Role of Smooth Muscle Cell Phenotype on the Function of an In Vitro Vascular Bilayer Model

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**Statement of Research**

This research was conducted in the Laboratory for Tissue Engineering and Organ Fabrication at Massachusetts General Hospital. The principal investigator of the lab was Cathryn Sundback and my direct supervisor was Emmanuel Ekwueme. I conducted research in this lab for 1 semester of 91R, 1 summer full-time, and 2 semesters of 99.

**Abstract**

**Introduction**

**Blood vessel structure and function**

Blood vessels are integral in circulating blood throughout the body and are responsible for transporting oxygen and nutrients to every tissue. To maintain their structural integrity and function, the tunica intima and tunica media of the blood vessel wall maintain constant communication. The tunica intima is comprised mainly of ECs, which line the inside of the blood vessel. This layer allows blood to pass through with minimal friction and without clotting, and regulates the exchange of substances from the circulatory system to the surrounding tissues and organs. The tunica media consists of a thick layer of SMCs and is responsible for maintaining proper blood pressure and circulation through vasoconstriction and vasodilation. The internal elastic lamina separates the tunica intima and tunica media so that they maintain their distinct functions. Importantly, the internal elastic lamina allows for significant cross-talk between the two cell types for dynamic regulation and adjustment of the blood vessel. [signal the importance of them further and why we should investigate them]

**Smooth muscle cell function**

Vascular SMCs contract and relax appropriately to regulate luminal diameter and blood pressure. SMCs *in vivo* have two distinct phenotypes: contractile and synthetic. In healthy blood vessels, the tunica media is comprised mainly of contractile SMCs that carry out the normal physiological functions of the blood vessels. However, synthetic SMCs are also present in the resting state and will increase in population to maintain and repair the vessel structure after vascular injury or during regular cell turnover. Morphologically, contractile SMCs appear more elongated and spindle-like. They contain contractile filaments and upregulate proteins like α smooth muscle actin, smooth muscle-myosin heavy chain, and smoothelin. By contrast, secretory SMCs appear more compact and form an epithelioid or rhomboid shape. Instead of contractile filaments, secretory SMCs have organelles used for protein synthesis. They also have higher growth rates, and have a higher migratory activity than contractile SMCs (Rensen et al., 2007).

These two phenotypes have distinct cellular characteristics and secretomes. As such, they provide different signals to the EC layer that ultimately affect the performance of the vasculature. As these interactions are vital for proper functionality of the blood vessel, it is crucial to gain a better understanding of them.

**Endothelial cell function**

The strength and proper functionality of the endothelium is largely dependent on the quality of its junctions. These junctions are formed through transmembrane adhesive proteins that adhere to the actin cytoskeleton. These proteins work in tandem with intracellular components to stabilize the junctions (Dejana et al., 1999). Endothelial cells form two major types of junctions: adherens junctions and tight junctions.

Adherens junctions form primarily through the transmembrane adhesion proteins of the cadherin family. Particularly, endothelial cells express vascular endothelial (VE)-cadherin (Dejana et al., 1995). These junctions are vital for the maintenance and normal functionality of the cell, regulation of cell growth and proliferation, and apoptosis (Caveda et al., 1996). Although tight junctions are the primary regulators of vascular permeability, adherens junctions are still significant in modulating the exchange of small molecules and ions. Interestingly, adherens junctions may play a role in tight junction formation and assembly. An intracellular adherens junction protein, β-catenin, has been shown to associate with the intracellular tight junction protein, zonula occludens-1 (ZO-1), early in tight junction assembly (Rajasekaran et al., 1996).

Tight junctions form through the transmembrane adhesion proteins of occludin, claudins, and junctional adhesion molecule-A. The most prevalent and well-documented intracellular component is ZO-1. Tight junctions serve to maintain cell polarity of the endothelial cells. Importantly, their main responsibility is to act as a barrier, strictly regulating the exchange of molecules and ions across the vessel wall (Bazzoni & Dejana, 2004). The density of tight junctions in the vasculature changes depending on the need of the specific organ or tissue. Denser tight junctions lead to more strict regulation, whereas less dense tight junctions lead to more permeable vessels that allow for more passage and exchange of molecules and ions (Truskey, 2010).

**SMC-EC communication**

Paracrine signaling is the primary pathway through which SMCs and ECs dynamically regulate each other’s functionality and phenotype. This type of signaling enacts local changes and exerts its effects on nearby cells rather than cells separated by a large distance. ECs regulate blood vessel diameter and blood pressure by releasing vasodilators and vasoconstrictors that may freely diffuse towards the SMC layer. Specifically, ECs can send out vasodilators like prostacyclin, endothelial derived hyperpolarizing factor, and nitric oxide to relax or dilate the SMC layer. Likewise, ECs can send out vasoconstrictors like endothelin and angiotensin II to constrict the SMC layer (Truskey, 2010b). Similarly, SMCs can send extracellular signals to the EC layer to regulate the release of vasoconstrictors and vasodilators (Di Luozzo et al., 2000).

Gap junctions are another frequently used mode of communication for SMCs and ECs. These junctions are primarily comprised of members of the membrane protein family connexin. Connexins associate into a hexameric structure termed a **connexon. Connexons** from two cells associate with each other to form a gap junction channel, which connects the cytoplasms of the two cells. Multiple gap junction channels can then aggregate to form gap junction plaques. These gap junctions allow electrical impulses and small molecules under 1 kilo-Dalton to move from one cell to another via passive diffusion. The size of the channel excludes larger molecules like nucleic acids and proteins from passing through (Kumar & Gilula, 1996). This type of junction is more direct than paracrine signaling, as factors can move specifically from the cytoplasm of one cell to the other.

**Vascular Tissue Engineering**

Multiple vascular models have been developed to better understand and evaluate the cross-talk between SMCs and ECs. In determining the best approach for this project, three types of models were considered: co-cultures with direct contact, co-cultures utilizing collagen gels, and co-cultures across porous membranes.

Direct contact co-cultures of ECs and SMCs are advantageous because they can closely mimic *in vivo* architecture. Evensen et al. (2009) observed that ECs form a system of interconnected capillaries that are stable for several weeks when in direct contact with vascular SMCs. Additionally, they noticed significant production of collagen IV, an integral component in the vascular basement membrane. Additionally, this model decreases diffusion distances for small molecules and substrates, and allows for increased myoendothelial junctions because of the proximity of the cell types (**citation)**. Direct contact co-cultures have been used in a variety of molecular studies, including evaluating tumor necrosis factor-α-mediated EC inflammatory response and uptake of lipoproteins (**insert Wallace/Truskey and Niwa)**. However, this approach makes it harder to separate the responses of the individual layer. While the layers can be separated, it is more difficult to manipulate and separate a mixed population.

Co-culturing ECs/SMCs across collagen gels or ECs on collagen gels containing SMCs provides a model that more accurately mimics the extracellular matrix and internal elastic lamina found *in vivo.* In this way, we can better investigate the cellular interactions in the extracellular matrix. This type of model can take on the form of some important architectural functions, such as Ganesan et al. (2017) demonstrated that the SMCs and ECs begin to produce their own extracellular interface, comprised mostly of laminin, collagen IV, and perlecan, comparable to the vascular basement membrane matrix *in vivo*. Basal mRNA expression of activation markers such as E-selectin and intercellular adhesion molecule I were similar to freshly isolated mouse inferior vena cava. **“**However, the major drawbacks of collagen gels are their low mechanical properties compared to synthetic materials13.”Ziegler et al. (1995) and van Buul-Wortelboer et al. (1986) showed that ECs tend to elongate under static co-culture conditions. Furthermore, ECs inhibit the growth and proliferation of the SMCs (van Buul-Wortelboer et al., 1986).

Membrane

* Advantage: you can easily manipulate either side and separate for analysis
* Disadvantage: stiff and and reduce amount of contact they have with each other
* Reason I chose this one: manipulating the SMC layer and then evaluating the EC layer so its easier to do it like that

Models that co-culture SMCs and ECs on opposite sides of a membrane offer a system that allows significant cross-talk between the cell types without fully direct contact. Chiu et al. (2003) showed that SMCs modulate gene expression in ECs. Specifically, under static conditions, SMCs impact the expression of intercellular adhesion molecule-1, vascular adhesion molecule-1, E-selectin, and endothelial nitric oxide synthase in ECs under static conditions. By contrast, Nackman et al. (1996) showed that ECs have a profound effect on SMC phenotype and characteristics. They showed that ECs in co-culture modulate SMC growth and proliferation; ECs release plasminogen activator inhibitor-1 to the SMC layer, which inhibits the activation pathway of transforming growth factor-beta 1. . Additionally, Fillinger et al. (1997) showed that compared to SMC monolayers, SMCs in co-culture with ECs showed increased cell density, increased growth and proliferation, decreased protein production, and yielded a more spindle-like morphology with filamentous projections. Furthermore, Hoganson et al. (2016) subjected this SMC-EC bilayer to physiological flow conditions and evaluated its ability to detect extravasation of red blood cells, a common feature of DIVI, from the lumen of the device into the SMC layer. They demonstrated that the functional responsiveness of the vascular model was similar to *in vivo* vasculature when treated with DIVI-causing drugs. “Because of the relative difference in the growth rates of ECs and SMCs and the need to easily separate the two cell types, most co-culture techniques involve the separate, but close, culture of the two cell types. Growing the two cell types on opposite sides of cell membranes is an easy way to permit separation of the cells and limit overgrowth while bringing ECs and SMCs within 10-50 μm of each other. The porous membrane also introduces a synthetic and stiffer surface between the ECs and SMCs than occurs in vivo and cell function appears to be very sensitive to the stiffness of the surface on which cells are grown 53. ECs and SMCs were seeded and grown separately on both sides of a microporous membrane so as to make it possible to analyze various functions of the two different types of cells separately.10–14

ECs cultured with SMCs also changed ECs from the normal polygonal morphology in vitro to an elongated shape 42 , increased EC gene expressions of TF 50, VEGF 54, adhesion molecules 38, growth-related oncogene-α and MCP-1 35. Co-culture of ECs with 10T1/2 cells, a smooth muscle-like cell line, produced increased localization of tight junction proteins to the junctions and increased permeability in a manner akin to the effect of cAMP 55, suggesting that SMCs play a critical role in regulating EC permeability 38 ,However, the membrane is elastic and has numerous pores whose diameters range from 2 to 7 µm,17 enabling a direct contact between the ECs coating the luminal surface of the vessel in a monolayer and the SMCs forming the media of the vessel.

Drug induced vascular injury (DIVI) occurs in about 10% of candidate drugs going through *in vivo* testing and serves as a major roadblock in innovating therapies and treatments for a plethora of diseases (Hoganson et al., 2016). Unfortunately, DIVI has been a complex issue to remedy due to a lack of diagnostic biomarkers and understanding of the mechanism of action (Louden et al., 2006). This is largely due to the fact that DIVI can manifest in a variety of forms. It causes damage to the blood vessel wall, affecting both endothelial cells (ECs) and smooth muscle cells (SMCs). Particularly, ECs can undergo harmful changes such as degeneration, apoptosis, and hypertrophy. Similarly, SMCs can undergo severe alterations like hyalinization, apoptosis, and necrosis (Mikaelian et al., 2014). Candidate drugs must be able to navigate and avoid these symptoms while maintaining their potency as a therapy.

Because of the lack of a robust *in vitro* model of DIVI, these damaging side effects often go unrecognized until preclinical animal testing, after much time, money, and effort have been expended. Developing a physiologically relevant *in vitro* model of human small diameter blood vessels would be an invaluable tool in early drug screening and studying the mechanism of action of DIVI. This may allow for earlier detection of complications and could minimize the amount of time problematic candidate drugs spend in development. In order to achieve such a model, it is imperative to gain a better understanding of the cross-talk between ECs and SMCs. Particularly, the impact of SMC phenotype on the EC layer and the functional response of thevascular bilayer must be investigated.

**Hypothesis and Experimental Summary**

It has been established that healthy blood vessels *in vivo* contain mostly contractile SMCs, but still are comprised partly of synthetic SMCs. These two phenotypes provide distinct secretomes that ultimately affect the blood vessel. However, the effect of SMC phenotype on ECs in an *in vitro* model has not been well characterized. Therefore, I hypothesize that the phenotype of SMCs has a profound effect on the ECs and functional response of the *in vitro* vascular bilayer model. I will evaluate my hypothesis using the following 3 aims:

1. Characterize contractile and synthetic SMC phenotypes
2. Evaluate the functional response of ECs in indirect culture with SMCs
3. Evaluate the functional response of bilayers consisting of ECs and SMCs

SMC phenotype will be modulated through different treatment conditions, which will yield either the synthetic or contractile phenotype. Phenotype will be confirmed based on phenotype-specific characteristics. These differentiated SMCs will then be cultured indirectly with ECs to evaluate the effect of soluble factors on adherens and tight junction formation. Additionally, these SMCs will be cultured in bilayer with ECs to assess the effect of contact signals on adherens and tight junction formation. Bilayers will also be evaluated on their viability as a model for DIVI.

This project will generate a better understanding of the effect of SMC phenotype on the cross-talk and functional response of an *in vitro* vascular bilayer. Knowing this information yields opportunities for creating more physiologically relevant vascular models and furthering our understanding of the mechanisms of action involved in DIVI.

**Methods**

**Part 1. Modulation and Characterization of SMC Phenotype**

*Experimental Overview*

To demonstrate that SMC phenotype direct EC formation and function, it was necessary to establish protocols for reliably producing contractile and synthetic phenotypes *in vitro.* To confirm the development of a specific phenotype, the cells were characterized based on five main features that differentiate contractile and synthetic phenotypes: morphology, proliferation rate, metabolic activity, protein expression, and gene expression.

Contractile SMCs have an elongated and spindle-like morphology. They contain contractile filaments and upregulate proteins like calponin, α-smooth muscle actin, smooth muscle-myosin heavy chain, and smoothelin. Additionally, contractile SMCs are characterized by slow growth rates and low migratory activity in comparison to synthetic SMCs. By contrast, synthetic SMCs are more compact and form an epithelioid or rhomboid morphology. Instead of advanced contractile filaments, synthetic SMCs have increased numbers of organelles used for protein synthesis. They have enhanced growth rates and heightened migratory activity (Rensen et al., 2007) (Rensen, Doevendans, and van Eys, 2007).

While primary SMCs offer a straightforward approach to deriving contractile and synthetic SMCs, this cell type comes with significant limitations. Depending on the donor, primary SMCs may be of low fidelity and ultimately affect the strength and reliability of results obtained using the bilayer (Gong & Niklason, 2008). Human mesenchymal stem cells (hMSCs) or induced pluripotent stem cell-derived mesenchymal stem cells (iMSCs) may prove to be a favorable alternative, as they have great self-renewal capabilities and are versatile in their application. [commericial – qc to minimize batch variation] Additionally, studies have shown the potential of hMSCs to differentiate into an SMC fate with some success. Notably, Narita et al. (2008) and Gong & Niklason (2008) showed that hMSCs can be differentiated into SMCs through treatment with exogenous factors such as TGF-β1. Gong & Niklason (2008) demonstrated that after 14 days of exposure to TGF-β1, hMSCs displayed significant amounts of the SMC specific markers calponin and α smooth muscle actin. Furthermore, they developed a hMSC-derived SMC vessel wall that demonstrated many of the key characteristics that were present in the positive control of coronary artery SMCs. Significantly, calponin and α smooth muscle actin were again present, as well as notable amounts of collagen IV?”??? in the extracellular matrix. However, the hMSC-derived SMCs did show some limitations, as they failed to express some of the late SMC markers.

Therefore, both human umbilical artery smooth muscle cells (hUASMCs) and iMSCs were assessed. Methods were established to reliably induce these two cell types into SMC contractile and synthetic phenotypes. They were subjected to specific conditions and were evaluated on their response and how well they acquired these phenotypes. They were characterized in a 4-day study based on morphology, proliferation rate, metabolic activity, protein expression, and gene expression, and were compared to known properties of synthetic and contractile SMCs.

The first condition for the cells was intended to generate the synthetic phenotype. The conditions for deriving this phenotype were based off the findings of Li et al. (1999), who demonstrated that SMCs grown in the presence of 10% fetal bovine serum (FBS) displayed the synthetic phenotype. As such, cells in the first group were treated with serum-containing medium. By contrast, the second group was designed to produce the contractile phenotype. Based off of the observations of Li et al. (1999) that SMCs demonstrated a contractile phenotype under serum withdrawal conditions, the cells in the second group were treated with serum-free medium. The third group was also intended to produce the contractile phenotype via different conditions. Hautmann et al. (1997) demonstrated that transforming growth factor beta 1 (TGF β1) treatment *in vitro* induced a contractile phenotype. Therefore, the third group was subject to both serum-free medium and TGF β1.

*Cell Culture*

hUASMCs (PromoCell**)** were grown in PromoCell Smooth Muscle Cell Growth Medium 2 (SMC Growth Medium) and used between passage 3 and 5. This medium was created by adding 5% fetal calf serum, epidermal growth factor, basic fibroblast growth factor, and insulin to PromoCell Smooth Muscle Cell Basal Medium (SMC Basal Medium). iMSCs (CDI**)** were grown in iMSC Growth Medium and used between passage 3 and 5. This media was created by adding 2% fetal bovine serum and fibroblast growth factor to Gibco Minimum Essential Medium Alpha (iMSC Basal Medium). For all assays in the characterization study (morphology, proliferation, metabolic activity, protein expression, and gene expression), hUASMCs and iMSCs were seeded at a density of 30,000 cells/cm2 into culture dishes. After 24 hours of incubation at 37° Celsius, cells were washed twice with 1X PBS and separated into 3 groups. Corresponding media was changed every other day. Each medium contained 1% Penicillin Streptomyocin.

hUASMCs:

1. Group 1 (SMC-G) was treated with SMC Growth Medium
2. Group 2 (SMC-B) was treated with SMC Basal Medium
3. Group 3 (SMC-T) was treated with SMC Basal Medium with 5 ng/ml of TGF β1 (SMC TGF β1 Medium)

iMSCs:

1. Group 1 (iMSC-G) was treated with iMSC Growth Medium
2. Group 2 (iMSC-B) was treated with iMSC Basal Medium
3. Group 3 (iMSC-T) was treated with iMSC Growth Medium with 5 ng/ml of TGF β1 (iMSC TGF-β1 Medium)

*Morphology*

Morphology was monitored through bright field images taken on days 2 and 4. Cells were seeded into a 6-well culture dish and subjected to the culture conditions noted above. Cells were imaged at 4X, 10X, and 20X magnification on a Nikon \_\_\_\_\_ inverted microscope.

*Proliferation*

The impact of culture conditions on proliferation was measured through the EdU assay on day 4. Cells were seeded into a 96-well culture dish and subjected to the culture conditions noted above. On day 3, media was removed and replaced with fresh media containing 10 μM F-para-EdU. After incubation at 37° Celsius for 24 hours, cells were fixed in 4% paraformaldehyde for 15 minutes, quenched with 1X PBS containing 50 mM glycine and 50 mM NH4Cl for 5 minutes, and washed once with 1X PBS. Cells were then stained with AlexaFluor 488 azide, 1 mM CuSO4, and 10 mM sodium ascorbate in PBS for 2 hours in the dark. They were then rinsed once with 1X PBS, permeabilized with 0.1% Trixon X-100 in PBS, and rinsed twice with 1X PBS. Cells were then incubated with DAPI in PBS for 15 minutes in the dark, rinsed 3 times with 1X PBS, and remained in 1X PBS for imaging. Cells were imaged at 10X magnification using a \_\_\_\_\_\_\_\_\_. 10 images per group were quantified automatically via ImageJ software, which included counting the number of EdU+ nuclei and total number of nuclei. For each group, the percentage of EdU+ nuclei was calculated by dividing the average number of EdU+ nuclei by the average total number of nuclei for that group.

*Metabolic Activity*

Metabolic activity was measured through the PrestoBlue assay (vendor) on culture day 4, following manufacturer’s instructions. Cells were seeded into a 96-well culture dish and subjected to the culture conditions noted above. PrestoBlue reagent was diluted 10-fold in fresh SMC/iMSC Basal Media to create a PrestoBlue workingsolution. Media was aspirated from each cell sample and replaced with 200 μL of the PrestoBlue solution. Samples of solely PrestoBlue solution were added to generate a baseline reading. The culture dish was then incubated for 1 hour in the dark at 37° Celsius. It was read for fluorescence under the conditions of excitation/emission 535-560 nm/590-615 nm. All readings from the cell samples were normalized to the baseline reading.

*Protein Expression*

Protein expression was measured through immunocytochemistry on culture day 4. Cells were seeded into a 96-well culture plate and subjected to the culture conditions noted above. Cells were fixed in 4% paraformaldehyde for 25 minutes, permeabilized with 0.1% Triton-X 100 and 1% bovine serum albumin for 1 hour, and then washed 3 times with 1X Phosphate Buffer Saline (PBS). Samples were then incubated with rabbit anti-calponin (1:500, Abcam) overnight at 4° Celsius, rinsed 3 times with 1X PBS, incubated with Alexa Fluor 532 goat anti-rabbit (1:500, Molecular Probes), and rinsed 3 times with 1X PBS. They were then incubated with Alexa Fluor 488 phalloidin (1:500, Invitrogen) for 1 hour in the dark and rinsed 3 times with 1X PBS. Cells were then incubated with DAPI (1:1000) for 15 minutes in the dark, rinsed 3 times with 1X PBS, and remained in 1X PBS for imaging. They were imaged at 4X, 10X, and 20X magnification.

*Gene Expression*

*put in biological replicates information*

Gene expression was measured through quantitative polymerase chain reaction (qPCR) on day 4. Cells were seeded into a 6-well plate and subjected to the culture conditions noted above. RNA isolation and purification was performed using the Qiagen RNeasy Mini Kit, following manufacturer’s instructions. Cells were lysed with RLT Plus Buffer with β-mercaptoethanol. The lysates were then passed through a gDNA eliminator column. The flow-through was treated with 70% ethanol and passed through an RNeasy spin column to wash away all contaminants. The RNA was then eluted with nuclease-free water. The concentration of the RNA was determined via Nanodrop and each sample was normalized to the lowest concentration. The RNA was then mixed with the RT Master Mix and placed on a thermal cycler to be reverse transcribed into cDNA. The cDNA was then mixed with SYBR Green Master Mix and primers for elastin, calponin, caldesmon, α smooth muscle actin, cyclin d1, smoothelin, PDGF, and SM22-alpha, and loaded into a reaction plate. Once the reaction plate was sealed, it was centrifuged and loaded into the reading instrument. The average CT values were normalized to housekeeping genes: SMC primers were normalized to Beta-Actin and iMSCs were normalized to Beta-2 microglobulin. **Why different b2m**

**Part 2. Evaluation of Functional Response of ECs in Co-Culture with SMCs**

*Experimental Overview*

With an established protocol for developing SMC phenotypes *in vitro*, the effects of those phenotypes on the EC layer were evaluated. Healthy vascular ECs display a contiguous monolayer with a cobblestone morphology under static conditions. [**elonagation under flow or when cocultured**]. Vital to this EC layer functionality is the quality of its junctional complexes. Adherens junctions are invaluable structures in stabilizing intercellular adhesion sites, proper tissue architecture, and intracellular signaling (Hartsock & Nelson, 2008; Meng & Takeichi, 2009). Tight junctions are vital in maintaining cell polarity and strictly regulating the passage of molecules and ions (Bazzoni & Dejana, 2004). Because of their importance in a variety of cellular functions, it is imperative to investigate how signals from the SMC layer can affect their integrity. [junctional complexes are often used to evaluate the strength of the vascular bilayer]

As paracrine signaling is a vital pathway through which cell types affect each other, it was very important to evaluate the effect of secreted and soluble factors from the SMC phenotype on the functionality of the EC layer. To evaluate the formation of adherens and tight junctions, induced pluripotent stem cell-derived ECs (iECs) were grown on fibronectin-coated tissue culture polystyrene and cultured in different types of conditioned media for 6 days. The conditioned media was created by capitalizing on the protocol developed in the previous section. hUASMCs/iMSCs were developed into either the contractile or synthetic phenotype and were then incubated with VascuLife Basal Medium (iEC Basal Media) to create the conditioned media. iECs cultured in conditioned medium were fixed and stained every 3 days for adherens and tight junction markers to evaluate the effects.

To further categorize the SMC phenotype effect on formation of adherens and tight junction and to evaluate the efficacy of each bilayer, iECs and hUASMCs/iMSCs were grown on opposite sides of track etched membranes (TEMs) for 13 days. The strength and integrity of the bilayer was evaluated via transepithelial electrical resistance on days 6, 9, and 12. Membranes were also stained for tight junction markers on day 13 to evaluate the effect visually.

*Conditioned Media Assay*

To create the hUASMC/iMSC conditioned media, hUASMCs/iMSCs were seeded into a 6-well culture dish at a density of 30,000 cells/cm2 (300,000 cells). After 24 hours of incubation, cells were washed twice with 1X PBS. hUASMCs were split into the same 3 groups as the characterization study: SMC-G, SMC-B, and SMC-T. iMSCs were split into 2 of the groups from the characterization study: iMSC-G and iMSC-T. iMSC-T showed more contractile SMC-like qualities than iMSC-B, so iMSC-B was not included in the following studies (Discussion Part 1). Cells were differentiated in their groups for 48 hours, washed twice with 1X PBS, and incubated with iEC Basal Medium for 24 hours at 37° Celsius. The conditioned media was collected from each group and centrifuged to remove cell debris. This yielded 3 groups of hUASMC conditioned media: SMC-G-Conditioned Medium, SMC-B-Conditioned Medium, and SMC-T-Conditioned Medium, and yielded 2 groups of iMSC conditioned media: iMSC-G-Conditioned Medium and iMSC-T-Conditioned Medium. All conditioned media was diluted in a 1:1 ratio with iEC Growth Medium to provide the necessary nutrients and factors for the iECs to continue to grow and proliferate when treated. iEC Growth Medium was created using the VascuLife Vascular Endothelial Growth Factor Endothelium Medium kit.

To visualize the effect of conditioned media on iECs, 96-well culture dishes were incubated in 3% fibronectin in 1X PBS for 1 hour at 37° Celsius to create a fibronectin coating. After the fibronectin solution was aspirated, iECs were seeded at a density of 30,000 cells/cm2 (10,000 cells). iECs were grown in iEC Growth Medium for 3 days until they reached confluence. iECs were washed twice with 1X PBS and split into treatment groups. Cells were treated with their corresponding media every other day in a 6 day study.

hUASMCs:

1. Group 1 (iEC-G) was treated with iEC Growth Medium
2. Group 2 (SMC-G-Cond) was treated with SMC-G-Conditioned Medium
3. Group 3 (SMC-B-Cond) was treated with SMC-B-Conditioned Medium
4. Group 4 (SMC-T-Cond) was treated with SMC-T-Conditioned Medium

iMSCs:

1. Group 1 (iEC-G) was treated with iEC Growth Medium
2. Group 2 (iMSC-G-Cond) was treated with G iMSC-Conditioned Medium
3. Group 3 (iMSC-T-Cond) was treated with T iMSC-Conditioned Medium

Junctional proteins were visualized through immunocytochemistry on treatment days 3 and 6. Cells were fixed in 4% paraformaldehyde for 25 minutes, permeabilized with 0.1% Triton-X 100 and 1% bovine serum albumin for 1 hour, and then washed 3 times with 1X PBS. Samples were then incubated with mouse anti-VE-cadherin (1:100, Santa Cruz Biotechnology) overnight at 4° Celsius, rinsed 3 times with 1X PBS, incubated with Alexa Fluor 488 goat anti-mouse (1:500, Invitrogen) for 1 hour in the dark, and rinsed 3 times with 1X PBS. They were then incubated with Alexa Fluor 594 phalloidin (1:500, Invitrogen) for 1 hour in the dark and rinsed 3 times with 1X PBS. Cells were then incubated with DAPI (1:1000) for 15 minutes in the dark, rinsed 3 times with 1X PBS, and remained in 1X PBS for imaging. They were imaged at 4X, 10X, and 20X magnification.

*Creation of Bilayer*

Track etched membranes were coated in both collagen and fibronectin to support the SMCs and iECs, respectively. **They were incubated with collagen for 1 hour and then 3% fibronectin in PBS for 1 hour at 37° Celsius**. The solutions were aspirated and TEMs were flipped upside down. hUASMCs/iMSCs were seeded onto the bottom side of the membrane and incubated at 37° Celsius for 3 hours to allow cells to attach. Membranes were then washed 1X PBS to get rid of debris. They were flipped right side up into a 24-well culture dish and incubated with SMC/iMSC Growth Medium for 24 hours. After incubation, cells were washed twice with 1X PBS. hUASMCs were split into the same 3 groups as the characterization study: SMC-G, SMC-B, and SMC-T. iMSCs were split into 2 of the groups from the characterization study: iMSC-G and iMSC-T (Discussion Part 1). Cells were differentiated in their groups for 48 hours, washed twice with 1X PBS, and incubated in SMC Basal Medium/iMSC Basal Medium. iECs were then seeded on the topside of the membrane at a density of 30,000 cells/cm2 (30,000 cells) and incubated in iEC Growth Medium. After 24 hours of incubation at 37° Celsius, all media was removed and replaced with a 1:1 ratio of iEC Growth Medium and SMC Basal Medium/iMSC Basal Medium. In addition to the bilayer groups, a control group of an iEC monolayer was cultured to serve as a baseline.

*TEER Assay*

On days 6, 9, and 12, TEER was measured for each bilayer using the Epithelial Voltohmmeter (EVOM2). The EVOM2 was disconnected from its charging cable, the machine was turned on and switched to ohms, and the electrode was connected. The electrode was placed 4-5mm from the bottom of the chamber. The bilayers and their corresponding media were brought to room temperature before measurement. To measure, 1.5 mL of media was added to the chamber, the bilayer was placed in the media, and media was added to the inside of the bilayer transwell to match the level on the outside of the transwell. The electrode was then placed on the inside of the bilayer insert and the value was recorded. All readings were normalized to the TEER value of a TEM transwell without cells.

*Protein Expression*

Junctional proteins were visualized through immunocytochemistry on treatment culture day 13. Previous work done in the lab found that tight junctions reach peak performance between days 12-13. Cells were fixed in 4% paraformaldehyde for 25 minutes, permeabilized with 0.1% Triton-X 100 and 1% bovine serum albumin for 1 hour, and then washed 3 times with 1X PBS. Samples were then incubated with mouse anti-ZO-1 (1:100, ???) overnight at 4° Celsius, rinsed 3 times with 1X PBS, incubated with Alexa Fluor 594 goat anti-mouse (1:500, Invitrogen) for 1 hour in the dark, and rinsed 3 times with 1X PBS. Cells were then incubated with DAPI (1:1000) for 15 minutes in the dark, rinsed 3 times with 1X PBS, and remained in 1X PBS for imaging. They were imaged at 4X, 10X, and 20X magnification.

**Results**

**Part 1. Modulation and Characterization of SMC Phenotype**

The synthetic (SMC-G) and contractile (SMC-B and SMC-T) groups showed significant differences across all characteristics evaluated. SMC-G appeared clumped together and compact morphologically, while SMC-B and SMC-T displayed a well spread out and elongated, spindle-like morphology (Figure 1A). Furthermore, these two groups exhibited distinction in metabolic activity. Relative to SMC-G, SMC-B and SMC-T showed a significant decrease in relative fluorescence units by 38.35% and 43.26%, respectively. Both contractile groups showed comparable values with each other (Figure 1B). This same trend remained consistent in the evaluation of proliferative capabilities. SMC-G displayed a significantly higher percentage of EdU+ nuclei than SMC-B and SMC-T by 36.11% and 35.00%, respectively. There was little variation between contractile groups, as SMC-B and SMC-T showed a 1.11% difference (Figure 2). SMC phenotypes were further distinguished by protein expression. SMC-G had relatively less intense and less frequent expression of calponin, with only a small subset of nuclei surrounded by the marker. They also showed a more diffuse signal for filamentous actin (F-actin). By contrast, SMC-B and SMC-T contained the marker for calponin around each nucleus, and displayed a more organized F-actin signal with more pronounced actin filaments (Figure 3). The gene expression profile also largely supported the phenotype differences. SMC-G had significantly smaller fold changes than both contractile groups for α smooth muscle actin and elastin. SMC-G also had the smallest fold change for calponin, which was significantly smaller than SMC-T. Similarly, SMC-G demonstrated a smaller fold change than SMC-T for smoothelin expression. By contrast, SMC-G displayed a significantly higher fold change than both contractile groups for cyclin d1 (Figure 4).

The iMSC groups showed similar trends to the SMC groups, in that the synthetic group (iMSC-G) demonstrated distinctly different profiles in many of the characteristics tested than the contractile groups (iMSC-B and iMSC-T). However, there were significant discrepancies between the two contractile iMSC groups. iMSC-G were densely packed and showed a compact, rhomboid morphology. By contrast iMSC-B and iMSC-T were more spread and elongated (Figure 5A). Additionally, there were significant differences in the metabolic activity between the synthetic and contractile iMSC groups. Relative to iMSC-G, iMSC-B and iMSC-T showed a significant decrease in relative fluorescence units by 74.02% and 51.87%, respectively. However, there was a significant difference between the contractile groups, SMC-B had an 85.26% smaller RFUs than SMC-T (Figure 5B). There was also a significant difference between the two phenotypes in terms of proliferative capability. iMSC-G demonstrated a significantly larger percentage of EdU+ nuclei than iMSC-B and iMSC-T by 62.04% and 75.82%, respectively. However, there was a significant difference between the contractile groups, as iMSC-T exhibited a 13.78% decrease in EdU+ nuclei compared to iMSC-B (Figure 6). The two phenotype groups demonstrated noticeable differences in terms of protein expression. iMSC-G displayed calponin and F-actin consistently across the cells with even overlap between the two markers, but the markers did not show clear cell boundaries. By contrast, iMSC-B exhibited calponin and F-actin expression that surrounded each nucleus in a rhomboid-like shape. Interestingly, while there is significant overlap between the markers in the merged image, there appeared to be varying ratios of calponin to F-actin in each cell. By contrast, iMSC-T had well-distributed calponin and F-actin expression that displayed an elongated, almost spindle-like form. The actin filaments also appeared more pronounced than the other two iMSC groups. Unlike the iMSC-B, the merged image shows even marker overlap across the cells (Figure 7). In terms of gene expression, iMSC-T had significantly larger fold changes than the other two groups for α smooth muscle actin, elastin and calponin. Both iMSC-B and iMSC-T show higher fold changes than iMSC-G. Lastly, iMSC-B significantly downregulates cyclin d1, while iMSC-G and iMSC-T show comparable higher values (Figure 8).

**Part 2. Evaluation of Functional Response of ECs in Co-Culture with SMCs**

*Conditioned Media Assay*

iEC-G served as a positive control group and was used as a reference for normal iEC development and maturation. By culture day 3, there were noticeable differences between the treatment groups. VE-cadherin in the control group was expressed consistently on the periphery of each cell, demonstrating a cobblestone morphology. SMC-G-Cond similarly showed VE-cadherin expression on the periphery, but with inconsistent intensity. Additionally, the cells appeared more elongated than the control. Similarly, SMC-B-Cond showed a more elongated form than the iEC-G. However, SMC-B-Cond displayed more consistent VE-cadherin expression across the monolayer. By contrast, SMC-T demonstrated the most comparable expression profile to iEC-G. VE-cadherin expression localized to the border of the cells and demonstrated a cobblestone pattern. By culture day 6, the differences between treatment groups became more apparent. iEC-G showed a contiguous monolayer with VE-cadherin expression shown consistently on the periphery of each cell in a cobblestone shape. SMC-G-Cond showed distinct differences from the control. Importantly, VE-cadherin was not expressed in all cells. While VE-cadherin was localized to the periphery in some cells, the morphology looked thin and elongated, almost spindle-like. SMC-B-Cond appeared more similar to the control than SMC-G-Cond. VE-cadherin was more consistently expressed in all cells and properly localized, but cells were noticeably elongated, although not entirely flattened like SMC-G-Cond. Interestingly, SMC-T-Cond continued to demonstrate a morphology comparable to iEC-G, with cobblestone morphology, consistent VE-cadherin expression on the periphery, and a contiguous monolayer.

The iMSC groups deviated from the trend set forth by the SMC groups. By culture day 3, iMSC-G-Cond had a comparable expression profile, as VE-cadherin was expressed consistently on the periphery of each cell with a cobblestone morphology. iMSC-T-Cond showed less intense signal on the periphery of each cell, but the cobblestone morphology was still apparent. By culture day 6, iMSC-G-Cond continued to demonstrate a morphology comparable to iEC-G, with cobblestone morphology, consistent VE-cadherin expression on the periphery, and a contiguous monolayer. iMSC-T-Cond recapitulated the iEC-G phenotype.

*TEER Assay*

After 6 days of culture, the SMC bilayers showed significant differences in their TEER values. The iEC monolayer was utilized as a control to evaluate how SMC phenotype affected the overall strength of the endothelial layer. In comparison to the iEC monolayer, the SMC-G/iEC bilayer exhibited a 59.02% decrease, the SMC-B/iEC bilayer presented a 4.92% increase, and the SMC-T/iEC bilayer showed a 9.84% increase in TEER values. This data displayed that the synthetic phenotype was associated with a significant decrease in TEER values, whereas the contractile phenotype was associated with an increase. This trend was also evidenced by the differences between the TEER values between the bilayers. Relative to SMC-G/iEC bilayer, the SMC-B/iEC bilayer and SMC-T/iEC bilayer groups had significantly larger TEER values by 156% and 168%, respectively.

After 6 days of culture, the bilayers showed significant differences in their TEER values. In comparison to the iEC monolayer, the iMSC-G/iEC bilayer exhibited a 5% increase and the iMSC-T/iEC bilayer showed a 20% decrease in TEER values. Relative to the iMSC-G/iEC bilayer, the iMSC-T/iEC bilayer displayed significantly lower TEER value by 23.81%.

*Protein Expression*

iEC monolayer was used as a positive control to show the normal growth pattern of iECs without influence of SMCs. ZO-1 expression for iEC monolayer showed localization to the cell periphery and displayed a cobblestone morpohlogy. You can see a contiguous monolayer without holes. iEC/SMC-G showed inconsistent ZO-1 expression with holes in th eexpression, denoting an inconsistent iEC layer. Its mostly just spread throughout the image in a disorganized fashion and you can’t really see each cell body. The ZO-1 does not localize as well to the periphery as in the iEC monolayer and in some cases, is randomly spread instead of organized. They look kinda elongated. SMC-B shows a more contiguous iEC layer and you can see localization to the periphery. SMC-T shows the most similar to the control, as it shows cobblestone morphology, a contiguous iEC layer and ZO-1 expression is localized to the periphery.

iEC monolayer was used as a positive control to show the normal growth pattern of iECs without influence of SMCs. ZO-1 expression for iEC monolayer showed localization to the cell periphery and displayed a cobblestone morpohlogy. You can see a contiguous monolayer without holes. iEC/iMSC-T shows a more contiguous iEC layer and you can see localization to the periphery, but there are some holes and the ZO-1 is a little less organized than would be expected. iMSC-G shows the most similar to the control, as it shows cobblestone morphology, a contiguous iEC layer and ZO-1 expression is localized to the periphery.

**Discussion**

*2.4.1 Successful Modulation of Phenotype with hUASMCs*

SMC-G display features characteristic of the synthetic phenotype for SMCs, suggesting that treating hUASMCs with SMC Growth Medium is sufficient to induce the synthetic phenotype. SMC-G displayed an epithelioid morphology and had significantly higher metabolic activity and proliferation than the contractile groups. Additionally, these cells downregulated contractile markers **like elastin, calponin, caldesmon, and α smooth muscle actin.**

By contrast, SMC-B displayed features characteristic of the contractile phenotype, which suggests that hUASMCs develop into the contractile phenotype under serum withdrawal conditions. SMC-B displayed a spindle-like morphology, and showed significantly lower metabolic activity and proliferation than the SMC-G. They significantly upregulated the contractile markers elastin, calponin, caldesmon, and α smooth muscle actin. Similarly, SMC-T displayed features that are characteristic of the contractile phenotype, indicating that hUASMCs can be effectively developed into the contractile phenotype under serum withdrawal conditions and with addition of TGF-β1. SMC-T displayed a spindle-like morphology and showed significantly lower metabolic activity and proliferation than the SMC-G. They significantly upregulated the contractile markers elastin, calponin, and α smooth muscle actin.

*2.4.2 Utilizing iMSCs as Alternatives to hUASMCs*

In this study, iMSCs showed possible capacity to emulate the synthetic SMC phenotype. G iMSCs displayed a more compact, rhomboid morphology, which is reminiscent of the SMC synthetic phenotype. They had significantly higher proliferation rates and metabolic activity than the B or T iMSCs, similar to the trend found in the hUASMC groups. In terms of protein and gene expression, G iMSCs upregulated the synthetic SMC marker cyclin d1 and downregulated the contractile SMC marker α smooth muscle actin. However, they upregulated the contractile marker calponin. G iMSCs share many of the same trends and features as G hUASMCs and synthetic SMCs, which suggests they may be a viable alternative.

In terms of creating a contractile SMC phenotype, iMSCs showed limited ability. B iMSCs displayed contractile features such as low proliferation rates, low metabolic activity, and downregulation of cyclin d1. However, B iMSCs downregulated important SMC contracftile markers like calponin and α smooth muscle actin. Additionally, they displayed an epithelioid, fatter morphology, which is inconsistent with the contractile phenotype. While B iMSCs display some characteristics of contractile SMCs, there is much room for improvement.

T iMSCs demonstrate a more promising choice for developing a SMC contractile phenotype. T iMSCs showed a more elongated, spindle-like morphology consistent with the contractile phenotype. They also showed low proliferation rates and metabolic activity. T iMSCs also showed potential in terms of protein expression, as immunocytochemistry images showed high calponin expression and actin filaments. They also showed higher expression than B iMSCs for contractile markers like calponin and α smooth muscle actin. However, they upregulate the synthetic marker cyclin d1. While the T iMSCs do not show a perfectly contractile SMC, the data suggests that T iMSCs are more similar to contractile SMCs than under B conditions, and have potential as alternatives for contractile hUASMCs.

Discussion Part 2

Overall, both B and T hUASMCs offered comparable statistics, indicating that either treatment is viable for differentiating hUASMCs into the contractile phenotype.

The conditioned media study showed differences between treatment groups, indicating that soluble factors in the SMC phenotype have a profound effect on iEC layer formation and maturity. VE-cadherin is a marker for adherens junctions and are a differentiation marker. In normal cells, it should be located to the periphery and show a contiguous monolayer in cobblestone formation. The normal growth formations mirror the expected results through day 6. However, the synthetic SMC-G demonstrates a distinct deviation from the expected phenotype. Cells began to inconsistently express VE-cadherin on day 3 and showed even less consistency in expression by day 6. They began to elongate and eventually by day 6 did not form a contiguous layer. Rather, there were holes and the cells were so deformed that they no longer resembled a healthy morphology. This indicates that the soluble factors in the SMC-G group had a profoundly negative effect on endothelial layer function. By contrast, SMC-B showed a healthier phenotype. SMC-B displayed more consistent VE-cadherin expression on the periphery of the cells. By contrast, SMC-T demonstrated the most comparable expression profile to iEC-G. VE-cadherin expression localized to the periphery and the cells demonstrated a cobblestone pattern. By culture day 6, SMC-B showed a deviation from the control, but it appeared more similar to the control than SMC-G. VE-cadherin was more consistently expressed in all cells, it localized to the periphery, but cells were still noticeably elongated, although not entirely flattened like SMC-G. This suggests that the contractile phenotype does less damage to the iEC health than the contractile group. Further, SMC-T demonstrate a morphology comparable to iEC-G on both time points. This shows that this contractile group was able to maintain the health and structure of the endothelium. It suggests that the SMC-T layer is the better contractile model so far.

This trend is remains consistent by the TEER data from the bilayers. The iEC monolayer was used as a baseline to see how SMC phenotype affects its resistance. The SMC-G/iEC bilayer showed significant decrease in value, indicating that the junctions were disrupted or there were holes in the layer. It indicates overall lack of strength in the bilayer and shows that the synthetic phenotype has a profound effect on the functionality and strength and maturation of the endothelium. By contrast, both contractile groups showed comparable values to the iEC monolayer, suggesting that the contractile phenotype allows for the healthy development and maturation of junctional complexes. Similar to the conditioned media study, the SMC-T bilayer shows slightly higher values than the SMC-B bilayer, which may be indicative of the effect from the soluble factors which showed a better morphology and localization ofhte VE-cadherin signal.

Furthermore, this can be seen in ZO-1 imaging. ZO-1 is an indicator of tight junction formation. In normal formation and in static culture they should be cobblestone and ZO-1 expression should be localized to the outside and it should be contiguous monolayer. The SMC-G bilayer shows partial localization to the outside, but shows messiness. It seems more disorganized and less well defined. It looks more like it is spread throughout the cell body rather than strictly adhering to the periphery. SMC-B bilayer helps recapitulate the phenotype and causes less dmage to the setrucutre than the SMC-G group. You can see localization to the periophery and although a bit elongated, somewhat of a cobblestone form. It does show some nonspecific binding or some stuff located to the middle. Note about nonspecific antibody binding in the results section. Most astounding is the SMC-T group. You can see the cobblestone morpohology and localization to the periphery and no holes in the bilayer. Overall, based on this data you can definitely say that SMC phenotype has a distinct effect on the endothelium. And we can say that the one most closely related to that one is the SMC-T.

The conditioned media study looked the same for all iMSC groups. VE-cadherin is a marker for adherens junctions and are a differentiation marker. In normal cells, it should be located to the periphery and show a contiguous monolayer in cobblestone formation. iMSC-G and iMSC-T demonstrated the most comparable expression profile to iEC-G. VE-cadherin expression localized to the periphery and the cells demonstrated a cobblestone pattern. Neither had an impact so nothing can be said for which is better yet.

After 6 days of culture, the bilayers showed significant differences in their TEER values. The iEC monolayer was utilized as a control to evaluate how SMC phenotype affected the overall strength of the endothelial layer. iMSC-G actually showed a better phenotype for the endothelial layer, whereas iMSC-T showed a significant decrease which is bad.

iMSC-G ZO-1 is better localized to the outside, no gaps. iMSC-T also no gaps but there is more spread out and not as localized as well.

Improvements

I would take more values for the TEER so that we could see functionally if there is a difference, but it broke so that’s an improvement. I would also stain more frequently so we can pinpoint at what day there is breakage.

Conclusions  
I would do a DIVI assay on the iEC/SMC-T bilayer because it showed the most promise in terms of creating a physiological bilayer. We showed that there is a profound effect from the SMC phenotype on the EC layer. It does matter how you capitulate that phenotype though.

, part of the [contractile](https://en.wikipedia.org/wiki/Muscle_contraction) apparatus in [muscle](https://en.wikipedia.org/wiki/Muscle) cells. It can be present as either a free [monomer](https://en.wikipedia.org/wiki/Monomer) called **G-actin** (globular) or as part of a linear [polymer](https://en.wikipedia.org/wiki/Polymer) **microfilament** called **F-actin** (filamentous), both of which are essential for such important cellular functions as the [mobility](https://en.wikipedia.org/wiki/Motility) and contraction of [cells](https://en.wikipedia.org/wiki/Cell_(biology)) during [cell division](https://en.wikipedia.org/wiki/Cell_division).

Actin participates in many important cellular processes, including [muscle contraction](https://en.wikipedia.org/wiki/Actin#Outline_of_a_muscle_contraction), cell [motility](https://en.wikipedia.org/wiki/Motility), cell division and [cytokinesis](https://en.wikipedia.org/wiki/Cytokinesis), [vesicle](https://en.wikipedia.org/wiki/Vesicle_(biology_and_chemistry)) and [organelle](https://en.wikipedia.org/wiki/Organelle)movement, [cell signaling](https://en.wikipedia.org/wiki/Cell_signaling), and the establishment and maintenance of [cell junctions](https://en.wikipedia.org/wiki/Cell_junction) and cell shape.

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