Enhanced elastin synthesis and maturation in human vascular smooth muscle tissue derived from induced-pluripotent stem cells

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Statement of Significance

Obtaining robust, mature elastic fibers is a key obstacle in tissue-engineered blood vessels. Human induced-pluripotent stem cells have become of interest due to their ability to supplement tissue engineered scaffolds. Their ability to differentiate into cells of vascular lineages with defined phenotypes serves as a potential solution to a major cause of graft failure in which phenotypic shifts in smooth muscle cells lead to over proliferation and occlusion of the graft. Herein, we have differentiated human induced-pluripotent stem cells in a pulsatile flow bioreactor, resulting in vascular smooth muscle tissue with robust elastic fibers and **enhanced functionality**. This study highlights an effective approach to engineering elastic functional vascular smooth muscle tissue for tissue engineering and regenerative medicine applications.

Abstract

Obtaining vascular smooth muscle tissue with mature, functional elastic fibers is a key obstacle in tissue-engineered blood vessels. Poor elastin secretion and organization leads to a loss of specialization in contractile smooth muscle cells, resulting in over proliferation and graft failure. In this study, human induced-pluripotent stem cells (hiPSCs) were differentiated into early smooth muscle cells, seeded onto a hybrid poly(ethylene glycol) dimethacrylate/poly (L-lactide) (PEGdma-PLA) scaffold and cultured in a bioreactor while exposed to pulsatile flow, towards maturation into contractile smooth muscle tissue. We evaluated the effects of pulsatile flow on cellular organization as well as elastin expression and assembly in the engineered tissue compared to a static control through immunohistochemistry, gene expression and functionality assays. We show that culturing under pulsatile flow resulted in organized and functional hiPSC derived smooth muscle tissue. Immunohistochemistry analysis revealed hiPSC- smooth muscle tissue with robust, well -organized cells and elastic fibers and the supporting microfibril proteins necessary for elastic fiber assembly. Through qRT-PCR analysis, we found significantly increased expression of elastin, fibronectin, **and collagen I**, indicating expression of necessary extracellular matrix components. Functionality assays revealed that hiPSC- smooth muscle tissue cultured in the bioreactor had an increased calcium signaling and contraction in response to a cholinergic agonist, significantly higher mature elastin content **and improved mechanical properties** in comparison to the static control. The findings presented here detail **an effective** approach to engineer elastic human vascular smooth muscle tissue with the **enhanced** functionality for tissue engineering and regenerative medicine applications.

Keywords: elastin, elastic fibers, human induced pluripotent stem cells, smooth muscle cells, bioreactors, tissue engineering, regenerative medicine

Introduction

Human induced-pluripotent stem cells (hiPSCs) have become of interest as patient-specific therapeutic tools to complement tissue engineering scaffolds. With the ability to differentiate to cells of the vascular lineage with defined phenotypes, hiPSCs could potentially be used to create patient-specific tissue-engineered blood vessels (TEBVs). However, hiPSC-derived vascular cells must exhibit high extracellular matrix (ECM) production and organization in order to withstand the hemodynamic stresses of the cardiovascular system, as well as the ability to respond to appropriate physiological stimuli in order to be considered for use in TEBVs [1]. In blood vessels, smooth muscle cells (SMCs) play a key role in adjusting the ECM in response to pulsatile flow and are essential for enhancing the mechanical properties of TEBVs to adapt to the hemodynamic stresses of the native tissue [2] and [3]. Vascular SMCs display a unique capability of shifting their phenotype along a continuum between a fully mature, contractile phenotype and a dedifferentiated synthetic phenotype. In the contractile phenotype, SMCs possess an elongated, spindle-like appearance [4], display reduced migrating and proliferative capabilities, focal adhesion molecules [5] that aid in the maturation of the elastic fibers in the ECM [6] and the contractile proteins necessary for interaction with the ECM and small molecular signals [4]. The synthetic phenotype is triggered by vascular lesions and characterized by losses of cell morphology, extensive rough endoplasmic reticulum to support high secretions of ECM proteins such as elastin and collagen and high proliferation and migration [4] and [7]. The ability to maintain the contractile phenotype is a major problem in TEBVs as a common cause for graft failure occurs when SMCs are recruited to grafts and begin to lose their contractile phenotype, leading to over proliferation of cells and restenosis of the blood vessel [7] and [8]. With these considerations in mind, it is crucial for TEBVs to have a well-defined medial layer consisting of vascular smooth muscle tissue with contractile SMCs embedded in a properly developed ECM. In the ECM of vascular SMCs, elastin is the predominant protein comprising up to 50% of the dry weight and contributes to the elasticity and compliance in blood vessels [9] and [10]. SMCs secrete the precursor molecule tropoelastin and organize it into aggregates along the cell surface. Tropoelastin aggregates are then formed into mature, insoluble elastic fibers upon interaction with the supporting microfibril proteins, fibrillin-1 and 2, and fibulin-4 and 5 [6]. **The fully mature elastic fibers are then organized transverse to the direction of flow to allow for restoration of original configurations in response to hemodynamic stresses [11].** The contribution of mature, insoluble elastin in blood vessels extends beyond structural roles as well organized elastic fibers inhibit SMC proliferation and is therefore a potential solution for the maintenance of the contractile phenotype [12]. While soluble, precursor elastin is produced in the proliferative, synthetic phenotype [13], it is through the maturation into elastic fibers that the maintenance of the contractile phenotype can occur [14]. Therefore, the synthesis of elastin and its assembly into mature, organized fibers in vascular smooth muscle tissue is crucial for maintaining the structural and morphological integrity of blood vessels.

The use of bioreactors in tissue engineering applications has recently increased due to their ability to expose samples to biophysical cues that mimic in vivo conditions, resulting in a more physiologically compatible tissue substitute [15]. Common approaches include seeding cells onto a scaffold in static conditions and then transferring the structure into a bioreactor that mimics physiological conditions. Biochemical cues from the media and physical regulatory signals through the dynamic culture conditions of the bioreactor can signal the cell to produce ECM and organize it in accordance to culture conditions [15].

Given the importance of mature elastic fibers in vivo and their potential use in vascular tissue engineering, smooth muscle tissue derived from hiPSCs must exhibit proper ECM production, maturation, and functionality in order to be considered for use in regenerative medicine. In order to achieve this goal, SMCs must produce and mature substantial amounts of elastin and its supporting microfibril proteins to allow for proper ECM development, and a proper bioreactor with the physiologically relevant biophysical cues must be used to induce ECM secretion and maturation into organized structures. By differentiating hiPSCs into smooth muscle cells, seeding them onto a hybrid poly(ethylene glycol) dimethacrylate/ poly(L-lactide) (PEGdma-PLA) scaffold, and introducing them to pulsatile flow in a bioreactor, we have engineered functional, mature human vascular smooth muscle tissue with abundant elastic fibers **and enhanced mechanical properties**. This study reveals further insight into the effects of biophysical cues on elastin synthesis and maturation and **highlights the potential use of vascular smooth muscle tissue with robust elastic fibers** in cardiovascular tissue engineering and regenerative medicine.

Materials and Methods:

3D Scaffold Fabrication

Electrospun scaffolds were fabricated according to a previously established protocol by dissolving equal proportions of PEGdma (687529; Sigma-Aldrich, Steinheim, Germany) and PLA (93578; Sigma-Aldrich) with 1% photoinitiator (410896; Sigma-Aldrich) in 1,1,1,3,3,3 hexafluoro-2-propanol (804515; Merck, Darmstadt, Germany) [16]. The polymer solution was then electrospun into a fiber mat employing a customized electrospinning device, a 20-gauge nozzle, a flat collector, an electrode distance of 18 cm and 18 kV. All scaffolds were cross-linked with ultraviolet light (365 nm) and coated with hyaluronic acid (HA) prior to cell seeding.

Cell Culture

Human induced pluripotent stem cells (hiPSCs) BC1 line were kindly provided by Dr. Cheng, JHU School of Medicine. Undifferentiated hiPSCs were maintained on an inactivated mouse embryonic feeder fibroblast feeder layer. Growth media for the hiPSCs comprised of 80% DMEM/F12 (GlobalStem), 20% KnockOut Serum Replacement (Invitrogen, Carlsbad, CA, USA) and 5ng/mL basic fibroblast growth factor (bfgf; Invitrogen). Cells were passaged every 3-5 days using 1 mg/mL Type IV collagenase (Invitrogen). Media changes were done daily.

Vascular Contractile Smooth Muscle Cell Differentiation

We followed an established differentiation protocol [17], [18], and [19]. The 30-day stepwise differentiation of hiPSCs was first performed with digestion with TrypleE (Invitrogen) and the use of a 40µm mesh strainer (BD Biosciences, San Jose, CA, USA) to separate colonies into individual cell suspensions. Cells were then seeded onto T-150 cell culture flasks (Falcon) previously coated with collagen type IV (R&D Systems, Minneapolis, MN, USA) at a seeding density of 5×10^4 cells/cm². The cells were then cultured for 6 days in a differentiation media comprised of base media alpha-MEM, 10% FBS (Hyclone), and 0.1 mM β-mercaptoethanol (Invitrogen) with daily media changes. On day 6, the cells were again digested with TrypleE,

separated with a 40µm strainer and seeded at a cell density of 1.25×10^4 cells/mL on previously coated collagen type IV T-75 flasks, and were further differentiated for an additional 6 days into smooth muscle like cells (SMLCs) in the same differentiation but supplemented with 1 ng/mL TGF-β1 (R&D Systems) and 10 ng/mL PDGF-BB (R&D Systems). On day 12, cells were digested with TrypleE, counted and then seeded onto PEGdma-PLA scaffolds mounted on CellCrown 6-well inserts (Sigma Aldrich, St. Louis, MO, USA) at a density of 1.5×10^6 cells/scaffold in quiescent media based on base media alpha-MEM, with 0.1 mM β-mercaptoethanol (Invitrogen), 1 ng/mL TGF-β1 (R&D Systems) beta mercaptoethanol (0.25 mL) and 0.5% FBS (Hyclone). The inserts were used for both static and dynamic controls and were used to help cells adhere onto the scaffolds. Prior to seeding, the scaffolds were first mounted onto the CellCrown inserts, sterilized for 15 minutes in 70% ethanol, rinsed three times with Dulbecco's Phosphate Buffered Saline (DPBS(-); Life Technologies, Carlsbad, CA, USA), then coated with a 1:5 mixture 1% HA (5011; Advanced BioMatrix Inc., San Diego, USA) and DPBS(-) and then incubated at 37 degrees centigrade for three hours. All samples were left to incubate for one day (day 13) after seeding in static conditions to allow for proper cell adhesion. After this incubation period (day 14), samples were evenly divided into two groups and either continued to be incubated under static conditions or subjected to peristaltic flow in the bioreactor system for the remainder of the differentiation (day 30).

Bioreactor System:

Bioreactors for the system were developed as previously described [20]. Shear stress distributions, Reynolds numbers and pump settings were previously calculated and simulated, with Reynolds number shown to be approximately 1, and shear stress between 3×10^{-4} and 11×10^{-4} dyne cm^{-2} at a flow rate of 0.74 mL min^{-1} [20]. In order to use the bioreactor system to our studies, several adjustments were made to the system so that it could be adapted to maintain the differentiation and survival of SMLCs. For each sample in the pulsatile flow group, the CellCrown insert was first removed and the sample was then carefully transferred to a one-chamber perfusion bioreactor connected to a closed tubing system, which included a 30 mL reservoir with attached filter for gas exchange (SLGP033RS; Life Technologies). Media was pumped throughout the system with a high precision, multi-channel, peristaltic pump (78023-02; Ismatec, Glattbrugg, Switzerland). Upon transferring to the bioreactor, the flow rate was initialized to 0.37 mL/min for the first day and then increased to 0.74 mL/min the following day. Cells seeded on the scaffolds in the flow group were directly exposed to pulsatile flow for the remainder of the differentiation period. Media changes were performed on the bioreactors every 6 days until conclusion of the experiment.

Immunohistochemistry staining and imaging

Samples were prepared for immunostaining by first fixing with 4% paraformaldehyde for 15 minutes, washing with DPBS(-), permeabilizing with 0.1% Triton-X (Sigma-Aldrich), washing again with DBPS(-) and then blocked for 30 minutes using a previously established blocking agent [21]. After blocking, samples were incubated overnight with anti-human elastin (1:200; Abcam), anti-human collagen I (1:100), anti-human collagen III (1:200), anti-human fibronectin (1:200), anti-human fibrillin-1 (1:1000), anti-human fibrillin-2 (1:1000), anti-human fibulin-4 (1:500), anti-human fibulin-5 (1:500), anti-human smooth muscle myosin heavy chain (1:100;

Dako, Glostrup, Denmark) and anti-human SM-22 α (1:200; Abcam). Samples were rinsed 3 times at 5 minutes per rinse with DPBS(-) and then incubated with Alexa 647 conjugated Phalloidin (1:250; Molecular Probes, Eugene, OR) and either anti-mouse Alexa 546 conjugate (1:250; Molecular Probes, Eugene, OR) or anti-rabbit Alexa 488 conjugate (1:250; Molecular Probes) for 45 minutes. Samples were then once again rinsed 3 times at 5 minutes per rinse with DPBS(-) and then incubated with DAPI (1:1000; Roche Diagnostics) for 15 minutes. After rinsing three more times with DPBS(-), samples were mounted with fluorescent mounting medium (Dako). Immunofluorescently conjugated samples were then examined using laser scanning confocal microscopy (LSM 780; Carl Zeiss, Jena, Germany).

Quantification of Cell Nuclei and Extracellular Matrix Alignment

The alignment of cell nuclei, cytoskeleton and ECM proteins for flow samples in response to peristaltic flow as well as those of the static control samples were analyzed through the ImageJ plug-in FibrilTool as the plug-in is capable of showing the average orientation of any region of interest in a raw image obtained from various forms of microscopy [22]. The program has been shown to work for microtubules and possesses the capabilities to quantitatively analyze the fibrillar structures in our studies such as elastin, fibrillin-1, fibrillin-2, fibulin-4, fibulin-5, collagen I, collagen III and fibronectin. Once an image was obtained through confocal microscopy, it was exported per instructions for analysis for Fibril-Tool and analyzed in ImageJ, where the elastin, collagen, fibronectin, f-actin, nuclei and microfibril (fibrillin or fibulin) orientations were then calculated. N=3 tissue samples were chosen, and 5 regions of interest were selected for each protein (n=5).

Calculation of tissue thickness

Thickness of each tissue sample was calculated by first taking into account the thickness of the scaffold. After imaging for immunohistochemistry, the depth of the Z-stacks was measured on Zen Black (Carl Zeiss AG, Oberkochen, Germany) to determine the contribution of the cells and ECM on the overall tissue thickness.

DNA Isolation and quantification

Samples were sectioned and weighed prior to extraction of **genomic** DNA. DNA was then extracted using the PureLink® Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's protocol. An ultraviolet spectrophotometer was then used to measure the DNA concentration and then normalized to the mass of the sample, thus expressing the concentration of DNA. We generated a standard curve for cell number by using defined quantities of day 30 differentiated SMCs to estimate cell number in the tissue samples.

RNA Isolation and quantitative PCR

Total RNA was extracted using TRIzol (Gibco, Invitrogen) according to manufacturer's protocol. An ultraviolet spectrophotometer was used to measure the RNA concentration for each sample and verify that there was no DNA contamination. RNA (1 µg per sample) was transcribed using the reverse transcriptase M-MLV (Promega Co., Madison, WI) and oligo(dt) primers (Promega), according to the manufacturer's protocol. TaqMan Universal PCR Master Mix and Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) was used for *ELN*, *LOX*, *FNI* and *GAPDH*. qRT-PCR using TaqMan reagents was performed with an Applied Biosystems Step-One Real Time PCR System (Applied Biosystems) according to the manufacturer's protocol. The relative quantity values were normalized to the amount of GAPDH in the same sample by using the standard curve provided. For each primer set, comparative tomography (Applied Biosystems) was used to calculate the expression differences between samples. Values of the expression levels were then calculated, normalized to the static control and graphed with the average and standard deviation displayed.

Imaging of Calcium Cycling

Tissue samples were incubated with 5µM of the membrane-permeable Ca²⁺-sensitive dye Fluo-4 AM (Invitrogen) and for 30 min at 37°C and 5% CO₂ in a humidified incubator. The solution was removed and then rinsed with working media and then left to incubate in media for an additional 30 minutes to allow for proper deesterification. Laser excitation was 488 nm and emission was recorded at 516 nm. Recordings for each replicate and cell type were obtained using the same confocal settings. The fluorescence response after addition of 10⁻⁵ M of carbachol was then recorded for 20 minutes [23]. Fluorescence responses were normalized to the baseline (F₀) and plotted for (n=25) regions of interest for each condition. The maximum fluorescence response (F_{max}) normalized to the baseline reading was also recorded for each sample.

Assessment of Contractility

Studies to analyze contraction were performed by analyzing the tissues' responsiveness to 10⁻⁵ M of the drug, carbachol (Calbiochem, Darmstadt, Germany). Calcein, a cytoplasm-viable fluorescence dye was used to visualize the cells and a series of time-lapse images using confocal microscopy. The change in tissue area then calculated by taking the percent difference in area at the introduction of drug and the end of the study (at time 20 min). In addition to taking the measurements at the tissue level, (n=25) individual measurements were taken at the cellular level for further quantification and comparison of results to previous studies.

*Quantification of **Precursor and** Mature Elastin*

The Fastin Elastin Assay (Accurate Chemical & Scientific Corporation, New York) was used to measure the insoluble (**mature**) elastin content in the same manner as a previous study, but with 2 extraction instead of 4 due to the negligible amount of elastin extracted after 2 extractions [24]. Samples were sectioned (5mm) and weighed in order to first record elastin concentration per milligram of tissue. Samples were digested with 0.25 M oxalic acid at 100°C for 1 hr twice. The assay was then performed according to the manufacturer's instructions and the elastin content was calculated per the standard curve and normalized to the wet tissue weight. Soluble

(precursor) elastin was measured from the media within the reservoir, proceeded according to the manufacturer's instructions and calculated from the generated standard curve.

Mechanical Testing

Mechanical properties of the tissue samples were characterized via uniaxial tensile testing and performed using a RSA-G2 Solids Analyzer (TA Instruments, New Castle, DE). Samples were sectioned into strips and the necessary dimensions were recorded prior to tensile testing. HA-coated acellular scaffolds were used as an experimental control. Stress-strain curves were generated for each sample and the tangential modulus was obtained by calculating the slope in the Hookean region. The maximum stress value upon fracture was recorded and defined as the ultimate tensile strength (UTS).

Statistical Analysis.

Image analysis, qRT-PCR and functionality assays were performed with at least 3 different tissue specimens (N=3) for both culture conditions. In the analysis of images via FibrilTool (n=5), readings were taken for each protein. For the contractility studies and calcium imaging, within each individual tissue sample, (n=25) readings were taken for analysis. Statistical analysis was performed on GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). One-way analysis of variance (ANOVA), unpaired two-tailed t-tests and Bonferonni corrections were performed when appropriate using GraphPad Prism 6 (GraphPad Software Inc.). Significance levels were set at *p<0.05, **p<0.01 and ***p<0.001. All graphical data is represented as mean $\pm$ standard error mean unless noted otherwise.

Results

Proper vascular smooth muscle tissue maturation in bioreactors. Human iPSCs were differentiated in culture for 12 days into SMLCs using the previously established protocol [17], [18] and [19]. The SMLCs were then harvested, seeded on PEGdma-PLA scaffolds coated with HA, and inserted into one-chamber perfusion bioreactor for continued differentiation in quiescent media up to day 30 (**Fig. 1**). These three-dimensional (3D) culture conditions, exposed the cells to $3\text{--}5.5 \times 10^{-4}$ dyne cm^{-2} shear [20] while in low serum media, and supported their differentiation and maturation into contractile SMCs.

Nuclear and the cytoskeletal alignment perpendicular to flow. Laser scanning confocal microscopy was used to qualitatively analyze the morphology of the cytoskeleton and nuclei in response to pulsatile flow. We observed that compared to the static control samples, samples exposed to flow exhibited organized cytoskeletal and nuclear alignment transverse to flow (**Fig 2A**). In addition, the cytoskeleton morphology appears more robust than that of the static samples. One of the defining characteristics of SMCs is their tendency to align perpendicular to an applied flow [25]. To determine the cellular alignment to flow, we next quantified the contact angle of the cytoskeleton and nuclei. We found that cytoskeleton and nuclei angle measurements for dynamic samples again differed significantly from those of static samples with average angle measurements of 92.9 ± 12.1 degrees and 91.2 ± 11.9 degrees for the cytoskeleton and nuclei respectively. Cytoskeleton and nuclei angle measurements for static samples also showed a high

variability between samples with 38.1 ± 23.9 degrees and 50.2 ± 18.0 degrees, indicating random alignment of the cells (**Fig. 2B**). Through analysis of the contact angles, it is evident that the use of the bioreactor aids in cell alignment, ensuring development in a manner similar to physiological conditions.

Insignificant differences in cell and DNA content with thicker tissue in the bioreactor. DNA and cell content were quantified in order to confirm that the changes observed in the study were not a result of the difference in cell number. Quantification of the DNA and cellular content reveal that at the end of the culture period, the cell number is comparable in both static and dynamic systems (**Fig. 2C**). Interestingly, the hiPSC-smooth muscle tissue generated in the bioreactor was thicker than the hiPSC-smooth muscle tissue cultured in the Petri-dish ($25.4 \pm 2.88 \mu\text{m}$ vs $16.4 \pm 4.56 \mu\text{m}$).

Organization of collagens, fibronectin, elastin and microfibrils transverse to flow. We continued to analyze the organization of the hiPSC derived smooth muscle tissue. We first analyzed whether our modified differentiation protocol, which include seeding of SMLCs on 3D scaffold and culturing in a bioreactor under pulsatile flow, did not compromise the differentiation outcome of the SMLCs. Indeed, much like previous studies [19], SMLCs matured on the 3D scaffold in static or dynamic conditions, express both SM-22 α and smooth muscle myosin heavy chain (SM-MHC; **Fig 3A**). We then continued to analyze the presence and morphology of the elastin and microfibril proteins. We found that elastin, collagen type I and III, and fibronectin are present in both static and dynamic conditions, further evidence for the contractile phenotype of the hiPSC-SMC derivatives on the 3D scaffold (**Fig. 3B**). However, we found that these ECM proteins produced by contractile SMCs exposed to flow were more robust and displayed an organized, fiber-like morphology (**Fig. 3B**). We further note that these ECM proteins are deposited extracellularly, specifically in the dynamic conditions (**Supplementary Figure 1**). The two components necessary for proper elastic fiber assembly are elastin as well as the microfibril proteins, fibrillin-1 and fibulin-4. Fibrillin-1 is thought to serve as a template for elastic fiber assembly while fibulin-4 is believed to act as a cross-linker for tropoelastin aggregates that may coalesce on the cell surface [6]. Consistent with the presence of elastin we found the expression of the supporting microfibril proteins necessary for proper mature elastic fiber assembly and maturation (**Fig. 3B**). The presence of these proteins in both culture conditions demonstrate that the hiPSC-SMCs are capable of producing the necessary components of the ECM consistent to that found *in vivo*. Overall, the fibrillar appearance of the various ECM proteins in the dynamic samples show that the use of pulsatile flow enhanced the formation of organized mature elastic fibers and the microfibrils transverse to flow (**Fig. 3B**). When quantitatively analyzing the alignment of elastin and the microfibrils of the dynamic samples, we found that the ECM proteins were aligned nearly perpendicular to flow with contact angles of (94.8 ± 13.9 , 94.5 ± 12.1 , 82.9 ± 11.0 , 86.6 ± 9.6 , 69.9 ± 8.3 , 84.4 ± 3.6 , 84.9 ± 2.9 , and 90.58 ± 2.8) degrees for elastin, fibrillin-1, fibrillin-2, fibulin-4, fibulin-5, collagen 1, collagen III and fibronectin respectively (**Fig. 3B**). The angles differed significantly for static samples with measurements of (41.8 ± 22.15 , 33.2 ± 16.5 , 29.9 ± 9.3 , 30.7 ± 19.1 , 33.8 ± 13.3 , 48.2 ± 7.8 , 59.4 ± 6.7 , and 42.5 ± 7.1) degrees for the same 5 proteins necessary for proper vascular ECM development (**Fig. 3C**). The higher variation in angle measurements for static samples denoted by the different average angle measurements and high standard error, indicates that the ECM is not an organized

architecture. It is evident that use the effect of pulsatile flow is significant in impacting the ability of ECM proteins to be arranged transverse to flow.

Upregulation of elastin, fibronectin and collagen I expression in response to flow. We next assessed whether the pulsatile flow bioreactors aided the cellular production of elastin and other key components of elastic fiber assembly. Elastin fiber assembly begins with the secreted monomer tropoelastin. Tropoelastin is primarily cross-linked by lysyl oxidase (LOX), by which it begins to mature into a mature elastic fiber [6] and [9]. Fibronectin (FN1) is a protein that serves as an adaptor between membrane spanning integrins and fibrillin-1, the major protein that serves as a scaffold for elastic fiber deposition [26] and [27]. **In addition to elastin, collagens types I and III (Col I and Col III respectively) are present in the ECM of vascular smooth muscle tissue and are important in conferring strength in vessel walls.** Measuring the mRNA expression levels of these essential ECM components for elastic fiber assembly, we found that the hiPSC derived vascular smooth muscle tissue **engineered in the bioreactors** have significantly higher expression levels of elastin, fibronectin and **Col I** compared to the static controls (**Fig. 4A,B and D**). There were no significant differences in expression levels of LOX **and Col III** between the two conditions (**Fig. 4C and E**).

Enhanced functionality of the hiPSC-derived smooth muscle tissue: Calcium cycling.

Functionality of the tissues was first analyzed using calcium intake in response to the cholinergic agonist, carbachol. Contractions in SMCs are known to be induced by a rise in the concentration intracellular calcium ions that interact with calmodulin to initiate contraction [28] and [29]. Using fluorescence imaging to examine the calcium cycling responses to the drug, we found that carbachol calcium cycling was increased in the dynamically cultured samples, which was confirmed through fluorescence intensities normalized to a baseline reading over time as well as a maximum fluorescence response normalized to the baseline reading (**Fig. 5A-B**). Dynamically cultured constructs had a significantly higher maximum response in comparison to the static controls (1.7 ± 0.2 vs. 1.2 ± 0.08). Greater fluorescence responses are correlated with intracellular calcium signaling, and from these studies it is apparent that dynamically cultured tissues exhibit a greater calcium intake on the molecular level. **Contractility.** The contractility of tissues from the different culture conditions was measured in response to carbachol. We found a significantly greater response from samples cultured in the bioreactor as shown by the greater changes in area on the tissue level (**Fig. 5C-D**). In assessing the functionality of SMCs, contractility in response to chemical stimuli is an important requirement for the long term success of the constructs in an *in vivo* environment. With the greater change in area on the tissue as well as cellular level, it is apparent that the hiPSC derived vascular smooth muscle tissue is more contractile and as a result, more functional on an intracellular level compared to samples cultured in the static environment. When analyzing the change in area on the tissue level, the flow samples displayed a more uniform contraction and when visualized with Calcein, revealed a highly organized structure with the appearance of tissue rather than a random arrangement of cells displayed by the tissues samples from the static conditions (**Video S1**). This uniform contraction and organized tissue structure will aid in higher likelihood of integration into the host tissue upon implantation. **Precursor and mature elastin concentrations.** We measured both the precursor (soluble) and mature (insoluble) elastin contents. **While no significant difference was observed in the precursor elastin concentrations between the static and dynamic cultures (Fig 5E),** it was found that **tissue engineered** in the bioreactor had significantly higher mature **elastic**

fiber concentrations than those found in the static tissue samples ($14 \pm 5 \mu\text{g}/\text{mg}$ tissue vs. $55 \pm 14 \mu\text{g}/\text{mg}$ tissue); (Fig 5F). **Mechanical testing.** Finally, we compared the mechanical features of the scaffold alone, constructs cultured in static conditions and in bioreactor. Uniaxial tensile testing revealed a significant improvement of the mechanical properties of the engineered tissue with the addition of cells and a further improvement of these properties when cultured in the bioreactor (Fig 5G). Constructs that were cultured in static conditions exhibited an improvement in stiffness ($215 \pm 53 \text{ kPa}$ vs. $70.6 \pm 4.2 \text{ kPa}$); (Fig 5H) and UTS ($408 \pm 21 \text{ kPa}$ vs. $166 \pm 5 \text{ kPa}$); (Fig 5I) compared to the acellular scaffolds. Constructs cultured in the bioreactors showed a greater increase in stiffness (339 ± 67), and a significant increase in ultimate tensile strength (670 ± 136) in comparison to the scaffold alone and static tissue respectively, demonstrating the merits of using dynamic culture conditions. The mechanical properties of the dynamically cultured tissue is comparable to murine blood vessels and even possesses some of the biomechanical properties found in human vasculature [30] and [31].

Discussion:

The objective of this study was to demonstrate a potential method for the engineering of vascular smooth muscle tissue with robust, mature elastic fibers. In the medial layer of blood vessels, SMCs produce a complex ECM comprised of both elastin and collagen which dictate the mechanical properties of blood vessels [2]. Previous studies have shown blood vessels with sufficient collagen production and maturation [32]. However, obtaining mature elastic tissue in vitro has proven very difficult and is a crucial component for the long-term patency of TEBVs [33]. Few studies have been able to show significant improvements in increasing the mature elastin concentrations in constructs [20], [24] and [34]. By using hiPSCs and introducing them to a dynamic culture environment with pulsatile flow along their development into contractile SMCs on 3D scaffold, we have demonstrated an approach to create human vascular smooth muscle tissue with a greater ECM content and assembly than that found in a previous study using mature vascular SMCs [20]. The use of hiPSCs in engineering smooth muscle resulted in a higher elastic fiber content found in a previous study [24], thus demonstrating the merits of using hiPSCs for patient-specific therapies. A plausible explanation for this finding may be due to the cumulative effect of pulsatile flow early in the differentiation of the SMCs, thus allowing for an increased elastic fiber content upon maturation. Further studies should be done to validate the effects of pulsatile flow along different time points of development in these cells.

When characterizing SMCs, the defining features of the contractile phenotype are a long spindle-like appearance, high expression of mature ECM proteins such as elastin, the collagens, fibronectin and the microfibrils, SM-MHC indicating the presence of the contractile apparatus within cells and the SMC marker SM-22 α . We first showed that the contractile hiPSC-smooth muscle constructs developed into highly aligned, and organized smooth muscle tissue in response to pulsatile flow. We then demonstrated the presence of contractile SMC phenotype markers, SM-22 α and SM-MHC in the hiPSC-smooth muscle constructs. Samples were expected to express the microfibril proteins, fibrillin-1, fibrillin-2, fibulin-4 and fibulin-5, which are necessary components for the assembly of mature elastic fibers [6]. By detecting the key proteins found in the ECM of vascular smooth muscle tissue, it is evident that the hiPSC-smooth muscle constructs have properly developed elastin, collagen as well as the necessary components for elastic fiber assembly. A common characteristic of SMCs in relation to shear stress is an

alignment transverse to flow [2] and [25]. Quantification of the alignment further demonstrate highly organized elastin, collagens - I and III, fibronectin, microfibril proteins and SMCs that align in the direction perpendicular to flow, further indicating proper formation of tissue structure in hiPSC derived vascular smooth muscle tissue.

We next examined the extent to which pulsatile flow impacted the system. We found significantly higher mRNA expression levels of elastin, fibronectin and Col I, suggesting that the hiPSC-vascular smooth muscle tissue is populated with SMCs expressing the proper components of vascular smooth muscle ECM. As the bioreactor culture did not improve the expression level of collagen III, additional studies should be performed to improve this aspect in engineered hiPSC- smooth muscle tissues. Several studies have suggested that fibronectin serves as a potent inducer of the synthetic phenotype in SMCs [35], [36] and [37]. However, it must be noted that the fibronectin being referred to in these studies is fibronectin derived from the plasma. Fibronectin plays a variety of roles depending on its origin whether it be in the plasma fibronectin derived from hepatocytes, or the cellular fibronectin synthesized in a variety of cell types [26]. Cellular fibronectin is deposited onto cell surface integrins where it plays a role in binding to ECM components such as fibrillin-1 and 2 [26]. Furthermore, it has been shown that the assembly of fibrillin-1 for elastic matrices is dependent upon a fibronectin network [27]. From our study it is evident that the fibronectin analyzed is not the type that promotes the synthetic phenotype, but rather the kind that promotes the assembly of mature elastic fibers, which will maintain the contractile phenotype [14]. Overall, our results show that culturing hiPSC-SMCs in a 3D scaffold under pulsatile flow enhanced the expression of key components of the vascular ECM.

In order to assess the functionality of the engineered tissue at both the tissue and cellular levels, intracellular calcium cycling, contraction in response to a cholinergic agonist, precursor and mature elastin quantification as well as mechanical properties were analyzed. As SMCs reside in a dynamic environment dictated by mechanical and chemical cues, it is crucial for engineered tissue to be able to respond to such stimuli. Functionality should entail the mature elastic tissue content, which correlates to proper ECM development [38] the ability to respond to the various cues found in the surroundings [7] as well as the mechanical properties to withstand the hemodynamic stresses of the cardiovascular system. As shown by analyzing the calcium cycling and measuring the change in area after stimulation with carbachol, the engineered hiPSC-smooth muscle tissue exposed to pulsatile flow exhibited significant improvements in responsiveness over the static control samples. Furthermore, we have shown that both individual cells and the entire tissue construct exhibited greater and more synchronous contractions when cultured in the bioreactor. For quantification of the elastin content, we used the Fastin Elastin Assay to quantify the precursor and mature elastin content of the engineered tissue. While the assay involves solubilizing the mature elastin, we have shown this differentiation and maturation approach yields a highly pure contractile phenotype population, which primarily displays the insoluble form of elastin. This, in addition to carrying the differentiation to 30 days allows us to make the claim that the elastin in the tissue is primarily mature insoluble elastin with amounts of soluble elastin below the detection limits of the assay [18]. In order to account for the soluble elastin content in our system, we also measured elastin readings from the media collected from media reservoirs or cell culture wells [24].

The results of the elastin quantification suggest that while the difference in concentration of the precursor **elastin** is not significant between the culture conditions, the use of pulsatile flow allows for the enhanced cross-linking of **tropoelastin**, leading to higher concentrations of mature elastin in the engineered tissues. **Significant differences in insoluble elastin concentrations in conjugation with insignificant differences in DNA, cellular content, and soluble elastin concentrations**, suggest that biophysical cues such as pulsatile flow aid in the cross-linking and maturation process of the elastin. In addition, insignificant differences in the expression of LOX further supports the claim that pulsatile flow aids in the cross-linking of precursor elastin into mature elastic fibers. A plausible theory may be that pulsatile flow enhances the cross-linking efficiency of elastin through stretching of the amorphous structure of tropoelastin, thus exposing additional binding sites for interaction with LOX and aiding its ability to be cross-linked into mature elastic fibers.

Mechanical testing revealed enhanced mechanical properties of the vascular hiPSC-smooth muscle tissue cultured in the bioreactor. As vascular smooth muscle tissue is primarily responsible for defining the mechanical characteristics blood vessels, it is imperative that the engineered tissues exhibit the proper strength and stiffness needed to withstand the physiological environment. With a significant improvement in the modulus and UTS with the addition of cells onto the scaffold, it is evident that the ECM synthesized by the hiPSC-SMCs in a tissue engineering constructs impacts the mechanical properties on a macroscopic level. Furthermore, similar cellular content yet differing tissue thickness as well as the limited contribution of SMCs in the mechanical properties of blood vessels [39] support the notion that the differences in the mechanical properties found amongst the three experimental conditions are a result of increased ECM deposition. With a greater improvement in mechanical properties found to be a result of the dynamic culture in the bioreactor, the ability to recapitulate features of the physiological environment *in vitro* provided the ability to enhance the functionality of the engineered tissue.

Further work should be done to address the stability of the scaffold for prolonged culture periods and **aid in its ability to be elongated greater distances**. In addition, other biophysical cues can be used to complement pulsatile and their effect on ECM production and maturation in an effort to further recapitulate the physiological environment of vascular SMCs. While we have engineered vascular smooth muscle tissue, we can now expand on the complexity of the system to include co-culture studies with endothelial cells in order to continue engineering vascular tissue **with additional functionality** that can withstand the physiological pressures and provide the vascularization needed for other tissue engineering constructs [40].

In conclusion, we have shown a novel approach to enhance elastic fiber expression and maturation in vascular smooth muscle tissue derived from hiPSCs. The findings of this study detail a method for creating vascular smooth muscle tissue with mature elastin that could contribute to the elasticity of TEBVs as well as ensure the contractile phenotype is maintained, thus reducing risks associated with over proliferation of vascular SMCs. Analyses of the alignment and morphology of the cytoskeleton, nuclei, elastin and microfibrils, the genetic expression and assessment of the functionality reveal contractile vascular smooth muscle tissue that is responsive to both mechanical and chemical cues, supporting the use of this physiologically relevant tissue for applications in regenerative medicine and vascular tissue engineering.

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Disclosures

The authors of this paper declare no conflicts of interest.

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Figure Legends:

Figure 1: Experimental set up. Schematic depicting the timeline for the formation of contractile 3D smooth muscle tissue derived from hiPSCs including the differentiation steps and bioreactor setup. Drawing not to scale.

Figure 2: hiPSC-SMCs align perpendicular to the flow. (A) 3D hiPSC-SMCs cultured in static and dynamic conditions were stained at the end of the 30 days for F-actin (left; in magenta) and nuclei (right, in blue) and (B) F-actin and nuclei angle of orientation was quantified in both conditions. (C) DNA and cellular content was quantified at the end of the 30-day differentiation for both conditions. White arrows indicate the direction of flow. Scale bars =50 μ m. Significance levels were set at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. All graphical data was reported.

Figure 3: hiPSC differentiation into contractile SMCs as well as elastin and microfibril organization. hiPSC -vascular smooth muscle tissue cultured in static and dynamic conditions were stained at the end of the 30 days for: (A) contractile markers including SM-22 α (in green) and SM-MHC (in red); and (B) (Tropo-)Elastin, Collagen I, Collagen III (all in red), Fibronectin, Fibrillin-1, Fibrillin-2, Fibulin-4, Fibulin-5 (all in green). White arrows indicate the direction of flow. Scale bars =50 μ m (C) Alignment of elastin and microfibril were quantified in both conditions. Significance levels were set at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. All graphical data was reported.

Figure 4: Upregulation of elastin and fibronectin expression under flow. qRT-PCR was performed on hiPSC-vascular smooth muscle tissue cultured in static and dynamic conditions for (A) Elastin, (B) Collagen I, (C) Collagen III (D) Fibronectin and (E) Lysyl Oxidase. Significance levels were set at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. All graphical data was reported.

Figure 5: Enhanced functionality of engineered tissue demonstrated by increased calcium intake, contraction upon stimulation, elastin content and mechanical properties.

Functionality studies performed on the 3D hiPSC-SMCs cultured in static and bioreactor conditions including calcium cycling measured using Fluo-4 AM, detecting (A) fluorescent response normalized to the baseline reading and recorded for a given region of interest; and (B) maximum fluorescent response to the drug, carbachol normalized to the baseline fluorescent reading; Contractility: (C) Change in area in response to carbachol at the tissue level and (D) Change in area in response to carbachol at the cellular level; Mature Elastin Quantification: (E) soluble (precursor) and (F) insoluble (mature) elastin content; Characterization of Mechanical Properties: (G) Representative stress-strain plots of the acellular scaffolds (black), static culture tissue, and the bioreactor tissue. (H) The modulus and (G) UTS of each experimental condition were also reported. Significance levels were set at: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. All graphical data was reported.

Figure(s)

Figure(s)

A

B

C

Figure(s)

Statement of Significance

Obtaining robust, mature elastic fibers is a key obstacle in tissue-engineered blood vessels. Human induced-pluripotent stem cells have become of interest due to their ability to supplement tissue engineered scaffolds. Their ability to differentiate into cells of vascular lineages with defined phenotypes serves as a potential solution to a major cause of graft failure in which phenotypic shifts in smooth muscle cells lead to over proliferation and occlusion of the graft. Herein, we have differentiated human induced-pluripotent stem cells in a pulsatile flow bioreactor, resulting in vascular smooth muscle tissue with robust elastic fibers and **enhanced functionality**. This study highlights an effective approach to engineering elastic functional vascular smooth muscle tissue for tissue engineering and regenerative medicine applications.