Changes in Passive and Dynamic Mechanical Environments Promote Differentiation to a Contractile Phenotype in Vascular Smooth Muscle Cells

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Changes in Passive and Dynamic Mechanical Environments Promote Differentiation to a Contractile Phenotype in Vascular Smooth Muscle Cells

A Dissertation
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Worcester Polytechnic Institute
In partial fulfillment of the requirements for the Degree of Doctor of Philosophy in
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Abbreviations

2D – Two-dimensional
3D – Three-dimensional
AFM – Atomic force Microscopy
ANOVA – Analysis of variance
CABG – Coronary artery bypass
DM – Differentiation medium
EC – Endothelial cell
ECM – Extracellular matrix
eNOS – Endothelial nitric oxide synthase
EGF – Epidermal growth factor
ERK – Extracellular signal-regulated kinase
FGF – Fibroblast growth factor
GM – Growth medium
GPa – Gigapascal
H&E – Hematoxylin and Eosin
hCaSMC – Human coronary artery smooth muscle cell
HDF – Human dermal fibroblast
HRP – Horseradish peroxidase
Hz – Hertz
IgG – Immunoglobulin G
ITS – Insulin, Transferrin, Selenium
ICC – Immunocytochemistry
IHC – Immunohistochemistry
kPa – Kilopascal
MQP – Major Qualifying Project
MHC – Myosin Heavy Chain
MMP – Matrix Metalloproteinase
MTM – Maximum Tangent Modulus
NO – Nitric Oxide
PAAM – Polyacrylamide
PBS – Phosphate buffered saline
PDGF – Platelet-derived growth factor
PGA – Polyglycolic acid
PLL A – Polylactic acid
PSS – Physiologic saline solution
Sulfo-SANPAH – sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate
SMαA – Smooth Muscle α-actin
SMC – Smooth Muscle Cell
TBS-T – Tris buffered saline with tween
TEBV – Tissue engineered blood vessel
TGF-β – Transforming growth factor-β
TGM2 – Transglutaminase 2
TNF-α – Tumor necrosis factor-α
UTS – Ultimate tensile strength
UV – Ultraviolet
VIC – Valvular interstitial cell
Abstract

Every year, 400,000 coronary artery bypasses (CABG) are performed in the United States. However, one third of all patients who need a CABG cannot undergo the procedure because of the lack of suitable autologous blood vessels. Both synthetic and tissue engineered vascular grafts have been used clinically for vascular grafts or other surgical applications, but no small-diameter engineered vessels have yet been successfully used for CABG. The success of vascular tissue engineering is strongly dependent on being able to control tissue contractility and extracellular matrix (ECM) production to achieve balance between tissue strength and physiological function. Smooth muscle cells (SMCs), the main contributor of contractility in blood vessels, retain phenotypic plasticity, meaning they possess the ability to switch between a contractile and synthetic phenotype. In 2D culture, a number of biochemical and mechanical cues have been shown to promote the switch to a contractile phenotype in SMCs. However, achieving a stable contractile phenotype in 3D tissue has proven difficult.

The work in this dissertation describes an investigation of how passive and dynamic environmental cues influence the smooth muscle phenotype. We studied the effects of substrate modulus in conjunction with changes in cell culture media composition on SMC phenotype in 2D and 3D cultures. Culturing SMCs in a low-serum culture medium resulted in an increase in SMC contractility in 2D cell culture but not in 3D cell-derived tissue. We found that, in SMCs cultured on soft substrates, the ability to modulate SMC phenotype in response to changes in media was diminished. Passively crosslinking the ECM of our cell-derived tissues with genipin resulted in modest increases in elastic modulus, though not enough to observe changes in SMC phenotype. Additionally, we investigated how dynamic cyclic mechanical stretch, in conjunction with cell culture medium, modified SMC contractility in cell and tissue cultures. SMCs increased contractile protein expression when exposed to dynamic stretch in 2D culture, even on soft substrates, which have previously been shown to inhibit phenotypic modulation. In 3D tissue rings, after mechanical stimulation, SMCs became more aligned, the tissue became tougher, and SMCs exhibited a measurable increase in contractile protein expression.

In summary, we found that increasing substrate modulus, culturing in low serum cell culture medium, and imparting cyclic mechanical stretch can promote SMC differentiation and cellular alignment, and improve tissue mechanical properties. This information can be used to more accurately recapitulate vascular tissue for use in modeling or in the creation of tissue engineered blood vessels.
Chapter 1: Overview

1.1 Introduction

Every year, vascular disease and dysfunction play a role in five of the top fifteen causes of death in the United States, contributing to 57.9% of deaths annually\(^1\). This is, in part, due to the dedifferentiation of smooth muscle cells (SMCs) in the blood vessel wall. SMCs are the main contributor of contractility in blood vessels and mature SMCs possess the ability to switch between a differentiated, contractile phenotype and a proliferative, synthetic phenotype\(^2\). In normal arteries, SMCs proliferate infrequently and express contractile proteins\(^2\). As SMCs begin to switch to a more synthetic phenotype, they proliferate more frequently and contractile protein production is downregulated\(^2-4\). Abnormal SMC phenotype has been implicated in the initiation or progression of atherosclerosis, hypertension, aneurysm, and some forms of cancer\(^5-8\).

When SMCs de-differentiate into a synthetic phenotype, they become more proliferative and less contractile, resulting in narrowing of the blood vessel, weakening of the tissue wall, or changes in vessel permeability due to changes in structural integrity\(^9-11\). This phenotypic shift is not always pathogenic; the ability to change phenotype quickly is useful immediately after injury, allowing some SMCs to become more synthetic, to migrate to the wound site, and proliferate to fill the defect\(^12\). In traditional in vitro culture, SMCs exhibit a synthetic phenotype, proliferating rapidly, with increased ECM deposition compared to contractile SMCs. Without intervention, the switch to a synthetic phenotype for primary SMCs in vitro happens within 1-2 passages following explantation\(^13\).

Although phenotypic plasticity in SMCs is well known, only some of the stimuli that promote this change are well understood\(^2\). Understanding the cues that promote this change in phenotype is important for the development of treatments for when the synthetic phenotype creates a pathogenic condition. For example, SMCs become more synthetic at the beginning of the formation of an aneurysm, eventually resulting in a breakdown of the vessel wall that can lead to dissection\(^4\). If we understand the cues that initiate this phenotypic shift, we may be able to develop treatments that can be used prior to the onset of symptoms to attenuate disease.
progression. Some of the extrinsic factors known to be important in modulating SMC phenotype are environmental cues, such as mechanical forces, cell-cell interactions, soluble biochemical environment, and extracellular matrix interactions\textsuperscript{14-16}.

The most common method to study the effects of exogenous stimuli on phenotype is to culture SMCs in two-dimensional (2D) cell culture, attached to tissue culture plastic, glass, or a hydrogel\textsuperscript{17-21}. However, replicating experiments in 3D culture that were initially performed in 2D culture does not always yield the same results\textsuperscript{22-26}. The difference in cell responses to exogenous stimuli in 3D v. 2D culture may be due to many factors, including cell-cell interactions, differences in substrate elastic modulus, and nutrient diffusion\textsuperscript{17,27,28}. However, in studies comparing 2D and 3D culture conditions, one of the main uncontrolled parameters is the substrate elastic modulus. Elastic modulus affects cell phenotype in many cell types\textsuperscript{29-33}, potentially including SMCs\textsuperscript{17,34-37}.

We have developed a 3D culture system formed from completely cell-derived, self-assembled vascular tissues using only SMCs for use in examining the effects of environmental cues on cellular response and tissue structure\textsuperscript{38,39}. This system enables creation of 3D SMC tissues from a suspension of cells, and can be used as a model system to study the effects of different stimuli on SMC phenotype and function.

In this dissertation we focused on studying the effects of environmental signals, including cell culture media composition, surface stiffness, and cyclic mechanical stretch, on SMC phenotype in 2D and 3D culture systems.

1.2 Overall Goal and Hypothesis

The goal of this dissertation was to investigate how passive mechanical modifications and dynamic mechanical conditioning affect SMC phenotype. We applied these findings to stimulate a phenotypic shift in SMCs in 2D and 3D culture.
We hypothesized that, in conjunction with a low serum cell culture medium, increasing the elastic modulus of the culture surface and applying a cyclic stretch would promote a switch to a more contractile phenotype in SMCs, both in 2D cell culture and 3D tissue culture.

To test this hypothesis, this dissertation is separated into two specific aims. In Specific Aim 1, we investigated the role of substrate elastic modulus and cell culture medium on SMC phenotype. This work was done in 2D cell culture and the findings were applied to the cell-derived, 3D tissue culture, focusing on the elastic modulus of the culture surface. In Specific Aim 2 we investigated how dynamic mechanical stimulation could be used to prompt SMCs to differentiate into a contractile phenotype.

1.3 Specific Aim 1: Determine the effects of substrate modulus on smooth muscle cell phenotype in 2D and 3D cultures

In recent years, many tissue engineers have begun to shift their focus from scaffold-based vascular tissue engineering strategies to a cell-based approach for the creation of blood vessels and for \textit{in vitro} vascular models\cite{40-43}. By harnessing a cell’s innate ability to generate tissue, we aim to improve the cellular microenvironment, including cell-cell contact and paracrine factors, to recapitulate functional vascular tissue, and create a completely autologous tissue for a patient\cite{42}. However, the ability to differentiate SMCs in this environment has remained elusive. The current cell-derived methods to produce vascular grafts produce tissue tubes with vascular cells. However, they do not produce tissues that can be used for \textit{in vitro} modeling of vascular behavior or functionally to mimic native vascular tissue due to poor mechanical properties or lack of SMC contractility\cite{40,42,44}. There are many possible reasons for this lack of contractile SMCs. One reason is when designing tissue-engineered blood vessels, contractility is not prioritized since it is not required for the vessel to carry blood.

The goal of this study was to investigate methods of promoting a contractile phenotype in SMCs cultured in 3D, cell-derived tissue. We focused on evaluating how changes to the passive mechanical environment can affect SMC phenotype. Specifically, we modified the cell culture medium, lowering serum content and removing growth factors, to promote contractility. SMC differentiation was quantified by measuring contractile protein expression and population
doubling time. In SMCs cultured in differentiation medium on glass coverslips, population doubling time decreased, and contractile protein expression significantly increased compared to SMCs cultured in a growth medium. However, when 3D, cell-derived SMC rings were cultured in differentiation medium, the tissue was thinner and stronger, but the SMCs did not upregulate contractile protein expression, potentially due to the low elastic modulus of the tissue. We hypothesized that SMCs cultured on soft substrates, with elastic moduli similar to the tissue rings would not respond phenotypically to changes in cell culture media. We evaluated the effect of elastic modulus on SMC differentiation in 2D culture on PAAM gels with varied stiffnesses and observed that SMCs cultured on soft surfaces did not exhibit a contractile phenotype while SMCs cultured on stiffer gels produced significantly more calponin compared to the stiffer controls.

Since an increase in elastic modulus of the culture substrate increased the contractile response of SMCs, when cultured on 2D PAAM gels, we hypothesized increasing the modulus of the tissue rings could increase the contractile protein expression of the SMC in the tissue. Tissue rings were passively crosslinked with a non-cytotoxic cross-linker to increase stiffness within the tissue. However, this increase in stiffness was not enough to promote a contractile phenotype in the SMCs.

1.4 Specific Aim 2: Quantify the effects of cyclic strain on tissue mechanics and SMC phenotype

In the previous aim we focused on passive methods to promote the differentiation of SMCs to a contractile phenotype and in this aim, we focused on using dynamic mechanical stimulation to promote SMC differentiation on soft culture substrates. By imparting dynamic mechanical conditioning in place of chemical crosslinking, the ECM is modified only by the cells, not exogenous materials, leaving it open to modification and remodeling. In this aim we focused on the role of cyclic stretch on SMC differentiation.

SMCs cultured on gels with stiffnesses corresponding to healthy vasculature showed increased contractile protein expression when compared to SMCs cultured on gels with stiffnesses similar to our cell-derived tissues. However, when these SMCs were exposed to cyclic stretch, the SMCs
cultured on the softer gels expressed contractile proteins at a similar rate compared to SMCs cultured on stiff gel. This method of promoting SMC differentiation was applied to our vascular tissue ring system. SMC tissues were stretched cyclically for nine days, resulting in a tougher ring with more contractile SMCs.

1.5 Conclusion
In this dissertation we investigated the intertwining effects of biochemical cues, and passive and active mechanical environments on SMC phenotype. The phenotypic effects of three different culture parameters (cell culture medium, elastic modulus, and cyclic stretch) were evaluated in 2D cell culture and 3D tissue culture. Our findings will be applied to the further development of functional tissue engineered blood vessels and vascular tissue models.

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29. Galie, P. A., Westfall, M. V. & Stegemann, J. P. Reduced serum content and increased


Chapter 2: Background

In this dissertation we investigated how different mechanical environments affect smooth muscle cell (SMC) behavior, both in two-dimensional (2D) cell culture and in three-dimensional (3D), cell-derived, vascular tissue culture. We aim to apply the knowledge we gain about SMC behavior in response to changes in mechanical stimuli to the culture of vascular constructs for tissue modeling and tissue engineered grafting.

2.1 Native Blood Vessel Physiology

The wall of a blood vessel is divided into 3 main layers: the tunica intima, tunica media, and tunica adventitia. Starting at the lumen of the blood vessel, the tunica intima is the innermost layer. It consists of endothelial cells and the underlying extracellular matrix (ECM), and is responsible for regulating nutrient transport to and from the bloodstream and controlling the thrombogenicity of the vessel lumen. The tunica media is the middle layer, between the intima and adventitia. It consists of smooth muscle cells (SMCs), contributes to the mechanical strength of the vessel, and is the sole layer mediating blood vessel tone and contractility\(^1\). Surrounding the outside of the vessel is the tunica adventitia, which is composed of fibroblasts and serves to supplement the mechanical strength of the media, and increases the overall strength of the tissue\(^2,3\).

In healthy tissue, all three tissue layers are in equilibrium, regulating tissue contractility and preventing blood clotting while retaining sufficient strength to withstand the pressures of the vascular system\(^3\). Endothelial and smooth muscle cells regulate each other’s function by cell-cell interaction and paracrine signaling, resulting in contractile, non-thrombotic tissue\(^4\). In healthy tissue, SMCs remain predominantly non-proliferative and contractile, maintaining the strength, structure, and contractility of the tissue\(^5\). However SMCs can exhibit an alternative phenotype, which can result in the degradation of vascular function or the progression of vascular disease. In addition to the direct effects of pathogenic SMC phenotype, when vascular tissue becomes non-contractile, the regulation of nutrient distribution and blood pressure is compromised\(^3\). In this dissertation we will focus on the tunica media and the phenotype of the SMCs within.
2.2 Smooth Muscle Cell Phenotype

Smooth muscle cells and ECM make up the medial layer of blood vessels, carry the majority of the pressure load, and also constitute all of the contractile force of the vessel\(^6\).\(^7\). Adult SMCs exhibit phenotypic plasticity, and have been shown to switch from a contractile phenotype (characteristic of healthy blood vessels) to a synthetic phenotype in response to injury or other conditions known to cause vascular disease (Fig. 2.1)\(^8\). An abnormal SMC phenotype has been implicated in the initiation or progression of some forms of cancer, atherosclerosis, hypertension, and aneurysm\(^9\)\(^-\)\(^12\). Remarkably, an SMC phenotypic shift plays a role in 5 of the top 15 causes of death in the United States, contributing to 57.9% of deaths annually\(^13\).

![Figure 2.1. Phase Images and Contractile Protein Staining of Synthetic and Contractile SMCs.](image)

The two main SMC phenotypes are synthetic (A,B) and contractile (C,D; smooth muscle α-actin = green; DNA = blue). Synthetic SMCs proliferate rapidly, downregulate contractile protein expression (B), and are small in total cell area (A). Contractile SMCs proliferate more slowly, produce contractile proteins (D) and exhibit a more spread morphology (C). B and D show smooth muscle α-actin staining. Scale = 50μm

2.2.1 Smooth Muscle Cell Phenotype In Vivo

Contractile SMCs proliferate infrequently and express contractile proteins, such as myosin heavy chain (MHC), smooth muscle α actin (SMαA), and calponin (Fig 2.1D)\(^8\). The contractile apparatus is formed by SMαA and myosin fibers aligning parallel to each other and calponin regulates the contractile process by blocking the actin binding sites on myosin. In contractile
SMCs these contractile proteins are arranged in intracellular fibers. As SMCs begin to switch to a more synthetic phenotype, the first contractile protein to be downregulated is MHC followed by calponin, and lastly SMαA (Fig 2.1B)\textsuperscript{8,14,15}. Overall, this decrease in contractile protein expression leads to a decrease in vessel contractility\textsuperscript{16,17}. One of the most direct ways to determine SMC phenotype is to examine contractile protein expression, which can provide a measure of the differentiation continuum due to the known order in which the contractile proteins are up or down regulated. Two other indicators of a synthetic SMC phenotype are increased proliferation and apoptosis\textsuperscript{15}. The ability of SMCs to move into a proliferative phenotype is beneficial when the vessel is injured and SMCs are needed to repopulate and heal the defect. Immediately after an injury, some SMCs modulate their phenotype to be more synthetic, migrate to the wound site, and proliferate. After the defect has been repaired, the excess SMCs become apoptotic\textsuperscript{18}. However, in the pathogenic conditions involving SMC phenotype shift, the proliferation and apoptosis exacerbate the condition\textsuperscript{19,20}. For example, in the progression of aneurysm formation, SMCs become more synthetic, proliferating and becoming apoptotic, as well as upregulating matrix metalloproteinases (MMPs) resulting in an unstable vessel wall\textsuperscript{21}.

Changes in SMC phenotype also affect the structure and ECM of the tissue. In native blood vessels, the most abundant ECM proteins are collagen type I and III, which contribute most of the tensile strength of the vessel; and elastin, which is responsible for the majority of the elasticity of the vessel\textsuperscript{22,23}. As SMCs switch to a more synthetic phenotype, overall matrix metalloproteinase (MMP) production is increased, specifically MMP-2 and 9, which degrade components of the existing ECM\textsuperscript{22}. MMP-2 and 9 are gelatinases, degrading gelatin, collagen types IV, V, VII, and X, as well as fibronectin and elastin\textsuperscript{24}. In a non-disease state, these proteinases are essential for regulation and remodeling of the ECM, however the increased degradation of these ECM proteins leads to increased brittleness of the vessel wall\textsuperscript{25}. Because of this decrease in elasticity in the blood vessel wall, the vessel may thicken or rupture in response to the pressure\textsuperscript{8,26}.

While the transcriptional regulation of SMC differentiation is not fully understood, several transcription factors have been implicated in SMC phenotypic modulation\textsuperscript{27}. Subramanian, et al.
found that when the nuclear binding proteins Pura, Purβ, and MSY1 bind upstream of the SMαA gene, SMαA expression is downregulated\textsuperscript{28}, and Mano, et al. found that when GATA-6 binds to DNA, SMC differentiation protein markers increase\textsuperscript{29}, possibly due GATA-6 regulation of SM MHC\textsuperscript{30}. Understanding how these transcription factors play a role in SMC phenotypic modulation may be useful in developing strategies to alter SMC phenotype, either through gene delivery or through the use of transcriptional factors.

### 2.2.2 Smooth Muscle Cell Phenotype In Vitro

A shift in SMC phenotype, from contractile to synthetic, is also observed when culturing primary SMCs. In 1976, Chamley-Campbell, et al. were the first to report this loss of the contractile phenotype in \textit{in vitro} cell culture\textsuperscript{5}. Currently, in most \textit{in vitro} culture systems, SMCs proliferate and exhibit characteristics of the synthetic phenotype, losing contractile protein expression and proliferating rapidly\textsuperscript{31}. It has been shown that \textit{in vitro} culture conditions are important in the maintenance of the contractile phenotype in SMCs, but the culture parameters that promote contractility are not completely understood\textsuperscript{8,32,33}.

### 2.3 Factors That Affect SMC Phenotypic Modulation

SMC phenotype is highly plastic and can be modulated with many environmental cues, such as mechanical forces, cell-cell interactions, cell culture media components, and extracellular matrix interactions\textsuperscript{34}. In this work we will focus on the environmental signals not regulated by cell-cell contact, including cell culture media composition, surface stiffness, and cyclic mechanical stretch.

#### 2.3.1 Cell Culture Media Composition

Since the discovery of different SMC phenotypes, researchers have looked for culture media compositions that can stimulate SMCs to switch from a synthetic state, common in \textit{in vitro} tissue culture, into a contractile state\textsuperscript{31}. In 1976 Fritz, et al., demonstrated that proliferation of SMCs logarithmically increased with an increase in serum in cell culture medium from 0.5% to 20\%\textsuperscript{32}. This rapid increase in proliferation would later be referred to as a key phenotypic marker, indicating a switch from a contractile phenotype to a synthetic phenotype. Since these early
studies were published, the composition of cell culture media has been adapted many times, working towards one that promotes the phenotype of choice for researchers\textsuperscript{35-40}. With the goal of promoting a phenotypic switch from synthetic to contractile, cell culture medium has been supplemented with platelet derived growth factor (PDGF)\textsuperscript{41}, transforming growth factor-beta (TGF-\(\beta\)1)\textsuperscript{42,43}, tumor necrosis factor-alpha (TNF-\(\alpha\))\textsuperscript{40}, and angiotensin II\textsuperscript{37,44}. PDGF, a growth factor that regulates cell growth and division, has been used to stimulate SMC proliferation and a synthetic phenotype, and SMC response to PDGF appears to be dependent on the stiffness of the culture substrates on which the SMCs are cultured\textsuperscript{41}. TGF-\(\beta\)1, a cytokine known to control proliferation and cellular differentiation, has been shown to upregulate contractile protein expression in SMCs in 2D and 3D in collagen gels\textsuperscript{42,43}. However, TGF-\(\beta\)1 interacts with many pathways, including MAPK, RhoA, Notch, and SRF, resulting in unpredictable results with regard to cell behavior and phenotype\textsuperscript{45}. This is thought to be caused by the inadvertent activation of disparate pathways, leading to unpredictable outcomes to a stimulus\textsuperscript{45}. TNF-\(\alpha\), an adipokine, involved in inflammation and immune response, has been used to induce differentiation into a contractile phenotype but this reaction is highly dependent on culture confluence, cell source, and passage, and either results in differentiation or apoptosis\textsuperscript{40}. Unfortunately, despite some promising evidence that angiotensin II, a vasoconstricting hormone, may stimulate the production of contractile proteins in SMCs, it has also been shown to stimulate apoptosis in SMCs that exhibit a more “epithelioid” cell shape\textsuperscript{37,44}. All of these biochemical cues modulate SMC phenotype, however, each of these molecules has a response that makes its use non-optimal for our purposes.

In 1983, Libby, et al. developed a serum-free cell culture medium for use in the culture of SMCs to promote cell contractility\textsuperscript{36}. As discussed above, the inclusion of serum in cell culture medium strongly increases cellular proliferation in SMCs and downregulates contractile protein expression\textsuperscript{32,46}. However, when serum is removed from culture medium, SMCs do not remain viable without added supplements. The supplements insulin, transferrin, and selenium were investigated by Libby, et al., and were shown to increase DNA content, protein production, and thymidine uptake when compared to non-supplemented Dulbecco's Modified Eagle Medium\textsuperscript{47}. These three supplements facilitate iron transfer, protein regulation, and serve to protect cell
viability. In order to perform endothelial-smooth muscle cell co-cultures, Lavender, et al. used this media composition to differentiate SMCs, and added 1% serum to facilitate endothelial cell survival. This media composition will be referred to as differentiation media (DM) in this work, as it stimulates protein expression characteristic of mature, quiescent SMCs. In the work reported here, it was used to stimulate SMCs into a contractile phenotype, and was used to investigate the stimuli needed to differentiate SMCs in 2D and 3D culture.

In comparison to DM, traditional SMC culture uses growth medium (GM) to promote cellular proliferation. The GM used in this dissertation contains 5 fold more FBS than DM (5% v 1%), the same concentration of glutamine (required for cellular metabolism), insulin (to increase protein synthesis), fibroblast growth factor (to promote cellular proliferation), epidermal growth factor (to increase proliferation), and ascorbate (to promote collagen production).

2.3.2 Material Elastic Modulus
Preliminary evidence indicates that changes in substrate stiffness can also have a modulatory effect on SMC phenotype in 2D cultures, similar to its observed effect on other cell types. Currently, most in vitro vascular biology studies utilize cells cultured in 2D, or cells suspended in gels. However, these in vitro techniques fail to accurately mimic in vivo SMC contractility and responses to biochemical stimuli. In this dissertation, when comparing 2D and 3D culture conditions, one of the main uncontrolled parameters was the substrate elastic modulus. Elastic modulus is the ratio of tensile stress to tensile strain, resulting in a measurement of the stiffness of a material. It is well established that substrate modulus affects the phenotype of many anchorage dependent cell types; including myofibroblasts, endothelial cells, and mesenchymal stem cells and that there are differences in cellular response to substrate modulus in 2D and 3D cultures. As for SMCs, evidence indicates that varying substrate modulus in 2D and 3D cultures can have a modulatory effect on phenotype. However, based on the range of stiffness magnitudes tested, materials chosen, and the geometry of culture surfaces, the response appears to vary. On polyacrylamide (PAAM) gels ranging in modulus from 2 – 84 kPa, SMCs exhibit increased cell area with increasing substrate modulus, an indicator of a more contractile phenotype. When cultured on PAAM gels from 25 – 135 kPa, cell-cell contact reduced the effect of substrate modulus on SMCs. On thin collagen films with increasing
modulus, SMCs exhibited increased proliferation, which was also seen in SMCs on tecophilic/gelatin electrospun fibers\(^{63}\). SMCs on the tecophilic/gelatin electrospun fibers also showed decreases in contractile protein expression (smooth muscle α actin (SMαA) and smooth muscle myosin heavy chain)\(^{63}\) indicating a more synthetic phenotype. However, when SMCs were treated with a constitutive upregulation of an actin cytoskeletal regulator (RhoA) and suspended in PEG-fibrinogen gels with a modulus of 5.4 kPa, the contractile proteins (SMαA and calponin) were upregulated when compared to gels with a modulus of 0.45 kPa. When comparing the groups, Peyton, et al. also observed no significant change in proliferation\(^{66}\).

Substrate stiffness may not only affect SMC phenotype with regard to contractile protein expression and proliferation, but may also affect how SMCs respond to other stimuli. Brown, et al. found that SMC phenotypic response to the addition of PDGF to the culture medium was modulated by the elastic modulus of the surface on which the cells were cultured\(^ {41}\). This synergistic effect of substrate modulus and other stimuli has also been shown for myofibroblasts in 3D gels\(^{52}\) and mesenchymal stem cells in 3D electrospun scaffolds\(^{55}\).

2.3.3 The Effects of Mechanical Stimulation on Phenotype

*In vivo*, SMCs are subjected to pulse pressure, which exposes the blood vessel wall to radial, longitudinal, and circumferential strain. SMCs deform in response to global strain via their focal adhesions as stretch deforms the ECM surrounding the cells\(^{67}\). It has been suggested that stretch triggers the ERK1/2 pathway, upregulating cell proliferation, while also activating Rho, the same pathway that increases the sensitivity of SMCs to changes in substrate elastic modulus\(^{66}\). Rho increases actin polymerization, causing SMCs to remain contractile while increasing proliferation\(^{68}\). *In vivo* the pulsatile, circumferential strain between diastole and systole varies from 2% - 15% by vessel location, type, and size\(^{69}\). However, the most widely used peak strain when investigating the effects of stretch on cells *in vitro* is 10%\(^ {70-72}\). The effects of strain on SMC phenotype are generally studied using a monolayer of SMCs seeded on a 2D, silicone membrane or on SMCs suspended in collagen gels\(^{70,72}\). When suspended in a collagen gel, SMCs upregulated SMαA production and increased cell number in response to a 10% strain compared to unstrained gels\(^{72}\). However, when seeded on a silicone membrane, cell proliferation decreased in response to stretch\(^{73}\). Also, when seeded on a silicone membrane and exposed to cyclic 10%
strain, SMCs increased production of MHC, SM\(\alpha\)A, and calponin\(^{74-76}\). Overall, this indicates a phenotypic shift from a more synthetic phenotype to a more contractile phenotype in response to mechanical stretch. However, exceeding 10% circumferential strain, as would occur during balloon angioplasty or after initiation of an aneurysm, can trigger SMCs to become more synthetic\(^77\).

### 2.3.3.1 Circumferential Stretch Bioreactors

In order to impart cyclic stretch to tissue engineered vascular grafts, many researchers have developed circumferential stretch bioreactors. Niklason, et al. created a 165 beat per minute bioreactor utilizing a pulsatile pump to cyclically stretch the scaffold, pumping cell culture media through the lumen of tissue engineered blood vessel (TEBV), consisting of a PGA scaffold seeded with bovine SMCs\(^78\). These tissues were cultured for 8 weeks at 5% strain, which resulted in increased suture retention, overall collagen content, burst pressure and elastic modulus compared to the static tissue controls\(^{78,79}\). Another method of imparting cyclic stretch is to use an inflatable latex or silicone mandrel and cyclically inflate the tissue. When this method was used with fibrin or collagen gel based tissues, the strength and elastic modulus of the tissue increased\(^{80-82}\). Seliktar, et al. found that an inflating silicone mandrel system that imparts 10% strain on SMC- and fibroblast-seeded collagen gels, increased MMP-2 expression compared to the static constructs\(^83\). This indicates that mechanical conditioning can increase the rate of tissue remodeling, as well as tissue mechanical strength and modulus\(^83\).

### 2.3.3.2 Mechanical Conditioning Effects on Cell-Derived Tissues

Gauvin, et al., and Weidenhamer, et al. have examined the effects of cyclic mechanical conditioning on cell-derived tissues\(^{16,17}\). Both groups worked with human dermal fibroblasts (HDF). Gauvin, et al., cultured HDF sheets and then stretched the sheets to 10% with a 10% duty cycle at 1 Hz, and Weidenhamer, et al., seeded HDFs directly onto silicone tubing and mechanically conditioned them by inflating the silicone tubing (5% stretch at 0.5 Hz). Both groups observed significant increases in thickness, ultimate tensile strength (UTS), collagen alignment, and modulus\(^{16,17}\). In native tissue, SMCs contribute to the strength of the tissue by producing ECM, contracting, and aligning in the direction of force\(^84\). However, currently, there is
no published research investigating SMC responses to mechanical conditioning in 3D, cell-derived tissues \emph{in vitro}.

As described above, changes in the passive and dynamic mechanical environment of SMCs may help modulate SMC phenotype. In this work, we investigated how changes in elastic modulus and mechanical conditioning can affect how SMCs phenotypically respond to changes in cell culture medium in both 2D cell culture and 3D tissue culture.

\section*{2.4 Vascular Tissue Engineering}
Every year, 400,000 coronary artery bypasses (CABG) are performed in the United States. However, one third of all patients who need a CABG cannot undergo the procedure because of the lack of a suitable autologous blood vessels\footnote{85}. Currently, there are three types of TEBVs that have moved into human clinical trials for vascular grafts; however, these either use a biodegradable polymer (PGA-PLLA) scaffold seeded with bone marrow mononuclear cells\footnote{86}, cellular and decellularized rolled sheets of dermal fibroblasts\footnote{87}, or decellularized PGA scaffolds post SMC culture\footnote{88}. Tissue engineering holds promise for creating small diameter vascular grafts, but these are studied as arteriovenous fistulas\footnote{87,88}, cavopulmonary connections\footnote{86}, and lower limb bypass\footnote{88} not as coronary artery bypass grafts.

Despite the need, creating advanced small-diameter TEBVs that are functionally similar to blood vessels has proved challenging. While there is modest success in the creation of tissue engineering blood vessel equivalents with contractile SMCs\footnote{78,89,90} none of these TEBVs prioritize the differentiation of SMCs or enhance the scientific knowledge of how to promote contractility in other vascular grafts or model systems. PGA scaffolds seeded with SMCs contracted with 5\% of the force of rabbit aortic controls\footnote{78}, while tubular tissue formed around silicone tubing implanted peritoneally demonstrated a contraction 10\% of an aortic control\footnote{91}. Each of these systems rely on specific fabrication techniques to create contractile tissues, and have not investigated what parameters are needed to modify SMC contractile phenotype.

However, despite these successes, there are currently no TEBV models that, using only cell-derived components, fully recapitulate the native biological structure and function of blood
vessels. *In vivo*, blood vessels have 5 main functions: carry blood, withstand blood pressures, regulate blood flow, regulate nutrient diffusion, and prevent blood clotting\(^1\). While, clinically, blood vessel substitutes have functioned as blood carrying conduits without SMCs, these substitutes neglect one of the major roles of blood vessels: being able to regulate blood flow through tissue contraction\(^85-88\). None of these TEBVs recapitulate native tissue, due in part to the difficulty of creating a contractile and entirely biological TEBV using SMCs. These approaches result in tissues that can function as TEBVs, but not as contractile vascular tissue models for *in vitro* drug discovery and disease modeling.

### 2.5 Cell-Derived Tissue

The field of tissue engineering is largely predicated on the concept that cells are seeded onto an exogenous scaffold material and cultured. However, the area of cell-derived, or scaffold free, tissue engineering is becoming more popular. As shown by Ahlfors, et al. and Adebayo, et al., statically cultured cell-derived tissues have significantly higher UTSs,\(^92\) a metric of strength, and MTM,\(^93\) a metric of stiffness, compared to tissue constructs fabricated from the same number of cells embedded in collagen or fibrin gels. Cell-derived tissue systems also have the advantage of producing tissues with high cell densities, similar to native tissue, and with no scaffold materials, allowing for more direct cell-cell interaction \(^94,95\). These vascular tissues have the potential to fully recapitulate native vascular tissue, using only human cell-derived materials.

In the field of tissue engineered vascular grafting, several research groups have worked to create a completely cell-derived vascular graft. In 1969, Sparks reported that they had harnessed the body’s native ability to encapsulate foreign objects to create a cell-derived tubular construct\(^96\). They implanted Dacron rods into the ribcage and, after either five-twelve weeks, explanted the tissue and found that the body had encapsulated the materials, forming a tissue tube around the rods\(^96\). Since the 1960s, several researchers have used this method of forming tubular constructs. In 1999, Campbell, et al., reported they had implanted a silicone rod into rat and rabbit peritoneal cavities and after 2, 3, and 4 weeks had harvested strong, aligned tissues\(^91\). These tissues were subsequently implanted as a carotid artery graft and remained patent for up to 4 months\(^91\). In 2004, Yakayama reported that they had subcutaneously implanted rods made of six different
types of materials and, after either one or three months explanted the tissue and found that the body had encapsulated the materials, forming tissue tubes around the rods with different stiffnesses, thicknesses, and strengths of tissue for each material\textsuperscript{97}. One material was chosen based on tissue thickness and strength and the tissue generated was later implanted into a rat carotid model with a patency rate of 67\% (4/6)\textsuperscript{98}.

L’Heureux, et al. created cell-derived TEBVs by culturing sheets of SMCs and fibroblasts for 30 days, harvesting them, rolling them around a mandrel, and allowing them to mature\textsuperscript{99}. Overall, this method created strong, non-thrombogenic tissues, but requires a culture time of >3 months\textsuperscript{99}. This process has been refined many times, resulting in the removal of the smooth muscle layer from the process\textsuperscript{100}. Another method used to produce cell-derived TEBVs is to fuse spheroids, either through “bioprinting”\textsuperscript{101} or by using a negative space mold in a bioreactor\textsuperscript{102}. Both of these methods are more tailorable than others in regards to shape of the final TEBV, creating tissue building blocks, and the ability to incorporate SMCs in the culture. While the cell-derived methods described produce tissue tubes with vascular cells, they do not produce tubes that can be used for \textit{in vitro} modeling of vascular behavior or functionally mimic native vascular tissue due to poor mechanical properties or lack of SMC contractility. The aim of this work was to investigate environmental cues that could be used to produce more contractile tissues.

To investigate scientific questions about how vascular tissue functions and reacts to various stimuli, including pharmacological agents, \textit{in vitro} vascular models have been developed as reviewed by Fernandez, et al.\textsuperscript{103}. There are two main methods of creating cell-derived tissues that have been used for 3D \textit{in vitro} vascular modeling: cell sheet manufacture and cellular spheroids\textsuperscript{90,104}. The cell sheet method uses the process described above to create medial mimics using SMCs. These tissues take over 3 months to culture but exhibit excellent tissue contraction in response to vasoconstriction agonists\textsuperscript{90}. These tissues also have limited tissue geometries due to the culture methods\textsuperscript{90}. The cellular spheroid method creates its tissues with suspensions of SMCs and ECs\textsuperscript{104}. Another method of creating vascular models is to use suspensions of SMCs and ECs, allowing them to aggregate and form spheroids. This method can be used to evaluate cell-cell interactions, however, in a spheroid, it is difficult to evaluate mechanical properties and cell alignment\textsuperscript{104}.  

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To address these shortcomings, we have developed a system that uses cellular self-assembly to create mechanically stable, cell-derived 3D SMC rings with the intent to use these for tissue engineering and in vitro modeling purposes\textsuperscript{105,106}. Our unique method offers many advantages, including the ability to evaluate functional properties, measure cellular response, and quantify mechanical properties. Our cell-derived tissues consist of only SMCs and the SMC-produced ECM. The tissue rings have a high cell density, produce collagen, and can be fused into a vascular tube. However, human SMC rings cultured in a growth factor-rich medium (growth medium: GM), needed for cell aggregation and ring growth, lack SMCs in a contractile phenotype. SMC rings seeded and cultured in growth medium appear to exhibit a de-differentiated, synthetic phenotype, with little evidence of contractile protein expression characteristic of mature vascular SMCs. Changes in culture environment such as material elastic modulus, cell culture media composition, and dynamic conditioning have shown some promise in modulating SMC phenotype. The goal of this dissertation was to investigate how different mechanical environments affect SMC behavior, in both 2D culture and in 3D, cell-derived, vascular tissue culture.

\textbf{2.6 Summary And Project Objectives}

The ability to modulate SMC phenotype is useful when culturing SMCs, allowing researchers to rapidly expand SMCs in culture and then switch the SMCs back to a contractile phenotype. This switch is helpful for engineered vascular tissue growth, but may ultimately impede construction of healthy and patent TEBVs and vascular models\textsuperscript{107}.

The field of vascular tissue engineering has advanced rapidly over the last decade; however, achieving SMC mediated contractility in vitro still remains elusive. For implantable TEBVs, this may not be a priority because vessel contractility is not strictly required to transport blood or eliminate thrombosis. However, creating TEBVs that fully integrate into the native tissue, including cell contractility, and developing vascular tissue models for in vitro study both require the presence of contractile SMCs. In this work we focus on methods to promote a contractile phenotype in tissue engineered vascular cultures.
In this dissertation, we addressed the lack of contractile tissue-engineered vascular tissue by incorporating mechanical and chemical stimulation of SMCs to promote a contractile phenotype in 3D tissues. Synthetic SMCs were differentiated to a contractile phenotype using three methods: differentiation in media as discussed above, increases in substrate elastic modulus, and cyclic mechanical stretch. These techniques were evaluated in 2D cell culture then applied to a cell-derived 3D tissue ring model. We hypothesize that the confluence of these stimuli will push previously synthetic and proliferative SMCs to differentiate into a contractile phenotype.

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Chapter 3: The Effects of Substrate Modulus on Smooth Muscle Cell Phenotype in 2D and 3D Cultures

3.1 Introduction

Currently, there are no tissue-engineered blood vessels (TEBVs) or in vitro vascular models that fully recapitulate the native biological structure and function of native vessels, specifically regarding smooth muscle cell (SMC)–mediated contractility. Despite the need, creating small-diameter TEBVs that are functionally similar to native vessels has proved challenging. Smooth muscle cells and extracellular matrix (ECM) make up the medial layer of blood vessels and carry the majority of the pressure load, as well as contributing all of the contractile force of the vessel\(^1,2\). Adult SMCs exhibit phenotypic plasticity, and have been shown to switch from a contractile phenotype characteristic of healthy blood vessels, to a synthetic phenotype in response to injury or other conditions known to cause vascular disease\(^3,6\). Contractile SMCs proliferate infrequently and express contractile proteins, such as myosin heavy chain (MHC), smooth muscle \(\alpha\) actin (SM\(\alpha\)A), and calponin\(^7\). In contrast, synthetic SMCs proliferate readily and decrease contractile protein expression starting with MHC, then calponin, and lastly, SM\(\alpha\)A\(^7,8\). In vitro, SMCs exhibit characteristics of the synthetic phenotype, which is helpful for SMC proliferation and TEBV growth, but may ultimately impede construction of healthy and patent TEBVs\(^9\).

Previous studies have shown that culturing primary SMCs in vitro in a traditional growth medium (GM; 5-10% serum, with growth factors) leads to dedifferentiation of SMCs from a contractile phenotype to a more synthetic phenotype within two passages\(^10\). However, culturing SMCs in differentiation medium (DM; low serum, without exogenously added growth factors\(^11\)) stimulates contractile protein expression and SMC differentiation into a more mature, contractile phenotype. SMCs cultured in DM exhibited protein expression (smooth muscle \(\alpha\)-actin and calponin) more characteristic of mature vascular SMCs compared to SMCs cultured in GM. Additionally, SMCs cultured in DM were able to support direct endothelial cell attachment,
creating a co-culture of these two vascular cell types\textsuperscript{12}. 

In addition to changes in cell culture media composition, another culture variable that may work to modulate SMC phenotype is substrate stiffness. Preliminary evidence indicates that changes in substrate stiffness have a modulatory effect on SMC phenotype in 2D and 3D cultures similar to other anchorage dependent cell types\textsuperscript{13-18}. The ability to switch SMCs within a TEBV from a proliferative, synthetic phenotype into a quiescent, contractile phenotype could enable creation of a functioning, contractile TEBV.

Our lab has developed a system to use cellular self-assembly to create mechanically stable, cell-derived, 3D SMC rings\textsuperscript{19,20}. Our unique method offers many advantages, including the ability to tune graft structural and functional properties in a model that is conducive to quantitative measurement of mechanical properties and contraction. In this dissertation, we observed that similar to 2D SMC cultures, human SMC rings cultured in growth factor-enriched medium, needed for cell aggregation and ring growth, lack contractile SMCs. SMCs rings seeded and cultured in GM appear to exhibit a de-differentiated, “synthetic” phenotype, with little evidence of contractile protein expression characteristic of mature vascular SMCs. While there are many environmental differences between 2D and 3D culture, one parameter that was clearly different between our 2D and 3D cultures was substrate stiffness.

The goal of this work was to investigate how SMCs respond to changes in cell culture medium and culture on surfaces of different elastic moduli in 2D and 3D. This was accomplished by culturing cells in GM and DM on culture substrates of varying stiffness and assessing the effects on contractile protein expression, tissue structure, and mechanical strength. We hypothesized that by tailoring the substrate elastic modulus in both 2D and 3D tissue culture to be closer to native arterial tissue, we can promote a more contractile phenotype in SMCs, leading to more functional, contractile, engineered vascular tissue.
3.2 Materials And Methods

3.2.1 Cell Culture

Human coronary artery SMCs (hCaSMCs) and growth medium were purchased from LifeLine Cell Technology. Cells and tissues were grown in growth medium (GM) or differentiation medium (DM; Fig. 3.1). Growth medium (GM) consists of Vasculife Basal Medium, 5% FBS, 10 mM glutamine, 1% penicillin-streptomycin (MediaTech), 50 µg/mL ascorbate, 5 µg/mL FGF, and 5 ng/mL EGF (all components from LifeLine Cell Technology unless otherwise specified). Differentiation medium (DM) consists of DMEM/F12 (MediaTech), 1% FBS (PAA Laboratories), 1% glutamine (MediaTech), 1% ITS (MediaTech), 1% penicillin-streptomycin (MediaTech), and 50 µg/mL ascorbate (Wako). Passage 6 or 7 hCaSMCs were cultured and seeded in GM for 2D or 3D culture experiments. In 2D experiments, SMCs were seeded at a density of 8,000 cells/cm² and cultured in GM for 24 hours, then cultured in GM or switched to DM for an additional 4 days (Fig. 3.1).

3.2.2 Polyacrylamide Gel Fabrication

Glass cover slips (22mm, Ted Pella) were activated for polyacrylamide attachment by soaking in a 1% (aminopropyl)trimethoxysilane solution (Sigma-Aldrich) for 30 minutes, rinsing with deionized water, and incubating in 0.5% gluteraldehyde solution (Sigma-Aldrich) for three hours at 4°C. Coverslips were dried using vacuum aspiration. Polyacrylamide (PAAM) gels were formed at 3 different stiffnesses (0.6 kPa, 4.8 kPa, and 153.6 kPa) by varying the concentration of acrylamide.
and bis-acrylamide (BioRad) in the formation of the gels (concentrations of acrylamide:bis-acrylamide 3%:0.06%, 7.5%:0.05%, 12%:0.58%,respectively) as described by Quinlan, et al.\textsuperscript{21}. All gels in this work were fabricated in the Billiar lab at WPI, the same lab that verified the modulus in Quinlan, et al.\textsuperscript{22}. After gel formation, gels were coated with 0.5 mg/ml Sulfo-SANPAH (Thermo Scientific), photoactivated with UV irradiation for 5 minutes, and collagen-coated with 100 µg/mL bovine collagen I (Biomatrix) for 2 hours at room temperature. Following collagen incubation, gels were rinsed with sterile PBS and soaked overnight in an antibiotic/antimyocytic cocktail containing 100 µg/mL penicillin–streptomycin (MediaTech), 2.5 µg/mL amphotericin B (Corning), 10 µg/mL ciprofloxacin (Alfa Aesar), and 100 µg/mL gentamycin (Alfa Aesar) described by Clement, et al.\textsuperscript{23}. Gels were rinsed 3 times with sterile PBS prior to cell culture. SMCs were seeded at a density of 20,000 cells/well on glass coverslips. SMCs were seeded at a density of 8,000 cells/well on PAAM gels of different stiffnesses and on the glass coverslips without gels. All 2D SMC experiments were seeded at a low density to keep the cultures subconfluent to limit the confounding effects of cell-cell interaction on SMC phenotype. All SMCs in 2D experiments were from the same donor and were seeded in GM to allow cell adhesion, then half of the gels were switched to DM after 24 hours and media was exchanged every 2 days on all coverslips. Cells were fixed on day 5 and probed for contractile proteins.

### 3.2.3 Self-Assembled Ring Creation and Culture

#### 3.2.3.1 Ring Seeding

For 3D cultures, self-assembled SMC rings were formed as previously described\textsuperscript{19}. Briefly, 400,000-1,000,000 SMCs were seeded into round-bottomed, annular, agarose (2%; Lonza) wells with a 2mm central post. The number of cells seeded per ring was dependent on the number of cells required to form a stable ring. This is due to the variation of SMCs from different donors. Within 24 hours, the SMCs contracted around the central post, forming a cohesive, cell-derived tissue ring. These rings were cultured for 14 days in GM, or for 1 day in GM and 13 days in DM as shown in Fig. 3.1. For all rings, culture media was exchanged every 2 days for 14 days. For tissue ring crosslinking experiments, the culture media was supplemented with 20 µM genipin (Sigma, cat#G4796) on the fifth day of culture. Genipin is a small (226 Da) crosslinking agent that creates a hemiaminal group with free amines found in tissues\textsuperscript{24}. Madhavan, et al. found that
the modulus of collagen/chitosan gels increased 4 fold with the addition of 25 μM genipin to the culture medium. Based on previous work in our lab, the collagen content of rat SMC tissue rings was found to be 350 μg per ring. When ring volume was estimated using the thickness measurements and the assumption that the tissue was toroidal, the collagen concentration was similar to that used by Madhaven, et al. Based on this finding we hypothesized that genipin could be used to increase the modulus of our cell-derived tissue rings.

3.2.4 Measuring Contractile Protein Expression

3.2.4.1 Immunohistochemistry

The presence of contractile proteins was determined in both 2D and 3D cultures using fluorescence immunostaining. For 2D cultures, cells were fixed in 10% neutral buffered formalin for 15 minutes. For 3D cultures, tissue rings were fixed in 10% neutral buffered formalin for 45 minutes, processed, and paraffin embedded for sectioning. After embedding, tissue rings were sectioned into 5 µm slices using a microtome and mounted on glass slides (VWR, Superfrost Plus). Tissues sections were deparaffinized in xylene, followed by rehydridation in a graded ethanol series. Both culture conditions, 2D and 3D, were blocked in 1.5% normal rabbit serum in PBS and immunostained for SMA and calponin using mouse anti-human primary antibodies (Dako, cat#M0851 and cat#M0851, respectively) against each protein, and a rabbit anti-mouse secondary conjugated to AlexaFluor 488 (Invitrogen, cat#A11059). For each protein, a non-specific mouse IgG primary antibody (Vector, cat# I-1000) was used to serve as a negative control. Sections of healthy human umbilical artery samples (obtained from the University of Massachusetts Obstetrics Department) served as a positive control. Representative images of stained positive control sections can be found in Appendix A. All samples were counterstained with Hoechst (Invitrogen, cat#H3570) for nuclear labeling.

3.2.4.2 Quantifying Contractile Cells

Calponin-positive cells were quantified after immunostaining by imaging 5 regions (or 25 cells if regions were sparsely seeded) at a 40x magnification each PAAM-coated coverslip or well (n=3-4 samples; 25 cells/sample), quantifying the Hoechst stained nuclei, and quantifying the cells that had visible positive staining. All quantification was completed by an observer blinded to the sample group or expected outcomes.
3.2.4.3 Western Blotting

For 3D experiments, tissue rings were lysed in a lysis buffer containing 40mM Tris, 150mM NaCl, 8% glycerol, 0.0127% Triton X-100, 0.005% Tween20, 0.02% NP-40 and a protease inhibition cocktail (AEBSF, Aprotinin, Bestatin, E64, Leupeptin, and Pepstatin A tablet from Thermo-Fisher) at a final concentration of 1%. Samples were sonicated for 21-1 second pulses at 30 watts (Misonix). After protein quantification with a BCA assay (Pierce), 5x Laemmli sample buffer and 2-Mercaptoethanol (5:1; BioRad) was mixed with each sample. Approximately 10 µg of total protein was loaded into each well in a 10% polyacrylamide gel and the sample was separated at 160V for 1 hour in an electrophoresis running chamber (BioRad). Protein was transferred onto polyvinylidene difluoride membranes (Millipore) at 390 mA for 1.5 hours. Membranes were then blocked in 5% dehydrated milk powder (Carnation) in tris-buffered saline and 0.1% Tween-20 (TBS-T) for an hour. Membranes were probed with smooth muscle α -actin (Dako, 1:500), calponin (Dako, 1:500), β -tubulin (Developmental Studies Hybridoma Bank, 1:300), or β -actin (Sigma, 1:25000) antibodies in 1% milk TBS-T overnight at 8° C. Bound primary antibody was detected with goat anti-mouse- IgG-HRP (BioRad,1:5000), developed with a Pierce Western Solution (Thermo Scientific), and imaged using a ChemiDoc MP western blot imaging system (BioRad).

3.2.5 Histology

Tissue sections were stained with a Picrosirius Red/Fast Green stain to detect interstitial collagen. After deparaffinization, sections were stained with Picrosirius Red/Fast Green (0.1% Fast Green FCF and 0.1% Direct Red 80 in picric acid; Sigma) for 30 minutes. Sections were rinsed, dehydrated in ethanol and xylenes and coverslipped. Other sections were stained with hematoxylin and eosin to visualize overall tissue morphology. After deparaffinization, sections were stained with Harris Hematoxylin (Richard Allan Scientific) for 5 minutes, rinsed in running water, differentiated in 1% HCl in 70% ethanol with 3 quick dips, and rinsed in running water again. They were then blued in 0.84% ammonia hydroxide for 1 minute, rinsed in running water, counterstained with eosin (Richard Allan Scientific) for 1 minute, then dehydrated for coverslipping.
3.2.6 Mechanical Characterization

3.2.6.1 Measuring Ring Thickness
Rings were harvested, transferred into a Petri dish in phosphate buffered saline, and imaged using a DVT camera as previously reported\textsuperscript{19}. Ring thickness was measured at four points around the ring using edge detection software (Frameworks) and averaged to calculate the mean wall thickness for each ring sample.

3.2.6.2 Mechanical Testing
Uniaxial mechanical testing was done as described previously by Gwyther, et al.\textsuperscript{20} to determine ultimate tensile strength (UTS), failure strain, and maximum tangent modulus (MTM). Briefly, tissue rings were mounted on custom grips on a material testing system (ElectroPuls E1000, Instron), preconditioned for 8 cycles to 50 kPa, and then pulled to failure at a rate of 10 mm/min. Pre-cycling is included prior to mechanical testing of many soft tissues to decrease hysteresis, allowing us to test the tissue in an elastic steady-state to acquire a reliable measurement\textsuperscript{26}. Sample stress vs strain curves can be found in Appendix E.

![Figure 3.2. Measuring Stiffness of 3D ring tissue with AFM. A segment of the ring was excised from the tissue (A) and stiffness measurements were collected on the edge and in the center. An example raw data plot is shown (B,C) with a circle around the analyzed portion (B).](image)

3.2.6.3 Tissue Elastic Modulus Measurement
A cylindrical cross section was removed from each cell-derived tissue ring by making two vertical cuts, 1 mm apart, through the tissue ring and the sample was laid flat on a Petri dish.
Cross sections were adhered to Petri dish using vacuum grease and PBS was applied to the sample for hydration. Tissue stiffness was measured using an atomic force microscope (Asylum Research). Samples were measured using a spherical, 5µm diameter probe (Nova scan), and a cantilever stiffness of 0.06 N/m was used. Samples were probed in two locations, roughly 50 µm away from the edge of the cross section and in the center of the cross section, to an overall displacement of 50nm (Fig. 3.2A). As observed with histological staining, tissue morphology varies throughout the thickness of the tissue rings and sampling two areas on each tissue allowed us to evaluate how the elastic modulus varied in different regions of the tissue. Tissue samples from the genipin experiment were too small to reliably take two measurements per tissue; therefore, only one area was probed. Ten force curves were taken in each location. Stiffness was determined through fitting data to a Hertz model using custom Matlab code (Mathworks), explained below (Fig. 3.2B,C). Stiffness values of each sample were averaged together to calculate sample stiffness.

\[
\frac{1}{E^*} = \frac{1 - v_1^2}{E_1} + \frac{1 - v_2^2}{E_2}
\]

The elastic modulus of the tissue \((E_2)\) was determined by measuring the elastic modulus of cantilever/tissue \((E^*)\) by moving the piezoscanner and measuring the deflection of the cantilever, and using the measured stiffness of the cantilever \((E_1)\), and the Poisson’s ratios \((v_1, v_2)\). For the purpose of calculation, the tissue was assumed to be linearly elastic, homogeneous, isotropic, and incompressible.

### 3.2.7 Statistical Analyses

To determine differences between experimental conditions in all experiments, groups were compared using one-way ANOVAs with significant differences between groups determined using Holm-Sidak post hoc analysis. For experiments with only two groups Student’s t-tests were performed. For all statistical analysis, significance was indicated by a \(p<0.05\).
3.3 Results

3.3.1 Differentiation Medium Increases SMC Contractile Protein Expression in 2D Culture on Glass

In order to evaluate how SMC phenotype changes in response to media composition, SMCs were cultured on glass cover slips in growth medium (GM) or differentiation medium (DM). SMC doubling times were significantly shorter in GM samples (23 ± 1 hours) compared to DM samples (47 ± 3 hours, n=3; *p<0.05). Cells grown in DM produced visually more smooth muscle α-actin (Fig. 3.3A, B) and calponin (Fig. 3.3C, D) than GM-treated cells, as seen with immunocytochemistry. This result was confirmed with Western blotting and quantified with densitometry (Fig. 3.4A). Cells cultured in DM expressed significantly more smooth muscle α-actin (Fig. 3.4B) and calponin (Fig. 3.4C) compared to cells cultured in GM.

Figure 3.3. SMαA and calponin expression in SMCs cultured on glass in GM and DM. Smooth muscle α-actin (A, B) and calponin (C, D) protein was detected by immunocytochemistry (ICC; green), and counterstained with Hoechst (blue). Scale = 50 μm.
When cultured in differentiation medium SMC tissue rings were morphologically different than rings cultured in growth medium with regards to ring structure, extracellular matrix composition, and mechanical properties. SMC tissue rings cultured in DM were significantly thinner after 14 days in culture than rings grown in GM (400 ± 30 µm v. 540 ± 90 µm, respectively; Fig. 3.5A-C). When evaluated via histology, rings cultured in DM also appeared to be more compact (Fig. 3.6A, B) and a visible increase in interstitial collagen staining was observed (Fig. 3.6C, D). Rings grown in differentiation medium had significantly higher ultimate tensile strength (133 ±

**3.3.2 Structural Changes in Tissue Rings Cultured in Differentiation Medium**

When cultured in differentiation medium SMC tissue rings were morphologically different than rings cultured in growth medium with regards to ring structure, extracellular matrix composition, and mechanical properties. SMC tissue rings cultured in DM were significantly thinner after 14 days in culture than rings grown in GM (400 ± 30 µm v. 540 ± 90 µm, respectively; Fig. 3.5A-C). When evaluated via histology, rings cultured in DM also appeared to be more compact (Fig. 3.6A, B) and a visible increase in interstitial collagen staining was observed (Fig. 3.6C, D). Rings grown in differentiation medium had significantly higher ultimate tensile strength (133 ±
47 kPa v 261 ± 18 kPa, Fig. 3.7A), higher maximum tangent modulus (304 ± 11 MPa v 790 ± 16 MPa; Fig. 3.7B), and lower maximum load (530 ± 12 mN v 360 ± 5 mN; Fig. 3.7C) compared to tissue rings cultured in growth medium for 14 days.

![Figure 3.6. Tissue morphology, collagen deposition in 3D cell-derived tissue rings grown in GM and DM.](image)

**Figure 3.6. Tissue morphology, collagen deposition in 3D cell-derived tissue rings grown in GM and DM.** Cell rings were stained with hematoxylin and eosin (A,B) and Picrosirius Red/Fast Green (C, D) indicating interstitial collagen (red). Images are oriented with the ring lumen on the right. Scale = 50 μm.

![Figure 3.7. 3D cell-derived tissue ring mechanical properties in GM and DM.](image)

**Figure 3.7. 3D cell-derived tissue ring mechanical properties in GM and DM.** Tissue rings cultured in differentiation medium had significantly higher ultimate tensile strength (A), maximum tangent modulus (B), and significantly lower maximum load (C) when compared to rings cultured in growth medium. n=5, *indicates significant differences between culture conditions by student’s t-test (p<0.05)
3.3.3 Contractile Protein Expression in Tissue Rings Cultured in Differentiation Medium

The SMCs grown on coverslips increased contractile protein expression in response to changes in cell culture medium (from GM to DM). However, despite the structural changes in the rings, differentiation medium produced small but significant changes in smooth muscle α-actin expression compared to rings cultured in growth medium as shown by IHC (Fig. 3.8A, B) and Western blotting (Fig. 3.8E, F). Very little calponin protein was detected by IHC or Western blotting (Fig. 3.8 C-E, densitometry not shown).

Figure 3.8. SMαA and calponin expression in SMCs grown in GM and DM in cell-derived 3D tissue rings
Rings cultured in GM and DM were immunostained for smooth muscle α-actin (A, B) and calponin (C, D). Densitometry analysis was done to quantify this expression (E, F); n=5; *indicates significant differences by student’s t-test (p<0.05) Densitometry data are complied from the Western blot shown and an additional blot shown in the appendix. Images are oriented with the ring lumen on the right. Scale = 50 μm.
3.3.4 Differentiation Medium and Culture on Stiff Substrates Increase Contractile Protein Expression

We hypothesized that difference in the substrate modulus of the 3D tissue rings compared to the glass coverslips in the 2D cell culture experiments may have been responsible for the observed differences in SMC contractile phenotype in response to DM. In order to assess whether substrate modulus has an effect on SMC phenotype, PAAM gels were made with increasing moduli and SMCs were seeded on the surface. In addition, atomic force microscopy was used to measure the elastic modulus of the SMC tissue rings on the interior edge and center of the tissue to quantify stiffness within the 3D rings, and to set a baseline for the 2D gel experiments. For rings cultured in GM, the mean elastic modulus is $0.41 \pm 0.04$ kPa at the edge of the tissue and $0.78 \pm 0.31$ kPa at the center, compared to the stiffer elastic modulus in the DM rings, measured as $1.71 \pm 1.86$ kPa at the edge and $1.87 \pm 1.59$ kPa at the center of the tissue (Fig. 3.9).

![Figure 3.9. Stiffness of 3D rings cultured for 14 days were measured with AFM. Stiffness measurements were collected as shown in Figure 2. n=3 (GM), 2 (DM).]
To investigate the effect of substrate stiffness in 2D culture, initially, three substrate stiffnesses were tested: 0.6 kPa (most similar to a tissue ring cultured in GM), 4.8 kPa (stiffness value Peyton, et al. found promoted the most caldesmon expression\textsuperscript{28}), 153.6 kPa (a stiffness value in the range of a stiffened artery\textsuperscript{29}), and a glass coverslip control (approximately 70 GPa). We visually observed SMCs cultured in differentiation medium appeared flatter and more spread on stiffer surfaces (Fig. 3.10E-H) while this trend was not seen in SMCs grown in growth medium (Fig. 3.10A-D). When SMCs were cultured in GM, no contractile protein expression was detected, regardless of substrate modulus (Fig. 3.11A-D). In differentiation medium, SMCs exhibited an increase in smooth muscle $\alpha$-actin production in response to increasing surface modulus (Fig. 3.11E-H).

**Figure 3.10. Cell morphology of SMCs on PAAM gels of increasing stiffness cultured in GM and DM.** After 5 days in culture, SMCs in GM (A-D) exhibit less spreading based on substrate stiffness compared to SMCs in DM (E-H) which became more contractile in appearance as the PAAM gel stiffness increased. Scale = 100 $\mu$m.
Figure 3.11. SMαA expression in SMCs on PAAM gels of increasing stiffness cultured in GM and DM. After 5 days in culture, ICC indicates an overall increase in SMαA expression (green) in SMCs in DM (E-H) but not GM (A-D) as the substrate stiffness increased. Calponin positive SMCs were more prevalent on stiffer PAAM gels (G, H) when cultured in DM but not in GM (C, D). Scale = 50 μm
In order to further investigate the role of substrate modulus when SMCs were cultured in differentiation medium, we tested seven different stiffnesses: 0.6 kPa, 4.8 kPa, 9.6, 19.2, 38.4, 76.8 (stiffness value in the range of a healthy artery\(^{30}\)), 153.6 kPa, and a glass coverslip control (approximately 70 GPa). The contractile protein, calponin, was visualized with

![Calponin expression in SMCs on PAAM gels of increasing stiffness](image)

**Figure 3.12. Calponin expression in SMCs on PAAM gels of increasing stiffness.** After 5 days in culture, SMCs cultured in DM on PAAM gels were immunostained for calponin (green). Scale = 50 μm

![Percent of Calponin Positive Cells vs. Substrate Modulus](image)

**Figure 3.13. Calponin expression in SMCs on PAAM gels of increasing stiffness.** SMCs were immunostained for calponin after 5 days in culture. The percentage of calponin positive cells was quantified and graphed as a function of stiffness. n = 4 coverslips with >10 cells quantified per coverslip.
immunocytochemistry as an indicator of smooth muscle cell differentiation. As the modulus of the PAAM gel increased, the SMCs exhibited increased calponin expression (Fig. 3.12A-H). The percent of SMCs expressing calponin was quantified (Fig. 3.13). On the softest substrate, 0.6 kPa, only 9.4 ± 5.3% of SMCs expressed calponin. Calponin expression increased in a parabolic response, with the peak around 76.8 kPa, which had 71.4 ± 5.0% of cells staining positive for calponin (Fig. 3.13). The modulus at this peak in calponin expression is in the range of the elastic modulus of a healthy artery\textsuperscript{29}.

![Figure 3.14. Tissue morphology and collagen deposition in 3D cell-derived tissue rings cultured with and without genipin.](image)

Cell rings cultured in genipin were thinner and more compact than rings cultured without genipin (A,C) and Picosirius Red/Fast Green staining (B, D) indicates interstitial collagen staining (red) in both groups. Images are oriented with the ring lumen on the right. Scale = 100 μm.
3.3.5 Tissue Rings Treated with Genipin Show Structural Changes

Our previous results showed that SMCs increase expression of contractile proteins as substrate elastic modulus increases up to 76.8 kPa. However, in cell-derived tissue rings, the elastic modulus was measured to be around 2 kPa when cultured in DM (Fig. 3.9). We hypothesized genipin would crosslink the ECM, resulting in an increase in UTS, MTM, and elastic modulus resulting in an increase in SMC contractile protein expression. To do this, we passively crosslinked the tissue with 20 μM genipin, an aglycone that creates bonds between free lysine and hydrolysine, which are common in collagen. As shown with histological analysis, tissue rings cultured in genipin for 9 days (Fig. 3.14 B, D) were visually thinner and more compact than control rings (Fig. 3.14 A, C) with both staining positive for collagen deposition (Fig. 3.14 C, D). When mechanically tested, tissue rings cultured in genipin were slightly thinner (Fig. 3.15A) with a higher UTS (Fig. 3.15B) and had a significantly higher maximum tangent modulus (Fig. 3.15C) compared to control rings.

Figure 3.15. Mechanical properties of 3D cell-derived tissue rings cultured with and without genipin. Cell rings cultured in genipin were slightly thinner (A) and stronger (B) than control rings and had a significantly higher maximum tangent modulus (C). The maximum strain of the genipin treated tissue was slightly lower than the control (D). n = 6 for control group, 8 for genipin group, *indicates significant differences between culture conditions by student’s t-test (p<0.05)
3.3.6 SMCs in Tissue Rings Treated with Genipin are not More Contractile

Tissue elastic modulus was measured with AFM and tissue rings treated with genipin showed a trend toward an increase in modulus (0.55±0.30 kPa (genipin) v 0.34±0.25 kPa (control); Fig. 3.16). Tissue rings were immunostained for contractile proteins, SMαA and calponin (Fig. 3.17) and total contractile protein content was analyzed with Western blotting (Fig. 3.18A). Densitometry revealed a slight increase in SMαA expression in genipin treated rings but calponin expression was so low it could not be quantified (Fig. 3.18B).

![Figure 3.16. Stiffness of 3D rings cultured with and without genipin measured with AFM. Stiffness measurements were collected as shown in Figure 2. n= 4 (control), 5 (genipin).](image)

![Figure 3.17. SMαA and calponin expression in SMC tissue rings cultured with and without genipin. Cell-derived rings cultured in genipin show no visual increase in smooth muscle α-actin (A,B) or calponin (C,D) compared to rings cultured without genipin. Images are oriented with the ring lumen on the right. Scale = 100 μm](image)
3.4 Discussion

The ability to differentiate SMCs into a more contractile state is imperative for creating functional in vitro vascular tissue models and grafts that mimic native structure and function. Differentiation medium (DM), referred to as quiescence medium, has been shown by Lavender, et al. to promote contractile protein expression in SMCs in 2D culture. We hypothesized that DM could be used to promote a contractile phenotype in the SMCs in cell-derived tissue rings. In this work we measured contractile protein expression as a marker of SMC differentiation instead of functional contraction because changes protein expression can be measured prior to functional contractility. Changes in contractile protein expression are frequently used as markers for differentiation and correlate with increases in functional contractility. When SMαA expressing bone marrow mononuclear cells were isolated and seeded into fibrin gels, SMαA and calponin expression correlated to tissue contractility. Specifically, we measure SMαA and calponin because they are commonly measured contractile proteins that appear on a continuum when SMCs are differentiating. Before using DM in 3D culture we ran a 2D control experiment to assess the strength of this phenotypic effect on human coronary artery smooth muscle cells (hCaSMCs). When cultured on glass coverslips, SMCs cultured in differentiation medium produced more smooth muscle α-actin and calponin than growth medium-cultured cells, as seen with immunocytochemistry, and quantified with Western blotting and densitometry (Fig. 3.4). This phenotypic response is consistent with previously reported findings. In this dissertation,
we focus on only one media composition for SMC differentiation. Other work in our lab will focus on tailoring this culture medium for our purposes including the addition of TGF-β, a cytokine shown to upregulate contractile protein expression in SMCs 34,35.

We hypothesized that to generate cell-derived vascular tissue that was populated with contractile SMCs, we could culture our 3D vascular tissue rings in DM, to promote a phenotypic shift from synthetic to contractile. We found when SMCs were pre-differentiated prior to seeding or seeded in DM, many rings did not form and those that did were too weak to remove from the wells at 14 days unlike those cultured in GM (unpublished observation). However, when SMCs were cultured in GM and rings were seeded in GM, the media could be switched to DM after rings formed (within 24 hours), and the rings retained their structure. We cultured cell-derived rings in GM or DM for 14 days and analyzed the phenotypic and structural changes in the tissue. When cultured in DM, 3D tissue rings became more compact and showed an increase in staining for collagen (Fig. 3.6). When mechanically tested, the rings cultured in DM were also significantly stronger and stiffer (Fig. 3.7). Additionally, in vivo, unloaded blood vessels have circumferential residual stresses that have a significant impact on biological responses26. We quantified these stresses by measuring ring opening angle. Rings were radially cut, allowed to contract in physiological saline for 30 minutes, and opening angle was measured (data can be found in Appendix B).

While we observed changes in tissue morphology, we did not observe changes in SMC phenotype in response to the differentiation medium. Unlike our results in 2D culture, tissue rings cultured in DM did not produce smooth muscle α-actin or detectible calponin, as measured by immunostaining and Western blotting (Fig. 3.8). This may be due to differences between 2D and 3D culture systems that may affect SMC phenotype including cell-cell contact36, nutrient diffusion33, extracellular matrix molecules37, and substrate stiffness38. We chose to investigate the role of substrate stiffness on the SMC phenotype.

To test this, we measured the elastic modulus of the cell-derived tissue rings, using atomic force microscopy (Fig. 3.9) and found that the modulus of the tissue was much lower than healthy native tissue, which may explain why SMCs in the 3D tissue did not express contractile
proteins. While AFM is a common method of measuring stiffness, the conclusions that can be made about how cells sense the substrate are limited. Measuring the elastic modulus of a substrate with AFM requires indenting the surface of the material with a 5 µm borosilicate glass sphere and the measurements are taken from a very small area. Therefore, the measured elastic modulus at each section of the tissue may represent the modulus of a portion of a cell, a collagen molecule, or other ECM materials. For example, in one tissue ring cultured in GM, we found that our elastic modulus measurements in the center of the tissue varied from 0.3 to 1.3 kPa indicating a non-homogenous material. Additionally, when measuring the elastic modulus of our tissues, we isolated a cylindrical section of the tissues and measured the interior of the tissues, potentially probing molecules that had been cut in the isolation process. Another technique used to measure the elastic modulus of tissue is rheometry, where the resistance to a shearing force is measured, potentially providing a more biologically relevant measurement of the microenvironment. However, we were unable to use this technique for our samples due to the size limitations.

The difference in our measured tissue modulus compared to healthy arterial tissue may explain why the SMCs in the 3D tissue rings do not exhibit a contractile phenotype, even when cultured in differentiation media. The tissue’s elastic modulus was two orders of magnitude lower that of healthy vascular tissue (0.6 kPa v. 76.8)30. We hypothesized that by increasing the elastic modulus of the material on which SMCs are cultured, SMC differentiation would increase. When SMCs were grown on PAAM gels of varying stiffnesses, and cultured in GM, no contractile protein expression was detected, regardless of substrate modulus (Fig. 3.11). When cultured in DM, as substrate elastic modulus increased to 76.8 kPa (within the range measured for native arteries29), calponin expression in the SMCs significantly increased (Fig. 3.12) indicating that the modulus of the culture substrate plays a role in how SMCs phenotypically respond to changes in cell culture medium. We observed variation in contractile protein expression in SMCs cultured in DM on glass, both within the same experiment, as well as between experiments. While, in general, we observed contractile protein expression increases in the SMCs cultured in DM compared to GM, the magnitude of this response and the variation may be due to several factors including cell density and cell-cell contact, both shown to affect SMC phenotype36,42.
Based on these findings, the 3D tissue is too soft to induce SMC differentiation in response to changes in culture media alone. We hypothesized that by exogenously crosslinking the ECM of the tissues, and thereby increasing the elastic modulus, we could promote the differentiation of the SMCs into a contractile phenotype. Genipin was chosen over other crosslinking agents due to the molecule’s small size (226 Da) and cytocompatibility. Treating with 0.3% genipin (~ 13 µM), a crosslinking agent, doubled the elastic modulus in treated aortic tissue, and stiffening is attenuated around 10 µM in collagen gel based vascular tissue. Wang, et al. found that genipin toxicity started at 0.5 mM in osteoblasts and chondrocytes but when exposed to higher than 25 µM of genipin, SMCs proliferation decreases. Treating with 20 µM genipin for 9 days resulted in a significant increase in MTM and an increase in elastic modulus of the tissue. However, this did not result in significant increases in SMαA or calponin as measured by immunohistochemistry or Western blotting. The elastic modulus of the genipin-treated rings only reached 0.55 kPa, well below the modulus needed to see maximum calponin expression in 2D culture, 76.8 kPa. This is consistent with other published work, indicating the stiffness of the culture substrate needs to be higher to observe any phenotypic modulation. Other crosslinking agents such as 500 µM transglutaminase have been used to stiffen tissue non-cytotoxically and can be investigated to further stiffen our tissue.

Alternately, we can target the way that SMCs sense substrate stiffness, triggering a response generally observed on stiff substrates when the SMCs are cultured in soft environments. This technique was explored in mammary epithelial cells (MECs) by Paszek, et al. By constitutively activating β1 integrin, MECs cultured on soft substrates and in 3D tissues exhibited a phenotype similar to that seen when MECs were cultured on stiff substrates.

### 3.5 Conclusion

In Chapter 3 we investigated the effects of low serum differentiation media and substrate stiffness on smooth muscle cells in 2D and 3D culture. In summary, we discovered that in 2D culture, SMCs seeded on glass coverslips and cultured in DM exhibit increased contractile protein expression when compared to SMCs cultured in GM. However, when SMCs were cultured in DM in self-assembled 3D tissues, this media change was not enough to promote a contractile phenotype potentially due to the low elastic modulus of the tissue. By exogenously...
increasing the elastic modulus of the tissue we found that SMCs did not differentiate into a contractile phenotype in the 3D tissue. We hypothesize that this is due to the low elastic modulus of the crosslinked tissue. Although the tissue ring was stiffened, the elastic modulus of the genipin treated ring was lower than the initial DM cultured ring in Fig. 3.9. Therefore, we cannot conclude if increasing tissue elastic modulus can influence SMC phenotypic expression. In this work we have shown that modulation of SMC phenotype is, in part, due to the elastic modulus of the culture surface in 2D cultures. This knowledge can potentially be used to tailor TEBV and vascular modulus to promote the most favorable culture environment for contractile SMC culture.

3.6 References

Chapter 4: Effects of Cyclic Strain on Tissue Mechanics and SMC Phenotype

4.1 Introduction

In Chapter 3 we focused on passive methods to promote the differentiation of SMCs to a contractile phenotype. We showed that increasing the elastic modulus of the substrate on which the SMCs were cultured (2D polyacrylamide gels) increased the percentage of SMCs in a contractile phenotype. However, when 3D cell-derived tissue was exogenously stiffened we did not observe an increase in contractile protein expression. In this chapter, we focus on using dynamic mechanical stimulation to promote SMC differentiation. Previous studies indicated that cells, when exposed to cyclic stretch, can overcome the inhibitory effects of soft substrates on contractile protein expression\(^1\). In this chapter we investigated how cyclic stretch affects SMC phenotype in 2D cell culture and 3D tissue constructs.

In vivo, SMCs are exposed to many mechanical forces, due in part to the pulsatile nature of the vascular system. In non-pathogenic conditions, as SMCs experience these forces, the contractile phenotype is maintained\(^2\). In addition to simple phenotypic maintenance, stretch has been shown to promote a switch to a contractile phenotype from a synthetic phenotype in SMCs in 2D culture on silicone membranes\(^3\). In vivo the pulsatile, circumferential strain between diastole and systole varies from 2\% - 15\% by vessel location, type, and size\(^4\). However, the most widely used peak strain when investigating the effects of stretch on cells \textit{in vitro} is 10\% \(^3,5,6\). When cultured on silicone membranes and exposed to cyclic 10\% strain, SMCs increased the production of MHC, SM\(\alpha\)A, and calponin, indicating a phenotypic shift from a more synthetic phenotype to a more contractile phenotype in response to mechanical stretch\(^7,9\). When suspended in a collagen gel, SMCs upregulated SM\(\alpha\)A production indicating phenotypic modulation\(^6\).

Mechanical conditioning has also been used in the field of vascular tissue engineering to increase graft strength, modulus, collagen deposition, and cellular alignment\(^10-16\). In these cases, bioreactors were used to impart a cyclic, circumferential stretch on tissue-engineered blood vessels (TEBVs). This can be accomplished by pumping cell culture media through a scaffold
seeded with cells\textsuperscript{17} or inflating an internal mandrel to stretch the surrounding tissue\textsuperscript{5,12,16}. Seliktar, et al. found that by using an inflating silicone mandrel system a 10\% strain of SMC and fibroblast seeded collagen gels resulted in increases in the mechanical strength and modulus\textsuperscript{15} and Gauvin, et al., and Weidenhamer, et al. observed significant increases in thickness, ultimate tensile strength (UTS), collagen alignment, and modulus in their cell derived fibroblast-derived tissues in response to stretch\textsuperscript{16,18}. Circumferential cellular alignment around the lumen is found in native blood vessels and is thought to contribute to the overall strength and stability of the vessel\textsuperscript{19}. When exposed to cyclic stretch, SMCs align circumferentially, in the direction of the force, strengthening the tissue\textsuperscript{20-22}.

Based on the findings in Chapter 3 we found that SMCs cultured on soft gels (elastic modulus of 4.8 kPa) were significantly less likely to express contractile proteins than SMCs cultured on stiffer gels (76.8 kPa). Quinlan, et al., found that when valvular interstitial cells (VICs) were cultured on soft gels they produced little SM\textalpha A compared to VICs cultured on stiff gels. However, cyclically applying biaxial stretch to the soft gels resulted in VICs that expressed more f-actin and had larger cell areas, similar to VICs cultured on the stiffer gels\textsuperscript{1}. Cui, et al., saw a similar response to stretch in fibroblasts cultured on soft silicone pillars compared to fibroblasts cultured on stiff silicone\textsuperscript{23}. They hypothesized that transient forces can compensate for a less rigid matrix, in part because of the overlap of the mechanotransduction pathways of rigidity sensing and stretch sensing, resulting in similar phenotypic responses\textsuperscript{23}.

The goal of this chapter of the dissertation was to investigate how SMCs respond to dynamic mechanical conditioning and how changes in culture substrate elastic modulus influence this response. We cultured SMCs on 2D PAAM gels undergoing cyclic biaxial stretch and in 3D tissue rings undergoing cyclic circumferential stretch in a custom bioreactor. After stretching the culture substrates in 2D and 3D environments, the phenotype of the SMCs was evaluated by measuring contractile protein expression, tissue structure, and mechanical strength. We hypothesized that cyclic stretch would increase SMC contractile protein expression in 2D cultures on soft gels and in 3D tissue rings cultured in differentiation medium compared to static controls, leading to more contractile vascular tissue.
4.2 Materials And Methods

4.2.1 Cell Culture

Human coronary artery smooth muscle cells (hCaSMCs; Lifeline Cell Technology) were cultured in Vasculife Growth Medium (GM) described in Chapter 3. Passage 6 or 7 hCaSMCs were seeded and cultured in GM for 2D or 3D culture experiments. In 2D experiments, SMCs were seeded at a density of 8,000 cells/cm² and cultured in GM for 24 hours to allow time for cell attachment, then switched to differentiation medium (DM; described in Chapter 3) for 4 days.

4.2.2 Biaxial Stretch in 2D Culture

4.2.2.1 Gel Fabrication

Polyacrylamide gels of predefined stiffnesses were anchored to flexible, silicone-bottomed plates (Bioflex Culture plates, Flexcell) as described by Quinlan, et al. Briefly, silicone-bottomed six well plates were activated by plasma etching for 2 minutes followed by a five-minute coating with propyl methacrylate (Sigma Aldrich). This modified the surface of the silicone, allowing for a PAAM gel to anchor to the surface. Two different stiffnesses of PAAM gel were used, 4.8 and 76.8 kPa, and were made as described in Chapter 3.

4.2.2.2 Stretch Regimen

After allowing 24 hours for cell attachment, membranes were biaxially stretched to 10% strain at 1Hz for 4 days using a sinusoidal stretch pattern with a Flexcell stretch system (Flexcell FX-4000T, Flexcell) which uses a vacuum to alternately stretch the silicone membrane over a solid, circular platen and release it, resulting in a cyclic 10% strain. For a static control, SMCs were cultured on non-stretched PAAM gels.
4.2.3 Circumferential Stretch Bioreactor and Ring Culture

In order to impart circumferential strain on 3D SMC rings, SMC rings were cultured in a custom bioreactor that was designed and validated in our lab by Jennifer Cooper. This bioreactor consists of a pressurized tubing/syringe system driven by rotating cams (Fig. 4.1). The entire bioreactor is portable and able to fit inside of a standard sized incubator shelf (Fig. 4.1C). A motor, mounted in a humidity protective case, spins an extended shaft at 1 Hz and as the shaft spins, the circular cam spins (Fig. 4.1C), repeatedly depressing the spring-loaded plunger of the syringe (Fig. 4.1B). Each time the plunger is depressed, the clamped silicone tubing attached to the end of the syringe is cyclically inflated, stretching the tissue placed around it (Fig. 4.1A). The silicone tubing with the tissues is located inside of a polyethylene chamber with an optically clear lid to make tissue monitoring possible.

This bioreactor system was validated by Cooper, et al. where the amount of stretch propagation through the tissue, stretch over time, and stretch imparted as a function of silicone tube location were measured. By using a high-density mapping system developed by the Gaudette lab at WPI, she found that, measuring the exterior side of the tissue, the tissue experienced around 75% of the stretch seen on an unloaded silicone tube and this amount of stretch decreased slightly over time when rings cultured for 6 days were compared to 2 day old rings.
Additionally, she found that stretch along the length of the silicone tube varied by approximately 1% strain except for the very end of the silicone tubing (longer than 5 cm) at which point the distension decreased significantly. All work in this dissertation was done within the first 0-5 cm section of tubing proximal to the syringe.

SMC tissue rings were cultured as described in Chapter 3. After 5 days in static culture in DM, all rings were transferred onto the silicone tubing of the bioreactor. Half of the rings were transferred onto the tubing in the bioreactor but not mechanically stimulated. The other half underwent cyclic circumferential stretch to a maximum of 10% at 1Hz for 9 days in culture. Half of the media was exchanged every 3 days during bioreactor culture. At the end of a culture period of 14 days (5 in the agarose mold and 9 on the bioreactor) the rings were slid off of the silicone tubing without resistance, indicating there was little to no cell adhesion to the silicone tubing.

### 4.2.4 Measuring Contractile Protein Expression

The presence of contractile proteins, smooth muscle α-actin and calponin was detected using immunostaining and Western blotting as described in Chapter 3.

### 4.2.5 Extracellular Matrix Evaluation

Tissue morphology was visualized with a hematoxylin and eosin stain and collagen deposition was visualized with a Picrosirius Red/Fast Green Stain. Both stains are described in Chapter 3.

### 4.2.6 Measuring Circumferential Nuclear Alignment and Cell Density

Nuclear alignment was measured as described by Cooper, et al. Briefly, nuclei of histological sections were fluorescently labeled with Hoechst. Four rings per treatment group were analyzed and three human umbilical arteries were used as controls. Using ImageJ, the color was thresholded in all Hoechst images to the same level, images were binarized, and nuclei were identified with the “Analyze Particles” feature. ImageJ isolated all nuclei, applied an ellipse to the shape of the nuclei, and measured the angle of the long diameter. This angle was then normalized to a line drawn manually to reflect the edge of the lumen. An angle of zero reflects a...
nucleus that was circumferentially aligned to the lumen and an angle of 90 reflects a nucleus aligned perpendicularly to the lumen.

Nuclear density of the tissues was also measured using Hoechst stained images. All nuclei measured above were counted and the total tissue area was measured. Using these two values we determined the nuclear density.

4.2.7 Mechanical Testing
Uniaxial mechanical testing was used to determine UTS, max strain, and maximum tangent modulus (MTM) of SMC tissue rings as described in Chapter 3. Additionally, tissue toughness was calculated for this work. Toughness is the measure of the amount of energy absorbed per volume prior to failure and is calculated as the integral of the stress vs strain curve or the area under the curve.

4.2.8 Statistical Analysis
To determine differences between experimental conditions in all experiments, groups were compared using two-way ANOVAs with significant differences between groups determined using Holm-Sidak post hoc analysis or student’s t-tests, depending on number of variable groups. Differences between groups will be considered significant at p<0.05.

4.3 Results
4.3.1 Cyclic Stretch Increases Contractile Protein Expression in SMCs Grown on Soft PAAM Gels
SMCs were cultured on soft (4.8 kPa) or stiff (76.8 kPa) polyacrylamide gels that were cultured statically or cyclically stretched to 10% strain. When SMCs were statically cultured on soft (4.8 kPa) gels few SMCs stained positive for calponin (31.9 ± 29.1%, Fig. 4.2A), compared to SMCs statically cultured on stiff (76.8 kPa) gels (59.6 ± 21.4 %, Fig. 4.2B). However, when SMCs were cultured on soft and stiff gels and cyclically stretched the SMCs on the soft gels increase calponin expression to 77.0 ± 20.9 % (Fig. 4.2C) while 54.4 ± 9.3% (Fig. 4.2D) of SMCs cultured on stiff gels expressed calponin. Calponin in SMCs on gels that were stretched was located in long fibrils (Fig. 4.3 B) compared to the diffuse calponin in SMCs cultured statically.
Fig. 4.3A). Calponin and SMαA were further quantified by Western blotting (Fig. 4.4A) and normalized to β-actin. Faint bands were detected for calponin but were not strong enough to measure. Densitometry revealed SMCs cultured statically on stiff gels produced statistically more SMαA than SMCs statically cultured on soft gels or SMCs cultured on stiff gels and stretched (Fig. 4.4B).

**Figure 4.2. Calponin expression in SMCs on static or stretched PAAM gels.** After 5 days in culture in DM, SMCs on softer, static PAAM gels do not express much calponin (green; A). Calponin expression increases when the gel is cyclically stretched (C). Cyclic stretch had little effect on SMCs cultured on stiff gels, both of which exhibited calponin expression (B, D). The percentage of calponin positive cells per coverslip was quantified (E). n = 6 coverslips, > 25 cells per coverslip. Scale = 50 μm *indicates significant differences by two-way ANOVA with Holm-Sidak post hoc analysis (p<0.05).
4.3.2 Cyclic Stretch Structurally Alters Tissue Rings

4.3.2.1 Tissue Ring Mechanical Properties

Dynamically cultured tissue rings did not have a significantly smaller tissue thickness (315.2 ± 60.6 µm v 331.6 ± 78.6 µm; Fig. 4.5A), higher ultimate tensile strength (86.6 ± 28.9 kPa v 73.1 ±...
21.5 kPa; Fig. 4.5B), or higher maximum tangent modulus (409 ± 11.2 kPa v 450 ± 27 kPa; Fig. 4.5C) compared to statically cultured rings. However, dynamically cultured tissue rings were significantly tougher compared to the static controls.

![Table](image)

**Figure 4.5.** 3D cell-derived tissue ring mechanical properties after static or dynamic culture. Tissue rings dynamically cultured did not have a significant change in thickness (A), ultimate tensile strength (B), or maximum tangent modulus (C). Rings cultured dynamically did show a significant increase in toughness (D) compared to statically cultured controls. n=6, *indicates significant differences by student’s t-test (p<0.05)

### 4.3.2.2 Structural Changes in Tissue Rings

Tissue rings that were cyclically stretched (Fig. 4.6B) were visibly denser than static controls (Fig. 4.6A) and Picrosirius Red staining indicates both stretched and static rings contain deposited collagen (Fig. 4.6C, D). Further structural analysis revealed dynamically cultured rings showed a trend toward circumferential nuclei alignment (Fig. 4.7C), which was less clear in statically cultured rings (Fig. 4.7A). Significantly more nuclei were aligned within 30° from circumferential alignment in the dynamically cultured rings compared to the static controls (47.0

![Images](image)

**Figure 4.6.** Tissue morphology and collagen deposition in 3D cell-derived tissue rings cultured statically or dynamically. Cell rings exposed to cyclic stretch (B) appeared to have greater cell density than rings cultured statically (A) and Picrosirius Red/Fast Green staining (C, D) indicates interstitial collagen staining (red) in both groups. Images are oriented with the ring lumen on the right. Scale = 100 μm.
± 6.9% v 37.9 ± 7.2%; Fig. 4.7B) and dynamically cultured rings had a significantly higher nuclear density (5440 ± 700 nuclei/mm² v 3560 ± 950 nuclei/mm²; Fig. 4.7D).

Figure 4.7. Nuclear alignment in cell-derived tissue cultured statically or stretched. Cell rings circumferentially stretched during culture showed a trend toward circumferential alignment around the lumen (C) compared to the nuclear alignment of statically cultured rings (A). Stretched rings had significantly more nuclei within 30° of circumferentially aligned compared to the static control (B) as well as an increase in nuclear density (D). The dashed line (B, D) indicates the values for human umbilical artery control. n = 4 * indicates significant differences by student’s t-test (p<0.05)
4.3.2.3 Contractile Protein Expression in Tissue Rings

Tissue rings cultured with cyclic stretch produced visually more smooth muscle α-actin (Fig. 4.8A,C) and calponin (Fig. 4.8 B,D) than statically cultured rings, as seen with immunocytochemistry. This result was confirmed with Western blotting, quantified with densitometry, and the protein expression was normalized to the amount of β-actin (Fig. 4.9A). Bands indicating calponin were faint but were able to be analyzed with ImageJ. Dynamically cultured rings expressed significantly more smooth muscle α-actin (Fig. 4.9B) and calponin (Fig. 4.9C) compared to statically-cultured rings.

![Image of protein expression](image)

**Figure 4.8.** SMαA and calponin expression in SMC rings dynamically cultured, compared to statically cultured rings. Dynamically cultured rings produced more smooth muscle α-actin (A,C) and calponin (B,D) than rings grown statically. Images are oriented with the ring lumen on the right. Scale = 100 μm.
In Chapter 3, we showed that SMCs cultured on soft PAAM gels (4.8 kPa) had very low levels of contractile protein expression when compared to SMCs cultured on stiffer PAAM gels (76.8 kPa). A similar phenotypic response was seen by Quinlan, et al. in valvular interstitial cells (VICs)\(^1\). The VICs showed strong SMαA staining when cultured on a stiff surface (50 kPa) and very little on the soft surface (0.3 kPa). However, this phenotype could be rescued by imparting a 10% cyclic stretch\(^1\). This response to stretch has also been seen in fibroblasts by Cui, et al.\(^23\). In Chapter 3 we demonstrated that SMCs behave similarly and the SMC contractile phenotype can be regained in SMCs cultured on soft substrates when they are exposed to cyclic stretch (Figs 4.2 and 4.4). We also observed a decrease in SMαA expression in SMCs cultured on stiff gels and stretched. This response may be a cellular response to reduce the overall tension experienced by the cell, similar to the decrease in contractile protein expression in SMCs cultured on glass coverslips compared to softer gels (Chapter 3). When cells were stretched on either gel, we observed a visible increase in contractile fiber formation, indicating the assembly of the contractile apparatus (Fig. 4.3).

As previously discussed in Chapter 3, the elastic modulus of the cell-derived tissue is below 2 kPa, much lower than healthy native tissue, possibly explaining why SMCs in the 3D tissue did not exhibit contractile proteins.\(^{26-28}\) In Chapter 4 we investigated how cyclic circumferential...
stretch would affect SMC phenotype and 3D tissue structure. We hypothesized that by exposing the tissue rings to a cyclic 10% stretch, we could increase contractile protein expression as seen in our 2D PAAM gel experiments. In prior work with the circumferential stretch bioreactor, no contractile protein expression was observed in SMC tissue rings grown in a high serum growth culture medium and cyclically stretched for 7 days to stretch magnitudes of 0%, 5%, 7.5%, 10%, 15% or cultured in agarose wells for the duration of culture. Since previous static culture studies had been completed in differentiation medium and cyclic stretch studies had been completed in growth medium, we hypothesized that by culturing SMC tissue rings in differentiation media while cyclically stretching the tissue, the SMCs will exhibit a more contractile phenotype. Without the biochemical cues of a differentiation medium the cells may not have received the optimal cues to create a strong, contractile tissue.

After culturing the tissue rings statically for 5 days, they were cultured statically or circumferentially stretched on a bioreactor to a 10% strain for 9 additional days. Although significant changes in thickness (Fig. 4.5A), ultimate tensile strength (Fig. 4.5B), or maximum tangent modulus (Fig. 4.5C) were not observed in dynamically cultured tissue rings compared to the static controls they were qualitatively different. Rings cultured dynamically were easier to remove from the bioreactor. This is reflected in the significant increase in toughness, the measure of the amount of energy absorbed per volume prior to failure. Based on others’ work, we expected to see increases in UTS, and modulus due in part to increased MMP-2 expression indicating that mechanical conditioning can increase the rate of tissue remodeling, as well as tissue mechanical strength and modulus. One reason for the difference in results may be the difference in culture time exposed to stretch. Many stretch experiments take place over 3 weeks or more before a difference is observed and in our work the tissue was only exposed to stretch for 9 days. Also, many of these experiments are done on bioreactors with optimized stretch magnitudes, and duty cycles specifically tailored to improve the collagen content and mechanical strength of the tissues.

In addition to mechanical properties, we also measured the nuclear alignment and cellular density, which both increased significantly in the stretched group. In vivo circumferential alignment of SMCs in blood vessels contributes to the overall strength and stability of the
Nuclear alignment has been seen in fibroblast-derived tissue after 3 days of dynamic culture, in collagen gel culture after 8 days in culture, and in several SMC 2D studies. Despite minimal changes in tissue structure, when contractile protein expression was assayed, significant increases were measured in both SMαA and calponin in the dynamically cultured tissue. In vivo, the induction of a pulsatile flow has been shown to promote SMC differentiation. However, only a small increase in SMαA was measured in stretched collagen gel TEBVs. Differentiating SMCs in 3D tissue has proven difficult and our future work will address iterations on our technique. Our bioreactor system can be easily modified to incorporate changes in duty cycle by modifying cam geometry or can be modified to incorporate incremental stretch by changing the cams throughout the culture time. A preliminary study on the effect of a 50% duty cycle can be found in Appendix C.

4.5 Conclusion

In this chapter we demonstrated that SMCs differentiated into a contractile phenotype when dynamically cultured on a soft 2D surface. The elastic modulus of our 3D tissues was reported in Chapter 3 to be lower than the threshold for SMC differentiation, thus providing a rationale for the low level of contractile protein expression. After mechanical stimulation, tissue rings became more aligned, tougher, and promote SMC differentiation. Based on our research, future work will focus on increasing the stretch magnitude over time, lowering the stretch duty cycle, and increasing the duration of the culture period.

4.6 References


23. Cui, Y., Hameed, F. M., Yang, B., Lee, K. & Pan, C. Q. Cyclic stretching of soft
Chapter 5: Conclusions and Future Work

5.1 Overview
The work in this dissertation describes an investigation of how passive and dynamic environmental cues influence the smooth muscle phenotype. The ability to promote a contractile phenotype in smooth muscle cells (SMCs) in three-dimensional tissue culture is integral to the successful creation of functional vascular models and can be used to further the creation of tissue-engineered blood vessels that recapitulate native tissue. We studied the effects of substrate modulus in conjunction with changes in cell culture media composition on SMC phenotype in 2D and 3D cultures. Following this, in an effort to modify the vascular tissue without adding exogenous materials, we quantified the effects of dynamic mechanical stimulation on tissue mechanics and SMC phenotype.

5.2 Results and Conclusions
5.2.1 Specific Aim 1: Determine the effects of substrate modulus on smooth muscle cell phenotype in 2D and 3D cultures
Based on previous work done by others investigating how modifications in cell culture medium affect SMC phenotype, we hypothesized that 3D tissue rings could be cultured in a low serum, differentiation medium (DM) to create a tissue ring composed of contractile SMCs. Traditionally, SMCs are cultured in growth medium (GM) with 5%-10% serum and growth factors (our laboratory uses epidermal and fibroblast growth factors) to promote a synthetic phenotype and increase proliferation. Our lab, as well as others, have shown that SMCs cultured in DM, with no exogenous growth factors and 1% serum, begin to differentiate into a more contractile phenotype, upregulating contractile protein expression and increasing doubling time\textsuperscript{1,2}.

The overall goal of this work was to promote a contractile phenotype in the SMCs cultured in our three-dimensional tissue ring system. We hypothesized that culturing SMC tissue rings in DM would be enough to promote SMC contractile protein expression in this system. We began by culturing our tissue rings in DM, and then over the culture period of 14 days, we measured
differences in tissue thickness and in mechanical properties such as maximum tangent modulus and ultimate tensile strength. However, counter to our hypothesis, we did not observe any measurable changes in contractile protein expression when compared to the tissue cultured in GM, indicating that the SMCs were not altering their phenotype in response to the change in culture medium.

We hypothesized that this discrepancy in the response of the SMCs in 2D cell and 3D tissue culture was due to differences in the elastic modulus of the substrate to which the SMCs were attached. In 2D cell culture, they were attached to glass coverslips with an elastic modulus around 70 GPa and in 3D tissue culture we measured the elastic modulus to be 0.6 kPa, a difference of 5 orders of magnitude. To investigate this hypothesis, we created polyacrylamide (PAAM) gels with different elastic moduli on which to culture the SMCs. When SMCs were cultured in growth medium, they did not produce contractile proteins, regardless of the elastic modulus of the gel on which they were cultured. However, when SMCs were cultured in DM, there was an increase in calponin expression in a dose dependent fashion as the elastic modulus of the culture surface increased. From this we hypothesized that the difference in the response of the SMCs in 2D compared to 3D cultures was due to differences in elastic modulus of the culture surface.

In order to evaluate the effect of tissue stiffness on SMC phenotype we passively cross-linked the 3D cell-derived tissue rings in 20 μM genipin, an exogenous cross-linker. In preliminary experiments we found this concentration to only slightly decrease total cell number and this is supported by the literature to be non-cytotoxic at this concentration. We found by passively cross-linking the tissue with genipin, we increased the elastic modulus of the tissue from 0.34±0.25 kPa to 0.55±0.30 kPa. Subsequently, we measured that the genipin treated rings were slightly thinner with a higher UTS and had a significantly higher MTM. In addition to these changes in material properties, we measured contractile protein expression but observed no significant differences in contractile protein expression in the stiffened tissue compared to the control.
Although the genipin-treated rings and the rings from the GM vs DM experiments were both human coronary artery SMCs, they came from different donors. All GM vs DM work was completed using SMCs from an 18 year old, black male who died of a gunshot wound while the genipin experiments were completed with SMCs from a 20 year old, Hispanic male who died from a cerebrovascular accident. Both cell lots were deemed to be from “healthy” donors by Lifeline Technologies. We have found that one of the limitations of our ring culture system is that the mechanical and structural properties of our cell-derived rings can vary from lot to lot. This may be the cause of the differences between the mechanical properties between experiments. While the genipin did not sufficiently stiffen the tissue to see a phenotypic response in our experiments, we hypothesize that with a different lot of cells we may be able to observe a difference in phenotype in response to tissue stiffening.

5.2.2 Specific Aim 2: Quantify the effects of cyclic strain on tissue mechanics and SMC phenotype

In Specific Aim 1 we found that by exogenously cross-linking the extracellular matrix of the tissue rings we promoted a differentiation of our SMCs toward a more contractile phenotype. However, we were interested in developing a method to increase the contractile phenotype of SMCs without exogenously modifying the tissue, resulting in a tissue where structural changes are due to cell-derived tissue remodeling.

To determine if cyclic stretch could modify SMC phenotype when cells were attached to soft surfaces, we created PAAM gels with elastic moduli of 4.8 kPa and 76.8 kPa, serving as our soft and stiff gels, respectively. We then exposed half of these gels to cyclic 10% stretch, leaving the other half as a static control. SMCs that were cultured on soft gels that underwent cyclic stretch significantly increased calponin expression, both when measured as percentage of calponin positive cells as well as overall protein content.

This method was applied to our tissue ring system, known to have a low elastic modulus, to determine whether cyclic stretch would affect the SMCs in 3D culture. SMC rings were circumferentially stretched to a 10% strain on a cyclic stretch bioreactor. We found that dynamic mechanical conditioning modified the structure of the tissue rings, increasing ultimate tensile
strength (UTS), maximum tangent modulus (MTM), and cellular alignment compared to the static controls. In addition to the structural changes we also measured significant increases in contractile proteins, SMαA, and calponin in the rings that were exposed to mechanical stretch.

Overall, these findings indicate that exposing SMCs to cyclic stretch can counteract the inhibitory effect of the soft culture surface on SMC contractile phenotype in both 2D and 3D culture.

5.3 Future Work
The overall goal of this dissertation was to develop a method to differentiate SMCs in a three-dimensional cell-derived tissue ring into a contractile phenotype. In accomplishing this goal, we investigated several methods to promote this differentiation including changes in media composition, mechanical properties, and mechanical stimulation. To further improve tissue contractility with the ultimate goal of creating a vascular tissue model, work can be done to further increase the elastic modulus of the tissue, and the mechanical conditioning parameters can be optimized. In addition to a vascular model, we aim to eventually develop a fully functional tissue-engineered vascular graft. To achieve this goal, there needs to be further development in culturing methods and additional testing of the tissue to create a strong, contractile, non-thrombogenic tissue for vascular grafting.

5.3.1 Further Increasing the Elastic Modulus of Tissue: Alternative Modification Methods for Tissue Culture
Based on published work, 20 µm of genipin can increase the modulus of blood vessels\textsuperscript{5} and collagen–chitosan–elastin gels\textsuperscript{6} up to 4-fold. In our work, prior to the treatment with genipin, a crosslinking agent, the elastic modulus of the tissue rings was approximately 0.34 kPa, as measured using atomic force microscopy. This was increased to 0.55 kPa (62% over the control) after the addition of 20 µM genipin to the culture medium. However, when compared to the 2D studies on PAAM gels, this was still in the low range of stiffnesses tested. In two dimensional studies on PAAM gels, we found that the contractile protein, calponin, was expressed by the highest percentage of cells (71.4 ± 5.0%) when the gels had an elastic modulus of 76.8 kPa, compared to 9.4 ± 5.3% of cells on the gels with a modulus of 0.6 kPa. Based on these findings,
we hypothesize that further increasing the modulus of the tissue will increase the differentiation of the SMCs in the tissue.

There are several methods that could be employed to further increase the elastic modulus of the tissues. Similar to the genipin treatment, transglutaminase (TGM2) could be used to non-cytotoxicly crosslink the extracellular matrix (ECM). TGM2 is an enzyme that binds a free amine group to an acyl group on a free glutamine and has been used to passively crosslink collagen-based substrates\(^7\). The Vorp group found that a concentration of 5,000:1 (w/w) collagen to transglutaminase was sufficient for maximum crosslinking, increasing the burst pressure by 54\% compared to the uncrosslinked control, without an increase in cell death\(^7\). Based on published data, we expect TGM2 alone to increase the elastic modulus of the cell-derived tissue; however, we can also use both crosslinking agents to increase tissue stiffness if one does not cause a large enough increase to see a change in the SMC phenotype.

Additionally, if the non-cytotoxic crosslinking methods do not stiffen the tissue enough, the tissue could be decellularized using sodium dodecyl sulfate washes, cross-linked using glutaraldehyde, and recellularized with SMCs to determine the effect of substrate stiffening in 3D.

### 5.3.2 Functional Analysis of Contractility

While measuring SMC contractile protein expression is important, ultimately, one of the main outcomes of this phenotypic shift is tissue contractility. Since contractile proteins are necessary for the assembly of the contractile apparatus, we did not measure cellular contractility directly in this dissertation due to the low levels of contractile protein expression observed\(^5\).

There are many methods employed to measure contractility in vitro including gel compaction\(^8\), deformation of the culture surface\(^9\), migration of a 3D tissue up a conical post\(^10\), and wire myography\(^11\). In the field of TEBVs, wire myography is most commonly used because of the fine time resolution as well as the ability to rapidly exchange agonists in the sample bath. Currently, some TEBV models have demonstrated contractility, however these are all significantly lower than native arteries\(^12,13\).
In future work, tissue contractility could be evaluated using wire myography. Briefly, tissue rings will be harvested and slid onto the tungsten wires of a myograph chamber. After submersion in oxygenated physiological saline solution (PSS), ring contractile force will be measured in response to agonists (60mM KCL and 1µM phenylephrine) as described by our collaborators, Craige, et al.\textsuperscript{11}

5.3.3 Investigation of Mechanical Conditioning Parameters: Creating a Strong, Contractile Tissue

In this dissertation we investigated how cyclic 10% stretch can alter SMC phenotype and tissue structure. Other researchers have shown that how this stretch is imparted in the tissue can change the structural and functional responses of the cells including stretch magnitude, duty cycle, and incremental increase of stretch magnitude.\textsuperscript{1,14,15} We aim to investigate how duty cycle and incremental stretch modify tissue mechanical properties and SMC contractility.

5.3.3.1 Modifying Duty Cycle to Increase Tissue Strength and Modulus

While most cyclic stretch is done with a 100% duty cycle, with the cyclic stretch being imparted constantly, it has been shown that continuously subjecting SMCs to mechanical stimulation may not allow the cells adequate time to respond to the stimulus. This is thought to be an effect of damage caused by the breakdown of cross-links within the matrix.\textsuperscript{14} Shortening the duty cycle or decreasing the duration of stretching per day may result in stronger, stiffer, more collagenous tissues.\textsuperscript{8,14-16} Joshi, et al found that fibroblasts seeded on uniaxially stretched thermoplastic polyurethane were sensitive to strain rate, strain amplitude, and duty cycle, with a maximum elastic modulus at a strain rate of 25%/s, strain magnitude of 2.5%, a constant frequency of 0.5 Hz, and rest times 4 seconds or shorter between each stretch.\textsuperscript{16} Balestrini, et al found that fibroblasts seeded in fibrin gels showed increase collagen density with a nominal increase in ultimate tensile strength (UTS) when exposed to only 6 hours of biaxial stretch per day compared to continuous stretch.\textsuperscript{8} When translated into vascular tissue constructs, research in which SMCs suspended in collagen gels underwent cyclic inflation with a stretch time of 0.125s and 0.25s resulted in constructs with a higher UTS and elastic modulus than those with stretch times of 0.375s and 0.5s.\textsuperscript{14} When rest time was modulated, a rest time of 3.75 seconds showed
the highest UTS and elastic modulus when compared to shorter rest times, although any non-zero amount of rest time significantly increased UTS and elastic modulus of the tissue\textsuperscript{14}. The most common duty cycle used in bioreactors is 12.5\% with a frequency of 0.5 Hz resulting in a maximum strain rate of 80\%/s when cycling to 10\% strain\textsuperscript{15,17}. Our bioreactor system can be easily modified to incorporate changes in duty cycle by modifying cam geometry (Fig. 5.1) and a preliminary study can be found in Appendix C.

\textbf{Cam Shape Stretch Profile}

\begin{itemize}
  \item 100\%
  \item 50\%
  \item 0\%
\end{itemize}

\textbf{Figure 5.1. Pear-shaped Cams for Modifying Duty Cycle.} Cam shape alters the stretch profile. Circular cams provide a duty cycle of 100\% and pear shaped cams can be modified for many duty cycles. The one above is designed for a 50\% duty cycle.

5.3.3.2 Investigating the Effect of Incremental Stretch on Vascular Tissue

In addition to controlling the duty cycle, it has been proposed that, by taking cues from developmental biology and incrementally increasing mechanical stimulation over time, future experiments could result in more mature tissues\textsuperscript{18}. Syedain, et al, demonstrated in fibrin gel based tissue constructs made with porcine valve interstitial cells that incremental increases in strain magnitude over 3 weeks from 5\% to 15\% in 2.5\% increments significantly increased the UTS and collagen content when compared to constructs stretched at a constant strain magnitude\textsuperscript{15}. The tissues that were incrementally stretched also showed an increase in ERK signaling compared to the constant stretch magnitude groups, activating a pathway known to be
stimulated by stretch, which suggests that the constant strain constructs had adapted to the strain magnitude $^{15}$.

The effects of incremental stretch can be investigated using our modular bioreactor system. Cams of varying diameters can be made to easily increase stretch magnitude on the rings over time. We hypothesize this work will increase the modulus and UTS of our tissues over the constant stretch and static controls$^{18}$.

5.3.4 Development of a Vascular Tissue Model System
Cell derived rings can serve as a model to assess the effects of environmental factors on tissue structure and function. Ultimately, culture conditions that stimulate strong, contractile rings will be translated into fabricating vascular tubes in order to make in vitro models and in vivo grafts. We aim to use our cell-derived ring system to form tissue-engineered blood vessels (TEBV) with no exogenous material. In addition to the work described in this dissertation, in order to create and validate a functional TEBV, we need to optimize tissue ring fusion, create non-thrombogenic endothelial luminal monolayers, and increase overall tissue strength.

5.3.4.1 Tissue Ring Fusion
Preliminary studies have shown that our tissue ring constructs have the ability to completely fuse together, to form a tissue tube, both with human cells and with rat$^{19}$. SMC rings were cultured in the agarose mold for 7 days, then were harvested and transferred onto silicone tubing with anchor supports holding the rings in place (Fig. 5.2A, B) and allowed to fuse over 7 more days (Fig. 5.2C). Future work will investigate the mechanical properties of the fused rings, including separation strength and burst strength.
5.3.4.2 Endothelialization

Endothelial cells (ECs) play an important role in the regulation of SMC phenotype that was not investigated in this work. Lavender, et al. have shown that ECs do not attach to synthetic SMCs when seeded directly on the SMCs\textsuperscript{20}. Therefore, it was our aim to work without ECs to create vascular tissue with contractile SMCs with the goal of seeding ECs on the contractile SMCs, creating a co-culture system. The formation of this culture system will be used to create functional TEBVs, with a non-thrombogenic luminal surface. In vivo interior surfaces of healthy blood vessels are lined with a contiguous layer of ECs, which help prevent thrombosis, regulate nutrient absorption, and regulate SMC phenotype. ECs have been shown to attach to ECM proteins such as collagen type IV, laminin\textsuperscript{21} and fibronectin\textsuperscript{22} as well as directly onto contractile SMCs\textsuperscript{23}. The composition of the differentiation medium used when culturing the SMC tissue...
rings is specially formulated to differentiate SMCs while containing sufficient amounts of serum to facilitate endothelial cell survival\(^1\). Simply seeding the ECs on the luminal surface may not be sufficient to create a healthy monolayer of endothelial cells. *In vivo*, when a healthy endothelium is exposed to luminal blood flow, ECs express endothelial nitric oxide synthase (eNOS), upregulating nitric oxide (NO) synthesis, which contributes to the regulation of a contractile phenotype in SMCs\(^{24}\).

5.3.4.3 Luminal Flow Bioreactor

*In vivo* the luminal wall of the blood vessel is constantly exposed to hemodynamic shear stress due to blood flow. The shear stress is sensed by the endothelial cells lining the vessel unless the endothelium is desquamated due to balloon angioplasty or other damage. When the endothelial layer is intact, endothelial cells respond strongly to increased shear stress and in turn strongly regulate the SMC phenotype\(^{25}\). Specifically ECs regulate SMC phenotype by upregulating nitric oxide, transforming growth factor-\(\beta\)1, and platelet derived growth factor, and downregulating endothelin-1, resulting in SMCs differentiating from a synthetic phenotype to a contractile phenotype\(^{26,27}\). Most studies done on the effect of sheared endothelial cells on SMCs have been done with both cell types adhered, in a monolayer, to porous polyethylene terephthalate, polycarbonate, or dialysis membrane, and with the induction of only constant, non-fluctuating shear stress instead of pulsatile flow creating fluctuating shear stress as seen *in vivo*\(^{28}\).
In order to impart this shear stress, we have developed and begun to validate a pulsatile, luminal flow bioreactor for our tissue system (Fig. 5.3). This bioreactor was created as part of an MQP done in the lab and uses a pulsatile pump (not pictured) to pump cell culture media through the stopper, through a blunt ended needle apparatus, through the lumen of the tissue, through the back of the apparatus, and out of the stopper on the top, keeping the flow loops separate in case the endothelial layer and smooth muscle layer need different media compositions. For ideal EC culture, the luminal flow needs to be laminar. The Reynold’s number, a dimensionless quantity that predicts flow patterns, was calculated based on the flow rate, structure of the bioreactor, and the properties of culture media. A Reynold’s number lower than 2100 is considered laminar. For this system, the Reynold’s number was calculated to be 380, indicating laminar flow.

5.4 Final Conclusions

Overall, this work has shown that two culture parameters, elastic modulus and cyclic stretch can be used to modulate smooth muscle cell phenotype in cell culture and have also demonstrated that these changes in culture environment only affect SMC phenotype in conjunction with a low
serum, differentiation medium. When creating a system to model vasculature for the purposes of testing pharmacological agents, the discrepancies between 2D and 3D culture outcomes can mean the difference between the success and failure of a drug. In this dissertation we have shown that changes in mechanical environment can direct SMCs toward a more contractile phenotype, information that can be used to more accurately recapitulate vascular tissue for use in modeling or in the creation of tissue engineered blood vessels.

5.5 References


Appendices

Appendix A: Immunohistochemical Staining in Human Umbilical Artery

All contractile protein immunostaining presented in this dissertation was stained with control slides, both positive and negative, insuring our antibodies bound to healthy vascular tissue. Representative stains are shown in Fig. A.1. Positive controls consisted of staining human umbilical artery samples with SMαA or calponin antibodies as described in Chapter 3 (Fig. A.1A,C). Negative controls were stained by substituting the same concentration of a non-specific IgG antibody for the primary antibody and following the remainder of the protocol normally (Fig. A.1B,D).

![Image of positive and negative controls for contractile protein expression in human umbilical artery]

**Figure A.1. Contractile protein expression in human umbilical artery.** Positive (A,C) and negative (B,D) control slides were stained for all IHC. Scale = 100 μm. Lumen on right.
Appendix B: Opening Angle Studies

Changes in the residual circumferential stress in the tissue rings in response to the culture medium were quantified by measuring ring opening angle. Rings were placed in a physiological saline solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 1.2 mM MgSO$_4$, 2.5 mM CaCl$_2$, 10 mM glucose; pH 7.4) for 30 min (protocol modified from Williams, et al.\textsuperscript{1}). One cut was be made through the wall of the tissue and each sample was allowed to equilibrate for 30 minutes when an image was acquired using the digital image acquisition system described in Chapter 3. Opening angle was measured as the value of the interior angle formed by the lines created by connecting the ends of the cut ring segments and the midpoint of the tissue. The opening angle significantly increased in rings cultured in DM (Fig. B.1D) compared to GM (Fig. B.1C) indicating an increase in residual stresses.

Figure B.1. Opening angle of rings cultured in GM and DM. Rings cultured in GM (A, C) and DM (B, D) were cut and allowed to relax for 30 minutes. Opening angle was measured and rings grown in DM showed a significant increase in opening angle at 30 minutes (E, *indicates significance measured with a student’s t-test, p < 0.05). n = 3, Scale = 2mm

Appendix C: Duty Cycle Studies

In a preliminary study, investigating the role of duty cycle on tissue strength, pear shaped cams that imparted a 50% duty cycle (Fig. C.1A) were used on the stretch bioreactor and compared to 100% duty cycle cams as described in Chapter 4 (Fig. C.1B), and a 0% stretch control. Stretch waveforms can be seen in Fig. 5.1C. Tissue rings were cultured in GM for 7 days as described in Chapter 3 and then transferred into the bioreactor where they were stretched for 7 days in GM. Both the 50% and 100% duty cycle groups were stretched to a maximum strain of 10% at 1 Hz.

Figure C.1. Mechanical properties after cyclic stretch with a 50% duty cycle
Rings were stretched at a duty cycle of 100% or 50% or cultured statically (D) with 100% duty cycle (A) or 50% duty cycle cams (B). Mechanical testing indicated that there were no detectable differences between the experimental groups in ring thickness (C), ultimate tensile strength (E), or maximum tangent modulus (F). n=5.
and the 0% control was loaded onto the bioreactor but not stretched. At the end of the culture period, with 7 days in static culture and 7 days in the bioreactor, the rings were mechanically tested. Testing indicated no detectable differences between experimental groups for ring thickness, UTS, and MTM (Fig. C.1 C, E, F). We hypothesized that we saw no detectable difference between experimental groups because the rings were cultured in growth media and were not receiving sufficient stimuli to switch to a contractile phenotype, or because a 50% duty cycle did not provide enough pause time for sufficient tissue remodeling. In future studies, we will test the effects of even shorter duty cycles (12.5% and 25%) on tissue mechanical properties and SMC contractile phenotype for rings cultured in DM.

**Appendix D: Supplemental Western Blots**

All Western blots were run with a human umbilical cord control (Cord) and a protein molecular weight ladder. A sample Western blot can be found in Fig D.1. All protein bands appeared in the anticipated molecular weight range (SMαA = 42 kDa, β-tubulin = 51 kDa, β-actin = 42 kDa, and calponin = 34 kDa).

![Sample Western blot with protein ladder](image)

**Figure D.1. Sample Western blot with protein ladder.** Sample stained western blotting membranes for SMαA (42 kDa; A), calponin (34 kDa; B), and β-Actin (42 kDa; C) showing the entire membrane and protein ladder.
Additionally, several Western blots for additional samples were not presented in the body of this dissertation but were included in the densitometry analysis of the work. These Western blots appear in Fig. D.2.

Figure D.2. Supplemental Western blots Western blot stained for SMαA, calponin, and β-tubulin in SMC cultured on glass coverslips for Fig. 3.4 (A), in SMC tissue rings for Fig 3.8 (B), and tissue rings for Fig. 3.17 (C).

Appendix E: Sample Stress vs Strain Curves

Rings were tested mechanically and a displacement over time curve and a stress vs strain plot are shown in Fig. E.1.

Figure E.1. Sample stress vs. strain curves for mechanical testing of cell-derived tissue rings. Tissue rings were precycled then pulled to failure (A) to measure stress and strain (B) in the tissues.