Tissue-Engineered Arterial Tunica Media with Multi-Layered, Circumferentially Aligned Smooth Muscle Architecture

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Abstract

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Blood vessels play an important role in drug screening in terms of permeability and control of blood flow through cellular responses. Three distinctive functional layers make up the architecture of blood vessels, including tunica intima, tunica media and tunica externa. Among all layers, the tunica media layer regulates vascular tone and circumferential alignment of smooth muscle cells in tunica media is crucial to constrictive performances of vessels. Although much research has studied the anisotropic alignment of smooth muscle cells, there is yet a method to fabricate anisotropic smooth muscle cells in a three-dimensional hydrogel to mimic native tunica media. This project addresses the need for an *in vitro* tissue-engineered tunica media model that replicates *in vivo* architecture of circumferentially aligned smooth muscle cells in tunica media that is robust and reproducible. The project is divided into three phases: (1) A robust method to fabricate three-dimensional tunica media with circumferentially aligned smooth muscle cells and (2) the characterization and assessment on functional properties of tunica media model. Ultimately, the success of this project allows formation of tunica media with native

functionalities through cellular remodeling and mechanical properties to serve as a model of tunica media tissue in blood vessels.

Table of Contents

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Chapter 1. Background and Significance

1.1 Vascular diseases, hypertension

Cardiovascular diseases have remained the number one major cause of death in the United States causing over 7 million deaths in 2013 and affect over 71 million people [1]. Among all surgeries performed to treat cardiovascular diseases, over 450,000 in patient bypass surgeries are performed, which involves an invasive procedure to replace damaged arteries. Vascular health deteriorates with advance in aging and inactive living lifestyles that lead to a growing concern over increase in population with hypertension. According to the Centers for Disease Control and Prevention, hypertension in blood vessels is the primary cause of death of 360,000 Americans in 2013 [2]. It is estimated that 23 million will die from hypertension related cardiovascular diseases by 2030. To address the rising clinical need to treat hypertension, much efforts has been allocated to model for hypertension and design of drugs to treat hypertension non-invasively. This calls for a need for an accurate model that recapitulate biomimetic architecture of vasculature that allows for blood vessel functional output measurement.

1.2 Native architecture of blood vessels

Blood vessels function as conduits in the circulatory system to allow for blood flow throughout the body. As demonstrated in Figure 1, the arterial blood vessels are generally characterized by a trilayer structure with distinctive functions with the tunica intima layering the luminal side of blood vessels, tunica adventitia on the outer diameter, and tunica media in the middle. The tunica intima is made up of endothelial cells which acts as a selective physical barrier to allow for specific nutrients or wastes to enter blood

flow or organ systems [3] [4]. Tunica adventitia is the outermost layer of a blood vessel and is made up of fibroblasts and extracellular matrix. The adventitia provides structural support to hold blood vessel in place. The tunica media is a muscular layer with multiple circumferentially aligned vascular smooth muscle cell sheets that determines compliance, contractility and mechanical properties of the vessel.

The tunica media is a highly cellular tissue with made up of circumferentially aligned smooth muscle cells responsible of regulating vascular tone and contract or relax in response to biological signals that can constrict or dilate blood vessels [3].Thickness of tunica media varies in vessel type, size and location [4]. For instance, the aorta is highly muscular to provide vascular responses of recoiling upon the difference in blood pressure. A small artery would be made up of three to five layers of vascular smooth muscle cells that provide adequate support to the vessel and effective vasoconstriction and vasodilation in response to vasoactive reagents. One single layer of vascular smooth muscle cells is found in arteriole control vascular tone by regulating luminal diameter and blood vessel resistance. Multilayer of vascular smooth muscle cells is found in arteries while one single layer of smooth muscle cells is found in arterioles.

Vascular smooth muscle cells are found in the tunica media which regulates vascular tone and blood pressure. Innervated by the autonomic nervous system, vascular smooth muscle cells are capable of responding to signals through contraction and relaxation regulated by hormones and vasoactive agents [5] [6]. Characteristics of smooth muscle biomechanics have been well-documented in the literature to study the mechanism

behind smooth muscle cell contraction. In smooth muscle cell, numerous myofilaments as contractile units are oriented along the longitudinal axis of the cell and are made up of mostly myosin and actin [7]. Similar to other muscle cell types, the sliding of actin filaments over myosin filaments in opposite directions during cross-bridge cycling determines the magnitude of mechanical forces [5] [8]. From myosin and actin cross-bridge cycling, actin filaments are connected to other cytoskeletal structures including vinculin, a-actinin, paxillin and transmembrane integrins. Integrins are transmembrane receptors that facilitate cell-extracellular matrix adhesion. Upon cross-bridge cycling, contractile units shorten while pulling cytoskeletal structural proteins closer and thus shorten the cell. The generated cellular contractile force is transmitted to the extracellular matrix through integrin, resulting in vasoconstriction [9]. With smooth muscle cell contraction, luminal diameter is decreased resulting in higher resistance in blood flow. In native arteries, the orientation of medial smooth muscle cells are highly organized that smooth muscle cells align circumferentially with the long axis of the cells tracking the circumference of a vessel [10]. Alignment of cells allow for unidirectional shortening of cell sheet around the vessel so as to produce efficient forces to cause vasoconstriction [11]. To accurately model a native tunica medial layer in blood vessels, global smooth muscle alignment and contractile filament alignment should be scrutinized to allow for accurate recapitulation of contractile properties of blood vessels.

Another important morphological feature of smooth muscle cell includes the phenotypic plasticity described in the Figure 2 [12] [13]. Unlike other mature muscle cells that are terminally differentiated, smooth muscle cells at mature state are capable of switching

phenotype in response to vascular injury and other environmental cues [14]. Under normal physiologic condition, smooth muscle cells are in contractile phenotype that are non-proliferative and contractile to regulate vascular tone [15]. Contractile smooth muscle cells express smooth muscle-specific markers including smooth muscle alpha actin and local tropoelastin production in the cell [16]. Morphologically, synthetic and contractile smooth muscle cells are inherently different that synthetic smooth muscle cells are rhomboid-shaped while contractile smooth muscle cells are spindle-shaped [9] [17] [18]. Such dynamic cellular repositioning of smooth muscle cells allows for structural remodeling of tunica media which has a macroscopic effect over the diameter of small resistance vessels. Based on the structural-functional relationships in contractile units in smooth muscle cells [19], considerations in cell morphology should be included in the design of a tunica media model.

1.3 Tunica media importance and role in hypertension models

The tunica media layer in blood vessels plays an important role in hypertension as smooth muscle cells regulate vascular tone. Under pathological conditions, synthetic phenotype of smooth muscle cells change vascular tone significantly that affect blood vessel resistance and increase in blood pressure [20]. While complete understanding of underlying mechanism of hypertension is yet to be discovered, it is generally postulated that prolonged vasoconstriction increases vascular resistance and remodels arteries [21]. On the molecular and cellular level of vascular hypertension, remodeling and stiffening of vessel wall during hypertension often involve changes in cytoskeletal structure, organization of cell-cell connections and proliferation of vascular smooth muscle cells [22].

Current studies on arterial hypertension relies heavily on studies on mouse models to simulate conditions of interests [23]. While biological features are maintained in an *ex vivo* artery, there remains questions regarding the study specific structural functional relationship of vascular smooth muscle cells in the pathology of hypertension.

1.4 Current state-of-the-art

To model for arterial tunica media, multiple approaches have been described to study vascular smooth muscle cells in a biomimetic manner, including the design of tissue-engineered vascular graft and aligned smooth muscle thin films.

Tissue-engineered vascular grafts

Tissue-engineered vascular grafts have gained its popularity to fabricate three-dimensional blood vessels that allow for functional measurements [24]. Multiple methods were introduced using natural scaffolds from tubular organs, mandrels and biodegradable scaffolds [25] [26] [27]. The use of fibroblast cells was first described by Niklason *et al*., to fabricate a completely biological tissue-engineered blood vessel [28]. The resulting vessel showed promising proof-of-concept to use extracellular-matrix-secreting cell type to produce native collagenous matrix as scaffolds for blood vessel implant. However, this model is incapable of modelling contractile responses of blood vessels due to the lack of smooth muscle medial layer [29]. Combining the advancement in cell sheet engineering and electrospinning technology, Atala *et al*., described a highly reproducible method to isolate smooth muscle cell sheet wrapped around an electrospun mandrel scaffold to form a tunica media model [30]. Although the method describes a method to create multilayer

smooth muscle cells wrapped around a scaffold, the method does not recapitulate the unique circumferential alignment architecture of smooth muscle cells in blood vessels.

Anisotropic smooth muscle cell sheets

Further approaches have been explored to engineer smooth muscle cell sheets with ordered architecture. For instance, Parker, et al. published a method to produce biohybrid thin films to measure contractility in engineered muscle [31]. The method uses micropatterning technique to fabricate a scaffold with topographical cues to guide smooth muscle cell alignment. Cells seeded on micropatterned PDMS can be detached through a thermoresponsive polymer as a single monolayer. This biohybrid muscle film can then be isolated for further characterization in measuring cell sheet contraction. Although the method provides the alignment of smooth muscle cells similar to native tunica media, the lack of three-dimensional structure of this approach does not account for the need to evaluate vasoconstriction in a mechanistic level that would allow for functional measurements such as burst pressure and intraluminal pressure.

Although multiple approaches have been made to recreate the architecture of vessel, there exists a knowledge gap to the design of a tunica media model that recapitulates the structure-functional relationships of blood vessels. The project addresses the need of a biological tunica media model that provides opportunities for functional measurements. Through the design of a fabrication method to tissue engineer a tunica medial layer using vascular smooth muscle cells,

With the goal to recreate a circumferentially aligned architecture of tunica media, nanoscale cues were used to guide vascular smooth muscle cell alignment. A gel casting method was implemented to create anisotropic multilayer smooth muscle cell sheets wrapped around a three-dimensional biodegradable hydrogel. The three-dimensional design is a crucial aspect in the project that it allows for the design of a tube architecture with open-lumen for further functional and hemodynamic measurements of blood vessels, such as bursting pressure and intraluminal pressure for the characterization of vascular tone.

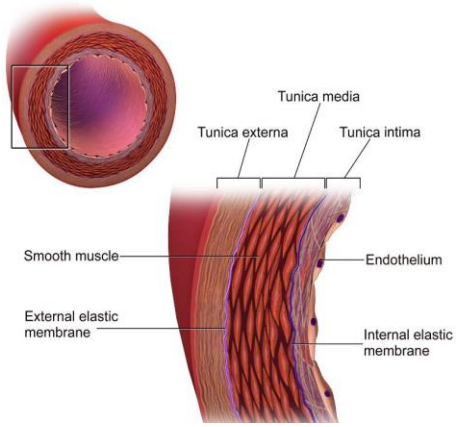

Figure 1. Illustration of architecture in native blood vessel

Image obtained from WikiJournal of Medicine, 2014

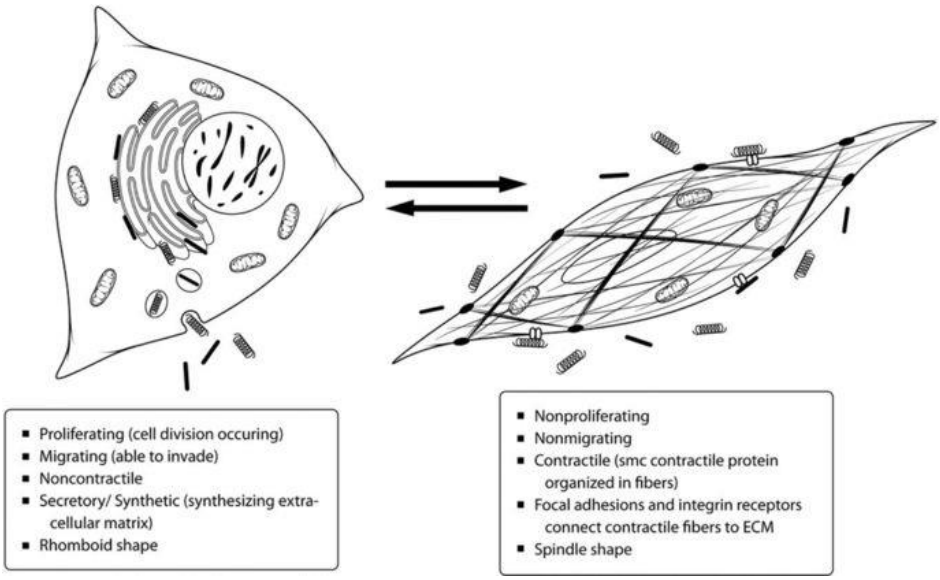

Figure 2. Phenotypic switching by vascular smooth muscle cells

Image obtained from Reid *et al*., Nature Genetics in Medicine, 2014

Chapter 2. Thesis outline

To design and fabricate a tissue-engineered three-dimensional tunica media model, a biomimetic approach was used to rebuild native architecture of the tunica media in blood vessels. Nanotopographical cues were presented on a flexible thermoresponsive nanofabricated substrate (fTNFS) to guide smooth muscle cell alignment and formation of a monolayer cell sheet to simulate the cell alignment observed in native tunica media. A gel casting method that was previously used with cardiomyocytes was implemented in this method to transfer cell sheet and create multilayer smooth muscle cell sheets that would then be wrapped around a biodegradable hydrogel to create a three-dimensional cell tube. Further characterization on smooth muscle cell morphology on cell tube, mechanistic properties were also performed to evaluate the tunica media model.

2.1 Hypothesis

It was hypothesized that through the design of a robust method to produce anisotropic smooth muscle cell sheets, multilayer stack of smooth muscle tissue wrapped around a three-dimensional hydrogel represents a biomimetic and functional model of tunica media layer in blood vessels. With nanotopographical cues, smooth muscle cells were guided to form an anisotropic cell sheet layer that resembles the *in vivo* alignment of cell orientation ideal for contractility. Aligned smooth muscle cell sheets were stacked through the fTNFS protocol to create trilayer stack of anisotropic smooth muscle cell sheets. A method was then introduced to allow for wrapping cell sheets around a biodegradable hydrogel to recreate an open lumen tubular architecture in blood vessels. The goal of the first phase of the thesis was the iterative design of method to fabricate a biological cell

tube with anisotropic smooth muscle cell sheets. Optimization of the cell sheet wrapping method was performed to create a robust method to produce tunica media models through three-dimensional design of mechanistic tools to perform the wrapping procedure. Validation of the model was performed in the second phase by characterizing smooth muscle cell marker expression, gene expression, as well as mechanical properties of the tunica media vessel.

2.2 Specific Aim 1: To design a method to create anisotropic smooth muscle cell sheets wrapped around a biodegradable hydrogel

The purpose of the first aim is to create a tunica media model with circumferentially aligned smooth muscle cells wrapped around a three-dimensional tubular hydrogel with open lumen. Cell sheet alignment, orientation and cell viability are evaluated by immunostaining and confocal microscopy of smooth muscle cells and image analysis performed on MATLAB and ImageJ. A hydrogel with physiological range properties is selected to provide structural support to the smooth muscle cell sheets. A cell sheet wrapping protocol is also described in this phase with optimization of mechanical tools designed to facilitate the wrapping, transfer and insertion of cell sheets involved in the protocol. The tools will be applied in the manipulation of cell sheets, stabilization of rolled cell sheet until inserted into a cylinder mold. Cell viability, cell alignment, mechanical properties of the cell tubes will be evaluated.

2.3 Specific Aim 2: To investigate and characterize properties of tunica media model

The purpose of this second aim was to carefully scrutinize the properties of the fabricated arterial tunica media model through characterization of cell tubes with cell orientation, cell remodeling on hydrogel, gene expression and functional properties of the cell. Cell alignment and orientation are evaluated through F-actin immunostaining of smooth muscle cells and quantified using a MATLAB script and ImageJ. Histology of the vessel is studied to study the circumferential alignment of smooth muscle cell nuclei around the hydrogel and extracellular matrix deposition from smooth muscle cells onto the hydrogel. Real-time PCR is performed to evaluate gene expression regulation by smooth muscle cells to observe upregulation of mature cell marker and contractile phenotype markers. Burst pressure of vessels after one week of culture is performed to evaluate mechanical and functional properties of the tunica media model. The output of Specific Aim 2 will contribute to the evaluation of the fabricated tunica media model with further possible avenues of usage of the model.

Chapter 3. **Specific Aim 1:** To design a method to create anisotropic smooth muscle cell sheets wrapped around a biodegradable hydrogel

3.1 Introduction

The goal of the Specific Aim 1 is to design a method to fabricate anisotropic smooth muscle cells sheets wrapped around a biodegradable hydrogel. Fabrication methods to align cells have been well documented in the literature but little has been shown to adopt circumferential alignment design in tissue-tissue engineered vascular grafts. The main deliverable of Specific Aim 1 was the proposal of a robust method to multilayer circumferentially aligned smooth muscle cell sheets wrapped around a 3D cylindrical biodegradable hydrogel.

The Kim Lab has previously studied nanoscale cues to rat myocardium cell orientation such that nanotopographical cues guide myocardial alignment during cardiovascular development. It is important for smooth muscle cells to be aligned in culture to maintain the contractility to recapitulate *in vivo* functions [32]. Two-dimensional mechanical and topographical features of cardiac matrix fibers were applied to a scaffold where cells are seeded onto. Results have supported that the nanopatterned scaffolds influence the cell morphology and genetic expression of cardiomyocytes such that the scaffold mimics native *in vivo* environment. The method of fabrication uses a silicon master of nanogroove features, 800nm 1:1 and through photolithographic methods, the pattern is transferred to

the scaffold made of PET film and polymer PUA. The fabrication method is of high accuracy in providing the nanotopographical cues on the scaffold. It is expected that the use of nanopatterned scaffold guides cell alignment and elongation, which is crucial for the project as the alignment of cells represents the architecture of native tunica media in vessel and provides unidirectional contractile force that will be measurable. In a study performed by Somali *et al*., it was shown that cell culture substrate stiffness had an effect in smooth muscle cell phenotypic differentiation that on softer and anisotropic substrate, an upregulation of gene expression related to contractile phenotype was observed [17]. Together, these studies suggested the need to guide smooth muscle cell alignment on a substrate of physiological range to promote contractile phenotype in smooth muscle cells.

Recent research from the Kim Lab has demonstrated engineered muscular tissue with well-defined anisotropy. Jiao *et al*., from the Kim Lab has developed a thermoresponsive nanofabricated substratum (TNFS) for the engineering of three-dimensional tissues by stacking nano-aligned cell sheets [33]. The use of the TNFS polymer, PNIPAM where cells are seeded onto, at temperature 37°C, it allows cells to adhere onto the surface a to develop a cell sheet layer. When the temperature drops, PNIPAM dissolves and the cell sheet layer detaches. Through the isolation of cell sheet layer, it is possible to transfer the layer to another cell sheet and culture for adhesion between the two layers. Currently, three to four layers of C2C12 cell sheets were stacked commonly to produce a small diameter vascular graft. This method enhances the feasibility of the project to develop multiple layers of cell sheets. Instead of C2C12, cell line of smooth muscle cell will be used in the project. Similarities between C2C12 and smooth muscle cell in terms of

proliferation, adhesion and alignment allows the employment of this method to create cell sheet stacks. The stacking method was employed to the project to achieve the goal of creating one to three layers of cell sheets, and the transfer of cell sheets on two-dimensional scaffold onto a three-dimensional gelatin tube for cell sheets reattachment.

It was hypothesized that smooth muscle cells on fTNFS would show uniaxial alignment and create a well aligned cells sheet layer along the nanopattern. The iterative design process in Specific Aim 1 would allow for effective detachment of viable smooth muscle cell sheet from fTNFS and transferred to another cell sheet to create multilayer constructs. It was hypothesized that after wrapping smooth muscle cell sheets around a hydrogel, smooth muscle cells remain circumferentially aligned on the exterior of the hydrogel gel, and that the MMP secreted from smooth muscle cells would not degrade the hydrogel that collapsed the open-lumen structure. A hydrogel of stiffness within physiological range was selected to provide structural support to the smooth muscle cell sheets and it was proposed that on a physiological range of tunica media at 5000Pa, smooth muscle cell sheets show better attachment onto hydrogel with higher cell viability.

3.2 Materials and Methods

3.2.1 Fabrication of flexible thermoresponsive nanofabricated substratum (fTNFS)

A flexible thermoresponsive nanofabricated substratum was fabricated using a method adapted from a protocol to fabricate thermoresponsive nanofabricated substratum published in 2014. Capillary force lithography was used to fabricate a UV-cured

poly(urethane acrylate) (PUA) mold to serve as a template for nanopatterned topography. Glycidyl methacrylate (GMA) (Sigma) was added to liquid PUA precursor (Norland Optical Adhesive 76) at 1% weight/volume and 10% weight/volume to allow for epoxy functionalization of the engineered substrate. The varying % weight/volume allows for different degree of functionalization to allow for efficient cell sheet detachment. The GMA-PUA prepolymer solution was then degassed under a vacuum for 1 hour to remove air bubbles. A roughly 50 µL droplet of GMA-PUA prepolymer solution was then applied to the center of a polyethylene terephthalate (PET) film (92 gauge, 23 µm thick, Skyrol Polyester Films). A PUA nanopatterned template of 800 nm wide and 500 nm deep parallel grooves and ridges was pressed into the prepolymer droplet deposited on the PET film and cured under 365 nm UV light for 5 minutes to initiate photopolymerization of the prepolymer solution. The PUA template was then peeled off from the PET film and the remaining GMA-PUA-PET nanopatterned construct was UV-cured overnight for complete polymerization.

3.2.2 PNIPAM Grafting to fTNFS

Amine-terminated poly(N-isopropylacrylamide) (Sigma) was dissolved in deionized water at room temperature at 33.3 mg/mL. PNIPAM solution was added to polymerized GMA-PUA-PET nanopatterned construct in a shaker at room temperature for 24 hours at 55 rpm to allow for thermoresponsive functionalization of the substrate. After full functionalization, the flexible thermoresponsive nanofabricate substrate (fTNFS) was then washed three times with deionized water and sterilized with 294 nm UV light for 1 hour prior to use in cell culture experiment.

3.2.3 Vascular smooth muscle cell culture

Vascular smooth muscle cells isolated from consenting human patients and mice were used in this project. Vials of cryopreserved cells were thawed and resuspended in a feeding medium with 4.5 g/L glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin streptomycin. Cells are fed with feeding medium every other day.

3.2.4 fTNFS cell culture system design

To allow for efficient seeding on fTNFS, a cell culture system with PDMS blocks on cell culture dish is designed. fTNFS was cut to smaller pieces of dimensions 1.25cm x 1.5cm. A silicone elastomer curing agent was mixed with an elastomer base (Sylgard 184) at a ratio of 1:10 and added to 35mm cell culture dish. A 3D printed block was designed and used to create the negative space in the polymer mixture. The mixture was allowed to degas in a vacuum for 2 hours then transferred to a 65 ^{o}C oven to cure overnight. The 3D printed block was then removed from the PDMS well. fTNFS and PDMS wells were sterilized a with 294 nm UV light for an hour prior to the attachment of fTNFS in the bottom of the PDMS well. Each PDMS well was designed to hold two pieces of fTNFS in place with 1 mL medium reservoir. fTNFS was attached to the bottom of the PDMS well with 5% gelatin crosslinked with transglutaminase. Fetal bovine serum was added to fTNFS in PDMS wells 2 hours prior to cell culture experiments.

3.2.5 Vascular smooth muscle cell seeding on fTNFS

Vascular smooth muscle cells were plated at 100,000 cells/cm^2 on fTNFS in 1 mL of medium. Single-layer cell sheets on fTNFS were maintained in culture in incubation at 37 ^oC with 5% CO_2 for 48 hours prior to transfer experiments. Cultures were monitored throughout this period, and any samples that failed to promote the formation of confluent monolayers were removed from subsequent experimentation.

3.2.6 Fabrication of trilayer smooth muscle cell sheets

fTNFS cultures were transferred to 35mm dish in room temperature phosphate buffered saline (DPBS; Life Technologies) which changes the conformation of PNIPAM layer and promoted smooth muscle cell sheet detachment for 20 minutes. DPBS was then aspirated and 2 mL of gelatin solution (7.5% weight-to-volume, Sigma) and incubated at 4 ^oC to encourage cell attachment onto solidified gelatin for 1 hour. The gelatin-cell constructs were cut out of the surrounding gelatin layer using a sterile scalpel, removed from the underlying fTNFS resulting in a construct with gelatin and smooth muscle cell sheet. To create multiple layers of cell sheets, the construct with gelatin and smooth muscle cell sheet is transferred to another cell sheet on fTNFS with two cell sheets stacked on top of each other. The constructs were incubated at 28 ^oC, 5% CO_2 to allow time for cells to adhere while simultaneously preventing the gelatin from melting. After 1 hour of incubation time, warm cell culture medium was added to the constructs to melt the gelatin in a 37 ^oC, 5% CO_2 incubator, leaving behind an oriented cell sheet adhered to the underlying substrate.

3.2.7 Immunocytochemistry

Cells were washed with PBS and fixed in 4% paraformaldehyde (Sigma) for 20 minutes at room temperature. Fixed cells were permeabilized and blocked with a solution of 0.2% Triton-X-100 solution and 5% bovine serum albumin (BSA; Sigma) in PBS for 1 hour at room temperature. Cell constructs were then incubated overnight at 4 ^{o}C in a primary antibody solution, consisting of desired primary antibodies diluted in PBS with 1% BSA. The primary antibodies used in this study were as follows: monocolonal anti-SMa-actin produced in mouse (Sigma), polyclonal anti-SMa-actin produced in rabbit (Abcam), polyclonal anti-tropoelastin produced in rabbit(Abcam), and polyclonal anti-collagen I produced in rabbit (Abcam). After primary antibody incubation, cells were washed 3 times in PBS and then incubated for 1 hour at 37 ^{o}C consisting of secondary antibodies diluted in PBS with 1% BSA. The secondary antibody solution consisted of anti-IgG antibodies bound to AlexaFluor green (488) and red (594) fluorescent conjugates. AlexaFluor 647-conjugated phalloidin (Invitrogen) was included in the secondary antibody step to facilitate visualization of F-actin fibers in cultures. All samples were then stained with a Hoechst stain (Sigma) to visualize nuclei for 15 minutes. After all incubation, cells were washed 3 times with PBS and mounted for imaging.

2D samples were mounted on microscope slides using a drop of mounting medium (Vectashield). 3D constructs were placed on a microscope slide pre-fabricated with a PDMS well to hold construct in place. A 2 mm thick PDMS well was placed on the microscope slide and sealed with optical clear nail polish. 3D constructs were placed in

the PDMS well and a drop of mounting medium was added to the well before a coverslip was placed over to press against the sample for high-resolution imaging.

3.2.8 Confocal microscopy

Images were taken at the Garvey Imaging Core at the University of Washington's Institute for Stem Cell and Regenerative Medicine using a Nikon A1 Confocal System on a Ti-E inverted microscope platform.

3.2.9 Fabrication of 3D smooth muscle cell tube

To generate a 3D smooth muscle cell tube, a method is developed in this project illustrated in Figure 13A. Cell sheet constructs attached to an fTNFS were physically wrapped end-to-end to create a tube with the cells oriented in a circumferential manner. The fTNFS tube was then inserted into a transparent polystyrene mold and a mandrill was inserted into the center of the mold to create a luminal space within the tubular structure. Soluble hydrogel was used to fill the remaining negative space which in this project 5% gelatin crosslinked with transglutaminase (MooGloo) or 20 mg/mL fibrinogen crosslinked with thrombin (Sigma) was used. Gelatin constructs were incubated at 4 ^{o}C while fibrin constructs were incubated at 37 ^{o}C to promote crosslinking and promotes hydrogel formation for 1 hour. The cell-hydrogel construct was then removed from the mold and mandrill and the remaining fTNFS was unwrapped from the construct, leaving the cell sheets adhered to the exterior of an open-lumen hydrogel cylinder. The tube structures were then placed on 3D printed structures to allow for stabilizing and suspending constructs during control in conventional 6-well plates. The constructs were

maintained under normal cell culture conditions for 7 days before being prepared for analysis.

3.2.10 Live/Dead Cytotoxicity assay

A Live/Dead Cytotoxicity Kit for mammalian cells was purchased from Thermo Fisher Scientific to assess smooth muscle cell viability. Calcein AM and Ethidium homodimer-1 from the kit were diluted to working dilution at 2 μM and 4 μM in feeding medium respectively. Cells were washed with PBS and incubated with the Live/Dead assay dye for 1 hour at 37 ^{o}C. After incubation, the dye solution was aspirated from cells and cells were washed with feeding medium and incubated with feeding medium and stored away from light before imaging.

3.2.11 Image processing and quantification through ImageJ

Cell segmentation was performed through identifying cell margin on ImageJ system and is quantified through output including Feret angle, aspect ratio and circularity of segmented cell.

3.2.12 Pixel gradient analysis on Matlab

A Matlab script developed by Kim Lab was used to study F-actin organization through pixel gradient analysis. The program uses a Gaussian low pass filter and edge-emphasized filter to extract horizontal and vertical edges of the fibers to calculate gradient magnitude of each pixel in the image. The principle orientation of cells was determined through the calculation of angle of the gradient of pixels orthogonal and perpendicular to

the X-axis of image. The output of the script includes a histogram of pixels aligned from -90 degrees to 90 degrees.

3.2.13 Force measurements of 3D vessels on myograph

Stretching behaviors and breaking forces of acellular cylindrical hydrogel were measured using a DMT Multi Wire Myograph System - 620M. Two pins of 400 um in diameter were used to hold hydrogel in in the well in myograph and apply forces. After calibration according to manufacturer's manual, the hydrogel tubes were wired onto the system with 2 pins holding against the interior lumen of the tube. A micropositioner was manually turned to increase the distance between two pins, and thus adding forces to the lumen and stretching the vessel wider. An increment of 50 um increase in lumen diameter was applied to the tube and force exerted on the pin by hydrogel was measured with the in-built force transducer in the myograph unit and data is acquired through PowerLab data acquisition system.

3.3 Results

3.3.1 Fabrication of flexible thermoresponsive nanofabricated substratum

Through capillary force lithography, a polymer made of poly(urethane acrylate) (PUA) with glycidial methacrylate (GMA) was fabricated and nanopatterned on a flexible PET film. A thermoresponsive polymer, PNIPAM, was grafted onto the surface of nanopattern to allow for subsequent cell sheet detachment. Details of fabrication process was shown in Figure 3a. The fabricated substrate is flexible as shown in Figure 3b that it allows for manual manipulation over the shape of substrate.

3.3.2 Smooth muscle cell morphology on fTNFS

Mouse vascular smooth muscle cells were cultured on fTNFS and observation and results from cell morphology and cell alignment was illustrated in Figure 4. The culture of mouse smooth muscle cells on fTNFS shows global alignment of cells compared to unpatterned thermoresponsive substrates. To quantify cell alignment and morphology, bright field images were transferred to ImageJ for cell segmentation and evaluation. Cell aspect ratio and degree of orientation were analyzed and the results showed that cell sheet alignment on fTNFS peaked at 0 degree with the nanopattern orientation, while cell sheet on fTNFS showed random alignment with no uniaxial direction of alignment (Figure 4B). Through measuring the cell aspect ratio of smooth muscle cells in Figure 4 ^{o}C, a higher aspect ratio in smooth muscle cell is observed from individual cell segmentation, indicating that the nanopattern on fTNFS not only guides cell patterning, but also cellular elongation along the direction of nanopattern.

Alignment of cytoskeletal structures contributing to contractile functions is a crucial factor to promoting contractile phenotype of smooth muscle cells and thus the functional potential of the cells. Through immunostaining, visualization of actin stress fibers in smooth muscle cells was achieved shown in Figure 5. From a qualitative perspective, it was observed that cells show higher form of unidirectional alignment in cells cultured on fTNFS compared to unpatterned thermoresponsive substrate. By quantifying F-actin stress fiber orientation in a MATLAB script developed in our lab previously, it was observed that a uniaxial directional alignment of stress fibers could also be achieved on

fTNFS culture (Figure 5B). Effects on guidance of nuclei orientation was also observed that nanopattern aligns the direction of nuclei orientation in cells on fTNFS (Figure 5C). In addition, smooth muscle alpha-actin and tropoelastin act as smooth muscle cell markers and both tropoelastin and SMa-actin are expressed in cultured vascular smooth muscle cells.

Global cell alignment is also observed on human smooth muscle cell cultured on fTNFS as illustrated in Figure 6. In addition to oriented F-actin stress fibers on fTNFS compared to unpatterned thermoresponsive substrate, smooth muscle cell nuclei are also well aligned in human smooth muscle cells illustrated through immunostaining of F-actin and DAPI in Figure 6B, C.

3.3.3 Fabrication of multilayer smooth muscle cell sheet stack

A gel casting method described previously by Jiao *et al*., was used to create multilayer smooth muscle cell sheets (Figure 7). With the goal to adapt the protocol with smooth muscle cells, cell sheet detachment with at the presence of thermoresponsive polymer, PNIPAM, was studied.

For human smooth muscle cell sheet detachment, we observed that smooth muscle cells tend to detach from fTNFS as single cell and balled up on the surface during detachment (Figure 8A). At 15 minutes after detachment at room temperature PBS, the monolayer of cell sheet is disintegrated and cells do not attach to one another as a monolayer. Although cells showed high viability when they were on scaffold, the lack of formation of cell sheet did not allow for successful cell sheet transfer and resulted in cell death post-transfer

(Figure 8B). Approaches to improve cell sheet detachment by prolonging smooth muscle cell culture on scaffold, increasing %GMA for more PNIPAM functionalization and grafting on fTNFS did not show improvements towards cell sheet detachment.

It was then hypothesized that, since contractile smooth muscle cells do not secrete abundant extracellular matrix, another cell source that can supply extracellular matrix to allow for better cell sheet formation could potentially improve cell sheet formation and detachment from fTNFS. A type of human bone marrow stromal cell, HS27A, was used in coculture with human smooth muscle cells at a ratio of 1:10 to form cell sheet. As seen in Figure 9. The approach did not facilitate in cell sheet detachment that at 15 minutes detachment in room temperature PBS, cells detach from one another and from fTNFS and was transferred as individual cell. The use of stromal cell did not help maintain cell-cell adhesion during detachment process.

Contrary to the expectation in human cells, mouse vascular smooth cells showed promising cell sheet detachment as a single monolayer with high cell viability. After cells are transferred onto gelatin, cell sheet organization was maintained as seen in Figure 10A, suggesting that cells are capable of detach as a single layer. Curling of edges of cell sheets were also observed during the process indicative of strong cell-cell adhesion to allow for peel off cell sheet layer from fTNFS. Through a cytotoxicity assay, cell sheets before and after transfer process both showed high cell viability. When a cell sheet layer stained with red membrane dye is stacked over another layer stained with green membrane dye, we could observe the stacking results under a confocal microscope

(Figure 10B). The image in Figure 10C suggests the stacking of two distinctive layer of different cell sheets and maintenance of separate cell sheet layer after transfer. The gel casting method was adapted to the use of mouse smooth muscle cells to fabricate multilayer smooth muscle cell sheets.

3.3.4 Selection of hydrogel with physiological stiffness and elastic properties

Hydrogel with features of tunable stiffness and thermoresponsive properties were evaluated as potential scaffold for cell sheets to be wrapped around. Among all, fibrin and gelatin hydrogel are ideal candidates due to its benign properties and availability. Mechanical stiffness of fibrin and gelatin gel at different concentration was evaluated and results were shown in Figure 11. To select an ideal candidate for the fabrication method, the hydrogel should fit within reasonable range with physiological stiffness of smooth muscle cells at 5 kPa, while allowing for adequate handleability to manually wrap and unwrap cell sheets during the process. With information from literature, we fabricated fibrin gel at a range of stiffness between 3 kPa and 13 kPa with and without collagen addition to the hydrogel. 5% Gelatin crosslinked with transglutaminase has a mechanical stiffness at 130 kPa. We observed that vessels made of 10 mg/mL fibrin or less tends to collapse and occlude during culture, and hydrogel softer than 20 mg/mL fibrin was difficult to manipulate manually.

Structural remodeling properties were inherently different between fibrin and gelatin. Through passive stretching studies of acellular hydrogel in a myograph system, vessel wall pressure was measured when vessel was being stretched manually by two pins. The

shape of graph in Figure 12 showed different remodeling responses from fibrin and gelatin gel that with increase in stretching force, fibrin gel could stretch and adapt to the increased lumen and restore wall pressure. There is less flexibility observed in gelatin tube that at shape deformation, wall pressure is also increased. Similar to observations in the literature, we fabricated fibrin hydrogel with high viscoelastic properties. Nonlinear elasticity was observed in the testing with shear or elongational strain was applied to fibrin gel, the more the material was deformed from its original shape.

Although gelatin has great benign properties and are widely used in tissue engineering, the stiffness at 5% gelatin crosslinked with transglutaminase averages at around 130 kPa, which is 20 times above the physiological range of smooth muscle cells. Fibrin at 20 mg/mL has a Young's modulus at 6.6 kPa and is the softest hydrogel that allows for manual manipulation without breaking and was used for subsequent experiments as the major hydrogel in the design of a tunica media model.

3.3.5 Fabrication of smooth muscle cell tubes as a tunica media model

Mouse vascular smooth muscle cell tubes are fabricated using the method described in Figure 13A. A culture system was designed to fabricate and culture cell tubes to allow for monitoring of cell tubes (Figure 13B, C). Under bright field microscope, differences in smooth muscle cell morphology was observed that edges of cell sheets tend to clump together and does not wrap uniformly across the exterior of the hydrogel compared to fibrin. To further characterize this observation, a cytotoxicity assay was performed to visualize and measure smooth muscle cell viability on hydrogel (Figure 14). Average

smooth muscle cell viability on fibrin was 79.4% while on gelatin was 61.4%. The difference in cell viability observed in fibrin and gelatin hydrogel could potentially be due to the difference in mechanical stiffness and crosslinking temperature of hydrogel that the crosslinking temperature of fibrinogen and thrombin is at physiological temperature at 37 ^{o}C while gelatin and transglutaminase is at 4 ^{o}C. With cell viability of 79.4% observed in fibrin gel construct, we were able to fabricate a viable tunica media model with circumferentially aligned smooth muscle sheets.

3.4 Discussion

Combining the results from Specific Aim 1, we successfully fabricated a thermoresponsive nanofabricated substrate with controlled global cell orientation and cytoskeletal contractile protein alignment with. We were able to adapt a gel casting protocol with smooth muscle cells to create multilayer of aligned smooth muscle cell sheets with high cell viability. The design of a three-dimensional open-lumen cylinder with fibrin as a mold to hold smooth muscle cell sheets at the exterior was also established in this method.

This study describes and implies a method that allows control of overall architecture of cell sheets around a biodegradable hydrogel. We were able to produce monolayer of smooth muscle cell sheets that were transferred to a 3D hydrogel with high maintenance over the architecture of cells a week. This observation suggests that no outgrowth of smooth muscle cells was observed in the vessels 1-week post culture. The highly ordered

circumferentially aligned orientation of cell sheets maintained in the tunica media captures native biomimetic feature of cellular orientation of smooth muscle cells and serves potential in long term culture of smooth muscle cells on 3D hydrogel.

Merits of the methods proposed include the simplicity and robustness of the fabrication process. 3D printing has become more available across different research setting and that the design of the 3D molds could be easily achieved through the match of desired tunica model dimensions and the changes in the printed molds. The method allows for efficient fabrication of tunica media models and the flexibility of producing multilayered constructs depending on users' interests in the design. This project demonstrated the fabrication of a tunica media layer of a small arterial blood vessel with lumen size smaller than 6 mm. To fabricate smaller tunica media model, a mandrill of smaller sizes can be obtained, for instance a choice of different gauge needles that are commercially available could act as the mandrill to create the open-lumen structure of the design. The thickness of the vessel could also be adapted to the model design. In order to decrease thickness of hydrogel in the vessel, a larger mandrill to create bigger lumen, or a smaller cylindrical mold to hold hydrogel could be printed to design tunica media model with variable dimensions to suit design interests.

Success of this model was demonstrated through the usage of mouse vascular smooth muscle cells. However, in order to capture the native human blood vessel responses, the use of human smooth muscle cells is crucial to recapitulate accurate responses from the model. To suit this process, preliminary data on human smooth muscle cell sheet transfer

onto fibrin hydrogel have been performed. We have obtained successful transfer of single layer smooth muscle cell sheet onto fibrin hydrogel although further characterization and iterative design has to be performed to create multilayer human smooth muscle cell sheet constructs. With the behavior of human smooth muscle cell sheet detachment as single cell from fTNFS, potential studies could be performed to evaluate cell-cell adhesion structures of smooth muscle cells and extracellular matrix deposition onto scaffold from the cells. Other approaches to modulate smooth muscle cell-cell adhesion could be incorporated into the design, such as extracellular matrix coating and patterning on fTNFS pre-seeding of smooth muscle cells due to the low extracellular matrix secretion behavior of contractile smooth muscle cells.

The proposed method to fabricate a tunica media model preserves cell sheet alignment on 3D hydrogel which suggests potential of a higher complexity model with controlled architecture. Circumferential alignment was a major emphasis on this project as smooth muscle cells are circumferentially aligned in native arteries. From the literature, it is also known that the endothelium in tunica intima layer of blood vessels are longitudinally aligned. The use of gel casting method in this project allows flexible control over the alignment of cell sheets being stacked onto one another to create tissue with higher complexity. This suggests future direction of the tunica media model being adapted to a tissue-engineered blood vessel model to incorporate the functional endothelium to the design.

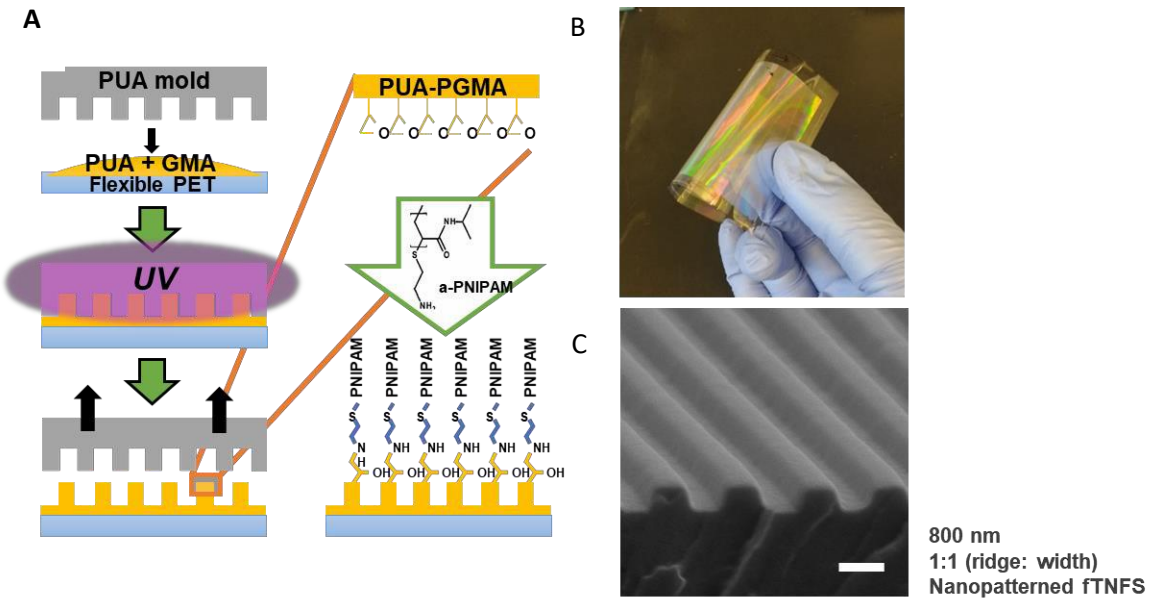

Figure 3. Fabrication of flexible thermoresponsive nanofabricated substratum (fTNFS)

(A) Schematic of capillary force lithography to create nanofabricated substratum and subsequent functionalization of the substratum with amine-terminated PNIPAM (a- PNIPAM)
(B) Photo of large-area, scalable TNFS with high flexibility
(C) SEM image of nanofabricated substratum showing the grooves and ridges on the surface of fTNFS

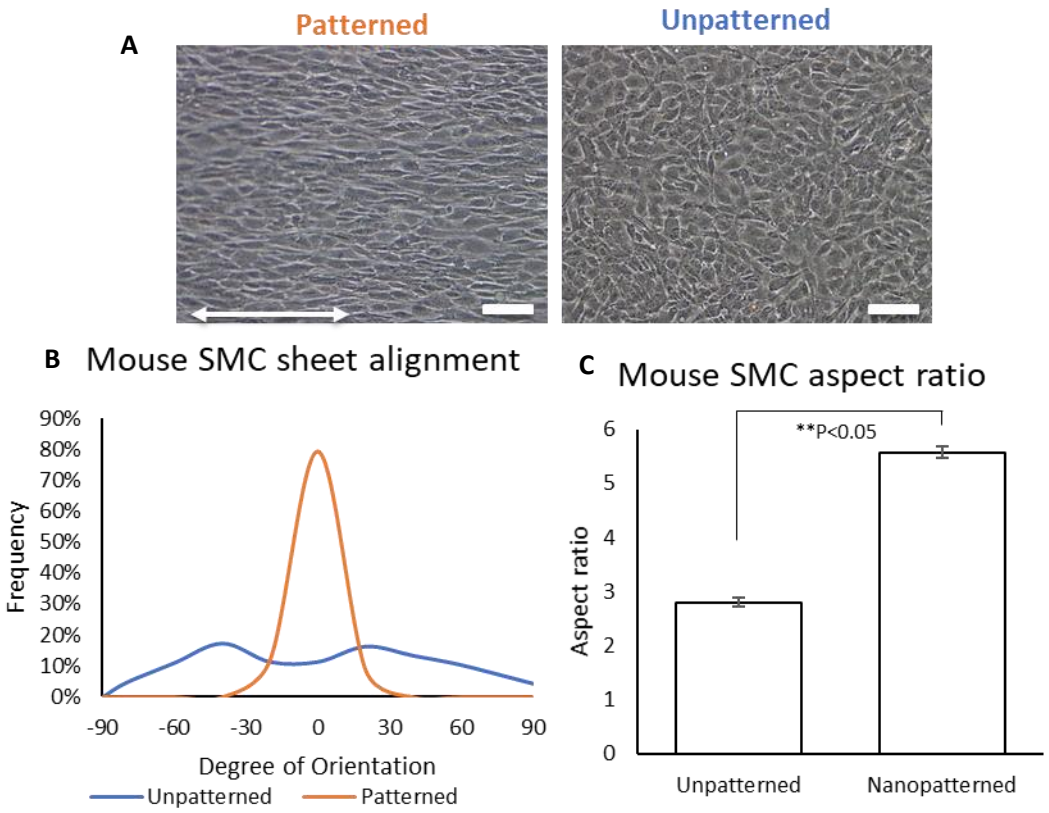

Figure 4. Mouse smooth muscle cell morphology on fTNFS

(A) Bright field image of mouse vascular smooth muscle cells cultured on patterned fTNFS (left) and unpatterned thermoresponsive substrate (right)
(B) Quantitative analysis of cell sheet through single cell segmentation on Image J and subsequent analysis on overall cell sheet alignment
(C) Quantitative analysis of cell sheet through single cell segmentation on ImageJ and subsequent analysis on cell aspect ratio. Scale bar, 100 μm

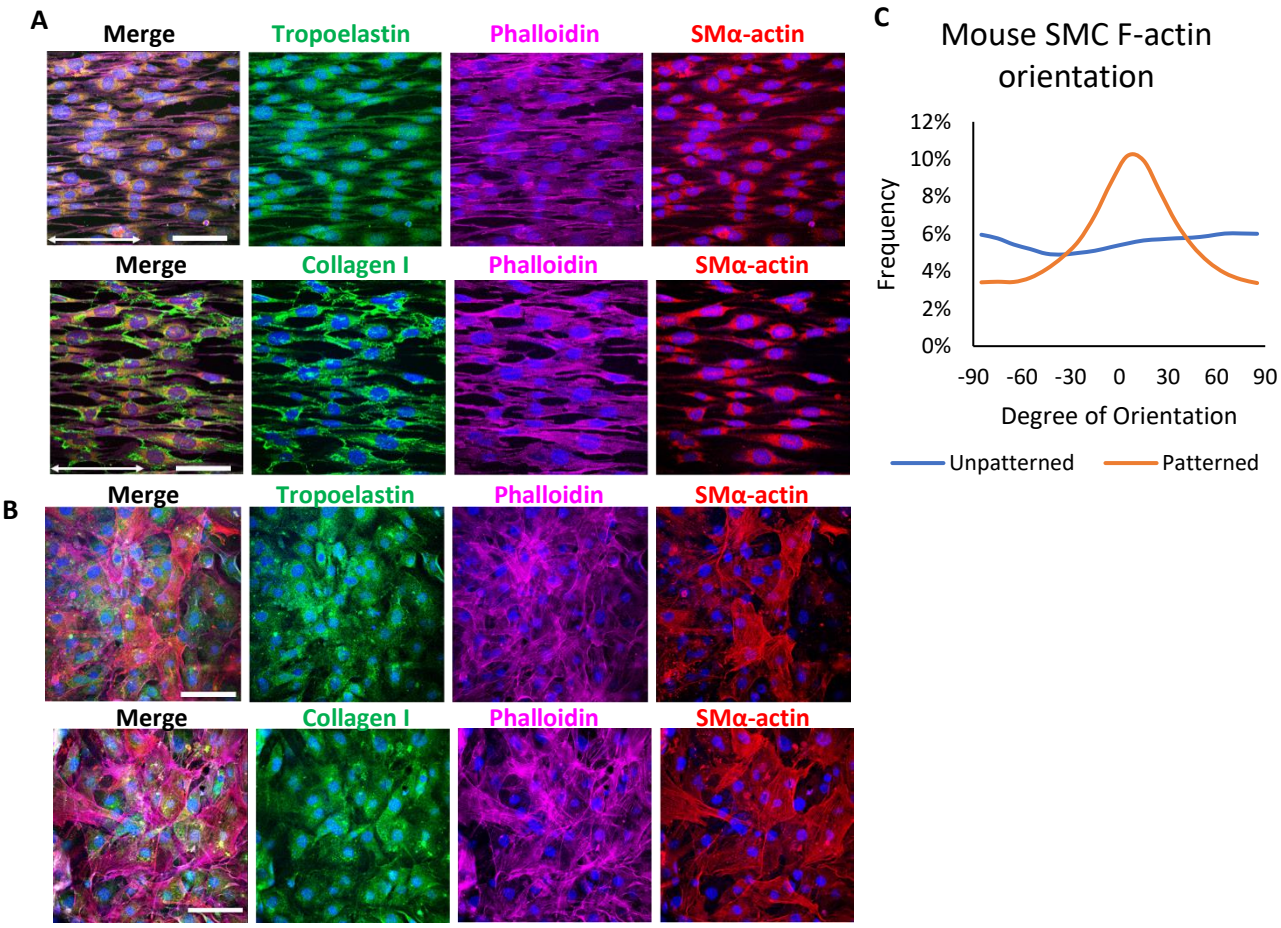

Figure 5. Immunostaining of mouse smooth muscle cells cultured on fTNFS

(A) Confocal images of patterned mouse vascular smooth muscle cell sheet stained with tropoelastin (top) and collagen I (bottom)

(B) Confocal images of unpatterned mouse vascular smooth muscle cell sheet stained with tropoelastin (top) and collagen I (bottom)

(C) Quantitative analysis of smooth muscle cell sheet alignment. Scale bar, 100 μm

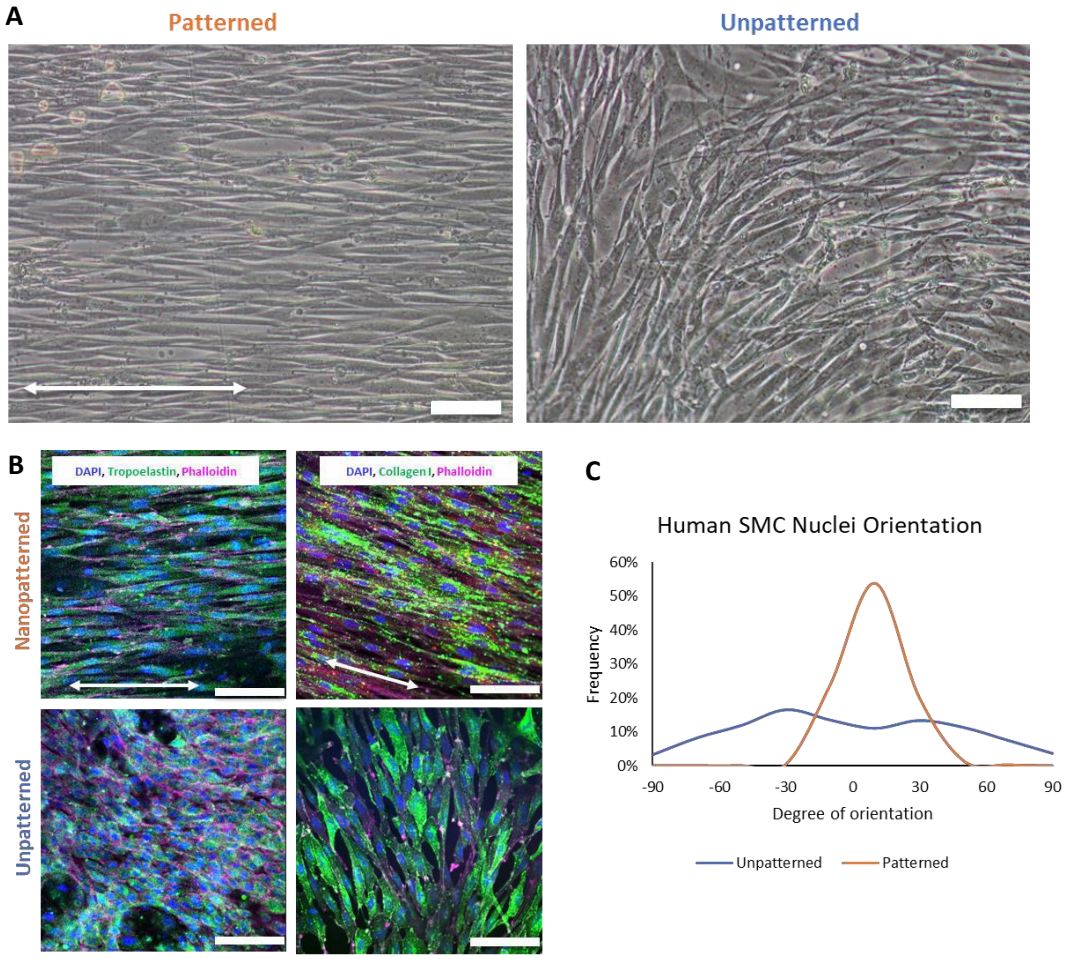

Figure 6. Human smooth muscle cells cultured on fTNFS

(A) Bright field images of human vascular smooth muscle cells cultured on patterned fTNFS (left) and unpatterned thermoresponsive substrate (right).
(B) Confocal images of human vascular smooth muscle cells cultured on fTNFS and unpatterned thermoresponsive substrate stained with tropoelastin and collagen I.
(C) Quantification of cell alignment through nuclei orientation distribution. Scale bar, 100 μm

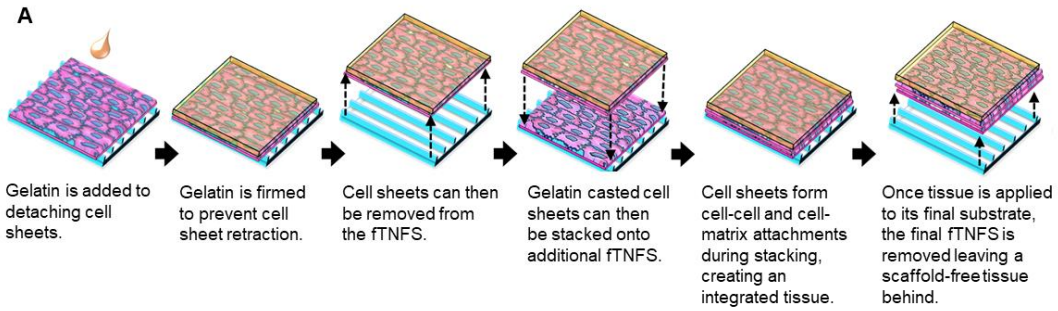

Figure 7. Gel casting method to produce multilayer smooth muscle cell sheets

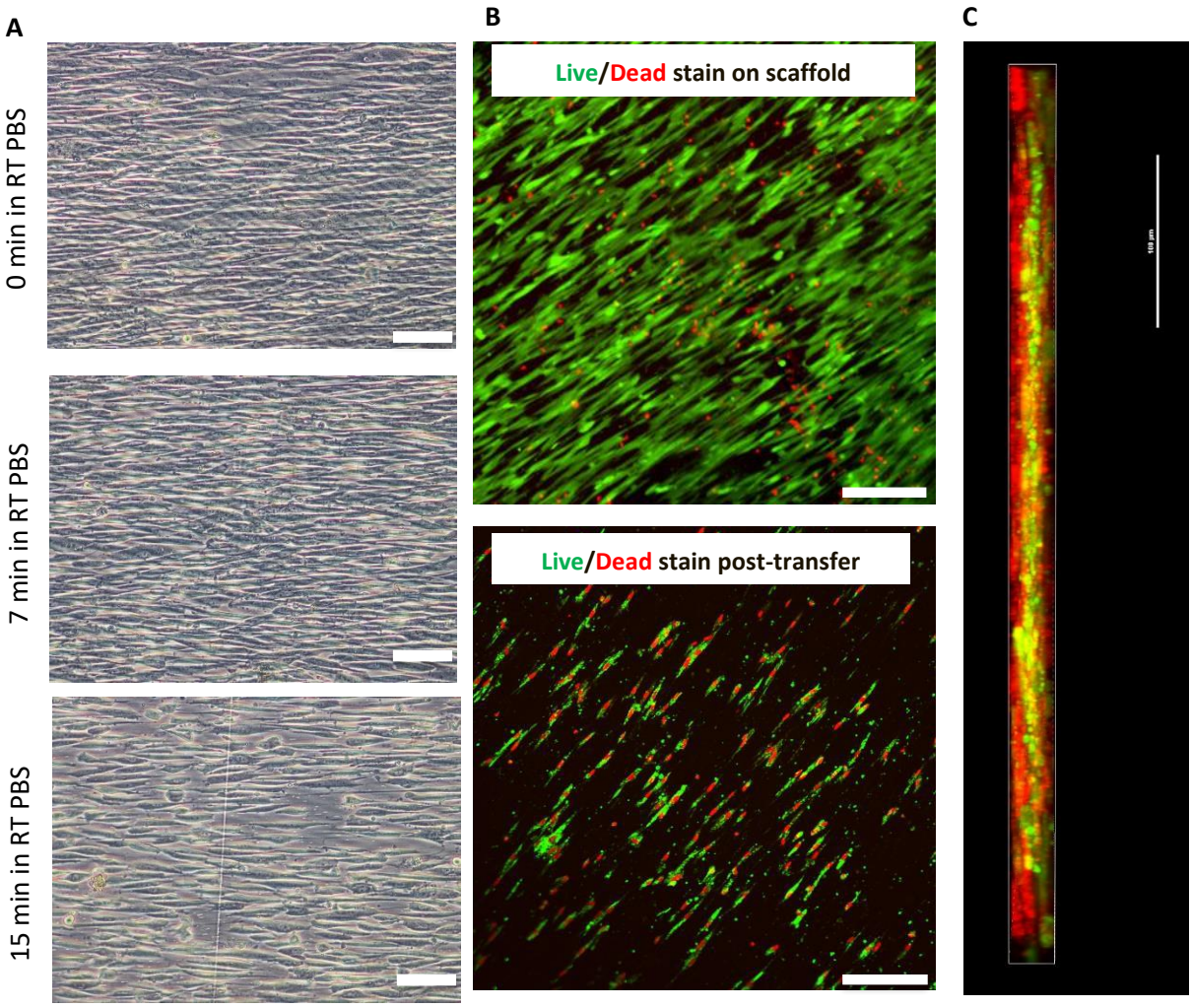

Figure 8. Human smooth muscle cell sheet detachment studies

(A) Bright field images of human smooth muscle cell sheet detachment for 15 minutes in room temperature PBS

(B) Images from live/dead cytotoxicity assay before and post transfer of cell sheets

(C) Confocal image of trilayer stacking of cell sheets using red and green membrane dye. Scale bar, 100 μm

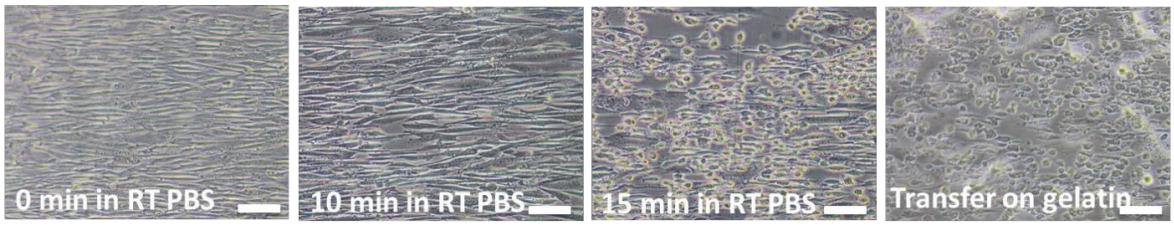

Figure 9. Detachment of cell sheet made of stromal cell HS27A and human smooth muscle cells

Scale bar, 100 μm

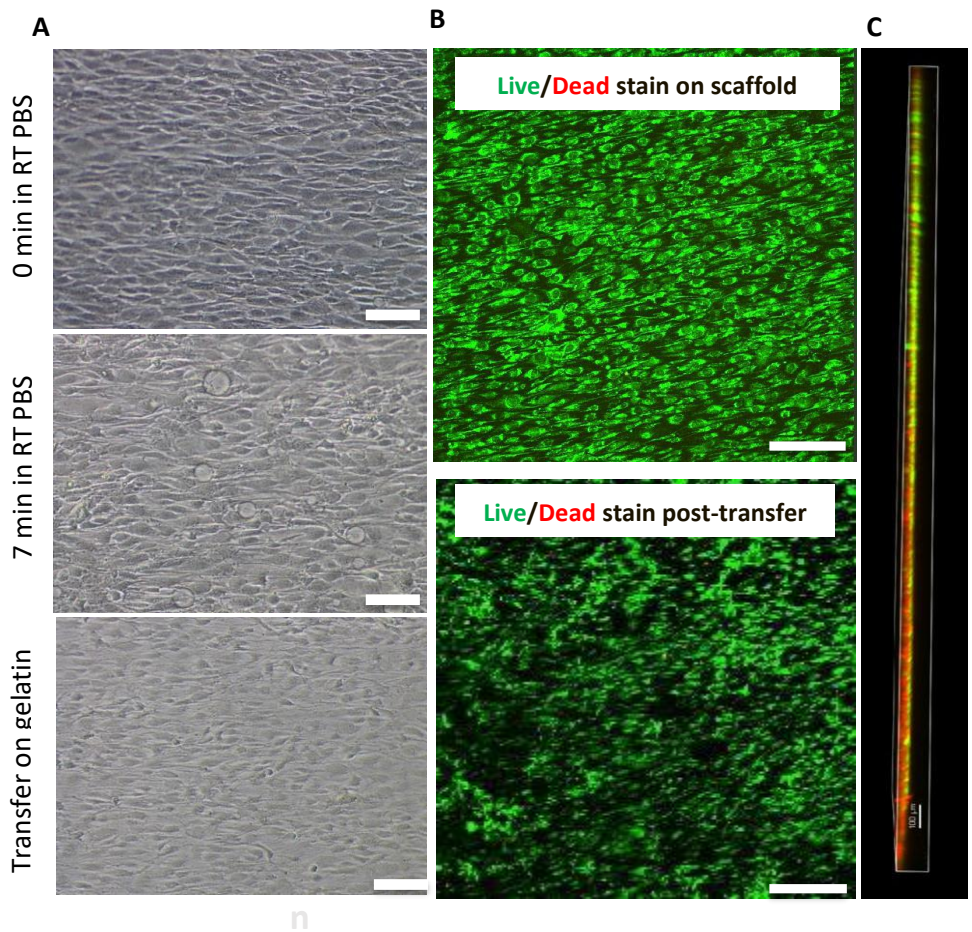

Figure 10. Mouse smooth muscle cell sheet detachment studies

(A) Bright field images of mouse smooth muscle cell sheet detachment for 5 minutes in 1X PBS at room temperature.
(B) Images from live/dead cytotoxicity assay before (top) and post (bottom) transfer of cell sheets.
(C) Confocal image of bilayer stacking of cell sheets using red and green membrane dye. Scale bar, 100 μm

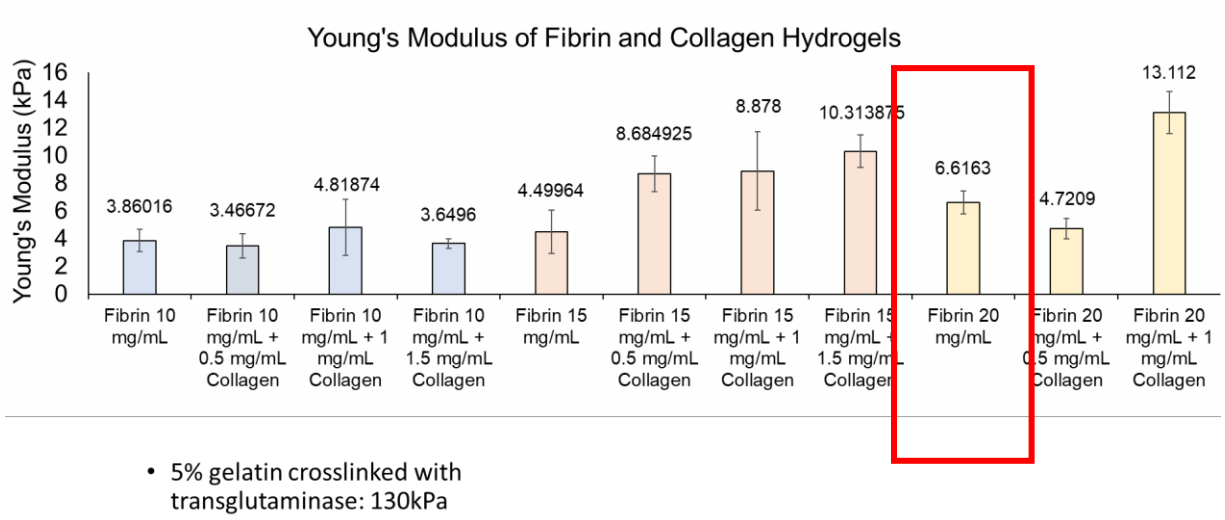

Figure 11. Mechanical stiffness of fibrin hydrogel at different concentration

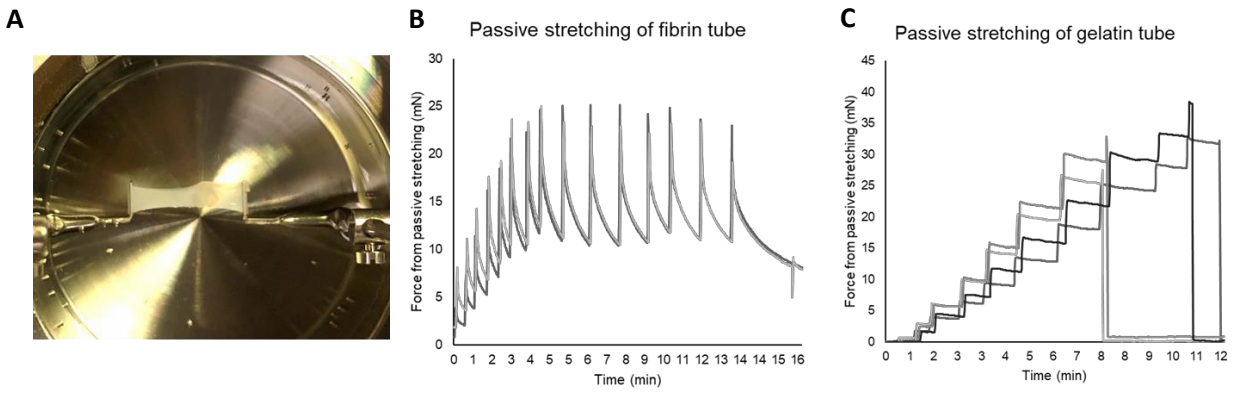

Figure 12. Viscoelastic properties of fibrin and gelatin hydrogel

(A) An image showing set up in myograph system with two pins holding and stretching the lumen of the acellular vessel
(B) Force measurement on fibrin vessels upon manual passive stretching
(C) Force measurement on gelatin vessels upon manual passive stretching

Chapter 4. **Specific Aim 2:** To characterize smooth muscle cell sheets cultured on three-dimensional hydrogel, vessel dimensions and mechanical properties

4.1 Introduction

The goal of Specific Aim 2 is to characterize and perform functional assessment of tunica media model fabricated in Specific Aim 1. Micropatterning methods were well established in the field of tissue engineering to create biomimetic tissue architecture. However, in the field of vascular tissue engineering, there has been a lack of design methods to create circumferentially aligned architecture. Through characterizing and assessing functional properties of our proposed tunica media model, we hoped to learn about cell maintenance in their circumferential alignment architecture, remodeling of hydrogel they are embedded in, and compare bursting pressure as a method to assess functions of the fabricated tunica media model.

Fibrin is a widely used polymer for tissue engineering purposes [34] [35]. Of many interesting properties of fibrin, we are interested in the mechanical properties of fibrin with stiffness being tunable and the crosslinking process happens at 37 ^{o}C to solidify the hydrogel [36]. Tawil *et al.*, reported Young's modulus of acellular three-dimensional fibrin hydrogel that ranges from 0.43 kPa to 11.47 kPa depending on the concentration of fibrinogen and collagen in the hydrogel [37]. Fibrin is widely documented in the literature as a biodegradable hydrogel for the culture of tissue-engineered vascular grafts.

Interesting properties of fibrin include the upregulation of contractile phenotype of vascular smooth muscle cells at the presence of diminished fibrin degradation products [29] [38] [39]. Tranquillo *et a*l., described an observation that at different level of concentration of fibrin degradation product, vascular smooth muscle cells express different levels of deposition of collagen and cross-linked elastin [36]. Through the fabrication of a smooth muscle cell tube with circumferentially aligned architecture, we are interested in studying smooth muscle cell morphology on fibrin hydrogel and respective cytoskeletal structure orientations.

Burst pressure strength is one of the fundamental characteristics of tissue-engineered vascular grafts to determine strength of vessels. Strength of tissue-engineered vascular graft depends on the ability of cells to deposit their own extracellular matrix which confers tissue with tensile strength [40]. Niklason *et al*., had successfully tissue engineered decellularized blood vessels from dermal fibroblasts and had fabricated vessels with above physiological range burst pressure [29] [41]. Without the incorporation of functional tunica medial layer, the vessel cannot perform specific functions such as control over vascular tone. However, these studies have shown the degree and importance of cellular remodeling of hydrogel that cells deposit their own extracellular matrix which could greatly enhance the biomimetic features of the tissue engineered model.

The deliverables of Specific Aim 2 would provide insights towards the cellular remodeling features of circumferentially aligned cell sheets on a fibrin hydrogel in a three-dimensional open-lumen cylinder. Vessel dimensions and smooth muscle cell morphology would

provide basic understanding towards the tunica media model. It was hypothesized that the cell sheets wrapped around fibrin hydrogel would remodel the fibrin hydrogel by depositing their own extracellular matrix, and the actual addition of trilayer cell sheets would intrinsically change the burst pressure of an acellular fibrin hydrogel.

4.2 Materials and Methods

4.2.1 Smooth muscle cell tube culture

Smooth muscle cell tube was cultured in feeding medium with 4.5 g/L glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin streptomycin in a system of 3D printed parts shown in Figure 13C.

4.2.2 Live/dead cytotoxicity assay

A Live/Dead Cytotoxicity Kit for mammalian cells was purchased from Thermo Fisher Scientific to assess smooth muscle cell viability. Calcein AM and Ethidium homodimer-1 from the kit were diluted to working dilution at 2 μM and 4 μM in feeding medium respectively. Cells were washed with PBS and incubated with the Live/Dead assay dye for 1 hour at 37 ^{o}C. After incubation, the dye solution was aspirated from cells and cells were washed with feeding medium and incubated with feeding medium and stored away from light before imaging.

4.2.3 Immunocytochemistry

Cells were washed with PBS and fixed in 4% paraformaldehyde (Sigma) for 20 minutes at room temperature. Fixed cells were permeabilized and blocked with a solution of 0.2%

Triton-X-100 solution and 5% bovine serum albumin (BSA; Sigma) in PBS for 1 hour at room temperature. Cell constructs were then incubated overnight at 4 ^{o}C in a primary antibody solution, consisting of desired primary antibodies diluted in PBS with 1% BSA. The primary antibodies used in this study were as follows: monocolonal anti-SMa-actin produced in mouse (Sigma), polyclonal anti-SMa-actin produced in rabbit (Abcam), polyclonal anti-tropoelastin produced in rabbit(Abcam), and polyclonal anti-collagen I produced in rabbit (Abcam). After primary antibody incubation, cells were washed 3 times in PBS and then incubated for 1 hour at 37 ^{o}C consisting of secondary antibodies diluted in PBS with 1% BSA. The secondary antibody solution consisted of anti-IgG antibodies bound to Alexa fluor green (488) and red (594) fluorescent conjugates. AlexaFluor 647-conjugated phalloidin (Invitrogen) was included in the secondary antibody step to facilitate visualization of F-actin fibers in cultures. All samples were then stained with a Hoechst stain (Sigma) to visualize nuclei for 15 minutes. After all incubation, cells were washed 3 times with PBS and mounted for imaging.

3D constructs were placed on a microscope slide pre-fabricated with a PDMS well to hold construct in place. A 2 mm thick PDMS well was placed on the microscope slide and sealed with optical clear nail polish. 3D constructs were placed in the PDMS well and a drop of mounting medium was added to the well before a coverslip was placed over to press against the sample for high-resolution imaging.

4.2.4 Confocal microscopy

Images were taken at the Garvey Imaging Core at the University of Washington's Institute for Stem Cell and Regenerative Medicine using a Nikon A1 Confocal System on a Ti-E inverted microscope platform.

4.2.5 Histology studies

Histology studies were performed at the University of Washington Histology and Imaging Core at South Lake Union. Cell tube samples were fixed in 4% paraformaldehyde for 24 hours before embedded in paraffin. Sectioning of paraffin block to show lumen cross section was performed to generate multiple microscopic slides. Sections were stained for Hematoxylin and Eosin, as well as trichrome for visualization of cells and deposition of extracellular matrix in hydrogel.

4.2.6 Burst pressure measurement

A setup to measure burst pressure consists of a custom measurement system obtained from Honeywell, ABPDJJT001PGAA5. The pressure sensor in the system measures fluid pressure in mmH_2O. Through suturing one end of the vessel to allow for fluid inflow from a Harvard Apparatus Pump 11 Elite and another end of the vessel closed, the accumulation of fluid in the tube adds pressure on the vessel wall until its breaking point. The pressure data was collected in a python script by Ardruino Uno.

4.3 Results

4.3.1 Smooth muscle cell morphology on fibrin tube

Human smooth muscle cells wrapped around a fibrin tube were formed and respective cell morphology was studied through immunostaining of tropoelastin, F-actin and nuclei. Human smooth muscle cells were stained positive for tropoelastin, where most of the tropoelastin were found in the cytosol of the cell (Figure 15A). Comparing between patterned and unpatterned cell tube, we saw that cells maintained their global alignment a week after culture and free from patterned guidance from fTNFS. Cell sheets wrapped around the fibrin hydrogel maintained their anisotropic features and such claim was quantified through measurements of nuclei and F-actin orientation. By isolating different channels to observe nuclei and F-actin respectively, it was found that the effect of patterning on fTNFS was maintained in the cell sheet layers post transfer and post wrapping (Figure 15B, 15C). The quantitative outlining of nucleus segmentation showed that smooth muscle cell nuclei were oriented along the nanopattern when cultured on patterned fTNFS when compared to a more random and non-unified angle of orientation for unpatterned cell tubes. A similar result was observed in F-actin orientation such that cells on patterned fTNFS showed uniaxial alignment along the nanopattern at 0 degree of orientation while unpatterned cell sheets were unaligned and distribute more evenly across a spectrum of degree of orientation.

Trilayer mouse smooth muscle sheets were wrapped around a fibrin hydrogel as well. When cells were stained for tropoelastin, F-actin and nuclei, mouse smooth muscle cells showed tropoelastin production in the cells (Figure 16). Internalized tropoelastin was found inside of individual smooth muscle cells, and there was some tropoelastin found at

spaces with extracellular matrix. Similar to the results found in human smooth muscle cell tubes, mouse smooth muscle cell tubes also showed a uniaxial alignment of nuclei and F-actin from patterned smooth muscle cell sheets when compared to unpatterned ones. Mouse smooth muscle cell sheets were able to maintain their global cellular alignment and subcellular structures alignment even a week after the nanotopographical cues were taken away from the cues.

4.3.2 Vessel dimension physical characterization

Dimensional information of vessels was obtained through histological sectioning and imaging on circumferential section of a vessel. Thickness of hydrogel, exterior diameter and internal diameter of vessels were measured on ImageJ by measuring the distance from center of the tube (Figure 17). The outer and inner diameter of a vessel averages at 800 um and 450 μm respectively. As the outer and inner diameters are controlled by the diameter of a commercially available stripette and straw for cell culture as the mold system in our design, it is within our expectation that the variability of diameter is very small. On the other hand, the thickness of gel averages at 400 μm.

Hematoxylin and eosin stain was used to visualize cell sheet layers wrapped around fibrin tube and allows for measurements of dimensional information regarding the vessel (Figure 18). Although much of the cell sheet layers had been scraped off unintentionally during the process of segmentation, we were still able to look at parts where cells were still wrapping around the fibrin gel. Nuclei were stained purple in a hematoxylin and eosin

stain and shows that the smooth muscle cells on the outer layer of cell tube showed in general, a flat orientation with a certain degree of nuclei elongation circumferentially.

To study the extracellular matrix deposition from smooth muscle cells on fibrin gel, a trichrome stain was performed to visualize collagen fibers from our samples (Figure 19). Similar to literature expectations, smooth muscle cells cultured on less fast degrading hydrogel would deposit less extracellular matrix while upholding a contractile phenotype. From the results of trichrome staining, little to no collagen fibers was observed from the histological slides. Cell dense portions were observed from histological slides and are believed to be scraped off portions from the patient's wound. Crosslinked mature collagen fibers were not found in the samples even at high cell density portions. Although smooth muscle cells were able to form a confluent monolayer of cell sheet, the collagen content in the tissue was not high. Such observation matches with the expectation of the nature of smooth muscle cells that contractile smooth muscle cells are not major extracellular matrix-secreting cells in the blood vessels and that we were able to maintain a more contractile phenotype in smooth muscle cells when we culture them in a fibrin tube with circumferential alignment.

4.3.3 Burst pressure measurement of smooth muscle cell tubes

Burst pressure of blood vessels was measured by flowing fluid to the lumen of the vessel while pressure measurements were taken based on the pressure in the tube with the two ends of the vessels were sutured. A summary of burst pressure of acellular fibrin gels, patterned trilayer smooth muscle cell tube and unpatterned trilayer smooth muscle cell

tube is shown in Figure 20. Average burst pressure of acellular fibrin tube is at 264 mmH_2O, while that of patterned trilayer mouse smooth muscle cell tube is at 447 mmH_2O. With difference between the two values being statistically significant, cellular remodeling of hydrogel that increases burst pressure in vascular graft could be one of the potential benefits of using cellular hydrogel over acellular hydrogel. The observed statistical difference between the patterned and unpatterned trilayer smooth muscle cell tubes were found to be insignificant. The average burst pressure of patterned cell tubes is higher than that in unpatterned cell tubes, suggesting potential benefits in the use of patterned cell sheets to upholding vessel structural integrity over unpatterned cell sheets.

4.4 Discussion

The deliverables from Specific Aim 2 include characterization of the tunica media model with studies on smooth muscle cell morphology on 3D hydrogel, vessel dimensions, smooth muscle cell remodeling on 3D hydrogel, and functional burst pressure measurements. Patterned smooth muscle cell tubes in culture maintained anisotropic alignment 1-week post detachment from fTNFS and the circumferential aligned architecture was preserved in the tunica media model. The method produced an open lumen cylinder model with lumen diameter smaller than 6mm which could be characterized as a small diameter arterial blood vessel. In addition, cellular and patterned smooth muscle cell tubes showed material integrity through higher burst pressure compared with acellular and unpatterned smooth muscle cell tubes. The characterization of tunica media model suggests the importance of preserving circumferential alignment

of smooth muscle cells around a 3D hydrogel for more biomimetic features and better functional properties similar to native tunica media.

Native blood vessels vary in size and dimensions depending on the location. Larger diameter vessels were usually found in peripheral arteries above the knee, while small diameter vessels that are generally smaller than 6mm in diameter were found as coronary arteries and peripheral arteries below the knee. The design of the tunica media model has a luminal diameter of 4.5 mm and can be comparable to smaller diameter vessels. Small diameter vessels are prone to effects of hypertension by arterial stiffening, stenosis of lumen due to the size and thus a tunica media model made of smooth muscle cells would provide insights over the control of vascular tone of small diameter blood vessels. As mentioned previously, the platform that was designed in this project could be adapted to vessel models of varying sizes and thickness that the study implies a versatile platform to fabricate blood vessels of different sizes for modeling purposes.

Mechanical strength of hydrogel and the model was demonstrated through burst pressure measurements of the designed vessel. We recorded a peak of burst pressure from patterned smooth muscle cell tubes at 447 mmH_2O. Niklason *et al*., studied the burst pressure of native blood vessels and decellularized tissue engineered grafts made from dermal fibroblasts which are major extracellular matrix secreting cell type in blood vessels for mechanical strength that they recorded burst pressures of native blood vessels and cellular tissue-engineered blood vessels at 2000 mmHg and 5000 mmHg. Although the absolute value of burst pressure between our tunica media model is not comparable

enough to the native blood vessel however, we were still able to demonstrate a comparable change in the burst pressure between acellular and cellular model, suggesting smooth muscle cell remodeling processes. As mature contractile smooth muscle cells are not major extracellular matrix secreting cell type in the vessel, it is suggested that a fully functional and implantable vascular graft will require alternative and extra layering of cell sheets to reproduce the tunica intima and tunica externa layers in the blood vessels.

With an open lumen cylindrical structure, the model presents a similar architecture as native blood vessels with opportunities to measure hemodynamics responses through the open lumen. Parker *et al*., had demonstrate the use of biohybrid muscle thin films to fabricate smooth muscle cell sheets on a patterned PDMS scaffold that allows for flexibility to measure contractile properties of smooth muscle cells. Upon the addition of vasoconstrictor, endothelin-1, and vasodilator, HA-1077, smooth muscle cell sheet exerts forces on the PDMS film that causes curling of edge upon contraction and flattening of cell sheet when relaxed. Although this method evaluates smooth muscle cell contraction, it is not capable of modelling subsequent changes in hemodynamics due to vasoconstrictor or vasodilator. To address this need, future direction of the project could make use of the tunica media model platform to study changes in vascular tone in response to vasoconstrictors and vasodilators. Using the proposed tunica media model, it is then possible to evaluate changes in blood vessel resistance, blood flow and pressure on vessel wall as the lumen allows for flow of fluid and measurement of intraluminal pressure.

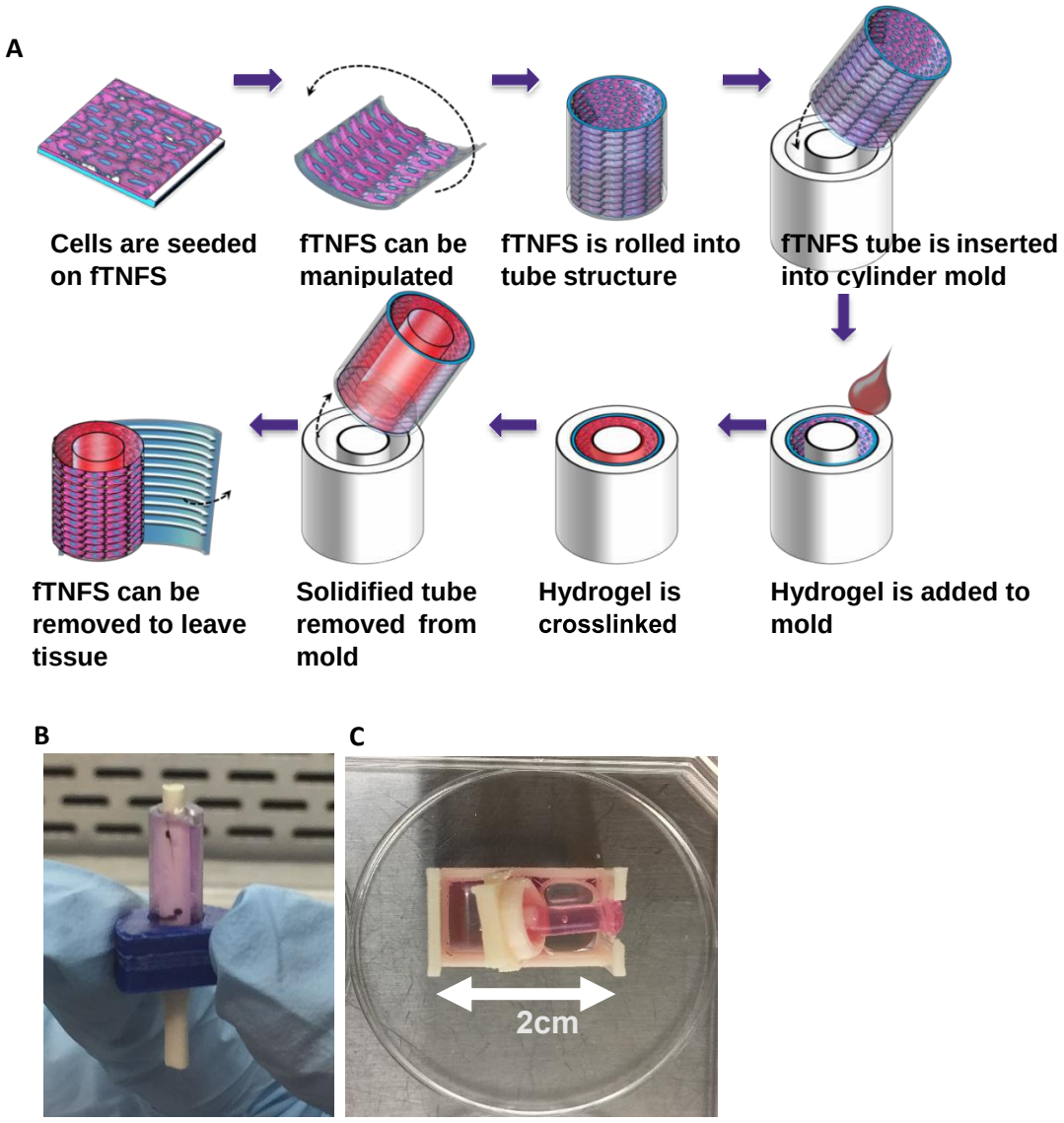

Figure 13. Fabrication of three-dimensional tunica media model

(A) Schematic of gel casting method to generate open-lumen cylindrical structures with smooth muscle cells

(B) Design of a mold to hold cell-hydrogel structure during curing process

(C) Design of a cell culture system to hold smooth muscle cell tube in place for culture

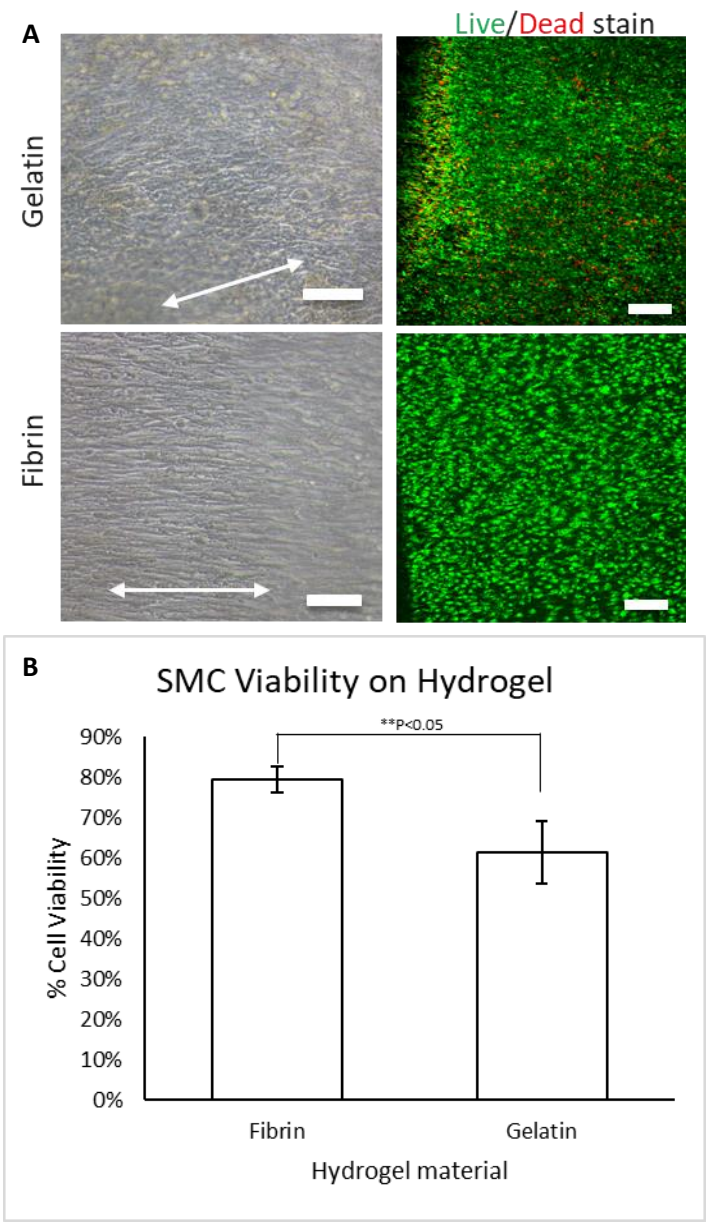

Figure 14. Mouse smooth muscle cell morphology on 3D hydrogel

(A) Bright field images of smooth muscle cells cultured on gelatin and fibrin hydrogel and respective live/dead cytotoxicity assay staining images (10X)

(B) Quantification of smooth muscle cell viability on hydrogel. Scale bar, 100 μm

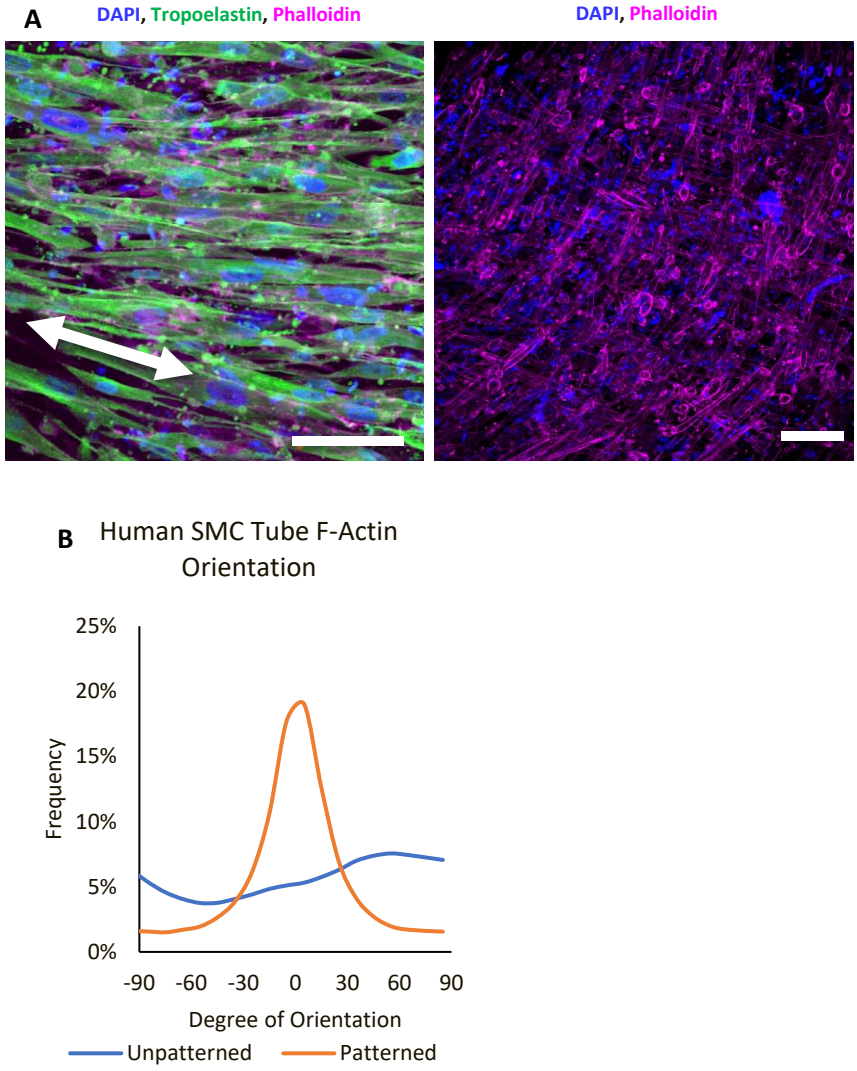

Figure 15. Multilayer human smooth muscle cells on 3D fibrin hydrogel

(A) Confocal image of multilayer human smooth muscle cells cultured on patterned fTNFS (left) and unpatterned thermoresponsive substrate stained with tropoelastin (green), phalloidin (pink) and nuclei (blue).

(B) Quantification of cell alignment through F-actin orientation. Scale bar, 100 μm

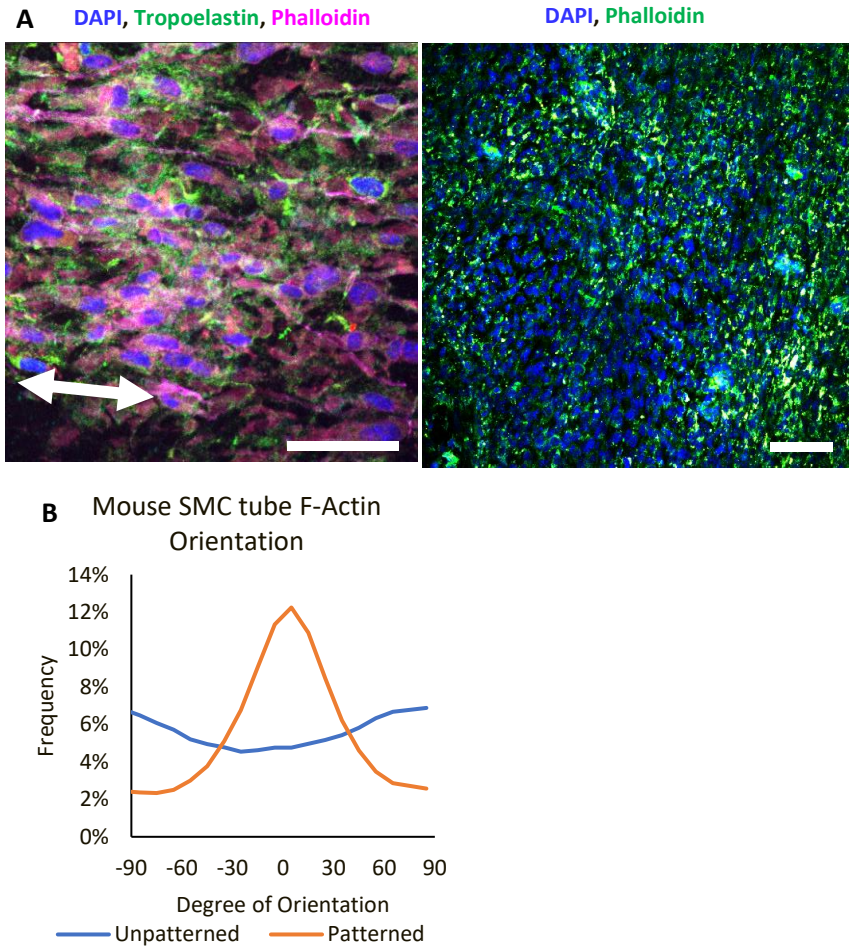

Figure 16. Multilayer mouse smooth muscle cells cultured on 3D fibrin hydrogel

(A) Confocal image of multilayer mouse smooth muscle cells cultured on patterned fTNFS (left) and unpatterned thermoresponsive substrate

(B) Quantification of cell alignment through nuclei orientation

(C) Quantification of cell alignment through F-actin orientation. Scale bar, 100 μm

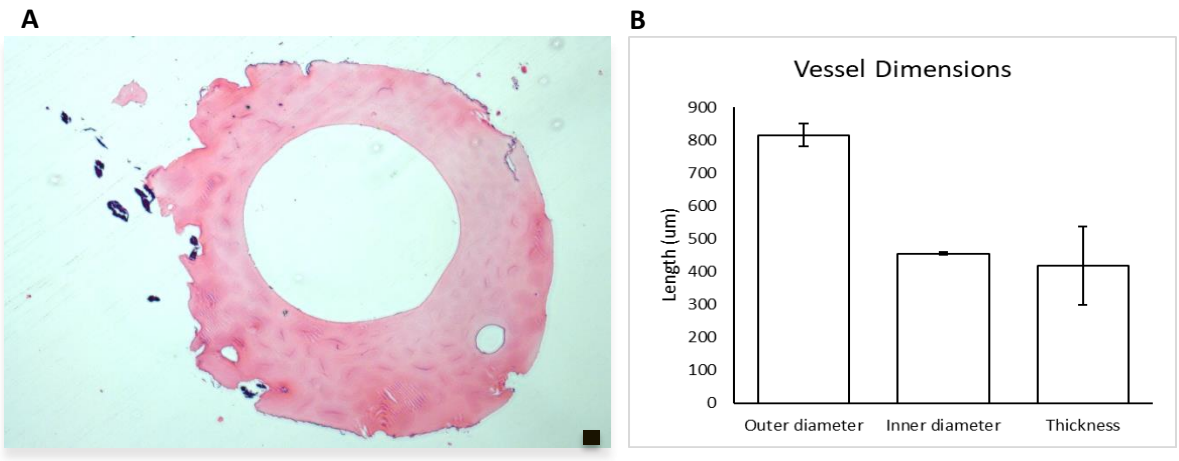

Figure 17. Histology of smooth muscle cell tube

(A) 2X Image of a cross section of cell tube stained with hematoxylin and eosin

(B) Quantification of vessel parameters and dimensions including outer diameter, inner diameter and thickness of fibrin. Scale bar, 100 μm

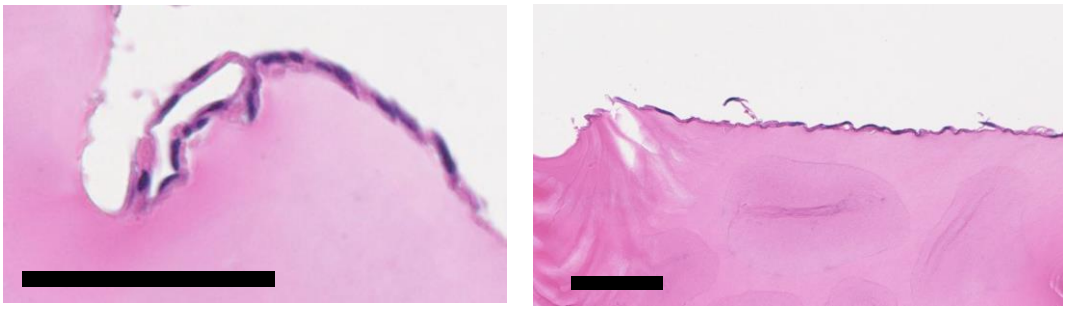

Figure 18. Images of hematoxylin and eosin staining of tunica media model consisting of smooth muscle cells wrapped around fibrin tube, 7 days in culture

Scale bar, 100 μm

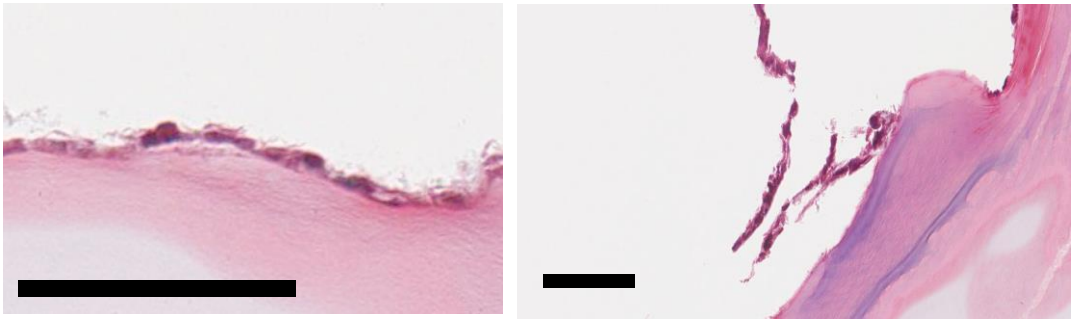

Figure 19. Images of trichrome staining of tunica media model consisting of smooth muscle cells wrapped around fibrin tube, 7 days in culture

Scale bar, 100 μm

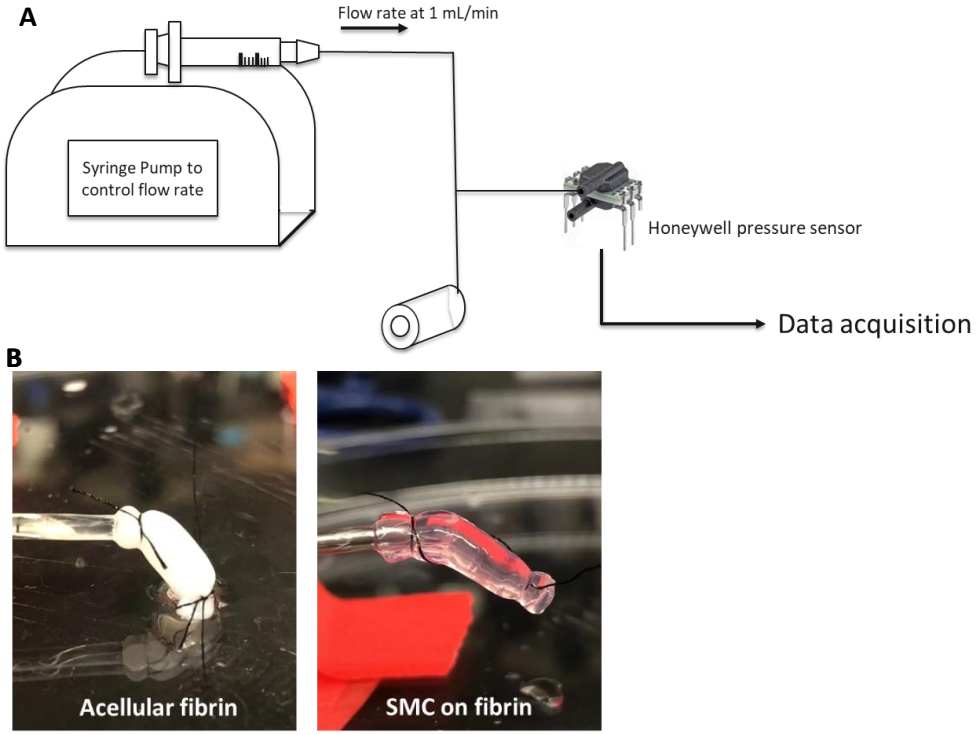

Figure 20. Setup to measure burst pressure of vessels

(A) A schematic diagram illustrating the setup to measure burst pressure using a syringe pump and pressure sensor for data acquisition

(B) Images showing acellular (left) and cellular (right) vessels were sutured on both ends to allow for a closed system to measure burst pressure

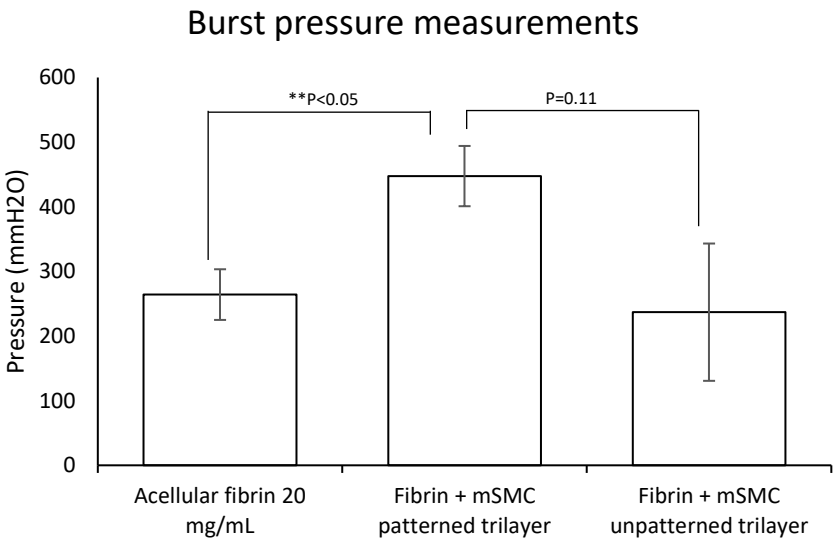

Figure 20. A bar graph showing burst pressure measurement of acellular, patterned trilayer and unpatterned trilayer vessels

Chapter 5. Conclusion

5.1 Summary of work

Based on the premise that tunica media in native blood vessel consists of highly ordered circumferentially aligned smooth muscle cells, the effects of nanotopography on smooth muscle cell sheet alignment and subsequent cytoskeletal structural orientation were investigated. Through the adaptation of a previously described protocol to fabricate thermoresponsive nanofabricated substrate, multiple layers of smooth muscle cell sheets were stacked to form multilayer structure to simulate multiple smooth muscle cells in tunica media in native blood vessels. A proposed method to wrap cell sheets around a three-dimensional hydrogel was proposed to fabricate a three-dimensional tunica media model with biomimetic features through cell alignment and hydrogel stiffness. This design served to address the current limitation in blood vessel modelling to precisely and accurately model the contractile component of blood vessels which control vascular tone and blood flow in vessel. It had been shown that nanotopography guides smooth muscle cell alignment and that upon transfer of cell sheets to a three-dimensional hydrogel, the anisotropy of cell sheets was well maintained. As a proof of concept, tunica media of a small diameter arterial vessel was modelled in this project with a lumen size of 450 μm and outer diameter of 800 μm. Smooth muscle cell remodeling of the fibrin hydrogel was demonstrated through differences in burst pressure comparing acellular and cellular models. It was shown that with patterned vascular smooth muscle cell, tube higher model integrity was observed compared with unpatterned cell tube or acellular vessel. By capturing the native architecture of tunica media, we have demonstrated success in the fabrication of a viable smooth muscle cell tube, controlled modelling of circumferentially

aligned smooth muscle cells in tunica media and shown preliminary results functional properties of tunica media models.

5.2 Future Directions

This project described a method to fabricate a tissue-engineered arterial tunica media using circumferentially aligned smooth muscle cells as proof of concept. Multilayer mouse smooth muscle cell sheets were wrapped successfully around fibrin hydrogel that match physiological stiffness range and preserved cell sheet alignment after culture. To further improve the model, multiple avenues could be investigated. For instance, design of gel casting method to create three-dimensional tube tissue could be optimized to fabricate smaller models with thinner vessel wall. By increasing the lumen of straw size which are commercially available, a lumen of larger diameter and thinner fibrin gel thickness could be fabricated. The tunica media model fabricated could serve as a potential disease model if suitable cell types, such as patient-derived smooth muscle cells or gene-edited smooth muscle cells were used to form cell sheet layers to provide insights in hypertension and other vascular diseases. To fully capture the functions of native blood vessels, further characterization on smooth muscle cell contractile phenotype on the model and subsequent vasoconstriction or vasodilation experiments could be performed using the model.

Chapter 6. References

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