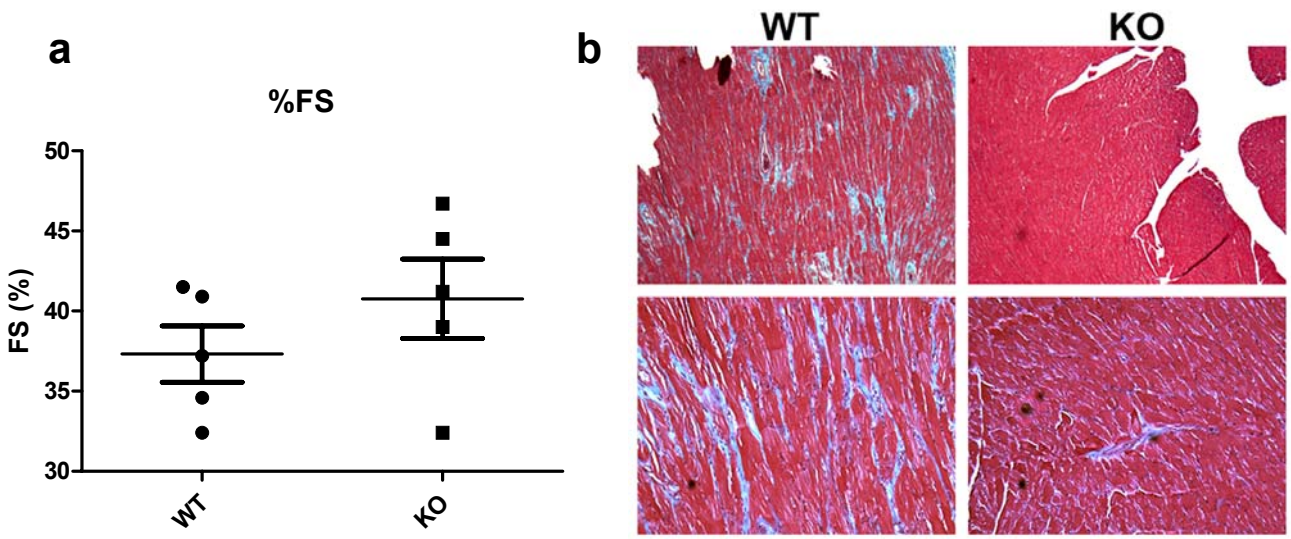


Figure 5-5

SPRR3 deletion leads to improved outcome in a mouse model of pressure-overload cardiac hypertrophy. Mice deficient for SPRR3 (KO) show greater percentage fractional shortening (a) and less fibrosis (b, blue denote collagen fibrosis, Masson's Trichrome Stain) compared to WT controls following transverse aortic constriction. Greater fractional shortening and decreased fibrosis indicate improved outcome in the SPRR3-null animals.

SPRR3: A lesson on the importance of the microenvironment

In recent years, growing emphasis has been placed on understanding tissue microenvironment of a given system, be it in a tumor, heart disease, or normal development.[192-194] The atherosclerotic plaque also has a unique mélange of ECM composition and compliance, cellular content, cytokines and other inflammatory mediators, dead and dying cells, and lipids.[195] So complex is this microenvironment that it cannot reasonably be completely recapitulated *in vitro*. In fact, the data presented in chapter III of this document showed a requirement for the presence of multiple factors in an *in vitro* model system in order to recapitulate the *in vivo* results. *In vitro* expression of SPRR3 in VSMCs occurs only when $\alpha 1 \beta 1$ integrins are expressed and bind type I collagen to sense cyclic strain. The absence of any of these components will prevent SPRR3 expression.[43] This demonstrates the need to recapitulate multiple features of the *in vivo* microenvironment when studying vascular biology *in vitro*. Not only should the cellular and ECM components be consistent with the physiologic and/or pathologic context, but the mechanical strains of the system should also be accounted for when working *in vitro*. This concept could be applied to studies of other vascular diseases including atherosclerosis, restenosis, and hypertension in addition to stem cell, developmental, and cancer biology. This underscores the growing body of evidence that *in vitro* cell culture-based work is limited in its inability to mimic the *in vivo* environment.[43, 193, 194]

SPRR3 as a biomarker

Outside of an understanding of its function, SPRR3 could prove very useful as a biomarker for atherosclerosis. As discussed in earlier chapters, expression of SPRR3 in both mouse and human vasculature is restricted to regions of atherosclerosis. Currently, clinically accepted surrogates of risk for cardiovascular disease are serum lipid measurements and assessment of the inflammatory marker, C-reactive protein (CRP).[196-199] However, these markers of atherosclerosis are somewhat non-specific and only partially predictive.[196] At their 57th Annual Scientific Session in 2008, the American College of Cardiology concluded that many of the currently accepted surrogate markers of cardiovascular events and atherosclerosis are not sufficiently predictive, and therefore bear further evaluation.[200] Furthermore, a single, definitive histological marker of atherosclerosis does not exist.[201]

Markers of atherosclerosis fall into three general categories: those used for histological detection (and therefore on non-living tissue), those detected in the peripherally circulating blood or urine, and those which are detected through various imaging modalities.[201] Tests which analyze proteins and molecules from peripheral blood or urine samples are most economical, least invasive, relatively fast, and are therefore preferred compared to imaging studies.[197] Ideally, development of new biomarker tests should meet the following criteria: 1) they should provide novel data beyond what can be determined by other tests, 2) the marker in question should be stable and not change from day to day nor with respect to time of day or diet, and 3) the marker should be reasonably simple and

inexpensive to test.[197] The tests which are currently used in a clinical setting measure serum lipid levels, specifically triglycerides, HDL, and LDL. Certain cutoff points have been established over which levels of these markers are considered to put an individual at risk for atherosclerosis, coronary artery disease, and myocardial infarction.[202] While these are statistically predictive of atherosclerosis and other clinical events, there remain individuals who had a lipid profile well within the normal range at the time of suffering a cardiovascular event.[197] Thus, other indicators of subclinical atherosclerosis have been sought, with CRP being the most successful marker identified to date.[196, 197, 199] As a marker of inflammation, CRP levels in the serum can reflect vascular inflammation, the most common cause of which is atherosclerosis. While it has been established that CRP does not significantly promote atherosclerosis, serum levels of CRP have been clearly correlated with increased risk of congestive heart disease and a clinically significant event.[199] Nevertheless, CRP is not a definitive marker of atherosclerotic burden, as CRP is a non-specific pro-inflammatory molecule and can be elevated by other inflammatory events.[196, 203] While CRP levels are a well-established marker of atherosclerosis and will undoubtedly continue to be used as a risk indicator, other biomarkers are being considered for development. Most of these are related to the inflammatory processes involved in atherosclerosis, such as CD40 ligand, IL-6, adiponectin, interleukin 18, and MMP9. However, none of these are yet as promising as CRP.[197] Therefore, the need for a highly specific atherosclerosis marker remains.

Imaging technology is a rapidly growing field and has been applied to studying atherosclerosis. While more expensive than clinical chemistry tests, imaging can provide more precise and accurate information such as location, size, and vulnerability of a plaque. Several invasive techniques such as angiography, intravascular ultrasound, thermography, and optical coherence tomography have been used for investigating the nature and extent of atherosclerotic plaques. However, these are expensive and complicated procedures. More desirable are the non-invasive approaches such as magnetic resonance imaging, CT scanning, and nuclear imaging.[201] Moreover, these modalities are being adapted to identify tracers targeted against specific molecules within the atheroma.[204] A highly specific marker for atherosclerosis would certainly aide in the development of an ideal imaging protocol, and could someday enable clinicians to determine an accurate measurement of plaque development in an individual.[201, 204]

As a highly specific marker of atherosclerosis, SPRR3 could theoretically be used as clinical marker, either in an assay to detect levels of SPRR3 in serum or urine, or in an imaging test. We currently have no data to indicate that SPRR3 is secreted and would therefore be found in the blood or urine. We have observed that SPRR3 may be released from VSMCs, probably as the cells die during plaque progression.[43] Therefore, it is possible that SPRR3 may be released from the vessel wall. Furthermore, SPRR3 could be used as a label for specific imaging of atheromas. Alternatively, work from our lab has also demonstrated specificity of integrin $\alpha 1\beta 1$ expression for VSMCs within

atheromas, and could conceivably also be used as a marker for atherosclerosis.[43] This is particularly appealing as integrins localize to the cell surface and therefore are more easily accessed by markers. Such work is still theoretical, but will hopefully be developed in the future.

Finally, it is our hope that SPRR3 will be adopted as a histological marker of atherosclerosis. While a trained pathologist can generally recognize moderate to advanced atheromas in a paraffin-embedded tissue section, early lesions can be more difficult to identify.[7] Since immunostaining against SPRR3 specifically highlights regions of atherosclerosis, even when a lesion would otherwise be difficult to find on an H&E-stained section (early lesions such as fatty streaks, as well as in vein graft atherosclerosis), SPRR3 could be developed for use as a histological marker of atheromas in tissue sections.[43, 164, 199]

Closing

Atherosclerosis is a major cause of mortality in the United States; therefore, gaining insight into the pathophysiology of the disease is of great interest.[205] The biomechanics of atherosclerotic initiation and progression is a new but growing field of research. SPRR3 expression in atheromas is regulated by cyclic strain as sensed through integrin $\alpha 1\beta 1$ binding to collagen I. These studies shed light onto the role of biomechanics in gene regulation, particularly in vascular pathologies such as atherosclerosis.

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