PERIVASCULAR CELLS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS
USING BIOCHEMICAL AND BIOMECHANICAL STIMULI

by
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ABSTRACT

Blood vessels play a vital role in the body because they deliver oxygen and nutrients to all organs and tissues. Vascular smooth muscle cells (vSMCs) and pericytes are the two major classes of perivascular cells (PCs) that encircle blood vessels in order to allow proper vascular function within the body. However, injury or disease may alter the function of these cells, which often compromises the stability of blood vessels. Vascular engineering using human pluripotent stem cells (hPSCs) represents a potential therapy that seeks to derive functional PCs, with the ultimate goal of clinical translation. The ability direct hPSCs to PCs relies on the utilization of different in vitro biochemical and biomechanical approaches that recapitulate in vivo environments.

We first sought to understand the development and differentiation of vSMCs derived from hPSCs by guiding the maturation of vSMC derivatives towards either a contractile phenotype or synthetic phenotype using biochemical cues. While the synthetic phenotype is usually associated with embryonic vSMCs, in healthy adult vessels vSMCs commit to the mature contractile phenotype. The long-term differentiation of hPSCs, including the integration-free-induced PSC line, in high serum with platelet derived growth factor-BB (PDGF-BB) and transforming growth factor-β1, allowed us to induce the synthetic vSMC (Syn-vSMC) phenotype with increased extracellular matrix (ECM) protein expression and reduced expression of contractile proteins. By monitoring the expression of two contractile proteins, smooth muscle myosin heavy chain (SMMHC) and elastin, we show that serum starvation and PDGF-BB deprivation caused maturation towards the contractile vSMC (Con-vSMC) phenotype. Con-vSMCs differ distinctively from Syn-vSMC derivatives in their
condensed morphology, prominent filamentous arrangement of cytoskeleton proteins, production and assembly of elastin, low proliferation, numerous and active caveolae, enlarged endoplasmic reticulum, and ample stress fibers and bundles, as well as their high contractility. When transplanted subcutaneously into nude mice, the human Con-vSMCs aligned next to the host’s growing functional vasculature, with occasional circumferential wrapping and vascular tube narrowing. We controlled hPSC differentiation into synthetic or contractile phenotypes by using appropriate concentrations of relevant factors. Deriving Con-vSMCs from an integration-free human induced pluripotent stem cell (hiPSC) line may prove useful for regenerative therapy involving blood vessel differentiation and stabilization.

Next, we studied human perivascular development and functionality by performing direct comparisons between perivascular cell derivatives with the same genetic background. Distinguishing between perivascular cell types remains a hurdle in vascular biology due to overlapping marker expressions and similar functionalities. We studied contractile vSMCs, synthetic vSMCs, and pericytes derived from a common human pluripotent stem cell source. Using in vitro cultures, we show unique cell morphology, subcellular organelle organization (namely endoplasmic reticulum, mitochondria, and stress fibers), and expression of smooth muscle myosin heavy chain and elastin for each cell type. While differences in extracellular matrix deposition and remodeling were less pronounced, the multipotency, in vivo, migratory, invasion, and contractile functionalities are distinctive for each cell type. Overall, we defined a repertoire of functional phenotypes in vitro specific for each of the human perivascular cell types, enabling their study and use in basic and translational research. Clarifying and defining heterogeneities in vitro among perivascular cells could lead to improved
cell-based tissue regeneration strategies and a better understanding of human developmental processes.

Finally, we studied the effect of biomechanical strain on the ECM expression of vSMCs derived from hPSCs. The effects of two types of tensile strain on hPSC vSMC derivatives at different stages of development were examined. The derivatives included smooth muscle-like cells (SMLCs), mature SMLCs (mSMLCs), and contractile vSMCs (Con-vSMCs). All vSMC derivatives were exposed to transforming growth factor (TGF-β1) and cyclic uniaxial strain at 1Hz and 7% elongation using a deformable silicone substrate. Additionally, a custom engineered bioreactor was used to propel pulsatile flow through silicone tubing with an inner diameter of 300μm in order to generate cyclic circumferential strain on the hPSC derivatives. Stimulated hPSC-derivatives were analyzed for cell alignment and the expression of extracellular matrix (ECM) genes. All vSMC derivatives including SMLCs, mSMLCs, and Con-vSMCs aligned perpendicularly to the direction of cyclic uniaxial strain. Serum deprivation and short-term uniaxial strain had a synergistic effect in enhancing collagen type I, fibronectin, and elastin gene expression of derivatives. Furthermore, long-term uniaxial strain deterred collagen type III gene expression while long-term circumferential strain upregulated both collagen type III and elastin gene expression. Long-term uniaxial strain downregulated ECM expression in more mature vSMC derivatives while upregulating elastin in less mature vSMC derivatives. Overall our findings suggest that in vitro application of both cyclic uniaxial and circumferential tensile strain on hPSC- vSMC derivatives induces cell alignment and affects ECM gene expression. Therefore, mechanical stimulation of hPSC- vSMC derivatives using tensile strain may be important in modulating the phenotype and thus the function of vSMCs in tissue engineered vessels.
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“When you learn, teach, when you get, give.”

-Maya Angelou
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Chapter 1

OVERVIEW

1

1.1 Perivascular cells

1.1.1 Introduction

The lack of blood perfusion ultimately limits tissue function. The emerging field of vascular tissue engineering focuses on the development of technologically advanced solutions to regenerate blood vessels and restore blood perfusion for vascular disease treatment as well as in engineered tissues [1]. Incorporation of healthy vascular cells at sites of injury or into engineered constructs is a promising option toward the goal of blood vessel regeneration.

The two critical components of the vascular cellular makeup are perivascular cells (PCs) and endothelial cells (ECs). Perivascular cells – such as pericytes and vascular smooth muscle cells (vSMCs) – surround the inner endothelial lining, conferring support and stabilization. During vessel development, both pericytes and vSMCs are recruited to stabilize newly formed vasculature. PCs wrap around blood vessels and promote vessel maturation by preventing hemorrhaging or leaking of blood vessels [2, 3]. Though they share similar functions, the two PC types localize to disparate vessels. Mature vSMCs surround and circumferentially wrap around the inner layers of larger arteries and veins including the aorta, carotid artery, and the saphenous vein. In contrast, pericytes surround smaller blood vessels or microvasculature, such as capillaries, in which a single EC makes up the inner perimeter of the blood vessel, precapillary arterioles, and postcapillary venules [4]. The greatest density of pericytes is found in microvessels of the central nervous system such as the retina and brain. In the brain, pericytes associate with the continuous endothelium that contains selective tight junctions to form and regulate the blood brain barrier [5]. Inclusion of these two cell types
is crucial for successful regeneration of blood vessels. Furthermore, understanding distinctions between vSMCs and pericytes will enable improved therapeutics in a tissue-specific manner.

Blood vessel growth occurs via vasculogenesis or angiogenesis. Vasculogenesis, the formation of blood vessels de novo, transpires mainly during embryo development. In the adult, circulating progenitor stem cells (such as endothelial progenitors, hematopoietic stem cells, or stromal stem cells) have also been noted for their ability to contribute to vasculogenesis [6, 7]. Angiogenesis occurs in both embryos and adults, with vessels forming from preexisting ones [8]. In adults, most vessels are quiescent; active angiogenesis occurs in the placenta during pregnancy, in the cycling ovary, during organ growth, and during wound healing [8]. Perivascular cells exhibit important functionalities in stabilizing vessels sprouted from either of these mechanisms. Indeed, nascent vessels regress without perivascular involvement [9].

Blood vessel regeneration has extensively emphasized EC behavior and assembly; owing to their importance in vascular stability and functionality, PCs play an equally significant role in the development of novel regenerative strategies to regenerate vessels of various sizes (pericytes for capillaries and small microvasculature and vSMCs for larger vessels). Here, we discuss vSMCs and pericytes to provide the groundwork for their use and importance in regenerative medicine pursuits: their cellular characteristics, stem cell sources, and their interactions with ECs, as well as approaches to understand their complex behavior.

1.1.2 Cellular properties of vascular smooth muscle cells and pericytes

Depending on the vascular tissue engineering application needed, either vSMCs or pericytes are better suited and thus it is important to understand differences between the two types of PCs. One of the foremost distinctions between vSMCs and pericytes is
their proximity to the endothelium. In larger vessels, the basement membrane and the inner elastic lamina that contains extracellular matrix (ECM) separate vSMCs from the endothelium [10]. In contrast, pericytes are typically in direct contact with ECs within the endothelial basement membrane, minimizing the diffusion distance from blood to tissue while maximizing the exchange of nutrients and oxygen (Table 1.1.2) [11, 12]. Vascular SMCs can be further sub-classified as either contractile or synthetic (Figure 1.1.2-1). During both neovascularization in the embryo [13] and vessel development, vSMCs take on a synthetic phenotype; in adult blood vessels, these cells are committed to a contractile phenotype, taking on the important task of vessel stabilization [14].

**Figure 1.1.2-1. Phenotypic plasticity of vSMCs.** Characteristics of the synthetic and contractile phenotypes – including morphology, proliferation, ECM and contractile protein expression, and phenotypic switch – are regulated by various biochemical and biomechanical cues.
Table 1.1.2. Comparison of cellular properties of perivascular cells

<table>
<thead>
<tr>
<th>Vascular SMCs</th>
<th>Pericytes</th>
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<tr>
<td>Separated from endothelium</td>
<td>In direct contact with ECs</td>
</tr>
<tr>
<td>Contractile</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Spindle shaped morphology</td>
<td>Elongated stellate morphology</td>
</tr>
<tr>
<td>Low proliferative capacity</td>
<td>Associated with microvessels (10–100 μm diameters) or capillaries (&lt;10 μm diameter)</td>
</tr>
<tr>
<td>Increased contractile protein expression (SMMHC)</td>
<td>Increased proliferation</td>
</tr>
<tr>
<td>Decreased contractile protein expression</td>
<td>Decreased contractile protein expression</td>
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<td>Functions: confer vessel contractility, control blood pressure</td>
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The principal function of contractile vSMCs is, as its name suggests, contraction. At a cellular level, the contractile vSMC phenotype is characterized by a spindle-shaped morphology; cell quiescence, marked by the cell’s low proliferative capacity; and increased contractile protein expression [15]. When surrounding vessels, contractile vSMCs replicate at the low frequency of 0.047 percent per day [16]. Cyclic AMP synthesis and signaling have been suggested to promote vSMC quiescence [14], while growth factors, as well as fetal calf serum, drive their proliferative capacity. Healthy blood vessels in the body contain vSMCs that exhibit the contractile phenotype. Thus, in the context of vascular engineering, the contractile phenotype is desired to generate stabilized and sturdy vasculature (Figure 1.1.2-2). The most mature marker that demarcates a contractile vSMC is smooth muscle myosin heavy chain (SMMHC), a protein that powers contraction. Two isoforms of SMMHC (SM-1 and SM-2) are distinct markers for vSMCs. Fetal tissue in both rabbits and humans contain the SM-1 isoform,
while the SM-1 and SM-2 isoforms exist in adult vSMCs [17]. Elastin, a non-basement membrane ECM protein that provides blood vessels with elasticity and resilience, has been shown to promote the contractile phenotype of vSMCs [18, 19]. Constituting 28 to 32 percent of major vascular vessels, elastin has been shown to induce actin stress fiber organization, regulate migration, and inhibit proliferation in a study using mouse vSMCs lacking elastin (Eln-/−) [19, 20]. A 3D topography has been suggested to promote elastin production. Lin et al. (2011) showed that vSMCs cultured in 3D scaffolds produced more elastin than vSMCs cultured in coverslips [21]. In healthy vessels, contractile vSMCs wrap circumferentially rather than longitudinally around blood vessels. The wrapping improves the mechanical properties of the vessel by providing circumferential force [10]. This orientation also helps to manage vasoactivity. Fibroblast growth factor 9 has been suggested to induce wrapping of vSMC around endothelial tubes, supporting their continuous stabilization [22].

**Figure 1.1.2-2.** Two distinct types of vascular smooth muscle cells serve two distinctive purposes. Contractile vSMCs wrap circumferentially around vasculature but switch to the synthetic phenotype in response to blood vessel injury or vascular disease.

The synthetic phenotype is prevalent in injured or diseased vessels or during vessel remodeling (Figure 1.1.2-2). For instance, vSMCs found in diseased
atherosclerotic plaques are mostly synthetic, exhibiting altered characteristics such as increased golgi and rough endoplasmic reticulum, decreased myofilaments, and altered lipid metabolism [23]. The synthetic vSMC phenotype is characterized by an in vitro “hill and valley” morphology, increased cell proliferation, and decreased contractile protein expression [15]. Synthetic vSMCs produce ECM proteins, such as fibronectin and collagen, as well as matrix metalloproteinases (MMPs) to aid in migration [24]. After vascular injury, vSMCs proliferate and migrate from the tunica media to the intimal layer of vessels causing intimal thickening [25]. Additionally, vSMCs found in aortic vessels that had experience aneurysms were found to experience a phenotypic change such as increased MMP2 and MMP9 [26]. After arterial injury of MMP9-/− mice, vSMCs harvested from the artery demonstrated significantly reduced proliferation and migration [27].

Typically, pericytes exhibit an elongated, stellate morphology and are associated with microvessels (10-100 µm diameters) or capillaries (<10 µm diameter). However, depending on their location, they can change their shape, size, distribution, attachments, and density [12]. For example, capillary pericytes align parallel to the longitudinal axis of the vessel; in microvessels, pericytes align circumferentially [28]. Pericyte coverage of the abluminal vessel ranges from 10 to 50 percent as a result of the various morphologies of pericytes, as well as the differences in the ratio of pericytes to ECs based on tissue type [29]. Functionally, pericytes confer vessel contractility as well as blood pressure control [30]. The contractility of pericytes has been reported to be regulated by Rho-GTPase-dependent signaling [31]. Bovine pericytes expressing the constitutively active GTP-bound Rho GTPase display a hypercontractile phenotype that includes numerous actin-enriched projections, as well as generation of a sufficiently large contractile force to deform silicon substrates [31]. In contrast, pericytes with Rho GTPase irreversibly locked in a GDP-bound state are polygonally shaped and exhibit
diminished contraction [31]. In the body, vessel injury prompts pericytes to switch from encircling vessels to migrating from their vascular locations [32, 33]. In addition, pericytes experience phenotypic changes during inflammation of vessels due to injury. In response to inflammation, inflammatory cells transverse the endothelium and the pericyte layer, resulting in the disruption of pericyte association with the basement membrane [34] as well as a pericyte phenotypic change to a relaxed state [35], likely due to a reduction of RhoA, which has been reported to induce a relaxed phenotype marked by loss of focal adhesion, stress fibers, and increased migration [35].

1.1.3 Perivascular marker expression profiles

A useful tool to isolate and distinguish vSMCs and pericytes in tissue engineering and regeneration employs marker expression profiles; because no single marker identifies either cell type, an array of markers is necessary and commonly utilized.

Contractile vSMC markers can be categorized according to their expression during development. Alpha smooth muscle actin (α-SMA) is expressed early in development, whereas transgelin (SM22α) is an early intermediate marker [15]. Basic calponin, caldesmon heavy chain, and smoothelin are expressed intermediately during development. SMMHC is expressed late during development; therefore, it functions as a mature vSMC marker [15]. Synthetic vSMCs are identified by caldesmon light chain, vimentin, non-smooth muscle myosin heavy chain B (SMemb), tropomyosin 4, and cellular retinol binding protein 1 [15].

The most common identification for pericytes is a CD146⁺PDGFRβ⁺CD34⁻CD31⁻ population. However, a variety of other markers are present on pericytes depending on their location in the body. The regulator G-protein signaling 5 (RGS5) designates an ‘activated’ pericyte, present in vascular remodeling and neovascularization [36]. The 3G5 antigen is widely accepted as a ubiquitous pericyte marker of the microvasculature
though also expressed by other cell types. The transmembrane chondroitin sulfate proteoglycan neuron-glial 2 (NG2 or cspg4) and αSMA help to distinguish pericytes in various types of vessels [30]. Namely, pericytes of the capillaries are NG2+αSMA-, of the venules are NG2-αSMA+, and of the arterioles are NG2+αSMA+. When cultured, pericytes are positive for NG2, αSMA, CD44, CD146, platelet derived growth factor β (PDGFRβ), and nestin and negative for CD56, CD34, CD31, and von Willebrand factor. Finally, pericytes also have mesenchymal stem cell (MSC) features [37, 38] and express MSC markers, such as CD44, CD73, CD90, and CD105.

1.1.4 Perivascular stem cell sources

Because PCs derived via biopsies may be diseased or have low proliferative capacity, it is imperative to have a source of PCs for tissue regeneration endeavors that is amenable to clinical translation. To this end, stem cell sources have been used to derive PCs, either for developmental studies or use in tissue-engineered constructs.

Adult stem cells are undifferentiated cells found in various tissues of the human body that can differentiate into specialized cell types of that tissue [39]. Specifically, MSCs serve as a cell source for the derivation of pericytes and vSMCs. These cells are advantageous for vascular engineering applications because they lack major histocompatibility complex II molecules, which incite an immune response [40]. Thus, allogeneic MSCs have the potential for transplantation with limited risk for immune rejection, though it is uncertain whether cells differentiated from MSCs are immunogenic. More detailed information about the relationship between PCs and MSCs can be found in refs. [40-42].

Pluripotent stem cells (PSCs), including human embryonic stem cells (hESCs) and induced PSCs (hiPSCs), can differentiate into all cell types of the body, including pericytes and vSMCs [43, 44]. ESCs are derived from a developing embryo, while iPSCs
are generated by reprogramming somatic or adult progenitor cells. Both have an unlimited ability to self-renew, making them easy to expand for therapeutic use [45, 46]. Collagen IV [47], retinoic acid [48-50], and the growth factors PDGF-BB [50-52] and transforming growth factor β1 (TGF-β1) [51] have been implicated in the derivation of vSMCs. We have previously demonstrated the derivation of smooth muscle-like cells (SMLCs) from hESCs in a two-dimensional, feeder-free approach (Figure 1.1.4-1) [51]. After supplementation with PDGF-BB and TGF-β1, SMLCs resembled mature vSMCs with respect to marker expression as well ECM deposition and contractility. Vascular SMCs have also been derived from hiPSCs from skin fibroblasts [53] and aortic SMCs [54]. Recently, Bajpai et al. (2012) differentiated hiPSCs toward contractile vSMCs through an intermediate population of clonogenic and multipotent intermediates (MSCs). The hiPSC-derived MSC intermediates were able to differentiate into osteogenic, chondrogenic, or adipogenic lineages [55]. Recently, Tang et al. have questioned the existence of the synthetic phenotype with the discovery of a new source of multipotent vascular stem cells present in the vessel walls [56]. From lineage tracking of SMMHC, they concluded that remodeling of blood vessels is not a result of the phenotypic switching of contractile vSMCs to a synthetic phenotype but rather the differentiation of MSCs to proliferative SMCs.

**Figure 1.1.4-1. Derivation of hPSC-SMLCs.** Previously published differentiation protocol to derive smooth muscle-like cells (SMLCs) using serum, TGF-β, and PDGF-BB [51].
The utilization of PSCs as a source of pericytes has also been demonstrated, though much less studied than vSMC differentiation. In a recent study by Dar and colleagues, human PSCs spontaneously differentiated via embryoid body formation were found to express a CD105+CD31- sub-population [57]. Following further culture of this sub-population, derived pericytes were found to express pericyte-specific markers, assemble with ECs to form a vascular network \textit{in vitro} and \textit{in vivo}, and exhibit mesenchymal differentiation potential. Flk1 has been noted as a progenitor marker of pericytes in mouse ESCs [58]. Flk1+ cells isolated from differentiating mouse ESCs were observed to differentiate into functional pericytes which could support EC tubes. \textit{De novo} differentiation of SMA+ pericytes in this system occurred even in the absence of exogenous growth factors in serum-free conditions. The differentiation of hPSCs toward either PC type has been comprehensively reviewed in [43].

Interestingly, many studies have suggested that pericytes themselves are multipotent vascular stem cells that migrate from their vascular niches to sites of injured tissues with the purpose of repairing these tissues [59]. Pericytes have been shown to produce progeny similar to multiligneage mesodermal progenitor cells [38], including, but not limited to, adipocytes, osteoblasts, chondrocytes, and odontoblasts [60].

\subsection{1.1.5 Interactions between perivascular cells and endothelial cells}

Perivascular cells are essential to prevent regression of assembled endothelial tubes, both \textit{in vivo} and \textit{in vitro}. To engineer a tissue for transplantation, its vasculature must be supported by PC types to facilitate and ensure its longevity and durability. Directing the complex process of vessel formation [12, 61], interactions between ECs and PCs have been examined in various \textit{in vitro} and \textit{in vivo} systems. In this section, we specifically focus on insights gained from \textit{in vitro} model systems developed to study the heterocellular crosstalk of the vasculature.
Vascular SMCs support the EC infrastructure [62, 63]. It has been demonstrated that co-cultures of ECs and SMCs alter expression of angiogenic factors VEGF, PDGF-AA, PDGF-BB, and TGF-β compared to monocultures [64]. SMLCs derived from hESCs have been shown to support cord-like structures of endothelial progenitors in a Matrigel system [51]. After 48 hours, cord structures created by endothelial progenitor alone collapsed, whereas those co-cultured with derived SMLCs were stabilized and created tubes with thicknesses between 20-30 µm. Co-cultures of ECs with a PC source (10T1/2, a multipotent mesenchymal cell) demonstrated that VEGF expression was dependent on heterocellular contacts. Inhibiting VEGF yielded a drastic increase in EC apoptosis. Thus EC survival seems dependent on perivascular-derived VEGF [65]. Another co-culture model was developed to mimic the arterial vessel wall [66]. Human umbilical artery SMCs were induced toward a contractile phenotype (via serum deprivation) and cultured with ECs, separated by a collagen gel layer. Under static conditions, α-SMA expression dramatically decreased, suggesting reversion to a synthetic phenotype, whereas under the influence of shear stress, α-SMA was maintained as in the contractile phenotype.

Co-cultures of ECs and pericytes or SMCs revealed that PCs inhibit EC proliferation; however, co-culture with fibroblast, epithelial, or 3T3 cells actually stimulated EC growth [67]. Though they limit proliferation, PCs seemingly do not impair endothelial ability to form tube structures, to which they are recruited and stabilize. By using defined conditions, the growth factors and cytokines necessary for vascular morphogenesis and pericyte stabilization have been realized; VEGF, fibroblast growth factor-2, stem cell factor, interleukin-3, and stromal-derived factor-1 are necessary for ECs to form vascular guidance channels to which pericytes home. Pericyte recruitment, triggered by EC-derived PDGF and epidermal growth factor [68], is necessary for the
formation of stabilized vessel structures as well as ECM deposition [61]. Overall, these systems have led to vital insights into the importance of the presence of PCs to recapitulate physiological processes.

1.2 Biomolecular and biomechanical regulators of perivascular cell behavior

Biomolecular understanding and manipulation of cells is another useful tool that has been utilized to study the complex behavior and multifaceted functionalities of PCs and their use in regeneration. Serum, growth factors, and microRNA (miRNA) have been the mostly widely studied regulators of perivascular properties.

1.2.1 Biochemical signaling: serum

For vSMCs, serum deprivation is known to induce a more contractile phenotype. After serum starvation, human umbilical arterial vSMCs adopt an elongated spindle shape, re-acquire contractility, and exhibit elevated α-SMA, SMMHC, calponin, and SM22α protein expression [69]. Similarly, after serum deprivation of cloned vSMCs from the human thoracic artery, random migration, ECM production, and proliferation decreased, and the vSMCs adopted an elongated spindle-shaped morphology. SMMHC was also shown to be upregulated after serum starvation [70].

The molecules that regulate the change to a contractile phenotype include serum response factor (SRF), myocardin, extracellular signal-regulated kinase 1/2 (ERK-1/2), E twenty-six (ETS)-like transcription factor 1 (ELK-1), and Kruppel-like factor 4 (KLF4). Studies have shown that almost all vSMC genes depend on motifs such as CC(AT)6GG, called CArG elements, found in the vSMC marker gene promoter on intronic sequences [14, 71]. CArG elements serve as binding sites for SRF [71]. The loss of SRF transactivation by suppression using RNAi induced synthetic phenotype characteristics, including decreased SMA expression, increased proliferation, and increased migration.
Myocardin, a muscle-specific SRF coactivator, forms a ternary complex with the bound SRF to activate SMC gene expression [71, 73]. Myocardin overexpression during embryoid body differentiation increased carbachol-induced contraction, as well as the number of cells positive for the contractile proteins SMA and SMMHC [74].

Contrastingly, the presence of serum induces a change to a more synthetic phenotype [75]. ERK1/2 has been shown to phosphorylate ELK-1. After phosphorylation, ELK-1 binds to SRF binding sites in CArG elements [76, 77]. The binding displaces myocardin and therefore represses contractile vSMC protein expression [77]. KLF4 represses the expression of multiple vSMC genes by both downregulating myocardin as well as preventing SRF/myocardin complexes from associating with vSMC gene promoters [78]. Additionally, proteasomal degradation of myocardin by urokinase-type plasminogen activator has been reported to lead to a synthetic phenotype [79].

The effect of serum has not been correlated with pericyte properties.

1.2.2 Biochemical signaling: growth factors
1.2.2.1 Contractile vascular smooth muscle cell phenotype

TGF-β1 and its receptor interactions play an important role in vessel formation. TGF-β1 binds to type I receptors, which form heterodimers with type II receptors, ultimately leading to the activation of SMAD transcription factors [80]. TGF-β1 is commonly reported to differentiate vascular progenitor cells into pericytes and vSMCs [49-51, 81]. Disruption of TGF-β in ECs reduces TGF-β1 availability and thus the ability to promote recruitment and differentiation of vSMCs [82]. TGF-β1 has also been found to increase contractile protein expression in vSMCs [83]. Sieczkiewicz et al. (2003) have shown that TGF-β1 reduces retinal pericyte proliferation and increases contractile protein expression.
1.2.2.2 Synthetic vascular smooth muscle cell phenotype

PDGF-B secreted by ECs promotes the recruitment of pericytes and vSMCs during vessel growth and remodeling through PDGF-B/PDGFR-β interactions. PDGF-B has been reported to repress the contractile phenotype, therefore making it a negative regulator of vSMC gene expression. After 24 hours exposure to PDGF-BB, mRNA expression of SMMHC decreased by 80 percent in rat vSMCs [84]. PDGF-BB treatment repressed α-SMA, SMMHC, and SM22α promoters of rat vSMCs [85]. Reportedly, activation of KLF4 is required for the PDGF-BB-induced effect of repressing the vSMC contractile phenotype [86].

1.2.2.3 Pericytes

PDGF is the most widely appreciated regulator of pericyte behavior. Endothelial-derived PDGF-BB has been demonstrated to promote pericyte recruitment and stabilization of nascent endothelial tubes [68]. Mouse embryos with PDGF-B deficiencies were found to lack pericytes; this resulted in the formation of tortuous capillaries, many of which ruptured at late gestation because they had formed microaneurysms [3]. The ECs of these pericyte-lacking sprouting capillaries were incapable of attracting PDGFR-β⁺ pericyte progenitor cells, which contributed to the deformation of the capillaries [3]. Similarly, Benjamin et al. (1998) found that adding exogenous PDGF-BB during vessel remodeling disrupted endogenous cues, impeding endothelial-pericyte interactions [87]. Proliferation of PDGFR-β pericytes and vSMC progenitors was observed at endothelial PDGF-B expression sites [88].

Other angiogenic growth factors have also been implicated in regulating pericyte activity. Though widely known for its mitogenic effects on ECs, exogenous VEGF has been demonstrated to stimulate pericyte migration in a dose-dependent manner, purporting it as a mitogen for pericytes as well [89]. TGF-β1 regulates the contractile
phenotype, induces SMA expression, and limits proliferation of pericytes [90]. TNF-α and IL-1β have been reported to induce a relaxed phenotype in pericytes yielding increased microvascular permeability in co-culture with microvascular ECs [91].

1.2.2.4 Common: Vascular smooth muscle cells and pericytes

Pericytes and vSMCs also express angiopoietin-1 (Ang-1) on their surfaces, and ECs express Tie-2 receptor [11]. The tight endothelial-pericycle interactions are a result of the binding of Ang-1 and Tie-2 [11]. Angiopoietin-2 (Ang-2), an antagonist on Ang-1 [92], is mainly expressed in ECs and vSMCs. Ang-1 and Ang-2 both bind to Tie-2, but only Ang-1 phosphorylates Tie-2. Ang-2 is thought to destabilize blood vessels by dissociating vSMCs and ECs. Ang-2 was found to inhibit EC-induced vSMC migration [93]. Direct injection of Ang-2 into the eyes or rats caused a dose-dependent loss of pericytes after seven days [94].

1.2.3 MicroRNA

Recently, microRNA (miR) has been implicated in controlling the phenotype of vSMCs. MicroRNAs are single-stranded noncoding RNAs that affect various cellular outcomes by altering messenger RNA (mRNA) function [95]. MicroRNA play different roles in vascular development of healthy compared to diseased vessels by altering gene function and therefore may serve as a potential therapeutic resource. For instance, both miR-143 and miR-145 cooperatively target KLF4, myocardin, and ELK-1 in order to promote differentiation from mouse ESCs and to repress proliferation as vSMCs mature [96]. Boucher et al. (2011) found that the activation of Notch signaling by Jagged-1 (Jag-1) in vSMCs resulted a contractile phenotype. Additionally, they reported that Jag-1/Notch signaling requires both miR-143 and miR-145 to promote the vSMC contractile phenotype [97]. The contractile vSMC markers SMA, calponin, and SMMHC were found
to be upregulated by the precursor to miR-145 (or pre-miR-145) [98]. Additionally, miR-195 reduced the proliferation and migration of vSMCs, indicating a contractile phenotype [99]. Similarly, miR-133a reportedly induced a contractile vSMC phenotype by inhibiting the transcription factor, specificity protein 1 (Sp-1) [100].

TGF-β and bone morophogenic protein (BMP) [101] signaling and subsequent SMAD protein binding was reported to induce a contractile phenotype via increased miR-21 and downregulation of programmed cell death 4 (PCDC4) [102].

However, unlike growth factor regulation of miR-21, hypoxia-induced upregulation of miR-21 has been reported to induce increased proliferation and migration, characteristic of the synthetic phenotype [103, 104]. This illustrates that the same microRNA may have different responses under different conditions. Increased proliferation of vSMCs was also observed with the increased expression of miR-221 and miR-222 by targeting p27 and p57, which are negative regulators of vSMC proliferation [105]. Knock-down of miR-221 and miR-222 reduced neointimal lesion formation caused by proliferating vSMCs [105]. miR-145 has also been found to be expressed in NG2+ pericytes via in situ hybridization on tissue sections [106]. In knockout mice with reduced pericyte-investment in microvessels, expression of miR-145 was reduced in harvested microvessels, whereas other miRNAs were not affected. Furthermore, altered levels of miR-145 diminished migration in vitro, suggesting its relevance to pericyte behavior.

1.3 Biomechanical regulators of perivascular cells
Technological advancements in recent years have enabled researchers to more accurately mimic the perivascular environment. In this section, we discuss the insights gained from the recreation of physiologically-relevant biomechanical forces in engineered niches.

1.3.1 Biomechanical approaches to study perivascular behavior

Pre-conditioning tissue-engineered constructs using mechanical forces may translate to improved cell adaptability when exposed to similar biomechanical forces once implanted in the body. Pericytes and vSMCs experience two major biomechanical forces: shear force generated from interstitial blood flow and uniaxial cyclic strain force generated from heart beats (Figure 1.3.1-1). In healthy vessels, pericytes and vSMCs are not in direct contact with the blood flow, unlike ECs. However, they experience low transverse shear stress resulting from differences in blood vessel pressure and tissue pressure [107]. Studies have approximated this interstitial shear stress at an average 1 dyn/cm² [108]. Exposure of aortic vSMCs to a shear stress of 0.05 dyn/cm² resulted in enhanced MMP-dependent vSMC motility [109] indicative of a synthetic phenotype. In contrast, vSMCs close to the internal elastic lamina may experience approximately 100 times higher shear than those away from the internal elastic lamina [110]. Additionally, after injury to the endothelium, vSMCs may endure higher shear resulting from exposure to blood flow [107]. In response to higher shear flow rates, canine vSMCs [111] and ovine vSMCs [112] aligned perpendicularly when a shear fluid force of up to 20 dyn/cm² was applied for between 48 and 96 hours. Proliferation of human vSMCs increased after exposure to shear stress of between 5 and 25 dyn/cm² for 24 hours [113]. Vascular vSMCs also have been reported to take on a more contractile phenotype after shear stress [112].
Various forces in the blood vessel act on the vSMCs, including shear stress, interstitial shear stress, pulsatile tensile force, and uniaxial tensile strain. Because PCs circumferentially predominantly surround vessel walls, they experience a predominantly uniaxial tensile force or strain. As the heart beats, it generates a pulsatile pressure resulting in an outward tension experienced by the vessel [114]. Since the heart beats rhythmically, the tension felt by the cells is also cyclic [115].

A number of researchers have mimicked these mechanical cues by applying strain to cultured vSMCs on two-dimensional (2D) surfaces. The expression of contractile vSMC proteins SMA, calponin, and SM22α were also shown to increase after cyclic strain [116, 117]. Vascular SMCs align perpendicular to the direction of uniaxial cyclic strain [118]. Uniaxial cyclic strain also regulates the expression of vSMC markers in MSCs by transiently increasing SMA and SM22α after one day [115]. After the return of these proteins to basal levels, the cells align perpendicularly to the direction of strain. Studies have shown that 2D uniaxial cyclic strain affects vSMC ECM deposition, marker expression, and alignment. Expression of ECM proteins, such as collagen I, collagen IV, and fibronectin increased under uniaxial cyclic strain [119, 120]. In contrast, equiaxial strain downregulates SMA and SM22α in MSCs.
To achieve 3D strain, pericytes and vSMCs are either embedded in a 3D matrix that is mechanically strained or the cells themselves are strained using pulsatile perfusion bioreactors. In these systems, vSMCs align parallel to the direction of strain [121, 122]. After five weeks of cyclic distension of adult SMCs embedded in collagen gels, the cells were aligned parallel to the direction of strain and elastin deposition was observed [121]. After eight weeks of 3D strain generated by pulsatile flow through a poly(lactide-co-caprolactone) scaffold seeded with rabbit aortic vSMCs, collagen production, proliferation, and SMA expression were enhanced [122]. Wang et al. (2010) subjected vSMCs derived from human adipose stem cells and seeded on polyglycolic acid (PGA) unwoven mesh scaffolds to eight weeks of pulsatile shear that was gradually increased from 75 to 150 mmHg [123]. Pulsatile stimulation increased SMA, calponin, and collagen expression [123]. The 3D strain resulting from the pulsatile stimulation of vSMCs has also brought about an increase in mechanical strength (tensile strength, suture strength, elastic modulus, and burst pressure), oriented vSMCs, and organized collagen fibers compared to the static controls [123, 124].

1.4 Thesis Overview

Perivascular cells play a pivotal role in the endurance and function of blood vessels, making them necessary for engineering the vasculature. Current difficulties facing the inclusion of PCs include their cryptic marker expression profiles, which limits efficient isolation and derivation techniques, misalignment of cells, and the ability of vSMCs to assume either a synthetic or contractile phenotype. The overall hypothesis of this thesis is that both biochemical and biomechanical cues can direct hPSCs into the distinct perivascular sub-types that can be used in engineered blood vessels to promote optimal perivascular function. The thesis can be divided into three specific aims:
Specific Aim 1: Study the effect of biochemical stimuli on vSMC differentiation and maturation

Control the fate decisions of hPSC Vsmc derivatives to guide their maturation towards a desired phenotype. Examine effect of growth factor supplementation and serum deprivation on vSMC differentiation and maturation. The study for this specific aim will be presented in Chapter 3.

Specific Aim 2: Directly compare between perivascular cell derivatives with the same genetic background

Compare cell morphology, subcellular organelle organization (namely endoplasmic reticulum, mitochondria, and stress fibers), and expression of smooth muscle myosin heavy chain and elastin for perivascular cells derived from hPSCs. Differences in extracellular matrix deposition and remodeling were less pronounced, multipotency, in vivo, migratory, invasion, and contractile functionalities are distinctive are examined. The study for this specific aim will be presented in Chapter 4.

Specific Aim 3: Study the effect of biomechanical strain on vSMC ECM production

To elucidate the effects of tensile strain on hPSC vSMC derivatives, cyclic uniaxial strain and circumferential strain are applied at various stages of vSMC development. Circumferential strain is applied using a custom engineered bioreactor. The study for this specific aim will be presented in Chapter 5.
Chapter 2

OVERVIEW OF EXPERIMENTAL METHODS

2

2.1 Cell Culture

All cells were cultured in humidified incubators, with atmospheres at 37°C and 5% CO₂.

**Human PSCs.** hiPSC line BC1 [125, 126] kindly provided by Dr. Cheng, SOM JHU and Human ESC line H9 (passages 15 to 40; WiCell Research Institute, Madison, WI) were grown on inactivated mouse embryonic fibroblast feeder layers (GlobalStem, Rockville, MD) in growth medium composed of 80 percent ES-DMEM/F12 (GlobalStem), 20 percent knockout serum replacement (Invitrogen, Carlsbad, CA), and 4 ng/ml basic fibroblast growth factor (bFGF; Invitrogen) for hEScs of 10ng/ml bFGF for hiPSCs, as previously reported [127]. Human iPSCs were passaged every four to six days using 1 mg/ml of type IV collagenase (Invitrogen). Media were changed daily.

**Human v-SMCs.** The control cell type used was human aorta v-SMCs (passages 4-7; ATCC, Manassas, VA). The cells were cultured in the specified ATCC complete SMC growth medium, composed of Kainghn’s Modification of Ham’s F-12 Medium (F-12K Medium; ATCC), 10% or 0.5% fetal bovine serum (FBS; Hyclone), 0.01 mg/ml transferrin (Sigma-Aldrich, St. Louis, MO), 0.01 mg/ml insulin (Sigma), 10 mM HEPES buffer (Sigma), 10 mM 2-(Tris(hydroxymethyl)methylamino)ethane-1-sulphonic acid (TES)(Sigma), 0.05 mg/ml ascorbic acid (Sigma), 10 ng/mL sodium selenite (Sigma), and 0.03 mg/ml Endothelial Cell Growth Supplement (Sigma). Human v-SMCs were passaged every three to four days using 0.25 percent trypsin (Invitrogen). Media was changed every two to three days.
**Human pericytes.** The control cell type used was human placental pericytes (passages 3-5; Promocell). The cells were cultured in the specified Pericyte Growth Media (Promocell) and were passaged every three to four days using a detachment kit (Promocell).

**vSMC differentiation protocol.** vSMCs were derived as previously described [128]. Briefly, hPSCs were collected through digestion with TrypLE (Invitrogen) and were seeded at a concentration of 5x10^4 cells/cm^2 onto plates previously coated with collagen type IV (R&D Systems, Minneapolis, MN). The hPSCs were cultured for six days in a differentiation medium, composed of alpha-MEM (Invitrogen), 10% FBS (Hyclone), and 0.1 mM β-mercaptoethanol (Invitrogen). Media were changed daily. On day six, the differentiated cells were collected through digestion with TrypLE (Invitrogen), separated with a 40-µm mesh strainer, and seeded at a concentration of 1.25x10^4 cells/cm^2 on collagen-type-IV-coated plates. The differentiating hPSCs were then cultured in differentiation medium; with the addition of 10 ng/ml PDGF-BB (R&D Systems) and 1 ng/ml TGF-β1 (R&D Systems) for additional 6 days (total of 12 days) for SMLCs. Media was changed every second day. Serum starved cells were passaged every 6-8 days with Tryple, using alpha-MEM (Invitrogen), 10% FBS (Hyclone), and 0.1 mM β-mercaptoethanol (Invitrogen) to neutralize Tryple but then seeded with 0.5% serum media.

**Pericyte differentiation protocol.** Pericytes were differentiated as previously described [67, 129]. Briefly, hPSCs were differentiated as described for vSMC differentiation above for the first 6 days. On day 6, cells were re-seeded at a concentration of 1.25x10^4 cells/cm^2 on collagen-type-IV-coated plates in endothelial cell growth media (ECGM) (PromoCell) supplemented with 2% FBS, 50ng/ml vascular endothelial growth factor (VEGF), and 10µM SB431542 (Tocris) for 6 days. Media was
changed every other day. On day 12, derived EVCs were collected through digestion with TrypLE and replated on tissue culture-treated six-well plates in medium composed of DMEM and 10% FBS. After 2–3 h, unattached cells were removed, and the medium was replaced. Cells were cultured for 6 d, with the medium changed every other day.

2.2 Immunofluorescent staining and imaging

Cells were prepared for immunofluorescence as previously described [128, 130]. Cells were fixed using 3.7% formaldehyde fixative for 15 minutes, washed with phosphate buffered saline (PBS), blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour minimum, permeabilized with a solution of 0.1% Triton-X (Sigma) for ten minutes, washed with PBS, and incubated for one hour with anti-human SMA (1:200; Dako, Glostrup, Denmark), anti-human NG2 (1:100; Santa Cruz), anti-human PDGFRβ (1:100, Santa Cruz), and anti-human SMMHC (3:100; Dako). For ECM staining, cells were incubated with anti-human fibronectin (1:200; Sigma), anti-human collagen1 (1:200; Abcam), anti-human collagen IV (1:100; Abcam), anti-human laminin (1:200; Abcam) or anti-human elastin (3:100 Abcam) for one hour. Cells were rinsed twice with PBS and incubated with Alexa 546 conjugated phalloidin (1:100; Molecular Probes, Eugene, OR) or anti-mouse IgG Cy3 conjugate (1:50; Sigma), anti-mouse FITC (1:50; Sigma), or anti-rabbit IgG Alexa Fluor 488 conjugate (1:1000; Molecular Probes, Eugene, OR) for one hour, rinsed with PBS, and incubated with DAPI (1:1000; Roche Diagnostics) for ten minutes. Coverslips were rinsed once more with PBS and mounted with fluorescent mounting medium (Dako). The immunolabeled cells were examined using fluorescence microscopy (Olympus BX60; Olympus, Center Valley, PA) and confocal microscopy (LSM 510 Meta; Carl Zeiss).
Table 2.2. List of antibodies and recombinant proteins

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<th>Vendor</th>
<th>Host species</th>
<th>Concentration</th>
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<td>Mouse anti-human</td>
<td>3:200</td>
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<td>Dako</td>
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2.3 Transmission electron microscopy (TEM)

Differentiated cells, placental pericytes, and aortic vSMCs were prepared for TEM analysis as described previously [131]. Serial sections were cut, mounted onto copper grids, and viewed using a Phillips EM 410 TEM (FEI, Hillsboro, OR, USA). Images were captured using a SIS Megaview III CCD (Lakewood, CO, USA).

2.4 Real-time quantitative RT-PCR
Two-step RT-PCR was performed on differentiated hPSCs at various time points as we previously described [128]. Total RNA was extracted by using TRlzol (Gibco, Invitrogen), as per the manufacturer’s instructions. All samples were verified as free of DNA contamination. The concentration of total RNA was quantified using an ultraviolet spectrophotometer. RNA (1 µg per sample) was transcribed using the reverse transcriptase M-MLV (Promega Co., Madison, WI) and oligo(dT) primers (Promega), as per the manufacturer’s instructions. The specific assay used was the TaqMan Universal PCR Master Mix and Gene Expression Assay (Applied Biosystems, Foster City, CA) for ACTA2, CNN1, CSPG4, PDGFRB, MYH11, COLA1, COL4A4, LAMC1, ELN, MMP14, CALD1, ALPNR, TCF21, KDR, PAX6, WNT1, SOX1, ACTB, and GAPDH, as per the manufacturer’s instructions. Assays on Demand Kits (Applied Biosystems, Foster City, CA, USA) was used for FN1 (Hs01549958) and the customized (Applied Biosystems, Foster City, CA, USA) ED-A spanning exons sequence primer was: Forward 5' - CCAGTGCACAGCTATTCCTG-3' and Reverse 5' - ACAACCACGGATGAGCTG-3' [132, 133]. The Taqman PCR step was performed with an Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer’s instructions. The relative expressions of the genes were normalized to the amount of ACTB or GAPDH in the same cDNA by using the standard curve method provided by the manufacturer. For each primer set, the comparative computerized tomography method (Applied Biosystems) was used to calculate the amplification differences between the different samples. The values for the experiments were averaged and graphed with standard deviations.

2.5 Flow cytometric analysis

Flow cytometry was performed as previously described [130]. Briefly, cells were incubated with PE-conjugated antigen specific antibodies for markers outlined in the text
including KDR-PE (1:10; BD), Nestin-PE (1:10; BD), CD56-PE (1:10; BD); SMMHC-PE (1:10; MYH11; Santa Cruz). To detect SMMHC -PE, cells were fixed with 3.7% formaldehyde for 10 minutes, washed, incubated with 0.1% Triton X for 10 minutes, washed, and finally incubated with SMMHC -PE for 45 minutes. All analyses were done using corresponding isotype controls. Forward-side scatter plots were used to exclude dead cells. User guide instructions were followed to complete the flow cytometry analysis via Cyflogic v1.2.

2.6 Western blot

We evaluated protein amounts from whole-cell lysates, quantified using the DC assay (BioRad, Hercules, CA, USA), and loaded a concentration of 50 mg of isolated protein from each of the indicated samples per well into a 12.5% SDS–PAGE gel (BioRad). Proteins were transferred to nitrocellulose membranes, blocked for 1 h and incubated overnight with constant shaking and primary antibody (antibodies indicated above). Membranes were washed and incubated for 2 h with either antirabbit HRP (1:1000; Cell Signaling Technology, Boston, MA, USA) or antimouse HRP (1:3000; Cell Signaling Technology), washed, developed using enhanced chemiluminescence (Pierce), and visualized using the ChemiDoc XRS+ System (BioRad). Images were acquired using BioRad Quantity One software.

2.7 Zymography

Zymography was performed to determine MMP activities as previously [134]. MMP1 was detected using SDS-Page casein zymography while both MMP2 and MMP9 were detected using SDS-Page gelatin zymography. Cells were cultured in serum free media for 72 hours. We collected the media of each sample and loaded the media of the samples per well into either a casein gel (BioRad) or gelatin gel (BioRad).
Quantification of protein was done using the Bradford Assay. After electrophoresis, the gels were renatured by washing in renaturation buffer (Invitrogen) and incubated at 37°C in denaturation buffer (Invitrogen) for 24h. The proteins were fixed in 50% methanol and 10% acetic acid for 30 min and then stained in 0.02% comassie blue (Sigma). Gels were destained in 20% methanol and 10% acetic acid and were visualized using the ChemiDoc XRS+ System (BioRad). Images were acquired using BioRad Quantity One software.

2.8 Functional contraction studies

Contraction studies in response to pharmacological drugs were done, as previously described [128, 135]. Briefly, perivascular cell derivatives were cultured, washed, and contraction was induced by incubating with 10^{-5} M carbachol (Calbiochem, Darmstadt, Germany) in DMEM medium (Invitrogen) for 30 minutes. The perivascular cell derivatives were visualized using cytoplasm-viable fluorescence dye, calceine. A series of time-lapse images were taken using a microscope with a 10X objective lens (Axiovert; Carl Zeiss). The cell contraction percentage was calculated by the difference in area covered by the cells before (at time zero) and after contraction (at time 30 minutes). Area analysis was performed with Adobe Photoshop CS5 (Adobe Systems Inc., Mountain View, CA). Each set of images was analyzed three times. The magic wand and measurement tools were used to calculate the area of the image not covered in cells, which was then subtracted from the total area of the image. This method improves upon our previously established procedure [135] by eliminating the need for image compression and by increasing the consistency of cell selection within each set of images.

2.9 Subcutaneous Matrigel implantation
hiPSC-derived perivascular cells were trypsinized, collected and stained with PKH26 (Sigma-Aldrich) membrane dye. We encapsulated a total of $0.5 \times 10^6$ PSC-vSMCs in reduced growth factor Matrigel (BD Biosciences) and 20 μL of EGM-2 media (endothelial growth media). The Matrigel, which contained 250 ng/mL of bFGF (R&D Systems), was loaded, along with the cell mixture, into a 1 mL syringe with a 22-gauge needle and injected subcutaneously into each side of the dorsal region of six- to eight-week-old nude mice. On day 7, we injected isolectin GS-IB4, an Alexa Fluor(R) 488 conjugate glycoprotein isolated from the Griffonia simplicifolia African legume (Invitrogen) through the tail veins of the mice. After 20 minutes, we euthanized the mice by CO2 asphyxiation and harvested the Matrigel plugs, which were fixed in 3.7 percent formaldehyde (Sigma-Aldrich) for one hour, and incubated with DAPI (1:1000; Roche Diagnostics) for ten minutes. A sequence of z-stack images was obtained using confocal microscopy (LSM 510 Meta, Carl Zeiss, Inc.). The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols.

2.10 Cyclic uniaxial strain application

hPSC vSMC derivatives were seeded on collagen IV coated polydimethylsiloxane chambers, which were inserted between two metal frames inside of a cyclic strain loading STREX instrument (STREX ST-140, Strex, Osaka, Japan). Uniaxial strain was achieved as the STREX instrument utilized a computer controlled step motor to drive two metal frames closer or further apart. The amplitude and frequency were controlled by the programmable microcomputer. In the present study we used a cyclic strain in the 5-10% range with a frequency of 1Hz. All strain experiments were performed with the supplementation of 1ng/mL TGF-β1 and were performed in humidified incubators, with atmospheres at 37°C and 5% CO₂.
2.11 Pulsatile flow loading bioreactor design

To examine the effect of circumferential strain on hPSC vSMC derivatives, a bioreactor was designed to allow pressurized flow into tubular silicone constructs (Silastic Laboratory Tubing, Dow Corning) and allow the radial distention of the constructs. The system was used inside a standard humidified incubator, with atmospheres at 37°C and 5% CO₂ and consists of a media reservoir, a programmable peristaltic pump (Ismatec), and a polydimethylsiloxane (PDMS) media bath. The peristaltic pump was operated at speeds between 0.11 – 11.25 rpm and propels culture medium in a cyclic flow pattern through a Kel-F hub 30 gauge needle (Hamilton) through the silicone construct secured on both ends using 6-0 nylon sutures (Henry Schein). The path of fluid flow continues through the silicone construct and out of a second Kel-F hub 30 gauge needle (Hamilton) and finally into a media reservoir where media collects and is recycled back into the pump. The 2 needles were pierced through the PDMS media bath at opposite ends in order to hold tubular samples 300μm in radial diameter and 3cm in axial length. A PDMS lid was used to ensure sterility of the media bath.

2.12 Statistical analysis

Real-time RT-PCR, functionality assays, flow cytometry and image analyses were performed in at least triplicate biological samples. Real-time RT-PCR analyses were also performed with triplicate readings. Statistical analyses were performed with GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, CA). Unpaired two-tailed t-tests and one-way ANOVA analysis and Bonferonni post tests were performed where appropriate using GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, CA). Significance levels were set at *p<0.05, **p<0.01, and ***p<0.001. All graphical data are reported as mean ±SEM.
Chapter 3

DERIVATION AND MATURATION OF SYNTHETIC AND CONTRACTILE VASCULAR SMOOTH MUSCLE CELLS FROM HUMAN PLURIPOTENT STEM CELLS

3

3.1 Introduction

The stabilization of blood vessels occurs by extracellular matrix (ECM) formation, as well as through the recruitment of mural cells, which include vascular smooth muscle cells (vSMCs) and pericytes. While pericytes are found in the microvasculature, such as capillaries, vSMCs surround larger vessels, such as arteries and veins. During angiogenesis, endothelial cells (ECs) proliferate; connect to preexisting blood vessels; and, through lumen formation, develop endothelial tubules, a process known as intussusception [136]. After the formation of the nascent tubes composed of ECs, surrounding undifferentiated mesenchymal cells are recruited and differentiated into proliferating vSMCs, which are needed to stabilize the formed tubules [137, 138]. Platelet-derived growth factor (PDGF-BB) [51, 88] and transforming growth factor (TGF-\(\beta\)1) [139, 140] act as signaling cues for the recruitment and differentiation of vSMCs. It has been suggested that, after birth, vSMCs become quiescent and take on the contractile phenotype found in adult vessels [141, 142].

During neovascularization in the embryo [13] or during vessel development, vSMCs have a synthetic phenotype, which is characterized by high proliferation, migration, and ECM protein production [14]. In adult blood vessels, vSMCs play an important role in vessel stabilization; therefore, they become committed to the mature contractile phenotype, which is characterized by low proliferation, expression of
contractile proteins — namely, smooth muscle myosin heavy chain (SMMHC) and elastin — and low synthetic activity [14].

Adult vSMCs wrap around the vessel layer of endothelial cells and contract to regulate and maintain blood vessel diameter in order to counteract the pulsatile blood pressure generated by the heart [143]. Remarkably, vSMCs do not stay in a particular terminally differentiated state. Instead, they exhibit plasticity and can reversibly take on either a contractile or a synthetic phenotype [14].

Pluripotent stem cells (PSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), serve as a reliable source for vSMCs because they can self-renew and proliferate. Pluripotent stem cells first differentiate into the mesoderm [144] and later into the vascular lineages, including vSMCs [145, 146]. Collagen IV [47], retinoic acid [147-150], and the growth factors PDGF-BB [51, 150-152] and TGF-β1 [51] have been implicated in the inducement of vSMC differentiation. Vascular SMCs have previously been derived from human iPSCs from skin fibroblasts [153] and human aortic smooth muscle [54]. To the best of our knowledge, no study demonstrated the regulation of both contractile proteins, SMMHC and elastin, along the differentiation and maturation of vSMC from PSCs.

Our previous studies demonstrated the derivation of vascular smooth muscle-like cells (SMLCs) from hESCs using monolayer cultures supplemented with PDGF-BB and TGF-β1 [51, 154]. This study hypothesizes that hPSC-derived SMLCs can be guided to acquire either a synthetic phenotype or a contractile phenotype.

3.2 Materials and Methods

3.2.1 Cell Culture
**Human PSCs.** We grew the hESC lines H9 and H13 (passages 15 to 40; WiCell Research Institute, Madison, WI) and the hiPSC lines MR31[155] and BC1[156, 157] (kindly provided by Dr. Cheng, JHU School of Medicine) on inactivated mouse embryonic fibroblast feeder layers using previously established methods [51, 154].

**Human vSMCs.** We used human aorta v-SMCs (ATCC, Manassas, VA) for the control cell type according to the manufacturer instructions.

**Vascular SMC differentiation protocol**

We collected human PSCs seeded them at a concentration of 5x10^4 cells/cm² onto collagen type IV (R&D Systems, Minneapolis, MN) coated plates. The hPSCs were cultured for six days in a differentiation medium and on day six, the differentiated cells were collected and seeded at a concentration of 1.25x10^4 cells/cm² on collagen-type-IV-coated plates in differentiation medium with the addition of 10 ng/ml PDGF-BB (R&D Systems) and 1 ng/ml TGF-β1 (R&D Systems) for six additional days (a total of 12 days) for SMLCs. We cultured hPSC-derived SMLCs for the time periods and with the media components detailed throughout this paper.

3.2.2 **Real-time quantitative RT-PCR**

Two-step reverse transcription polymerase chain reaction (RT-PCR) were performed on differentiated hPSCs at various time points, as we have described previously [158]. Total RNA was extracted using TRIzol (Gibco, Invitrogen), as per the manufacturer’s instructions. We verified that all samples were free of DNA contamination. We quantified the concentration of total RNA using an ultraviolet spectrophotometer. RNA (1 μg per sample) was transcribed using the reverse transcriptase M-MLV (Promega Co., Madison, WI) and oligo(dT) primers (Promega),
following the manufacturer’s instructions. The specific assay used was the TaqMan Universal PCR Master Mix and Gene Expression Assay (Applied Biosystems, Foster City, CA) for SMA, CALPONIN, SM22, SMMHC, COL1, FN1, ELN, MMP1, MMP2, MT1-MMP, SRF, MYOCARD, ERK, YAP1, SMAD3, β-ACTIN, and GAPDH, as per the manufacturer’s instructions. The Taqman PCR step was performed with a StepOne Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer’s instructions. We normalized the relative expressions of the genes to the amount of β-ACTIN or GAPDH in the same cDNA by using the standard curve method provided by the manufacturer. For each primer set, we used the comparative computerized tomography method (Applied Biosystems) to calculate the amplification differences between the different samples. The values for the experiments were averaged and graphed with standard deviations.

3.2.3 Immunofluorescence

Cells were fixed using 3.7 percent formaldehyde fixative for 15 minutes, washed with phosphate-buffered saline (PBS), permeabilized with a solution of 0.1 percent Triton-X (Sigma-Aldrich, St. Louis, MO ) for ten minutes, washed with PBS, and incubated for one hour with anti-human SMA (1:200; Dako, Glostrup, Denmark), antihuman calponin (1:200; Dako), anti-human SM22 (1:200, Abcam, Cambridge, MA), and anti-human SMMHC (3:100; Dako). For ECM staining, we incubated cells with antihuman fibronectin (1:200; Sigma-Aldrich), anti-human collagen (1:200; Abcam), or antihuman elastin (3:100 Abcam) for one hour. For proliferation, we incubated cells with anti-human Ki67 (1:50, Invitrogen) for one hour. Cells were rinsed twice with PBS and incubated with FITC-conjugated phalloidin (1:40; Molecular Probes, Eugene, OR), antimouse IgG Cy3 conjugate (1:50; Sigma-Aldrich), or anti-rabbit IgG Alexa Fluor 488 conjugate (1:1000; Molecular Probes) for one hour. Cells were rinsed with PBS and
incubated with DAPI (1:1000; Roche Diagnostics) for ten minutes. Cover slips were rinsed once more with PBS and mounted with fluorescent mounting medium (Dako). We examined the immunolabeled cells using fluorescence microscopy (Olympus BX60; Olympus, Center Valley, PA).

### 3.2.4 Western blot

Whole-cell lysates were performed in either a tris-Triton X buffer (1 percent Triton X, 150 mM NaCl, 50 mM tris, pH 7.5) or in RIPA buffer (150 mM NaCl, 1.0 percent Triton X, 0.5 percent sodium deoxycholate, 0.1 percent SDS, 50 mM tris, pH 8.0) containing 1x protease inhibitor cocktail (Pierce, Rockford, IL). We evaluated protein amounts from whole-cell lysates, quantified using the DC assay (BioRad, Hercules, CA), and boiled at 95 °C for five minutes in Laemmli buffer (BioRad) with or without BME. We loaded a concentration of 50 μg of isolated protein from each of the indicated samples per well into a 12.5 percent SDS PAGE gel (BioRad). Proteins were transferred to nitrocellulose membranes, blocked for one hour in 3 percent nonfat milk, and incubated overnight at 4 °C, constantly shaking with primary antibody (antibodies indicated above). Membranes were washed three times in tris buffer saline containing 0.1 percent Tween-20 (TBST) for 15 minutes each and incubated for two hours at room temperature, constantly shaking with either anti-rabbit HRP (1:1,000; Cell Signaling Technology, Boston, MA) or anti-mouse HRP (1:3,000; Cell Signaling Technology). Membranes were washed three times in TBST, developed using enhanced chemiluminescence (Pierce), and visualized using the ChemiDoc XRS+ System (BioRad). Images were acquired using BioRad Quantity One software.

### 3.2.5 Functional contraction studies
We performed contraction studies in response to pharmacological drugs, as we previously described [51]. Briefly, we cultured hPSC derivatives (as detailed elsewhere in the paper), washed them, and induced contraction by incubating them with $10^{-5}$ M carbachol (Calbiochem, Darmstadt, Germany) in DMEM medium (Invitrogen) for 30 minutes. We visualized the cells using a cytoplasm-viable fluorescence dye, calcein. A series of timelapse images were taken using a microscope with a 10X objective lens (Axiovert; Carl Zeiss, Thornwood, NY). We calculated the cell contraction percentage as the difference in area covered by the cells before (at time zero) and after contraction (at time 30 minutes). We performed area analysis with Adobe Photoshop CS5 (Adobe Systems Inc., Mountain View, CA), analyzing each set of images three times. We used Photoshop’s magic wand and measurement tools to calculate the area of the image not covered in cells, which we then subtracted from the total area of the image. This method improves upon our previously established procedure5, 10 by eliminating the need for image compression and by increasing the consistency of cell selection within each set of images.

3.2.6 Subcutaneous Matrigel implantation

We trypsinized and collected PSC-derived vSMCs; we stained the pellet with PKH26 (Sigma-Aldrich) membrane dye. We encapsulated a total of $0.5 \times 10^6$ PSC-vSMCs in reduced growth factor Matrigel (BD Biosciences) and 20 μL of EGM-2 media (endothelial growth media). The Matrigel contained 250 ng/mL of bFGF (R&D Systems). Matrigel and cell mixture were injected subcutaneously into each side of the dorsal region of six- to eight-week-old nude mice. On day 7, we injected isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor(R) 488 conjugate (Invitrogen) through the tail veins of the mice prior to their euthanization. After 20 minutes, we euthanized the mice by CO2 asphyxiation and harvested the Matrigel plugs, which were fixed in 3.7 percent
formaldehyde (Sigma-Aldrich) for one hour. A sequence of z-stack images was obtained using confocal microscopy (LSM 510 Meta, Carl Zeiss, Inc.). Vessel diameters from the short axes of the lumen of the vessel were determined from the three-dimensional confocal images. The lumen diameter of vessel that contained areas with and without PSC-vSMC wrapping was measured using ImageJ [National Institutes of Health (NIH)] and known pixel:length ratios. The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

3.2.7 Histology

After confocal analysis, the fixed construct explants were dehydrated in graded ethanol (70 to 100 percent), embedded in paraffin, serially sectioned using a microtome (5 μm), and stained with either hematoxylin and eosin (H&E) or immunohistochemistry for anti-human elastin (Dako, Glostrup, Denmark). Mouse and human tissue samples were used as controls.

3.2.8 Transmission electron microscopy (TEM)

Differentiated cells, as detailed below, were prepared for TEM analysis as previously4. Briefly, cultures were fixed with 3.0 percent formaldehyde, 1.5 percent glutaraldehyde in 0.1 M Na cacodylate, 5 mM Ca2+, and 2.5 percent sucrose at room temperature for one hour and washed three times in 0.1 M cacodylate/2.5 percent sucrose (pH 7.4) for 15 minutes each. The cells were postfixed with Palade's OsO4 on ice for one hour, rinsed with Kellenberger's uranyl acetate, and then processed conventionally through Epon embedding. Serial sections were cut, mounted onto copper
grids, and viewed using a Phillips EM 410 TEM (FEI, Hillsboro, OR). Images were captured using a SIS Megaview III CCD (Lakewood, CO).

3.2.9 Statistical analysis

All analyses were performed in triplicate for n=3 at least. One Way ANOVA with Bonferroni post-hoc test were performed to determine significance using GraphPad Prism 4.02. (GraphPad Software Inc., La Jolla, CA). Significance levels were set at *p<0.05, **p<0.01, and ***p<0.001. All graphical data are reported ±SEM.

3.3 Results

3.3.1 Long-term culture in high serum and PDGF-BB and TGFβ1 induces synthetic phenotype

Our previous studies established a simple step-wise monolayer differentiation protocol, where we differentiated hPSCs in monolayers and supplemented with PDGF-BB and TGF-β1, resulting in highly purified cultures of SMLCs [51, 154]. The current study ultimately aimed to mature these SMLCs to contractile phenotype vSMCs. Two principal strategies for the maturation of SMLCs (Day 12 of differentiation) were examined: continuous culture in differentiation medium and the effect of deprivation of serum and growth factors during the culture period. The molecular analysis of ECM, cytoskeleton, and contractile proteins enabled the monitoring of the various stages of the maturation process. The aortic vSMC line, which exhibited high expression levels of the contractile proteins, was chosen as the control for mature human vSMCs (Figure 3.3.1-1).
Figure 3.3.1. SMLC compared to human aortic vSMCs. Quantitative RTPCR analysis shows the relative mRNA expression of the relevant cytoskeleton and ECM genes of the differentiated SMLCs compared to human aortic vSMCs.
In the first stage, we examined the effect of long-term culture using the differentiation medium. SMLCs (Day 12 of differentiation) were cultured for an additional 18 days in differentiation medium containing 10% serum, 10 ng/mL PDGF-BB, and 1 ng/mL TGF-b1. Interestingly, the 30-day differentiated cells took on a synthetic vSMC (Syn-vSMC) phenotype compared with SMLCs, including (i) a decrease in calponin mRNA expression, no significant difference in SMA and SM22 mRNA expression, and a decrease in the mRNA expression of SMMHC (Figure 3.3.1-2A and B); (ii) an increased expression and production of collagen I and fibronectin and a decreased expression of elastin (Figure 3.3.1-2C and D); (iii) and an increased expression of membrane type 1 matrix metalloproteinase (MT1-MMP), MMP1, and MMP2 (Figure 3.3.1-2E). These data proved consistent among the different hPSC lines examined. Because it has been suggested that PDGF-BB interferes with vSMC maturation [85, 86, 159, 160], we attempted to induce maturation by exposure of Day 12 differentiated SMLCs to TGF-β1 or to no growth factors at all. We found some detectable increase in the expression of SMMHC and elastin, especially when no growth factors were added (Figure 3.3.1-3). Although these culture conditions did not induce significantly improved contractility, they suggest that a lessening of signaling activation may induce contractile maturation.
Figure 3.3.1-2. SMLCs take on a synthetic phenotype. On Day 12 of culturing SMLCs, they were moved to differentiation medium containing 10% serum with PDGF-BB and TGF-β1 for an additional 18 days. (A) Quantitative RT–PCR revealed no significant changes in the expression of SMA and SM22 and showed a decrease in calponin and SMMHC between SMLCs and Syn-vSMCs. (B) Western blot analysis confirmed the protein expression of SMA, calponin, and SM22 in SMLCs, Syn-vSMCs, and aortic vSMCs. We found (C and D) increased expression of ECM proteins, collagen I, and fibronectin and a decrease in the expression of elastin in Syn-vSMCs compared with SMLCs. (E) Up-regulation in the expression of MT-1 MMP, MMP1, and MMP2 in Syn-vSMCs. Scale bars are 100 mm. *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 3.3.1-3. SMLCs differentiation. SMLCs were cultured (from day 12) in differentiation media containing 10% serum with PDGF-BB and TGF-β1, with TGF-β1 only, or without any growth factors.
3.3.2 Serum starvation and PDGF-BB deprivation induce contractile phenotype

The proposed association of quiescence with the contractile phenotype of vSMCs after birth [161, 162] led us to examine the effects of serum starvation and growth factor depletion during the differentiation of SMLCs. At first, we tested the Syn-vSMC derivatives for an additional 6 days in culture in a medium containing 10% serum plus TGF-β1 or 0.5% serum plus TGF-β1. We could not detect up-regulation in the expression of contractile proteins, specifically SMMHC and elastin, under either of the conditions (Figure 3.3.2-1A). This implied that Syn-vSMC derivatives had already committed to the synthetic phenotype. Thus, we attempted to mature the SMLCs (Day 12) in the same conditions. Indeed, in medium containing 0.5% serum plus TGF-β1, matured SMLCs (mSMLCs) were detected with significantly up-regulated expressions of the contractile proteins SMMHC (~40-fold) and elastin (approximately eight-fold) and with no significant change in the expression of early cytoskeleton markers (i.e. SMA, calponin, and SM22) and ECM proteins (i.e. collagen and fibronectin; Figure 3.3.2-1B). The mSMLCs began to acquire a more filamentous cytoskeleton organization, as observed with F-actin, SMA, calponin, SM22, and occasionally SMMHC; they also began to produce elastin (Figure 3.3.2-1C). These data were consistent among the different hPSC lines examined. Culturing the mSMLCs in media containing high concentrations of serum for 6 days resulted in down-regulation of both elastin and SMMHC (data not shown). It should be noted that attempts to differentiate SMLCs in medium without serum (0% serum) could not support cell growth, resulting in extensive cell death after 6 days. Re-adding serum to the mSMLCs for another 6 days resulted in down-regulation of SM-MHC and elastin (data not shown).
Figure 3.3.2-1. Serum starvation and PDGF deprivation induce contractile maturation. (A) Syn-vSMCs and (B) SMLCs were cultured for six additional days in media containing 10% serum with PDGF-BB and TGF-b1, 10% serum with TGF-b1, and 0.5% with TGF-b1; they were analysed for the mRNA expression of relevant cytoskeleton and ECM genes. (C) Immunofluorescence analysis of mSMLCs demonstrates the expression and organization of various cytoskeleton proteins, including calponin, SMA, SM22, phalloidin, and SMMHC, as well as the expression of elastin. Scale bars are 100 mm. *P < 0.05, **P < 0.01, and *** P < 0.001.
3.3.3 Serum starvation and PDGF-BB deprivation induces contractile maturation

To achieve the maturation of Con-vSMCs from PSCs at levels comparable with those in the body, we examined the effect of short-term (6 days) and long-term (12 days) culture in media containing 0.5% serum with and without TGF-β1. First, as expected, we noticed that the growth rate decreased along the culture period in low serum. The continuous differentiation of mSMLCs for an additional 6 days in either set of conditions was not sufficient to induce maturation (Figure 3.3.3-1A). Continuous differentiation of the mSMLCs in low serum medium for 12 days (a total of 30 days of differentiation) induced Con-vSMC maturation, namely the up-regulation of SMMHC and elastin, with slightly different responses to the addition of TGF-β1 among different hPSC lines (Figure 3.3.3-1B; Figure 3.3.3-2). Nonetheless, we found that levels of SMMHC expressed in Con-vSMCs were slightly higher than those of aortic vSMCs, while elastin levels were inconsistent in the cell lines but were, overall, higher than in the Con-vSMCs (Figure 3.3.3-3). Notably, culturing these Con-vSMCs in low-serum medium with TGF-β1 for up to 18 days maintained high levels of SMMHC and elastin expression with decreasing proliferation rates, whereas culturing them in high-serum medium reduced the levels of SMMHC and elastin expression with increasing proliferation rates (data not shown). An up-regulation in the expression of myocardin, a serum response factor (SRF) coactivator, through ERK was found to correlate with Con-vSMCs maturation (Figure 3.3.3-1C). The activation of the pathway proved more prominent in the hESCs than in the integration-free hiPSCs (Figure 3.3.3-4). Both SMAD3 and YAP1 were up-regulated in the Syn-vSMCs compared with Con-vSMCs. Interestingly, these data also suggest that TGF-b1 is imperative for the proper regulation of those pathways (Figure 3.3.3-1C).
Figure 3.3.3-1. Mature SMLCs acquire a contractile phenotype, with or without the addition of TGF-β1. H9-hESC-derived mSMLCs were cultured in medium containing 0.5% serum, with and without TGF-β1, for (A) an additional 6 days and (B) an additional 12 days and analyzed for the mRNA expression of relevant cytoskeleton and ECM genes. (C) Comparison of the mRNA expression of pathway regulators during the stages of differentiation and maturation. *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 3.3.3-2. Maturation of hiPSC lines. Mature SMLCs derived from (A) BC1 and (B) MR31 were cultured in media containing 0.5% serum, with and without TGF β1, for an additional 12 days and analyzed for the mRNA expression of relevant cytoskeleton and ECM genes.
Figure 3.3.3. Con-vSMCs compared to human aortic vSMCs. Quantitative RT-PCR analysis shows the relative mRNA expression of the relevant cytoskeleton and ECM of the differentiated SMLCs compared to human aortic vSMCs.
An up-regulation in the expression of myocardin, a serum response factor (SRF) coactivator, through ERK was found to correlate with Con-vSMCs maturation (Figure 3.3.3-1C). The activation of the pathway proved more prominent in the hESCs than in
the integration-free hiPSCs (Figure 3.3.3-4). Both SMAD3 and YAP1 were up-regulated in the Syn-vSMCs compared with Con-vSMCs. Interestingly, these data also suggest that TGF-β1 is imperative for the proper regulation of those pathways (Figure 3.3.3-1C).

3.3.4 Characterization of Syn-vSMC and Con-vSMC derivatives

We continued by characterizing the Con-vSMCs and Syn-vSMCs. Both are spindle shaped, with the Syn-vSMCs more elongated (Figure 3.3.4-1A). Filamentous cytoskeleton organization of F-actin, SMA, calponin, SM22, and SMMHC was observed in the Con-vSMC, but to a lesser extent in the Syn-vSMCs (Figure 3.3.4-1A). The production of elastin was detected in the Con-vSMCs but not in the Syn-vSMCs (Figure 3.3.4-1A), and the assembly of elastin was further detected after several days in culture (Figure 3.3.4-1B). Con-vSMCs proliferated slower than Syn-vSMCs (14.15+ 4.20 vs. 83.19 +10.22%; Figure 3.3.4-1C). Finally, TEM analysis revealed that the Con-vSMCs have more (and more active) caveolae than the Syn-vSMCs, which have fewer caveolae (Figure 3.3.4-1Di-ii). The Con vSMC has a larger endoplasmic reticulum (ER) than the Syn-vSMC (Figure 3.3.4-1Dii; Figure 3.3.4-2), while plentiful actin stress fibres (with occasion bundles) were observed in the Con-vSMCs (Figure 3.3.4-1Diii; Figure 3.3.4-2).
Figure 3.3.4-1. Characterization of Con-vSMCs and Syn-vSMCs. (A) The two phenotypes were characterized for morphology, using light microscopy (LM), and the organization of various cytoskeleton proteins, including calponin, SMA, SMMHC, SM22, and phalloidin, as well as the expression of elastin, all using immunofluorescence staining. (B) Con-vSMCs cultured for 6 days were analysed for elastin assembly using immunofluorescence staining. (C) Proliferative cells were detected using immunofluorescence staining of Ki-67 in both cell phenotypes. Scale bars are 100 mm. (D) TEM analysis showed (i) caviolae (arrowheads) with occasional fusion (asterisk); (ii) ER; and (iii) stress fibres (arrows). ER, endoplasmic reticulum.
Figure 3.3.4-2. TEM analysis and functionality of hPSC derivatives. (A) High resolution imaging of organelles in Syn-vSMCs and Con-vSMC show (i) caviolae (arrowheads) ER, and stress fibers (arrows); (ii) actin bundles (asterisks). ER=endoplasmic reticulum; N=nucleus. (B) Subcutaneously transplanted Syn-vSMCs migrated to newly formed blood vessels within the Matrigel, as indicated by representative confocal images. Syn-vSMCs in red; mouse endothelial cells in green; and nuclei in blue.)
3.3.5 Functionality

To determine functionality, we first measured contractility in vitro. Contraction studies indicated that Con-vSMCs contract significantly better than Syn-vSMCs; aortic vSMCs and Syn-vSMCs contract better than SMLCs; and Con-vSMCs contract similarly to the human aortic vSMC line (Figure 3.3.5-1A). Our earlier studies demonstrated that vSMC derivatives of human PSCs, which were synthetic by nature, migrate towards and support vasculature both in vitro [51] and in vivo [163]. Here, we examined Syn-vSMC and Con-vSMC interaction with newly formed functional blood vessels. We observed transplanted Syn-vSMCs and Con-vSMCs migrating to the vasculature and locating in the outer layers of the mouse blood vessels that penetrated into the matrigel plug (Figure 3.3.5-1Bi; Figure 3.3.4-2). In the case of Con-vSMCs, human elastin was further detected around some of the smaller mouse blood vessels that penetrated into the matrigel plug (Figure 3.3.5-1Bii-iii). On some occasions, the human Con-vSMCs were found to wrap the smaller mouse vasculature circumferentially (Figure 3.3.5-1C), narrowing the endothelial tube (Figure 3.3.5-1D). These were not observed with the Syn-vSMCs. Hence, to achieve the contractile or synthetic maturation of differentiating hPSCs, we propose the use of a stage-specific differentiation practice, with appropriate concentrations of factors known to control these developmental steps in the early embryo and in adulthood (Figure 3.3.5-2). Moreover, individual hPSC lines require the optimized administration of TGF-β1 for efficient maturation of contractile vSMCs. Such an approach enables the acquisition of the morphological features, cytoskeleton expression, and contractility typical for the contractile phenotype.
Figure 3.3.5-1. Functionality of hPSC derivatives. (A) Contractility rates of SMLCs (n=9), Syn-vSMCs (n=12), Con-vSMCs (n=9), and human aortic vSMCs (n=12). (B) Subcutaneously transplanted Con-vSMCs migrated to newly formed blood vessels within the Matrigel, as indicated by representative (i) confocal images; and (ii–iii) light microscopy images of human elastin-stained sections. Scale bars are 50 mm. (C) Occasionally, Con-vSMCs were found wrapping around the functional vasculature. The high-magnification inset shows the boxed area. (D) (i) 3D reconstruction of confocal microscopy images revealed an event of tube narrowing of small vessels by the transplanted Con-vSMCs further demonstrated by (ii) measurements of adjusted areas within the vessel with and without Con-vSMC wrapping. Scale bars are 20 mm. For confocal images, Con-vSMCs in red (some indicated with white arrows); mouse endothelial cells in green; and nuclei in blue. *P < 0.05, **P < 0.01, and ***P < 0.001.
3.4 Discussion

Our previous studies demonstrated that we could derive vascular lineages from hESCs by administering angiogenic growth factors using a two-dimensional (2D) monolayer differentiation protocol or by isolating vascular progenitor cells or CD34^+ cells from ten-day-old EBs, followed by selective induction into either endothelial-like cells (using vascular endothelial growth factor (VEGF) or SMLCs (using PDGF-BB) [154, 164]. More recently, we built on these initial studies, establishing a simple step-wise monolayer differentiation protocol that differentiated hPSCs in monolayers and supplemented them with PDGF-BB and TGF-β1, which resulted in highly purified cultures of SMLCs [51]. These SMLCs were .98% positive for SMA, calponin, and SM22 and _50% positive for SMMHC. They produced collagen and fibronectin, and they contracted in response to carbachol. Further in vitro tubulogenesis assays revealed that these hPSC-derived SMLCs interacted with human endothelial progenitor cells to support and augment the formation of cord-like structures [51]. The current study sought to determine how these SMLCs make the synthetic vs. contractile phenotype decision.
Synthetic-vSMCs produce ECM proteins, such as collagen and fibronectin, as well as MMP proteins, in order to aid in cell migration [24]. We first demonstrated that long-term (up to 30 days) culture of the differentiated SMLCs in high serum with PDGF-BB and TGF-β1 resulted in maturation towards a synthetic phenotype, reducing the expression of contractile proteins and increasing the expression of ECM proteins, collagen, fibronectin, and MMPs. Indeed, both of these growth factors were suggested in early stages of differentiation [51, 154, 164]. Attempts to eliminate only PDGF-BB or both growth factors from the culture media somewhat increased synthetic phenotype characteristics (i.e. SMMHC and elastin expression), suggesting that this strategy may prove useful for guiding the contractile phenotype. Nonetheless, after their long-term exposure to PDGF-BB and TGF-β1, these Syn-vSMCs seemed unable to acquire a contractile phenotype when deprived of serum and growth factor, suggesting a terminal synthetic phenotype.

To mimic the native state of vSMCs in vessels, we wanted to switch to a quiescent and contractile state [161]. Quiescence is marked by the reduction of the proliferative capacity of a cell. Vascular SMCs in vessel walls replicate at the low frequency of 0.047% per day [165]. In this low proliferative state, the vSMC becomes committed to its contractile function [14, 162]. Growth factors, as well as foetal calf serum, drive the proliferative capacity of vSMCs [166]. However, we still do not know how the proliferative state of native vSMCs becomes suppressed. Moreover, it has been suggested that PDGF-BB interferes with vSMC maturation [85, 86, 159, 160]. SMMHC has a high specificity for SMCs and is also considered a mature marker indicating a contractile phenotype [14]. The ECM protein elastin is also expressed in the contractile state [18, 19]. In adult vSMCs, elastin acts as an autocrine regulator and also determines mechanical responsiveness [167]. Indeed, when SMLCs were matured in
media containing low concentrations of serum and supplemented with TGF-β1, we saw the up-regulation of SMMHC and elastin in the mSMLCs. These mSMLCs seem to retain plasticity, as indicated by down-regulation of the contractile proteins SMMHC and elastin when differentiated in media containing high concentrations of serum.

Continued quiescence of mSMLC in media containing low concentrations of serum and supplemented with or without TGF-β1 induce additional upregulation in the expression of contractile proteins. These Con-vSMCs maintained their contractile phenotype when cultured in low-serum conditions; they exhibited plasticity with the down-regulated expression of contractile protein when cultured in high-serum concentrations.

Myocardin, a potent SRF coactivator that is expressed exclusively in vSMCs and cardiomyocytes [168], reportedly promoted SMC differentiation through transcriptional stimulation of SRF-dependent smooth muscle genes including SMMHC [169, 170]. A recent study demonstrated that myocardin-/- mouse ESCs differentiate to vSMCs, suggesting the dispensability of myocardin for the development of vascular SMCs [171]. In support of this observation, our data using hPSCs shows that upregulation of SRF and myocardin is not necessarily associated with the contractile state of the differentiating vSMCs. In support of this observation, we report that deprivation of TGF-β1 seems to affect the activation of the different pathways, although up-regulation of contractile proteins was observed. Overall, our data using hPSCs show that up-regulating the myocardin pathway was not necessarily associated with the contractile state of the differentiating vSMCs. Finally, both YAP-1 and SMAD3 have been suggested as regulators important for inducing the synthetic phenotype in vSMCs [172, 173]. Our data suggest that these also get up-regulated during the synthetic phenotype.
maturation of hPSC derivatives. Here as well, deprivation of TGF-β1 seems to affect the activation of these pathways in contractile maturation. Additional studies to delineate the specific mechanism underlying these observations in hPSCs.

Comparing Con-vSMC and Syn-vSMC derivatives, we observed that both acquire a more spindle-shaped morphology than SMLCs. More prominent filamentous organization of the various cytoskeleton proteins was found in Con-vSMC than in Syn-vSMC derivatives. Interestingly, both cell derivatives showed increases in contractility: Syn-vSMCs showed some increased contractility, which may be attributed to the needed optimization of the culture period and to cell confluence; Con-vSMCs exhibit a rather greater increase in contractility than human aortic vSMCs, most likely due to higher SMMHC expression. Reducing the serum concentrations in media of SMLCs markedly decreased the proliferation rates of the cells and was accompanied by an increase in the contractile phenotype. Indeed, the Con-vSMC phenotype was marked by a reduced proliferative capacity, unlike the Syn-vSMC phenotype, which exhibited a high proliferative capacity. Finally, high-resolution analysis further revealed profound differences previously observed between the two phenotypes [174]. Unlike Syn-vSMCs, Con-vSMCs exhibited numerous and active caveolae with enlarged ER and abundant stress fibres and bundles, underlining the distinctive shift between two major differentiated states with distinct morphological and functional properties.

Researchers envision human iPSCs — which can be derived directly from a patient, thereby reducing the risk of immunogenicity upon transplantation — as dramatically revolutionizing cell-based therapies for regenerative medicine. Since Takahashi and Yamanaka’s pioneering discovery [45], hiPSC technology has evolved rapidly. While the hiPSC technologies initially reported have several obvious shortcomings, many of these have recently been overcome. This study tested MR31 —
a hiPSC clone derived from the IMR90 line, which was derived from normal, foetal lung fibroblasts using lentivirus to deliver three reprogramming factors (Oct-4, Sox2 and Klf4) [155] — and BC1, which was induced using CD34+ blood cells from bone marrow using plasmids encoding all four reprogramming factors [156, 157]. We have shown that hiPSCs respond to the differentiation protocol similarly to hESCs and can mature into the synthetic and contractile phenotypes of vSMCs. The mSMLCs derived from all the hPSCs examined exhibited comparable expression levels of both SMMHC and elastin. We observed some differences during their long-term exposure to serum starvation with and without TGF-b1. Specifically, when culturing mSMLCs derived from MR31 in a low concentration of serum, with or without TGF-b1, we detected up-regulated elastin expression and down-regulated expression of SMMHCs. The derivation of vSMCs from the BC1 line, an integration-free-induced PSC line [156, 157], offers a practical approach for using this clinically relevant technology for vascular regeneration. Thus, it seems apparent that hiPSCs have immense potential for providing effective treatments or cures for vascular diseases, which warrants further investigations and improvements.

Previous studies have suggested that vSMCs wrap circumferentially rather than longitudinally around blood vessels [175, 176]. Some have suggested that this wrapping improves the mechanical properties [161, 177] of the vessel wall while also managing proper vasoactive activity [161]. In early studies, we demonstrated the contribution of vSMC derivatives of a synthetic nature to growing vasculature [51, 161]. The current study tested whether the Con-vSMCs could still migrate towards a growing vessel, as well as begin wrapping. Utilizing a subcutaneous transplantation model assay, we have shown that Con-vSMCs encapsulated in Matrigel plugs migrate to sites near newly grown functional vasculature where they produce elastin that stabilizes those
vasculatures. Moreover, the Con-vSMCs were sometimes found wrapping and even narrowing the host vessels. Such Con-vSMCs offer opportunities to use such derivatives to enhance the stabilization and maturation of new blood vessels in regenerating tissues.

In summary, the findings reported here demonstrate fate decisions in vascular smooth muscle phenotypes during the differentiation of hPSCs. By monitoring the expression of SMMHC and elastin, we demonstrate the possibility of generating synthetic or contractile phenotypes from different hPSC lines with appropriate concentrations of factors known to control these developmental steps in the early embryo and in adulthood. These findings highlight the importance of designing stage-specific differentiation strategies that follow key developmental steps to exploit cellular plasticity for vSMC phenotypic decisions. Finally, contractile hPSC-vSMCs derived from the integration-free hiPSC line BC1 may prove useful for regenerative therapy involving blood vessel differentiation and stabilization.
4

4.1 Introduction

The vasculature is a multicellular system in which each cell type plays an important and indispensible role in its function. The inner lining of endothelial cells (ECs), which are in direct contact with the blood, is surrounded and supported by perivascular cells – either vascular smooth muscle cells (vSMCs) or pericytes. Vascular SMCs surround larger vessels such as arteries and veins, whereas pericytes typically surround smaller microvessels and capillaries [4]. The disparate vessel locations for each perivascular cell type suggests that further differences exist that should be investigated and better understood in vitro in order to appropriately rebuild blood vessels for therapeutic applications [43, 178].

As the vasculature’s support system, perivascular cells are primarily responsible for imparting contractility and producing and depositing extracellular matrix (ECM) proteins. Both cell types migrate to sites of angiogenesis, the growth of blood vessels from pre-existing ones, to help stabilize and mature nascent endothelial tubes. Whether pericytes and vSMCs function similarly in these regards and to what extent has been unclear.

Along with the aforementioned functional similarities, perivascular cell types also exhibit overlapping marker expression. Adding to this complexity, neither perivascular cell type can be distinguished by one marker alone; instead, a combination of markers is needed for their identification. For example, both cell types have been demonstrated to
express alpha smooth muscle actin (α-SMA). The expression of α-SMA and the transmembrane chondroitin sulfate proteoglycan neuron-glial 2 (NG2) help distinguish pericytes in different vessel types [30]; pericytes of the capillaries are NG2+αSMA−, of the venules are NG2-αSMA+, and of the arterioles are NG2+αSMA+. When cultured in vitro, however, pericytes are positive for both of these markers. Other markers that are expressed on both perivascular cell types include calponin and PDGFRβ [179, 180].

Examining differences in perivascular cell types is further complicated by added heterogeneities within the subtypes [181, 182]. Two distinct vSMC phenotypes have been elucidated: synthetic and contractile [127, 181, 183]. Both participate in neovascularization, but synthetic vSMCs predominate in the embryo and in diseased or injured adult vessels while contractile vSMCs predominate in healthy adult vessels.

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced PSCs (hiPSCs), have been widely used to study somatic cell types due to their ability to obtain cell derivatives of identical genetic backgrounds. They are known for their ability to self renew indefinitely in culture and to differentiate toward every cell type, including perivascular cells [43]. Human iPSCs are derived from a patient’s own cells, and thus can yield derived cell populations that are patient specific, providing a clinically-relevant pluripotent cell source for therapeutic use. Indeed, we and others have examined the derivation of both vSMCs [127, 184, 185] and pericytes [129, 180, 186].

Using a stepwise differentiation protocol, we have demonstrated the maturation of smooth muscle-like cells (SMLCs; [135]) to synthetic vSMCs (syn-vSMCs) and contractile vSMCs (con-vSMCs) from both hESCs and hiPSCs [127]. Using a similar but
distinct stepwise differentiation protocol, we have also demonstrated the derivation of pericytes from various hPSC lines [129].

Building off of our previous studies, we sought to comprehensively define differences between con-vSMCs, syn-vSMCs, and pericytes derived from a common hPSC source in order to uncover cellular and functional differences *in vitro*, toward the long term goal of rebuilding vasculature for therapeutic applications. For example, the quality of tissue-engineered blood vessels is dependent on the characteristics of the *in vitro* perivascular cells used. Current challenges of engineering blood vessels include precise mechanical requirements and tissue-specific cell types [187]. The *in vitro* characterization of our hPSC-derived perivascular cells may mediate the production of tissue-engineered blood vessels that have the patency and mechanical responsiveness equivalent to the native tissue [161]. Of clinical relevance, hiPSC-BC1 line is used as the hPSC source for our studies. BC1 is derived without viral integration and has been fully genetically sequenced [125, 126]. Here we focus on differences in perivascular cells derived from BC1 cells with respect to cellular characteristics, protein expression, ECM deposition and remodeling, migration, invasion and contractility.

### 4.2 Methods and Materials

#### 4.2.1 Cell Culture

All cells were cultured in humidified incubators, with atmospheres at 37°C and 5% CO₂.

**Human PSCs.** hiPSC line BC1 [125, 126] kindly provided by Dr. Cheng, SOM JHU and Human ESC line H9 (passages 15 to 40; WiCell Research Institute, Madison, WI) were grown on inactivated mouse embryonic fibroblast feeder layers (GlobalStem, Rockville, MD) in growth medium composed of 80 percent ES-DMEM/F12 (GlobalStem), 20 percent knockout serum replacement (Invitrogen, Carlsbad, CA), and 4 ng/ml basic
fibroblast growth factor (bFGF; Invitrogen) for hESCs of 10ng/ml bFGF for hiPSCs, as previously reported [127]. Human iPSCs were passaged every four to six days using 1 mg/ml of type IV collagenase (Invitrogen). Media were changed daily.

**Human v-SMCs.** The control cell type used was human aorta v-SMCs (passages 4-7; ATCC, Manassas, VA). The cells were cultured in the specified ATCC complete SMC growth medium, composed of Kaighn’s Modification of Ham’s F-12 Medium (F-12K Medium; ATCC), 10% or 0.5% fetal bovine serum (FBS; Hyclone), 0.01 mg/ml transferrin (Sigma-Aldrich, St. Louis, MO), 0.01 mg/ml insulin (Sigma), 10 mM HEPES buffer (Sigma), 10 mM 2-(Tris(hydroxymethyl)methylamino)ethane-1-sulphonic acid (TES)(Sigma), 0.05 mg/ml ascorbic acid (Sigma), 10 ng/mL sodium selenite (Sigma), and 0.03 mg/ml Endothelial Cell Growth Supplement (Sigma). Human v-SMCs were passaged every three to four days using 0.25 percent trypsin (Invitrogen). Media was changed every two to three days.

**Human pericytes.** The control cell type used was human placental pericytes (passages 3-5; Promocell). The cells were cultured in the specified Pericyte Growth Media (Promocell) and were passaged every three to four days using a detachment kit (Promocell).

**vSMC differentiation protocol**

vSMCs were derived as previously described [128]. Briefly, hPSCs were collected through digestion with TrypLE (Invitrogen) and were seeded at a concentration of 5x10^4 cells/cm^2 onto plates previously coated with collagen type IV (R&D Systems, Minneapolis, MN). The hPSCs were cultured for six days in a differentiation medium, composed of alpha-MEM (Invitrogen), 10% FBS (Hyclone), and 0.1 mM β-mercaptoethanol (Invitrogen). Media were changed daily. On day six, the differentiated
cells were collected through digestion with TrypLE (Invitrogen), separated with a 40-µm mesh strainer, and seeded at a concentration of 1.25x10^4 cells/cm^2 on collagen-type-IV-coated plates. The differentiating hPSCs were then cultured in differentiation medium; with the addition of 10 ng/ml PDGF-BB (R&D Systems) and 1 ng/ml TGF-β1 (R&D Systems) for additional 6 days (total of 12 days) for SMLCs. Media was changed every second day. Serum starved cells were passaged every 6-8 days with Tryple, using alpha-MEM (Invitrogen), 10% FBS (Hyclone), and 0.1 mM β-mercaptoethanol (Invitrogen) to neutralize Tryple but then seeded with 0.5% serum media.

**Pericyte differentiation protocol**

Pericytes were differentiated as previously described [67, 129]. Briefly, hPSCs were differentiated as described for vSMC differentiation above for the first 6 days. On day 6, cells were re-seeded at a concentration of 1.25x10^4 cells/cm^2 on collagen-type-IV-coated plates in endothelial cell growth media (ECGM) (PromoCell) supplemented with 2% FBS, 50ng/ml vascular endothelial growth factor (VEGF), and 10µM SB431542 (Tocris) for 6 days. Media was changed every other day. On day 12, derived EVCs were collected through digestion with TrypLE and replated on tissue culture-treated six-well plates in medium composed of DMEM and 10% FBS. After 2–3 h, unattached cells were removed, and the medium was replaced. Cells were cultured for 6 d, with the medium changed every other day.

**4.2.2 Stress fiber quantification**

The number of stress fibers per cell was quantified using line intensity profiles of cells in ImageJ [188]. Stress fibers were labeled with fluorescent Alexa-488 phalloidin and imaged at 20x and 40x. A line intensity profile across a single cell was generated with each peak representing a single stress fiber.
4.2.3 Invasion toward ECs

A downward invasion assay was used to assess invasion of perivascular cells. Human umbilical vein ECs were seeded on 16 well detachable wells (Fisher). After 24 h, 150 uL of collagen gel (150 uL) was added on top of the ECs. After 1h, hiPSCs perivascular cells were seeded on top of the gels. After 48h the gels were fixed, stained with toluidine blue dye and cross-sections of the gels were imaged and quantified.

4.2.4 Flow cytometry

Flow cytometry was performed as previously described [130]. Briefly, cells were incubated with PE-conjugated antigen specific antibodies for markers outlined in the text including KDR-PE (1:10; BD), Nestin-PE (1:10; BD), CD56-PE (1:10; BD); SMMHC-PE (1:10; MYH11; Santa Cruz). To detect SMMHC -PE, cells were fixed with 3.7% formaldehyde for 10 minutes, washed, incubated with 0.1% Triton X for 10 minutes, washed, and finally incubated with SMMHC -PE for 45 minutes. All analyses were done using corresponding isotype controls. Forward-side scatter plots were used to exclude dead cells. User guide instructions were followed to complete the flow cytometry analysis via Cyflogic v1.2.

4.2.5 Immunofluorescence

Cells were prepared for immunofluorescence as previously described [128, 130]. Cells were fixed using 3.7% formaldehyde fixative for 15 minutes, washed with phosphate buffered saline (PBS), blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour minimum, permeabilized with a solution of 0.1% Triton-X (Sigma) for ten minutes, washed with PBS, and incubated for one hour with anti-human SMA (1:200; Dako, Glostrup, Denmark), anti-human NG2 (1:100; Santa Cruz), anti-human PDGFRβ (1:100, Santa Cruz), and anti-human SMMHC (3:100; Dako). For ECM staining, cells
were incubated with anti-human fibronectin (1:200; Sigma), anti-human collagen1 (1:200; Abcam), anti-human collagen IV (1:100; Abcam), anti-human laminin (1:200; Abcam) or anti-human elastin (3:100 Abcam) for one hour. Cells were rinsed twice with PBS and incubated with Alexa 546 conjugated phalloidin (1:100; Molecular Probes, Eugene, OR) or anti-mouse IgG Cy3 conjugate (1:50; Sigma), anti-mouse FITC (1:50; Sigma), or anti-rabbit IgG Alexa Fluor 488 conjugate (1:1000; Molecular Probes, Eugene, OR) for one hour, rinsed with PBS, and incubated with DAPI (1:1000; Roche Diagnostics) for ten minutes. Coverslips were rinsed once more with PBS and mounted with fluorescent mounting medium (Dako). The immunolabeled cells were examined using fluorescence microscopy (Olympus BX60; Olympus, Center Valley, PA) and confocal microscopy (LSM 510 Meta; Carl Zeiss).

4.2.6 Cellular characterizations

The nuclei size of cells was quantified in ImageJ by thresholding fluorescence intensities of DAPI. The cellular area was quantified by thresholding the fluorescent intensities of the membrane dyes FM464. The percentage of replicating cells was quantified in ImageJ by taking the ratio between the number of Ki67 fluorescent positive cells and the fluorescent DAPI. At least three fields of view were imaged at 10x for each sample.

4.2.7 Transmission electron microscopy (TEM)

Differentiated cells, placental pericytes, and aortic vSMCs were prepared for TEM analysis as described previously [131]. Serial sections were cut, mounted onto copper grids, and viewed using a Phillips EM 410 TEM (FEI, Hillsboro, OR, USA). Images were captured using a SIS Megaview III CCD (Lakewood, CO, USA).

4.2.8 Real-time quantitative RT-PCR
Two-step RT-PCR was performed on differentiated hPSCs at various time points as we previously described [128]. Total RNA was extracted by using TRIzol (Gibco, Invitrogen), as per the manufacturer’s instructions. All samples were verified as free of DNA contamination. The concentration of total RNA was quantified using an ultraviolet spectrophotometer. RNA (1 µg per sample) was transcribed using the reverse transcriptase M-MLV (Promega Co., Madison, WI) and oligo(dT) primers (Promega), as per the manufacturer’s instructions. The specific assay used was the TaqMan Universal PCR Master Mix and Gene Expression Assay (Applied Biosystems, Foster City, CA) for *ACTA2, CNN1, CSPG4, PDGFRB, MYH11, COLA1, COL4A4, LAMC1, ELN, MMP14, CALD1, ALPNR, TCF21, KDR, PAX6, WNT1, SOX1, ACTB, and GAPDH*, as per the manufacturer’s instructions. Assays on Demand Kits (Applied Biosystems, Foster City, CA, USA) was used for FN1 (Hs01549958) and the customized (Applied Biosystems, Foster City, CA, USA) ED-A spanning exons sequence primer was: Forward 5’-CCAGTGCACAGCTATTCCTG-3’ and Reverse 5’-ACAACCACGGATGAGCTG-3’ [132, 133].

The Taqman PCR step was performed with an Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer’s instructions. The relative expressions of the genes were normalized to the amount of *ACTB* or *GAPDH* in the same cDNA by using the standard curve method provided by the manufacturer. For each primer set, the comparative computerized tomography method (Applied Biosystems) was used to calculate the amplification differences between the different samples. The values for the experiments were averaged and graphed with standard deviations.

4.2.9 Zymography
Zymography was performed to determine MMP activities as previously [134]. MMP1 was detected using SDS-Page casein zymography while both MMP2 and MMP9 were detected using SDS-Page gelatin zymography. Cells were cultured in serum free media for 72 hours. We collected the media of each sample and loaded the media of the samples per well into either a casein gel (BioRad) or gelatin gel (BioRad). Quantification of protein was done using the Bradford Assay. After electrophoresis, the gels were renatured by washing in renaturation buffer (Invitrogen) and incubated at 37˚C in denaturation buffer (Invitrogen) for 24h. The proteins were fixed in 50% methanol and 10% acetic acid for 30 min and then stained in 0.02% comassie blue (Sigma). Gels were destained in 20% methanol and 10% acetic acid and were visualized using the ChemiDoc XRS+ System (BioRad). Images were acquired using BioRad Quantity One software.

4.2.10 Mesenchymal differentiation (adipogenic and osteogenic)

We followed our previously published protocol for mesenchymal differentiations (Kusuma et al., 2013). For adipogenic differentiation [189], we cultured derived pericytes at 10,000 cells/cm² in media comprised of DMEM, 10% FBS, 1% Penicillin/Streptomycin, 200µM Indomethacin, 500 µM 3-Isobutyl-1-methyl xanthine (IBMX), and 5 µg/ml Insulin (all from Sigma) for 4 weeks. To assess adipogenic potential, cells were fixed with 3.7% formaldehyde, then dehydrated with 60% isopropanol for 5 minutes. Cells were incubated with Oil Red O (Sigma) at 1.8 mg/ml in 60/40 isopropanol/DI H₂O, for 10 minutes and imaged using an inverted light microscope (Olympus).

For osteogenic differentiation [190], we cultured derived pericytes at 5,000 cells/cm² in media comprised of low glucose DMEM, 10% FBS, 1% Penicillin/Streptomycin, 10mM β-glycerophosphate, 100nM dexamethasone, and 50 µM
ascorbic acid (all from Sigma) for 2 weeks. Media were prepared fresh weekly. To assess osteogenic potential, samples were fixed with 3.7% formaldehyde, and washed with DI H₂O. Samples were incubated with Alizarin Red S (40mM in DI H₂O, pH ~4.2; Sigma) for 10-20 minutes.

4.2.11 Subcutaneous Matrigel implantation

hiPSC-derived perivascular cells were trypsinized, collected and stained with PKH26 (Sigma-Aldrich) membrane dye. We encapsulated a total of 0.5×10⁶ PSC-vSMCs in reduced growth factor Matrigel (BD Biosciences) and 20 μL of EGM-2 media (endothelial growth media). The Matrigel, which contained 250 ng/mL of bFGF (R&D Systems), was loaded, along with the cell mixture, into a 1 mL syringe with a 22-gauge needle and injected subcutaneously into each side of the dorsal region of six- to eight-week-old nude mice. On day 7, we injected isolectin GS-IB4, an Alexa Fluor(R) 488 conjugate glycoprotein isolated from the Griffonia simplicifolia African legume (Invitrogen) through the tail veins of the mice. After 20 minutes, we euthanized the mice by CO2 asphyxiation and harvested the Matrigel plugs, which were fixed in 3.7 percent formaldehyde (Sigma-Aldrich) for one hour, and incubated with DAPI (1:1000; Roche Diagnostics) for ten minutes. A sequence of z-stack images was obtained using confocal microscopy (LSM 510 Meta, Carl Zeiss, Inc.). The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols.

4.2.12 Wound healing assay

Migration of the derived hiPSC perivascular cells was assessed using a wound healing assay [191]. Cells were cultured to a confluent monolayer in a 6 well plate. Cell monolayers were wounded by scratching a strip of cells with a 200uL pipette tip. After the detached cells were removed and the cells were washed, fresh medium containing
0.5% serum was added. Cells were incubated in a humidified incubator coupled to a microscope, which took a series of images of the migration of the cells into the gap every 10 min for 24 h. Migration trajectories and speed was calculated using the MTrackJ plugin of ImageJ (NIH).

4.2.13 Functional contraction studies

Contraction studies in response to pharmacological drugs were done, as previously described [128, 135]. Briefly, perivascular cell derivatives were cultured, washed, and contraction was induced by incubating with $10^{-5}$ M carbachol (Calbiochem, Darmstadt, Germany) in DMEM medium (Invitrogen) for 30 minutes. The perivascular cell derivatives were visualized using cytoplasm-viable fluorescence dye, calceine. A series of time-lapse images were taken using a microscope with a 10X objective lens (Axiovert; Carl Zeiss). The cell contraction percentage was calculated by the difference in area covered by the cells before (at time zero) and after contraction (at time 30 minutes). Area analysis was performed with Adobe Photoshop CS5 (Adobe Systems Inc., Mountain View, CA). Each set of images was analyzed three times. The magic wand and measurement tools were used to calculate the area of the image not covered in cells, which was then subtracted from the total area of the image. This method improves upon our previously established procedure [135] by eliminating the need for image compression and by increasing the consistency of cell selection within each set of images.

4.2.14 Statistical analysis

Real-time RT-PCR, functionality assays, flow cytometry and image analyses were performed in at least triplicate biological samples. Real-time RT-PCR analyses were also performed with triplicate readings. Statistical analyses were performed with
GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, CA). Unpaired two-tailed t-tests and one-way ANOVA analysis and Bonferroni post tests were performed where appropriate using GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, CA). Significance levels were set at *p<0.05, **p<0.01, and ***p<0.001. All graphical data are reported as mean ±SEM.

4.3 Results

4.3.1 Pericytes and vSMCs differ in morphological features and proliferation rates

Perivascular cells were derived from hPSCs using differentiation protocols from previous studies [127, 129, 135]. Using this protocol, hPSC SMLCs subjected to long-term differentiation and in the presence of high serum and growth factors were guided toward a synthetic fate, whereas deprivation of serum and growth factors yielded a contractile fate (Figure 4.3.1-1A; [127]). Early pericytes were derived as part of a bicellular population with early ECs named early vascular cells (EVCs) and further differentiated toward mature pericytes by a selective plating strategy (i.e. removal of collagen IV substrate and constrained adhesion time) in the presence of high serum (Figure 4.3.1-1A; [129]). For pericytes, EVCs highly express CD105 and CD146 while pericycle derivatives express NG2, PDGF-receptor β (PDGFRβ), CD44 and CD73 (Figure 4.3.1-1A; [129]).
Figure 4.3.1. Characterization of cellular properties of perivascular derivatives. (A) Schema for the differentiation procedure to derive perivascular cells from hPSCs. (B) Flow cytometry analysis of day 6 differentiating cells, EVCs, and SMLCs. Isotype control in gray. Results shown are representative of three independent experiments. CIV, collagen IV.
To better understand progression in differentiation, we examined marker expression at different time points along the differentiation of hPSCs. Over the first 6 days of differentiation, the three classes of perivascular cells underwent identical differentiation conditions. During embryonic development, vSMCs may arise from a number of precursors from different germ layers [192, 193]. We found that expression of mesodermal genes KDR, APLNR, and TCF21 increased over the first six days of differentiation in both BC1 and H9 cell lines whereas expression of neural crest markers SOX1, PAX6, and WNT1 remained stagnant (Figure 4.3.1-2A). Additionally, WNT1 was not expressed in BC1 differentiating cells. These data suggest the emergence of a mesodermal population. Furthermore, our day 6 differentiating cells were assessed for the expression of markers indicative of several intermediate lineages: neuroectoderm (Nestin), lateral plate mesoderm (KDR), paraxial mesoderm (Pax1) [193], early mesoderm (CD56) [194] and general mesoderm (CD73) [195, 196]. We found that a small fraction of day 6 differentiating cells was positive for Nestin (Figure 4.3.1-1B). KDR was only slightly expressed. Pax1 was not detected via PCR or by immunofluorescence (data not shown). Mesoderm markers CD56 and CD73, however, exhibited more pronounced expression; day 6 differentiating cells were 72% positive for CD73 and >95% positive for CD73. To distinguish the subsequently derived cell populations (i.e. EVCs and SMLCs), we performed flow cytometry analysis for the aforementioned markers. After differentiation toward EVCs, we observed that Nestin expression was completely abolished; however, a small fraction of SMLCs remained Nestin+ (Figure 4.3.1-1B). Similarly to day 6 differentiating cells, EVCs exhibited >95% positive expression for CD73, whereas CD73 expression decreased to ~70% in SMLCs. An important differentiator between EVCs and SMLCs is the presence of VEcad. Our previous studies demonstrated that BC1-EVCs exhibited approximately 30% VEcad+ cells [129]. Contrastingly, we could not detect VEcad expression on SMLCs (Vo et al.,
A similar trend of marker expression was observed at different time points along the differentiation of hESC line H9 (Figure 4.3.1-2B).

Figure 4.3.1-2. Marker assessment of perivascular derivatives. (A) Quantitative real time RT-PCR analysis of mesoderm (in solid lines) and neural crest (in dashed lines) genes at days 0, 3 and 6 along differentiation for BC1 and H9 (n=3 biological replicates). (B-C) H9 differentiating cells analyzed for (B) marker expression by flow cytometry analysis of day 6 differentiating cells, EVCs, and SMLCs (isotype control in gray) and (C) perivascular cell and nuclei area. Results shown are representative of three independent experiments. All graphical data are reported as mean ±SEM.
We next evaluated the differences in proliferation rates among the hPSC-derived mature perivascular cells. As expected, hiPSC con-vSMCs exhibited low proliferation rates as they are cultured in media containing low serum (0.5% serum) (Figure 4.3.1-1B) [127], while hiPSC syn-vSMCs exhibited high proliferation rates as they are cultured in media containing 10% serum (Fig. 2A). Although hiPSC pericytes were cultured using media containing 10% serum, they exhibited contact inhibited proliferation and grew in cell colonies (Figure 4.3.1-3A-B). Next, we evaluated the morphological features after culture on 2D surfaces (Figure 4.3.1-3B). While both hiPSC vSMC types spread evenly throughout the Petri-dish, hiPSC pericytes arranged themselves into colony-like structures. hiPSC con-vSMCs displayed the largest cell areas and nuclei sizes compared to hiPSC syn-vSMCs and hiPSC pericytes (Figure 4.3.1-3C). Human ESC-derivatives exhibited the same trend (Figure 4.3.1-2C). Indeed, a phenotypic switch from syn-vSMCs to con-vSMCs has been correlated with smaller, nuclei and a decrease in proliferation [197].
Figure 4.3.1-3. Analysis of hPSC-derived perivascular cells. Derivatives were analyzed for (A) proliferation using (i) stain for Ki67 (red; nuclei in blue) and (ii) corresponding image quantification, (B) morphology using (i) light microscopy and (ii) FM4-64 membrane stain (red) and DAPI (blue), (C) corresponding image quantification of (i) cell and (ii) nuclei area, and (D) subcellular organelle organization using transmission electron microscopy. CIV, collagen type IV; ER, endoplasmic reticulum; mitoch, mitochondria. Scale bars in (D) are 1 mm. Results shown from three independent experiments. All graphical data are reported as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

There were stark differences in endoplasmic reticulum (ER), mitochondria location, stress fibers, and the presence of autophagosomes observed between pericytes and vSMCs. As previously described, hiPSC con-vSMCs contain dilated ER while hiPSC syn-vSMCs contain non-dilated ER [127] (Figure 4.3.1-3D). On the other hand, hiPSC pericytes (as well as cell line placental pericytes) contained both dilated
and non-dilated ER (Figure 4.3.1-3D; Figure 4.3.1-4). The mitochondria of hiPSC pericytes were in close proximity to the nucleus in contrast to vSMCs, whose mitochondria were located further away from the nucleus. With respect to stress fibers, only con-vSMCs had stress fibers located throughout the entire cell body. Both hiPSC syn-vSMCs and hiPSC pericytes primarily had stress fibers located at the basal lateral surface (Figure 4.3.1-3D). Pericytes also had autophagosomes present whereas both hiPSC syn-vSMCs and con-vSMCs did not (Figure 4.3.1-3D; Figure 4.3.1-4).

Placental pericytes

![Control pericytes](image)

**Figure 4.3.1-4. Control pericytes.** Placenta pericytes were analyzed for sub-cellular organelle organization using TEM.

4.3.2 Differential expression of perivascular markers

We next examined the expression and localization of specific cytoskeleton proteins that have been reported to distinguish vSMCs and pericytes. Stress fibers are bundles of actin filaments that are important in mechanotransduction of adherent cells byanchoring to substrates and creating isometric tension [198]. Perivascular cell contraction is associated with a more filamentous cytoskeleton within the cells. Accordingly, the hiPSC con-vSMCs demonstrated elevated stress fibers per cell compared to both hiPSCs syn-vSMCs and hiPSC pericytes, which had significantly lower stress fibers per cell (Figure 4.3.2-1Ai-ii; additional fields of view in Figure 4.3.2-2). While differences in α-SMA expression and organization could not be observed, calponin expression was upregulated in hiPSC pericytes (Figure 4.3.2-1Ai, iii; Figure 4.3.2-2).
Figure 4.3.2-1. Differences in stress fiber and contractile marker expression. 
Perivascular derivatives were assessed for (A) (i) organization of stress fibers (confocal z-stacks), a-SMA, and calponin (in red; phalloidin in green; nuclei in blue); (ii) stress fiber number and (iii) a-SMA and calponin expression via quantitative real time RT-PCR; (B) (i) organization of NG2 (green) and a-SMA (red; nuclei in blue) and (ii) NG2 expression using quantitative real-time RT-PCR; (iii) (C) (i) PDGFRβ (green) and SMMHC (red; nuclei in blue) and (ii) PDGFRβ and SMMHC expression using quantitative real-time RT-PCR; (iii) flow cytometry analysis of SMMHC (isotype control in gray); and (D) caldesmon expression via quantitative real-time RT-PCR. Results shown from three independent experiments; each RT-PCR sample was run with three technical replicates. All graphical data are reported as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001
Figure 4.3.2-2. Differences in stress fiber and contractile marker expression. Perivascular derivatives were assessed by immunofluorescence for αSMA and calponin (in red; phalloidin in green; nuclei in blue). Figures shown are low magnification representative images of 4.3.2-1A.

The cell surface proteins NG2 and PDGFRβ are also commonly associated with perivascular cells. The proteoglycan NG2 has been shown to be widely expressed by perivascular cells in both vasculogenic and angiogenic vasculature [199]. While NG2 is an appropriate marker for identifying pericytes in microvessels, it is merely a supplemental vSMC identifier considering the variety of vSMC markers [199]. Interestingly, profuse stress fibers were observed with the expression of NG2 protein only in hiPSC con-vSMC cultures (Figure 4.3.2-1Bi). Indeed, NG2 mRNA expression in hiPSC con-vSMCs was significantly elevated compared to hiPSC syn-vSMCs and hiPSC pericytes (Figure 4.3.2-1Bii). Similarly, aortic vSMCs exhibited NG2 expression with profuse stress fibers compared to placental pericytes (Figure 4.3.2-1Bii; Figure 4.3.2-3A). We observed differences in the expression of PDGFRβ in our hiPSC derivatives. Human iPSC con-vSMCs exhibited elevated PDGFRβ mRNA expression that had a
punctuate membrane expression as well as nuclear expression (Figure 4.3.2-1Ci,ii) comparable to aortic vSMCs (Figure 4.3.2-3B).

Figure 4.3.2-3. Perivascular marker expression in control cells. Organization of (A) SMA (red) and NG2 (green; nuclei in blue) and (B) SMMHC (red) and PDGFRβ (green; nuclei in blue) in aortic vSMCs and placenta pericytes. (C) Expression of NG2, SMMHC, PDGFRβ and caldesmon in the different cell types compared to aortic vSMCs starved in low serum using quantitative real time RT-PCR. Results shown from three independent experiments; each RT-PCR sample was run with three technical replicates. All graphical data are reported as mean ±SEM. *P<0.05; **P<0.01; ***P<0.001.
Mature vSMC marker SMMHC is associated with the contractile vSMC phenotype [167, 200]. Human iPSC con-vSMCs exhibited elevated SMMHC expression compared to hiPSC syn-vSMCs and pericytes (Figure 4.3.2-1C). Correspondingly, aortic vSMCs exhibited elevated SMMHC expression and SMMHC stress fibers while placental pericytes did not (Figure 4.3.2-1C; Figure 4.3.2-3). We further evaluated mRNA and protein expression of SMMHC on our perivascular cell derivatives. Human iPSC con-vSMCs exhibited the greatest expression of SMMHC mRNA compared to all other cell types tested (Figure 4.3.2-1Cii). At the protein level, SMMHC was only detected on control aortic vSMCs and hiPSC con-vSMCs ((Figure 4.3.2-1Ciii); hiPSC syn-vSMCs were negative for SMMHC. Interestingly, SMMHC was not detected in either hiPSC pericytes or control placental pericytes (Figure 4.3.2-1Cii-iii). Finally, the expression of caldesmon, which plays an important role in the perivascular contraction function, was assessed in the different types of perivascular cells. We found that caldesmon was elevated in con-vSMCs compared to syn-vSMCs and pericycle derivatives ((Figure 4.3.2-1D). We note that the expression of perivascular markers in aortic vSMCs cultured in low serum (0.5%) conditions was slightly altered yet exhibited a similar trend of aortic vSMCs cultured in 10% serum and hiPSC con-vSMCs (Figure 4.3.2-3C).

4.3.3 ECM protein production

A primary function of perivascular cells is the deposition of ECM proteins to help stabilize vasculature. Because the ECM composition of various vessel types differs, we next assessed the different perivascular cell types for expression and production of ECM proteins collagen I, collagen IV, fibronectin, laminin, and elastin in vitro (Figure 4.3.3-1). We found that both phenotypes of vSMCs exhibited concentrated perinuclear collagen I expression while hiPSC pericytes demonstrated diffuse expression of collagen I around
the cytoplasm (Figure 4.3.3-1Ai). hiPSC syn-vSMCs and hiPSC pericytes exhibited similar extracellular and globular expression of collagen IV, while hiPSC con-vSMCs had abundant fibrous intracellular and extracellular collagen IV (Figure 4.3.3-1Aii). Human iPSC con-vSMCs deposited abundant fibronectin extracellularly (Figure 4.3.3-1Aiii); though abundant fibronectin production was detected by hiPSC syn-vSMCs and pericytes, we observed fibronectin production was primarily intracellular (Figure 4.3.3-1Aiii). Laminin appeared perinuclearly around the three perivascular derivatives (Figure 4.3.3-1Aiv). However, hiPSC pericytes exhibited a punctate expression compared to the diffuse laminin protein expression in vSMC derivatives (Figure 4.3.3-1Aiv). Comparable deposition of ECM proteins was observed in aortic vSMCs placental pericytes (Figure 4.3.3-2A).
Figure 4.3.3-1. Differential ECM and MMP expression by perivascular derivatives. (A and B) Perivascular derivatives were examined for the production of ECM proteins (i) collagen I, (ii) collagen IV, (iii) fibronectin, (iv) laminin, and (v) elastin (all in green; phalloidin in red; nuclei in blue) after 6 days in culture (A), and their relative expression via quantitative real-time RT-PCR is shown (B). (C) Perivascular derivatives were compared for (i) the production of MMP2 and MMP9 using zymography, and (ii) the relative expression of MMP14 using quantitative real-time RT-PCR is shown. Results from three independent experiments are shown; each RT-PCR sample was run with three technical replicates. All graphical data are reported as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.
Figure 4.3.3. ECM in control cells.
(A) Deposition of the ECM proteins collagen I, collagen IV, fibronectin, and laminin (all in green; phalloidin in red; nuclei in blue) in placenta pericytes and aortic vSMCs. (B) Elastin organization in aortic vSMCs (in green; phalloidin in red; nuclei in blue) showing intercellular (left column) and extracellular (right column) deposition. (C-D) Expression of fibronectin, ED-A fibronectin and elastin, in the different cell types (compared to aortic vSMCs starved in low serum) using quantitative real time RT-PCR. Results from three independent experiments are shown; each RT-PCR sample was run with three technical replicates. All graphical data are reported as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.
A mature vSMC marker implicated in the mechanical responsiveness of vSMCs [167, 200], elastin was expressed primarily intracellularly and diffusely within con-vSMCs with some cells exhibiting perinuclear characteristic disordered elastin expression (Figure 4.3.3-1Av). Correspondingly, aortic vSMCs exhibited elastin expression with both intercellular and extracellular deposition (Figure 4.3.3-2B). We could not detect elastin in either hiPSC syn-vSMCs, or hiPSC pericytes (Figure 4.3.3-1Av).

Corroborating our immunofluorescence data, we performed RT-PCR analysis on the tested perivascular cells (Figure 4.3.3-1B). We found an increased expression of collagens I and IV and elastin by hiPSC con-vSMCs. Surprisingly, hiPSC-pericytes demonstrated the highest expression of fibronectin and its extra domain A (ED-A fibronectin; Figure 4.3.3-2C), suggesting the propensity of the derived pericytes to produce fibronectin under amenable in vitro culture conditions. Human iPSC con-vSMCs had higher levels of both fibronectin and ED-A fibronectin than syn-vSMCs. Con-vSMCs and aortic vSMCs exhibited the greatest expression of both laminin and elastin compared to the other tested perivascular cell types. Interestingly, aortic vSMCs had lower expression of collagen I compared to hiPSC con-vSMCs; placental pericytes exhibited lower expression of collagen IV and fibronectin compared to hiPSC pericytes (Figure 4.3.3-1; Figure 4.3.3-1C). Finally, the hiPSC con-vSMCs expressed elastin mRNA 100 fold more than the hiPSC syn-vSMCs and hiPSC pericytes (Figure 4.3.3-1B). We note that elastin expression in aortic vSMCs was higher compared to hiPSC con-vSMCs and increased when aortic vSMCs were cultured in low serum demonstrating the importance of culture conditions for the derivation of perivascular cell types from hiPSCs (Figure 4.3.3-1B; Figure 4.3.3-2D).

In the vasculature, there exists a wide range of matrix metalloproteinases (MMPs), which are proteolytic enzymes that degrade the ECM and remodel the
architecture of associated vessels. The degradation of ECM allows perivascular cells to migrate and proliferate [201]. Via zymography analysis, we found that only hiPSC syn-vSMCs produce pro-MMP9. Both hiPSC syn-vSMCs and hiPSC pericytes produce pro-MMP2 and its active form, while hiPSC con-vSMCs produce only the pro-MMP2 (Figure 4.3.3-1Ci). We could not detect MMP1 expression using zymography (data not shown). Molecular analysis revealed that hiPSC pericytes have greater mRNA expression while syn-vSMCs have the lowest expression (Figure 4.3.3-1Cii).

4.3.4 Functionality

In the body, the cellular dynamics of perivascular cells can provide information regarding whether a vessel is emergent, pathogenic, remodeling, or at a stable steady state. Of particular functional importance are multipotency, in vivo behavior, migration, invasion, and contractility of perivascular cells.

Multipotency

A major feature of pericytes is their ability to behave as mesenchymal precursors [202]. Indeed, our previous studies have demonstrated that pericyte derivatives could be differentiated to adipocytes and osteoblasts [129]. Contrastingly, neither hiPSC con-vSMCs nor syn-vSMCs demonstrated the potential to differentiate toward adipogenic or osteogenic lineages (Figure 4.3.4-1A).
Figure 4.3.4-1. Comparison of functionalities demonstrated by perivascular derivatives.

(A) Differentiation potential of perivascular derivatives into mesenchymal lineages including adipocytes (oil red O stain) and osteoblasts (alizarin red S stain). (B) One-week subcutaneously transplanted perivascular derivatives (in red; PKH26) migrated to newly formed host blood vessels (mouse ECs in green [Alexa 488-conjugated isolectin IB4]) within Matrigel, as indicated by representative confocal images. White arrows indicate occasions of circumferential wrapping of vasculature by the transplanted hiPSC derivatives; nuclei are indicated in blue (DAPI). (C) Migration potential via a wound-healing assay. Shown are (i) phase-contrast images quantified for (ii) cell trajectories and (iii) speed. (D) Downward invasion of hiPSC perivascular cells through collagen gels toward ECs was examined after 48 hr via (i) cross section of toluidine blue-dyed cells and (ii) quantification of the cell number, distance of invasion, and (iii) the average distance traveled. (E) Contraction was induced by 105 M carbachol, and percent contraction was quantified. Results shown from at least three biological replicates using three independent fields of view in each. All graphical data are reported as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.
In vivo integration

To compare in vivo functionality, we employed a Matrigel plug assay using our hiPSC perivascular cells. After one week of subcutaneous transplantation, all three types of perivascular cells aligned next to the host’s growing functional vasculature, with occasional circumferential wrapping observed by con-vSMCs and pericytes (Figure 4.3.4-1B), and vascular tube narrowing observed only by con-vSMCs [127, 129].

Migration

Mechanisms that induce cell motility include chemokinesis, chemotaxis, responses to interactions with ECM, and random increases such as in wound healing [203]. In a wound healing assay, hiPSC pericytes and hiPSC syn-vSMCs migrated inward from the wound margin (Figure 4.3.4-1Ci). Human iPSC pericytes exhibited a significant number of trajectories that were not perpendicular to the wound margin, whereas hiPSC con-vSMCs followed oriented trajectories predominantly toward the right. Indeed, after 24 hours, wound closure was observed only with the hiPSC syn-vSMCs (Figure 4.3.4-1Ci-ii). Overall, the hiPSC con-vSMCs exhibited the slowest migration speed (Figure 4.3.4-1Ciii).

Invasion

Invasion is the cell motility associated with ECM degradation. To assess the ability of perivascular cells to invade toward ECs, we cultured a monolayer of ECs beneath a three-dimensional collagen gel. Each perivascular cell type was cultured atop the collagen gel and migration was measured after 48 hours. Human iPSC syn-vSMCs exhibited increased invasion toward ECs after 48h compared to hiPSC con-vSMCs and hiPSC pericytes (Figure 4.3.4-1Di), Quantification of this dynamic behavior further
revealed that not only more hiPSC syn-vSMCs invaded the collagen gels compared to the other perivascular cells but also invaded to a deeper distance (Figure 4.3.4-1Dii-iii).

**Contractility**

In healthy blood vessels, perivascular cells provide stability to vessels by contracting to counteract the pulsatile force generated by heart-beats. Examining the contractility of the three perivascular derivatives in response to the cholinergic agonist drug carbachol, we found that hiPSC con-vSMCs contracted significantly more than both hiPSC pericytes and hiPSC syn-vSMCs (Figure 4.3.4-1E).

### 4.4 Discussion

The major function of both pericytes and vSMCs is to stabilize blood vessels and thus, both exhibit a great deal of similarities. Distinguishing between the three perivascular cells will facilitate their use in tissue engineering applications. Because pericytes are found in capillaries (<10 μm diameter) and microvessels (10-100 μm diameter), while vSMCs are found in larger vessels (>100 μm diameter), we sought to investigate methods that could elucidate similarities and differences between pericytes and vSMCs *in vitro*.

In previous studies, we derived pericytes [129] and both hiPSC syn-vSMCs and hiPSC con-vSMCs [127]. In performing direct comparisons between these perivascular cell derivatives, we observed numerous differences that enable the study of human perivascular development and functionality and may shed light on means to not only distinguish between them but also clearly define their functionality for future use in tissue regenerative strategies. Summary of key features compared among the perivascular derivatives from hiPSC-BC1 is shown in Table 4.4.
Table 4.4. Summary of features compared among hPSC-derived perivascular cells. Note that values are relative to the three cell types examined.

<table>
<thead>
<tr>
<th>Cell type/Features</th>
<th>Con-vSMCs</th>
<th>Syn-vSMCs</th>
<th>Pericytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td>Large, Spindle-like</td>
<td>Small, Spindle-like</td>
<td>Colony-like Flat, polygonal</td>
</tr>
<tr>
<td><strong>Proliferation rate</strong></td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cell size</strong></td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Endoplasmic reticulum</strong></td>
<td>Dilated</td>
<td>Non-dilated</td>
<td>Dilated+ Non-dilated</td>
</tr>
<tr>
<td><strong>Markers</strong></td>
<td>SMA, Calponin, NG2, PDGFRβ, SMMHC, Elastin</td>
<td>SMA, Calponin, NG2, PDGFRβ, SMMHC low</td>
<td>SMA, Calponin NG2, PDGFRβ</td>
</tr>
<tr>
<td><strong>ECM mRNA expression</strong></td>
<td>Collagen I&lt;sub&gt;high&lt;/sub&gt;, Collagen IV&lt;sub&gt;high&lt;/sub&gt;, Fibronectin, Laminin</td>
<td>Collagen I, Collagen IV, Fibronectin, Laminin</td>
<td>Collagen I, Collagen IV, Fibronectin&lt;sub&gt;high&lt;/sub&gt;, Laminin</td>
</tr>
<tr>
<td><strong>ECM deposition</strong></td>
<td>Collagen I, Collagen IV&lt;sub&gt;fibrous&lt;/sub&gt;, Fibronectin, Laminin&lt;sub&gt;diffuse&lt;/sub&gt;, Elastin</td>
<td>Collagen I&lt;sub&gt;low&lt;/sub&gt;, Collagen IV&lt;sub&gt;globular&lt;/sub&gt;, Fibronectin, Laminin&lt;sub&gt;diffuse&lt;/sub&gt;</td>
<td>Collagen IV&lt;sub&gt;globular&lt;/sub&gt;, Fibronectin, Laminin&lt;sub&gt;punctate&lt;/sub&gt;</td>
</tr>
<tr>
<td><strong>MMPs</strong></td>
<td>14 (MT-1)</td>
<td>2,9</td>
<td>2,14</td>
</tr>
<tr>
<td><strong>Mesenchymal differentiation</strong></td>
<td>--</td>
<td>--</td>
<td>Adipogenic, osteogenic</td>
</tr>
<tr>
<td><strong>In vivo integration</strong></td>
<td>Alignment Circumferential-wrapping Tube narrowing</td>
<td>Alignment</td>
<td>Alignment Circumferential-wrapping</td>
</tr>
<tr>
<td><strong>Migration</strong></td>
<td>-/+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Invasion</strong></td>
<td>-/+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Contractility</strong></td>
<td>+++</td>
<td>+</td>
<td>-/+</td>
</tr>
</tbody>
</table>

By assessing marker expression from day 6 differentiating cells compared to derived EVCs and SMLCs, we were able to label our day 6 cells as early mesoderm, characterized by expression of CD56 and CD73. Via EVC differentiation, day 6 cells
differentiate into VEcad+ and PDGFRβ+ cells [129]; in SMLC differentiation, day 6 cells were induced to differentiate into cells positive for SMMHC, SM22, and calponin [135].

In vivo perivascular cell characteristics are dependent on the local 3-dimensional cellular environment, which is comprised of cell-cell interactions, surrounding ECM, local mechanical conditions, and chemical cues. While contractile vSMCs are quiescent in the body, synthetic vSMCs display increased proliferation in order to remodel vessels in response to growth factors and cytokines released during vessel injury or disease [204]. The in vitro cell culture supplement, serum, contains numerous cytokines and growth factors among other chemokines such as hormones, lipids, attachment factors, etc. [205]. Therefore, hiPSC syn-vSMCs responded in vitro just as the synthetic phenotype in the body with high proliferation rates as they were exposed to these different chemokines in serum. Although endothelial-pericyte interactions have been studied extensively, pericyte-pericyte interactions are not well understood. Here we show the colony-like growth of hiPSC pericytes, suggesting that profound gap junction interactions are needed to activate contact inhibited proliferation of our hiPSC pericytes [206].

The endoplasmic reticulum (ER) is a major organelle involved in cell protein synthesis. Although in vivo synthetic rat vSMCs have been shown to increase their secretory apparatus that included extensive active ER and golgi complexes compared to contractile vSMCs, in vitro we observe significant differences in ER size [207]. An in vivo study using mice with knockout miR-143 and miR-145, microRNAs that promote the contractile vSMC phenotype, reported that mice aortas exhibited synthetic vSMC phenotypes and dilated ER containing flocculent material [208]. In contrast, we show in vitro that hiPSC con-vSMCs have dilated ER, hiPSC syn-vSMCs have non-dilated ER, and hiPSC pericytes have both types of ER. Expansion of the ER in mammalian cells has been reported to be necessary in order to accommodate increasing luminal content,
mostly unfolded proteins, as a result of ER stress or improper ER function [209, 210]. Consequently, the observed ER expansion may indicate that serum deprivation activates vSMC contraction signaling while halting ER dependent protein synthesis, folding, and transport signaling; thus creating a bottleneck in the ER machinery giving rise to the dilated appearance. Because their ER machinery is a low priority, hiPSC con-vSMCs therefore have accumulated unfolded proteins in their dilated ER and are functionally dedicated to cell contractility. Likewise, ER stress in the endothelium has also been linked to the increased contraction of isolated mouse aortas [211]. Liang et al. demonstrated that there was greater phenylephrine-induced vasoconstriction in the aortas of AMP-activated protein kinase knockout mice (AMPKα2/-/−), which exhibit aberrant ER stress in ECs, compared to WT mice [211]. The study may suggest that vSMCs not only functionally contract more as a result of their own ER stress, but may also respond in a similar manner in response to EC ER stress. This correlation between ER stress and vSMC contractility may additionally have a role in vessel diseases such as atherosclerosis in which vSMCs take on a synthetic phenotype and contraction is greatly reduced [212]. For instance, apoptosis of vSMCs as a result of vSMC ER stress has been implicated in collagen production in atherosclerotic lesions [213]. Therefore, differences in ER signaling cues as well ER structure in our hiPSC con-vSMCs and hiPSC syn-vSMCs may be helpful in studying the diseased syn-vSMCs in vitro. Finally, the proximity of mitochondria to the nucleus as well as the presence of autophagosomes in both placental and hiPSCs pericytes but not in hiPSC vSMCs may also indicate differences in the cellular machinery of pericytes.

We also found clear differences in the amount of stress fibers between the perivascular derivatives. Abundant stress fibers were found in con-vSMCs located throughout the entire cell body. While hiPSC syn-vSMCs had fewer stress fibers,
pericyte derivatives demonstrated the lowest levels of stress fibers per cell and had stress fibers located at the basal lateral surface. All perivascular cells expressed α-SMA in similar levels while calponin was found to be highly expressed in hiPSC pericytes, suggesting that this typical early vSMC marker may also be helpful to identify pericytes. Our findings also indicate that SMMHC is a distinctive marker for con-vSMCs. Upregulated expression and stress fiber organization of SMMHC were observed in hiPSC con-vSMCs. The markers NG2 (or CSPG4) and PDGFR-β are widely utilized markers to identify pericytes; however, vSMCs also express these markers making it difficult to distinguish which cell type is actually represented [214]. NG2 has been observed to be expressed by both pericytes and vSMCs in arterioles and capillaries but not beyond post-capillaries (along venules) in rats [214]. Here we showed that con-vSMCs can be distinguished from pericytes and syn-vSMCs by colocalization of NG2 with stress fibers.

The *in vitro* production and expression of ECM proteins collagens I and IV and laminin differed between the perivascular derivatives. In the body, pericytes produce ECM in the subendothelial basement membrane of capillaries, while both vSMCs and pericytes produce ECM in the tunica media layer of larger blood vessels [215]. Collagen I, a fibrillar collagen, is a substantial component of the interstitial connective tissue in contrast to collagen IV, which is present in all basal lamina, forming the basic irregular fibrous 2-D network of vasculature [216]. Similarly, laminin is an indispensable component of the vascular basement membrane, the primary site where collagen IV and laminin form an interdependent network [216]. In human arteries, collagen I, deposited between vSMCs, was reported to exhibit sparse thin fiber morphology in the media of small arteries while being organized in fibrillar structures in larger arteries. We report that *in vitro*, hiPSC pericytes, associated with small vasculature have a greatly diminished
collagen I expression compared to both hiPSC syn-vSMCs and hiPSC con-vSMCs, found in larger vessels [217]. Both our *in vitro* findings and *in vivo* studies illustrate that perivascular cells associated with larger vessels express more collagen I. From our *in vitro* study, we also observed that a morphologically distinct high density globular collagen IV expression is deposited by both hiPSC syn-vSMCs and pericytes, while a more fibrous collagen IV deposition as well as increased collagen IV expression is exhibited by hiPSC con-vSMCs. Similarly, *in vivo*, collagen IV deposition varies between the two phenotypes of vSMCs. For instance, fibrous plaques of atherosclerotic human arteries, known to mainly contain syn-vSMCs, have been reported to have greatly decreased collagen IV deposition and increased collagen I deposition around vSMCs compared to healthy arteries containing contractile vSMCs [217-219]. However, the loci of the plaques have been shown to have large quantities of collagen IV, correlating with the vSMCs being surrounded by layers of basement membrane material in this region [217, 218]. Additionally, the *in vitro* laminin expression was different in hiPSC pericytes compared to both phenotypes of hiPSC vSMCs. hiPSC con-vSMCs had diffuse cytoplasmic expression of laminin while hiPSC pericytes had punctate expression around the cell membrane. hiPSC syn-vSMCs had mostly diffuse cytoplasmic expression of laminin with few instances of punctate expression. *In vivo*, rat synthetic vSMCs lost the ability to produce laminin unlike contractile vSMCs [207]. Fibronectin was expressed and deposited by all tested perivascular cell types, with hiPSC pericytes expressing the highest fibronectin mRNA levels. Human iPSC con-vSMCs expressed higher levels of fibronectin mRNA compared to hiPSC syn-vSMCs. Similarly, the hiPSC con-vSMCs also expressed higher levels of ED-A fibronectin compared to the hiPSC syn-vSMCs, while *in vivo*, ED-A fibronectin was suggested to be associated with the synthetic phenotype [220]. Examination of the expression pattern of ED-A fibronectin in differentiating stem cells highlights the need for further investigation of stem cell
derivatives and the conceivable differences between *in vitro* and reported *in vivo* phenotypes, thus warranting additional studies correlating fibronectin slice variants to perivascular phenotypes both *in vitro* and *in vivo*. Elastin was also highly expressed by con-vSMCs compared to all other perivascular cell types. Not only is elastin production characteristic of the contractile phenotype, but interestingly, *in vivo* studies demonstrated that increasing elastin production itself promoted a contractile vSMC phenotype by inhibiting vSMC proliferation [19, 221].

Pericytes and vSMCs have been known to express the gelatinases MMP2 and MMP9 needed to degrade basement membranes during vessel remodeling [222-224]. Accordingly, *in vitro*, hiPSC syn-vSMCs expressed MMP2 and MMP9, corresponding to previous observations that this phenotype is associated with vessel remodeling, which includes ECM degradation. The expression of MMP2 and MMP9 in hiPSC pericytes coincided with pericytes’ close contact with basement membranes of vessels. A membrane-associated MMP, *MMP14* was more greatly expressed by derived pericytes, compared to control placental pericytes. MMP14 is known for its ability to degrade various ECM proteins; thus, we had expected that control pericytes would express this MMP type more greatly given the abundance of these ECM proteins in microvessels and capillaries. We suspect the discrepancy may be due to a loss of this site-specific feature due to *in vitro* culture of harvested pericytes, emphasizing the advantages of derived perivascular cell types over primary cells.

Not surprisingly, only hiPSC-derived pericytes had the potential to differentiate to mesenchymal lineages, including adipogenic and osteogenic, while neither hiPSC-vSMC types could differentiate. *In vivo*, all transplanted perivascular cells aligned next to the host vasculature, with both pericytes and con-vSMCs occasionally wrapping the microvasculature. These differences correlated with *in vivo* phenotypes of these various
perivascular cell types; both pericytes and con-vSMCs support vasculature in vivo and are thus closely associated with the endothelial lining, providing support. Syn-vSMCs, alternatively, are known for their role in remodeling vasculature. Differences in cellular mechanics may also be used to distinguish between the three different perivascular cell types. Human iPSC syn-vSMCs were able migrate in response to wounding and invade through ECM toward ECs whereas hiPSC con-vSMCs were not. Interestingly, while the hiPSC pericytes migrated in response to wounding, they failed to invade through ECM toward ECs, indicative on their short-distance migratory nature. This result coincides with the fact that pericytes have a close spatial relation to ECs in vessels [225].

Finally, hiPSC con-vSMCs can be characterized by their elevated cellular contractility (as demonstrated by both carbachol treatment and increased expression of contractile protein, caldesmon), while hiPSC syn-vSMCs are characterized by increased speed, migration, and invasion. These results are in agreement with previous observations that vSMCs switch to a synthetic phenotype in the body in response to injury to aid in tissue repair [203].
Chapter 5

BIOMECHANICAL STRAIN INDUCES ELASTIN AND COLLAGEN PRODUCTION IN HUMAN PLURIPOTENT STEM CELL DERIVED VASCULAR SMOOTH MUSCLE CELLS

5

5.1 Introduction

Vascular diseases such as coronary artery, cerebrovascular, and peripheral artery disease affect various organs primarily because they are all attributable to the dysfunction blood vessels which are ubiquitously located throughout the body [226]. Cell based tissue engineered blood vessels promise to be a viable treatment for such vascular diseases and offer to address the functional limitations of synthetic and autologous grafts [227, 228]. Vascular SMCs are a crucial component of the cell based engineered vessels as they provide structural support by circumferentially surrounding the inner endothelial lining. Additionally, these vSMCs should provide enough vessel stability when transplanted and integrated with host vasculature to be able to maintain blood pressure.

Healthy contractile vSMCs are characterized by their deposition of the elastin extracellular matrix (ECM) protein and their contraction of the vessel wall in order to counteract the pulsatile force generated by the beating heart. Not only do vSMCs deposit elastin but other ECM proteins such as collagen type I, collagen type III, collagen type IV, and fibronectin, which play integral roles in maintaining the mechanical integrity of blood vessels. While elastin contributes to vessel elasticity, both collagen type and I collagen III bear vessel tensile forces and set the limit of vessel elasticity[216].
Additionally, changes in ECM protein expression often provide insight into the pathological state of cells. For instance, decreased elastin expression in vSMCs is indicative of a phenotypic switch from the contractile state to a synthetic phenotype. This switching is often triggered by vessel remodeling, injury, or disease in vivo, although it generally occurs when vSMCs are cultured in vitro [229]. The switch from the desired contractile phenotype to the synthetic phenotype presents a challenge when engineering vessels as the inadequate elastin and collagen deposition by synthetic vSMCs often compromises the mechanical integrity of vessel constructs [161].

The use of human pluripotent stem cells (hPSCs) is advantageous in generating vSMCs because of the ability to direct the differentiation of these cells towards the desired vSMC phenotype in vitro using various stimuli [127, 230]. In vivo mechanical stimuli plays a major role in the embryonic differentiation and later in the adult as blood vessels exist in extremely dynamic tissue environments where cells are exposed various mechanical forces [231, 232]. While adult vSMCs are exposed to interstitial shear stress from blood flow, they primarily experience a circumferential tensile stretch or strain as a result of the radial distention of vessels [178, 233, 234].

Since vSMCs are constantly exposed to tensile strain, they have the ability to sense biomechanical signals and respond by modulating intracellular pathways in a process called mechanotransduction [234]. This mechanotransduction process may, additionally, be altered depending on cell exposure to the various sub-types of tensile strain including uniaxial, equibiaxial, and circumferential tensile strain. And while all the sub-types characteristically cause cell elongation, where there is an extension of a cell’s original length to a larger cell length, they all have distinctive directions of stress. It is therefore important to incorporate relevant biomechanical strain as a part of engineering vessels from hPSCs in vitro. Accordingly, there have been various studies that
investigate the effect of the various tensile strains on adult vSMCs, however, much remains unknown regarding how tensile strain affects vSMCs at different stages of development [235-237].

In previous studies we have established a step-wise differentiation protocol for the adherent and biochemically controlled derivation of vSMCs through different stages of maturation including smooth muscle like cells (SMLCs), mature SMLCs (mSMLCs), and contractile-vSMCs (Con-vSMCs) [127]. In this study, we examined the ability of these hPSC derivatives to sense and respond to external tensile strain through changes in cell orientation and ECM gene expression. Although, all hPSC derivatives aligned perpendicular to the direction of cyclic uniaxial strain in adherent cultures, SMLCs tended to orient parallel to the direction of cyclic uniaxial strain when encapsulated in 3-D collagen matrices. Additionally, short-term cyclic uniaxial strain and long-term cyclic circumferential biomechanical strain of hPSC mSMLCs induced elastin and collagen deposition. These data suggest that exposing hPSC-vSMCs derivatives to biomechanical strain may improve mechanical integrity of implantable engineered vessels by increasing extracellular matrix protein production.

5.2 Methods

5.2.1 Cell Culture

All cells were cultured in humidified incubators, with atmospheres at 37°C and 5% CO₂. **Human PSCs.** hiPSC line BC1 [238, 239]kindly provided by Dr. Cheng, SOM JHU and Human ESC line H9 (passages 15 to 40; WiCell Research Institute, Madison, WI) were grown on inactivated mouse embryonic fibroblast feeder layers (GlobalStem, Rockville, MD) in growth medium composed of 80 percent ES-DMEM/F12 (GlobalStem), 20 percent knockout serum replacement (Invitrogen, Carlsbad, CA), and 4 ng/ml basic
fibroblast growth factor (bFGF; Invitrogen) for hESCs of 10 ng/ml bFGF for hiPSCs, as previously reported [127]. Human iPSCs were passaged every four to six days using 1 mg/ml of type IV collagenase (Invitrogen). Media were changed daily.

**Human v-SMCs.** The control cell type used was human aorta v-SMCs (passages 4-7; ATCC, Manassas, VA). The cells were cultured in the specified ATCC complete SMC growth medium, composed of Kaighn’s Modification of Ham’s F-12 Medium (F-12K Medium; ATCC), 10% or 0.5% fetal bovine serum (FBS; Hyclone), 0.01 mg/ml transferrin (Sigma-Aldrich, St. Louis, MO), 0.01 mg/ml insulin (Sigma), 10 mM HEPES buffer (Sigma), 10 mM 2-(Tris(hydroxymethyl)methylamino)ethane-1-sulphonic acid (TES)(Sigma), 0.05 mg/ml ascorbic acid (Sigma), 10 ng/mL sodium selenite (Sigma), and 0.03 mg/ml Endothelial Cell Growth Supplement (Sigma). Human v-SMCs were passaged every three to four days using 0.25 percent trypsin (Invitrogen). Media were changed every two to three days.

**vSMC differentiation protocol**

vSMCs were derived as previously described [127, 230]. Briefly, hPSCs were collected through digestion with TrypLE (Invitrogen) and were seeded at a concentration of 5x10⁴ cells/cm² onto plates previously coated with collagen type IV (R&D Systems, Minneapolis, MN). The hPSCs were cultured for six days in a differentiation medium, composed of alpha-MEM (Invitrogen), 10% FBS (Hyclone), and 0.1 mM β-mercaptoethanol (Invitrogen). Media were changed daily. On day six, the differentiated cells were collected through digestion with TrypLE (Invitrogen), separated with a 40-μm mesh strainer, and seeded at a concentration of 1.25x10⁴ cells/cm² on collagen-type-IV-coated plates. The differentiating hPSCs were then cultured in differentiation medium; with the addition of 10 ng/ml PDGF-BB (R&D Systems) and 1 ng/ml TGF-β1 (R&D Systems) for additional 6 days (total of 12 days) for SMLCs. Media were changed every second day. Serum starved cells were passaged every 6-8 days with Tryple, using
alpha-MEM (Invitrogen), 10% FBS (Hyclone), and 0.1 mM β-mercaptoethanol (Invitrogen) to neutralize Tryple but then seeded with 0.5% serum media.

5.2.2 Cyclic uniaxial strain

hPSC vSMC derivatives were seeded on collagen IV coated polydimethylsiloxane chambers, which were inserted between two metal frames inside of a cyclic strain loading STREX instrument (STREX ST-140, Strex, Osaka, Japan). Uniaxial strain was achieved as the STREX instrument utilized a computer controlled step motor to drive two metal frames closer or further apart. The amplitude and frequency were controlled by the programmable microcomputer. In the present study we used a cyclic strain in the 5-10% range with a frequency of 1Hz. All strain experiments were performed with the supplementation of 1ng/mL TGF-β1 and were performed in humidified incubators, with atmospheres at 37°C and 5% CO₂.

5.2.3 Pulsatile flow loading bioreactor design

To examine the effect of circumferential strain on hPSC vSMC derivatives, a bioreactor was designed to allow pressurized flow into tubular silicone constructs (Silastic Laboratory Tubing, Dow Corning) and allow the radial distention of the constructs. The system was used inside a standard humidified incubator, with atmospheres at 37°C and 5% CO₂ and consists of a media reservoir, a programmable peristaltic pump (Ismatec), and a polydimethylsiloxane (PDMS) media bath. The peristaltic pump was operated at speeds between 0.11 – 11.25 rpm and propels culture medium in a cyclic flow pattern through a Kel-F hub 30 gauge needle (Hamilton) through the silicone construct secured on both ends using 6-0 nylon sutures (Henry Schein). The path of fluid flow continues through the silicone construct and out of a second Kel-F
hub 30 gauge needle (Hamilton) and finally into a media reservoir where media collects and is recycled back into the pump. The 2 needles were pierced through the PDMS media bath at opposite ends in order to hold tubular samples 300\(\mu\)m in radial diameter and 3cm in axial length. A PDMS lid was used to ensure sterility of the media bath.

5.2.4 **Cell seeding around the silicone tubes**

A 3cm silicone tube segment was attached to the two Kel-F hub 30 gauge needles inside the media bath and secured by sutures. After the assembly was autoclaved, the silicone tubing was coated with a 2.5mg/mL collagen gel solution to facilitate cell seeding. After a 30 min incubation of the coated silicone tube in the incubator, 1x10^6 SMLCs in 2mL of media were added into the media bath. The media bath was then placed on an orbital shaker (Sigma) for 24h to allow the full coverage of cells around the tube. After 6 days of serum starvation and supplementation with 1ng/mL TGF-\(\beta\)1, the media bath was connected to the pump for circumferential strain studies.

5.2.5 **Radial distention measurement**

Radial distention of the silicone tubes was quantified optically by flowing 2\(\mu\)m fluorescent blue latex polystyrene beads (1:100; Sigma) using fluorescent stereoscope (AxioZoom V16, Zeiss) and taking time-lapse streaming videos (Axiocam MRm, Zeiss) of silicone tube expansion. Radial distention was optically measured and quantified as the ratio of the instantaneous outer diameter to original unpressurized outer diameter of the silicone tubes. Iterative Particle Image Velocimetry (PIV) was used to generate a bead displacement field for each set of bead displacement images with the PIV plugin in ImageJ software (National Institutes of Health, USA).

5.2.6 **Immunohistochemistry**
Cells were prepared for immunofluorescence as previously described [127]. Cells were fixed using 3.7% formaldehyde fixative for 15 minutes, washed with phosphate buffered saline (PBS), blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour minimum, permeabilized with a solution of 0.1% Triton-X (Sigma) for ten minutes, washed with PBS, and incubated overnight with anti-human calponin (1:100; Dako, Glostrup, Denmark). For ECM staining, cells were incubated with anti-human collagen type III (1:200; Abcam), or anti-human elastin (3:100 Abcam) overnight. Cells were rinsed twice with PBS and incubated with Alexa 488 conjugated phalloidin (1:100; Molecular Probes, Eugene, OR) or anti-mouse IgG Cy3 conjugate (1:50; Sigma), or anti-rabbit IgG Alexa Fluor 546 conjugate (1:1000; Molecular Probes, Eugene, OR) for two hours, rinsed with PBS, and incubated with DAPI (1:1000; Roche Diagnostics) for ten minutes. Silicone tubes and stretch chambers were rinsed once more with PBS and imaged immediately. The immunolabeled cells were examined using fluorescence microscopy (Olympus BX60; Olympus, Center Valley, PA) and confocal microscopy (LSM 510 Meta; Carl Zeiss).

5.2.7 Cell orientation analysis

Cell orientation was analyzed using three randomized images using ImageJ (NIH). Image thresholding was used to highlight cell boundaries and the particle analysis tool was used to give a best-fit ellipse for each cell. Cell orientation either to the direction of uniaxial strain or to the longitudinal axis of the silicone tube was then determined.

5.2.8 Real-time quantitative RT-PCR

Two-step RT-PCR was performed on differentiated hPSCs at various time points as we previously described [127]. Total RNA was extracted by using TRIzol (Gibco, Invitrogen), as per the manufacturer’s instructions. All samples were verified as free of DNA contamination. The concentration of total RNA was quantified using an ultraviolet
spectrophotometer. RNA (1 μg per sample) was transcribed using the reverse
transcriptase MMLV(Promega Co., Madison, WI) and oligo(dT) primers (Promega), as
per the manufacturer’s instructions. The specific assay used was the TaqMan Universal
PCR Master Mix and Gene Expression Assay (Applied Biosystems, Foster City, CA) for
COL1A1, COL3A1, COL4A1, FN1, ELN, ACTB, and GAPDH, as per the manufacturer’s
instructions. The Taqman PCR step was performed with an Applied Biosystems
StepOne Real-TimePCR System (Applied Biosystems), in accordance with the
manufacturer’s instructions. The relative expressions of the genes were normalized to
the amount of ACTB or GAPDH in the same cDNA by using the standard curve method
provided by the manufacturer. For each primer set, the comparative computerized
tomography method (Applied Biosystems) was used to calculate the amplification
differences between the different samples. The values for the experiments were
averaged and graphed with standard deviations.

5.2.9 Statistical analysis

Real-time RT-PCR and image analyses were performed in at least triplicate
biological samples. Analyzed images were representative of independent experiments.
Real-time RT-PCR analyses were also performed with triplicate readings. Statistical
analyses were performed with GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla,
CA). Unpaired two-tailed t-tests and one-way ANOVA analysis and Bonferonni post tests
were performed where appropriate using GraphPad Prism 4.02. Significance levels were
set at *p<0.05, **p<0.01, and ***p<0.001. All graphical data are reported as mean ±SEM.

5.3 Results and Discussion

5.3.1 Vascular SMC-derivatives.

Human PSC vSMC derivatives were generated at different stages of development as
previously described [51, 127, 230]. Briefly, using step-wise, two-dimensional
adherence culture protocol, hPSCs were differentiated to mesodermal cells after six days in high-serum medium. These Day6 mesodermal cells were then supplemented with PDGF-BB and TGF-β1 for an additional 6 days in order to generate Day12 smooth muscle-like cells (SMLCs), which expressed comparable levels of α-SMA, calponin, SM22α to the human aortic vSMCs control cell line, and low levels of the mature vSMC marker, smooth muscle myosin heavy chain (SMMHC). Day 12 marked the branching point in differentiation scheme where SMLCs were either cultured in high-serum media to derive vSMCs exhibiting the synthetic phenotype or serum-deprived media to derive vSMCs exhibiting the contractile phenotype. To derive synthetic vSMCs (Syn-vSMCs), we cultured SMLCs for an additional 18 days using PDGF-BB and TGF-β1 supplemented high serum culture conditions. These Day30 Syn-vSMCs expressed significantly reduced levels of SMMHC as well as increased collagen I and fibronectin when compared to the SMLCs. In contrast, to derive contractile vSMCs (Con-vSMCs), we first cultured Day12 SMLCs using TGF-β1 supplemented serum deprived culture conditions for six days in order to generate an intermediary contractile phenotype named Day18 mature SMLCs (mSMLCs) which expressed increased SMMHC and elastin when compared to the SMLCs. Further serum deprivation of the mSMLCs for an additional 12 days generated mature Day30 Con-vSMCs, which expressed elevated SMMHC when compared to the SMLCs and comparable elastin levels to the human aortic vSMCs control cell line. Additionally, the Day30 Con-vSMCs displayed significant proliferation, morphological, and functional differences compared to the Day30 Syn-vSMCs [230]. For instance, Con-vSMCs exhibited a higher number of stress fibers, increased contraction, lower migration rates, and lower proliferation rates when compared to Syn-vSMCs [230]. Subsequent strain experiments were performed.
on PSC vSMC derivatives using their respective high serum or serum deprived culture conditions supplemented with TGF-β1.

Figure 5.3.1-1. vSMC-derivatives organization and alignment after short-term cyclic uniaxial strain. A) A schematic representation of hPSC vSMC maturation after days of in vitro culture and B) displacement vectors of hESC vSMC-derivatives and aortic vSMCs control cell line exposed to uniaxial strain at 7% elongation for 24h: C) Light microscopy images, D) immunofluorescence images of phalloidin (green) and calponin (red; nuclei in blue), and E) quantification of cell orientation before (white) and after (black) uniaxial strain. Arrows indicate direction of strain. All graphical data are reported as mean ±SEM. *p<0.05, **p<0.01, and ***p<0.001.
5.3.2 Cyclic uniaxial strain and actin fiber orientation at different stages of vSMC development.

To determine the conditions that promoted hPSC vSMC alignment, hPSC-SMLCs were initially exposed to uniaxial strain at a constant frequency of 1Hz and various elongations. SMLCs were exposed to cyclic uniaxial strain at elongations of 5, 7, and 10% for 24h as cell alignment in mouse embryonic cells was reportedly observed when elongations between 4-12% were used [240]. After uniaxial strain at a 5% elongation, SMLCs exhibited randomly oriented actin filaments with a mean angle of 47.11° ±0.7° and lacked cell alignment (Figure 5.3.2-1). In contrast, while SMLCs exhibited reorientation of actin filaments perpendicular to strain with a mean angle of 56.24° ±0.7° after 10% elongation, they also displayed a decrease in stress fiber assembly (Figure 5.3.2-1). Cell alignment and stress fiber organization was observed at 7% elongation and it was therefore selected as the elongation magnitude for our studies. These results indicate that there exists a lower elongation limit that developing vSMCs begin to alter cytoskeletal structure and an upper limit in which stress fiber assembly is interrupted. Although the reorientation of cells perpendicular to uniaxial cyclic strain direction has been widely observed in a variety of cells and therefore does not seem to be cell dependent, the contractility level of stress fibers have been implicated in directing this cell alignment [241, 242]. Since vSMCs vary in number of stress fibers and contractility depending on the stage of development and phenotype, we exposed hPSC vSMC derivatives to uniaxial strain to determine whether the stage of development had an effect on cell alignment. After 24h exposure to uniaxial cyclic strain in high-serum conditions, the human aortic vSMCs control cell line, exhibited cell and actin filament reorientation to a mean angle of 57.9° ±0.5°, which was perpendicular to the strain and
parallel to the direction of minimum strain, when compared with mean orientation of 46.9° +/-0.7° before strain (Figure 5.3.1-1B-D). A similar cell alignment and cell orientation of 57.0° +/-0.5° was induced in hPSC-SMLCs cultured and stretched in high-serum (Figure 5.3.1-1B-D). After 24h exposure to uniaxial cyclic strain in serum-starved media both mSMLCs and Con-vSMCs exhibited cell and actin filament alignment perpendicular to the strain. It is worthwhile to note that the synthetic vSMCs (Syn-vSMCs) similarly aligned perpendicular to the direction of strain at a mean angle of 60.2° +/-0.8° (Figure 5.3.2-2A-B). All derivatives at different stages of development were positive for the early vSMC marker, calponin after strain (Figure 5.3.1-1C; Figure 5.3.2-2). Quantification of cell orientation before and after strain showed significant changes in the organization of all cell types from random to a perpendicular orientation to strain (Figure 5.3.1-1D; Figure 5.3.2-2), illustrating that both hPSC vSMC developmental stage and phenotype specification do not alter the strain based mechanotransductive response of cytoskeleton remodeling.
Figure 5.3.2-1. hESC-SMLC organization and alignment at various magnitudes of strain elongation. Smooth muscle-like cells after 24h of uniaxial strain at 5, 7, and 10% elongation: A) Light microscopy images, B) immunofluorescence images of phalloidin (green) and calponin (red; nuclei in blue), and C) quantification of cell orientation after (black) uniaxial strain. Arrows indicate direction of strain. All graphical data are reported as mean ±SEM. *p<0.05, **p<0.01, and ***p<0.001.
Figure 5.3.2-2. hESC-Syn-vSMC organization and alignment after short-term cyclic uniaxial strain. Synthetic vascular smooth muscle cells after 24h of uniaxial strain at 7% elongation: A) Light microscopy images, B) immunofluorescence images of phalloidin (green) and calponin (red; nuclei in blue), and C) quantification of cell orientation before (white) and after (black) uniaxial strain. Arrows indicate direction of strain. All graphical data are reported as mean ±SEM. *p<0.05, **p<0.01, and ***p<0.001.

Although there have been studies reporting the alignment of adult vSMCs perpendicular to the direction of strain [243, 244], the 2-D in vitro environment does not accurately mimic the in vivo environment in which vSMCs form concentric layers surrounded by a 3-D ECM and are arranged in circumferential spirals or diagonal to the direction of strain [245]. When hPSC SMLCs were embedded in a dense 3-D collagen matrices and subsequently stretched for 24h and 48h uniaxially, cells and stress fibers tended to align and orient parallel to direction of principal strain illustrating that cell-matrix contact plays an important role in the vSMC mechanosensing response of actin fiber organization (Figure 5.3.2-3). It should be noted that although mechanically stimulating cells that are encapsulated in 3-D matrices is more representative of the in vivo physiological environment, the issue of selecting suitable materials with optimum
matrix stiffness or density arises. For instance, collagen gel density has been reported to affect the direction of stress fiber orientation [246]. Similarly, Foolen et al. report that the existence of synergy between matrix density and Rho-kinase signaling that controls cyclic stretch induced actin stress fiber reorganization [247].

**Figure 5.3.2-3. 3-D Cyclic uniaxial Strain.** Actin fiber labeled with Alexa-488 conjugated phalloidin (green) of hESC SMLCs embedded in collagen gel after 24h of static culture and after 24h and 48h of uniaxial strain.

5.3.3 *Short-term cyclic uniaxial strain and vSMC-derivatives ECM gene expression*

To investigate whether hPSC vSMC derivatives exhibited mechanotransductive ECM production responses, we exposed cells to uniaxial strain for 24 and 48h and quantified the gene expression of collagen I, fibronectin, and elastin. After SMLCs were exposed to uniaxial stretch for 24 and 48 h in high-serum media, there was no significant change in the gene expression of collagen I and fibronectin ECM. However, there was a significant decrease in the elastin mRNA levels when compared to static equivalents (Figure 5.3.3-1A). hPSC Syn-vSMCs exposed to uniaxial strain for 24 and 48 h in high serum conditions exhibited a significant upregulation in the expression of collagen I, fibronectin, and elastin (Figure 5.3.3-2). These results show that in high-serum media, ECM genes are upregulated in response to uniaxial strain in vSMCs with the synthetic phenotype. After 6 days of serum starvation, the serum starved mature SMLCs, or
mSMLCs, exhibited a significant upregulation in the gene expression of collagen I and fibronectin in addition to elastin, compared to static equivalents (Figure 5.3.3-1B). Similarly, when the hPSC Syn-vSMCs were serum starved for 6 days and subsequently exposed to 24 h of uniaxial strain, an increase in collagen I, fibronectin, and elastin gene expression was observed compared to static equivalents (Figure 5.3.3-2). Notably, when the control human aortic vSMCs control cell line were serum starved and exposed to uniaxial strain for 24 h in serum-starved media, there was a significant decrease in the ECM gene expression of collagen I and elastin (Figure 5.3.3-1B). Collectively, these results indicate that uniaxial strain induces upregulation of ECM protein expression in synthetic vSMCs. Furthermore, both serum starvation and uniaxial strain combined contribute to the increased ECM gene expression of maturing hPSC vSMCs. It is likely that intercellular serum sensitive molecules known to regulate vSMC maturation, such as serum response factor, may be modulated by a membrane-bound mechanosensor to activate ECM deposition pathways [248].
Figure 5.3.3-1. ECM gene expression after short-term cyclic uniaxial strain. Quantitative real time RT-PCR gene expression of collagen type I, fibronectin, and elastin of A) hESC SMLCs after 24h and 48h of uniaxial strain in high-serum conditions (strain conditions in black and static equivalents in white) and B) serum starved SMLCs (mSMLCs) and aortic vSMCs after 24h of uniaxial strain in serum deprived conditions (strain conditions in blue and static equivalents in light blue). All graphical data are reported as mean ±SEM. *p<0.05, **p<0.01, and ***p<0.001.
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5.3.2. hESC-Syn-vSMC ECM gene expression after short-term cyclic uniaxial strain. hESC Syn-vSMCs collagen type I, fibronectin, and elastin gene expression after 24h and 48h of uniaxial strain in A) high serum conditions (strain conditions in black and static equivalents in white) and B) after serum deprivation for 6 days and 24h of uniaxial strain (strain conditions in green and static equivalents in light green) using quantitative real time RT-PCR. All graphical data are reported as mean ±SEM. *p<0.05, **p<0.01, and ***p<0.001.

5.3.4 Long-term cyclic uniaxial strain and vSMC-derivatives ECM gene expression

To determine whether the uniaxial strain based increase in ECM was a transient or long-lasting response, we exposed SMLCs and mSMLCs to six days of uniaxial strain.
Throughout the culture period, we observed cell detachment from the silicone uniaxial strain chambers, resulting in a decrease in cell concentration after 72 hours (Figure 5.3.4-1). It is worthwhile to note that static equivalents seeded on the culture plates expressed similar vSMC protein levels as those seeded on silicone stretch chambers.

![Figure 5.3.4-1. Uniaxial Strain induced cell detachment. Representative light microscopy images of hiPSC BC1 SMLCs after application of uniaxial strain. Scale bar = 200 μm.](image)

hPSC mSMLCs were used as the static equivalents of hPSC SMLCs exposed to six days uniaxial strain in serum-starved media or Mech-mSMLCs (Figure 5.3.4-2A). These Mech-SMLCs exhibited significant increase in elastin mRNA levels compared to mSMLCs but no significant change in collagen I, -III, -IV, and elastin was observed. Similarly, the more mature mSMLCs exhibited increase not only in elastin, but also in collagen III, and fibronectin gene expression after exposure to uniaxial strain for only 24h (Figure 5.3.4-2B). In contrast to the mSMLCs exposed to uniaxial strain for 24h, after long-term uniaxial strain of 6 days, the mSMLCs exhibited no change in elastin mRNA levels, and a significant decrease in collagen I, -III, and -IV (Figure 5.3.4-2B). These results suggest that vSMCs produce more elastin in response to long-term uniaxial strain earlier in development rather than when they are more mature and contractile since SMLCs exposed to long-term uniaxial strain expressed increased elastin while the more mature mSMLCs expressed decreased elastin after exposure to long-term uniaxial strain when compared to static equivalents (Figure 5.3.4-2B). The results also indicate that as
vSMCs mature, they respond to long-term uniaxial strain by downregulating ECM proteins. For instance, while mSMLC gene expression of all tested ECM proteins was downregulated in response to long-term uniaxial strain, elastin and fibronectin gene expression was upregulated in mSMLCs in response to short-term uniaxial strain. Additionally, ECM gene expression by hPSC vSMC derivatives in response to long-term uniaxial strain seem to mirror developing vSMCs in vivo where most ECM gene expression is dynamic [249]. For instance, in mice, there is an initial increase of ECM gene expression in the embryo followed by a brief decrease postnatally, followed by a steady rise for 2 weeks and a final decline to low levels in the adult [249].
Figure 5.3.4-2. ECM gene expression of hiPSC vSMC derivatives after long-term cyclic uniaxial strain. A) A schematic representation of long-term uniaxial strain experiments. B) hiPSC SMLCs and hiPSC mSMLCs collagen type I, collagen type III, collagen type IV, fibronectin, and elastin gene expression after 6 days of long-term uniaxial strain in serum deprived media using quantitative real time RT-PCR. All graphical data are reported as mean ±SEM. *p<0.05, **p<0.01, and ***p<0.001.
5.3.5 Long-term cyclic circumferential strain and vSMC-derivatives ECM gene expression

Although uniaxial strain closely resembles the tensile strain of blood vessels, a more physiologically relevant tensile force that takes into account the curvature of vessels is a circumferential strain (Figure 5.3.5-1A). Thus, we established a bioreactor that enables pressurized flow into tubular silicone constructs which in turn allows the radial distention of the constructs (Figure 5.3.5-1B-C).

Figure 5.3.5-1. Experimental setup used to study the effect of circumferential strain using a pulsatile flow bioreactor. A) A schematic representation of circumferential strain displacement vectors. B) Schematic representation and C) actual experimental setup of pulsatile flow bioreactor where media is propelled inside system tubing by a peristaltic pump and travels into a 30 gauge needle then into a silicone construct where circumferential strain is generated. The media then travels from the silicone construct and out of a secondary 30 gauge needle and is finally recycled in a media reservoir. D) The silicone construct is immersed in a media bath to ensure cells receive appropriate nutrients.
The circumferential strain was assumed to be uniform longitudinally because the constant thickness of the homogeneous and incompressible silicone tubes. Additionally, although the tensile stress is largest in the inner surface of the silicone tube compared to the outer surface, the cells were assumed to experience uniform circumferential strain because they were seeded as a monolayer on the outer surface of the silicone tubes [250]. hPSC mSMLCs were used to study the effect of long-term circumferential strain on ECM genes since we observed that long-term tensile uniaxial strain had a significant effect on most of the ECM genes (Figure 5.3.5-2A). After 24h after seeding, silicone tubes were fully covered with hPSC mSMLCs (Figure 5.3.5-2B). Cell concentration on the tubes was greater than the uniaxial strain samples as a result of different seeding methods. While the cells on the tubes were seeded using an orbital shaker to ensure tube coverage, cells on the 2-D uniaxial strain samples were allowed to seed on a stationary surface. Orbital shaking was used to resolve difficulties in uniform cell attachment as there were greatly reduced cells present on the silicone tubes when they were statically seeded. An elongation magnitude consistent with the elongation used for the uniaxial strain experiments was used for the circumferential strain experiments. Radial distension was measured optically using digital calipers by tracking the diameter of the silicone tubes [96, 251]. Using the radial distention measurements, an elongation of 7% at a mean pulsatile flow of 4.3 mL/min +/- 0.04 was used to elongate silicone tubes. After circumferential strain of mSMLCs for six days, there was a significant increase in the expression of collagen III and a significant decrease in fibronectin mRNA (Figure 5.3.5-2C).
Figure 5.3.5-2. ECM gene expression after long-term cyclic circumferential strain. A) Schematic representation of long-term uniaxial strain experiments. B) Confocal images of a cross section and 3D reconstruction of a flexible silicone tube seeded with hPSC mSMLCs prior to pulsatile flow: phalloidin (green), nuclei (blue). C) hiPSC mSMLCs collagen type I, collagen type III, collagen type IV, fibronectin, and elastin gene expression after 6 days of long-term circumferential strain in serum deprived media using quantitative real time RT-PCR (strain conditions in red and static equivalents in light red). D) Confocal images comparing mSMLCs (i) and Con-vSMCs (ii) elastin deposition without strain and mSMLCs elastin deposition (i) and alignment (iv) after circumferential strain for 6 days. mSMLCs collagen type III deposition after circumferential strain for 6 days (iii and iv). All graphical data are reported as mean ±SEM. *p<0.05, **p<0.01, and ***p<0.001.
Elastin gene expression was also significantly upregulated after circumferential strain and although mSMLCs deposited small amounts of elastin when compared to the mature Con-vSMCs prior to exposure to circumferential strain, they deposited more elastin protein after circumferential strain when compared to the non-mechanically stimulated but mature Con-vSMCs (Figure 5.3.5-4C-Di-iii). These results indicate that the application of circumferential strain during vSMC development results in highly increased elastin deposition. Occasionally, cells seemed to form contracted structures and exhibited alignment parallel to the direction of strain (Figure 5.3.5-4Div). This parallel alignment may also be attributed to the higher concentration of cells present in the circumferential strain samples when compared to the uniaxial strain samples. The higher concentration would allow a higher number of physical cell-cell contacts, in which case the cells are less likely to respond individually and instead exhibit a group response. Samples exposed to circumferential strain similarly deposited significant collagen type III protein (Figure 5.3.5-4E). Several studies have shown this similar increase in vSMC elastin expression after exposure of vessel constructs to cyclic loading or pulsatile flow [236, 252]. Overall, the production of these ECM proteins by vSMCs is highly desirable because mechanical integrity of blood vessels is largely attributed to collagen and elastin deposition. For instance, collagen type I and III are major constituents of the interstitial connective tissue in vessels that functionally regulate vessel tensile forces by limiting vessel expansion [216]. On the other hand, elastin contributes to vessel elasticity and resilience which is necessary in order to accommodate vessel dilation and expansion [216]. It should be noted that while elastin and collagen type III expression decreased after exposure to long-term uniaxial strain, both ECM molecules were increased after exposure to long-term circumferential strain. These conflicting changes in the gene expression of elastin and collagen type III
observed in response to long-term uniaxial compared to circumferential strain may suggest that vSMCs mechanosense the two forces differently. These results suggest that curvature effects, absent when stretching the cell uniaxially play an important role in promoting ECM expression in the developing vSMC. Long-term mechanical stimulation of vSMC-derivatives using circumferential strain may therefore improve mechanical properties of vascular constructs more efficiently than long-term uniaxial strain by upregulating collagen and elastin.
Chapter 6

CONCLUSIONS AND FUTURE WORK

6

6.1 Conclusions

Human pluripotent stem cells (hPSCs) growth and differentiation are influenced by biochemical and biomechanical cues of the surrounding milieu. To enable the use of hPSCs in vascular engineering applications, it is essential to understand how in vitro biochemical and biomechanical stimuli dictate differentiation, and to control it to guide perivascular cell (PC) maturation and functionality. Therefore, in order to derive functional PCs from hPSCs and understand the effects of biochemical and biomechanical stimuli of PC maturation, approaches in both vascular biology and stem cell engineering should be utilized. We first sought to derive and characterize v-SMCs from hESCs and hiPSCs, followed by the study of the effect of biochemical stimuli on v-SMC differentiation and maturation. We then investigated the differences between three distinct PCs derived from hPSCs. Finally, we examined the effect of biomechanical forces, specifically the effect of uniaxial and circumferential strain on maturation of the vSMC-derivatives. Towards this, we designed a bioreactor system mimicking a blood vessel in order to study the effect of circumferential mechanical strain on the phenotype of vSMC-derivatives.

In Chapter 3, we demonstrate fate-decisions in vascular smooth muscle phenotype along the differentiation of hPSCs. By monitoring the expression of SMMHC and elastin, we show that it is possible to control synthetic or contractile phenotype from different hPSC lines with appropriate concentrations of factors known to control these developmental steps in the early embryo and in adulthood. These findings highlight the importance of designing stage-specific differentiation strategies that follow key
developmental steps to exploit cellular plasticity for vSMC phenotypic decision. The contractile hPSC-vSMC derived from integration-free hiPSC line, BC1, may prove useful for future regenerative therapy involving blood vessel differentiation and stabilization.

In Chapter 4, we generated human perivascular cells including contractile vSMCs, synthetic vSMCs, and pericytes with identical genetic backgrounds, which offers unprecedented opportunities to study the development and functionality of well-defined human perivascular derivatives from healthy and disease hiPSCs. These perivascular derivatives demonstrate an important building block toward not only reconstructing physiologically-relevant vasculature but also the study of developmental processes and diseases implicating these cell types. Important elements of our system are the several noted discrepancies between our in vitro results and in vivo phenotypes, alluding to the complexity of the field. A major challenge of correlating in vitro results to phenotypes observed in vivo is the presence of heterogeneous vSMC subpopulations of unknown origin that lie between the contractile and synthetic phenotypic spectrum in vivo. Unanswered questions particularly regarding the in vivo synthetic phenotype still remain. For instance, ECM deposition of syn-vSMCs located in vastly different environments such as normal developing or remodeling vessels compared to diseased or injured vessels remains unknown. To further add to the complication, by utilizing SMMHC lineage tracing, Tang et al. reported that synthetic vSMCs actually arise from multipotent stem cells lying within vessels and not phenotypic switching by vSMCs in vivo [185, 253]. The advantage of our study is the derivation and comparison of synthetic and contractile vSMCs from a known common early smooth muscle precursor. In fact, some of our study’s in vitro results actually yield a more useful phenotype for engineering blood vessels, such as increased ECM production from the hiPSC derivatives compared
to control cell lines. By employing a viral integration-free and fully genetically sequenced hiPSC line, BC1, we anticipate that these findings hold translational importance.

Finally, in Chapter 5, we demonstrated that hPSC-derived vSMCs display mechanotransduction that affects cytoskeleton organization and ECM gene expression in response to tensile strain. All vSMC derivatives including SMLCs, mSMLCs, and Con-vSMCs aligned perpendicularly to the direction of cyclic uniaxial strain. Serum deprivation and short-term uniaxial strain had a synergistic effect in enhancing collagen type I, fibronectin, and elastin gene expression of derivatives. Furthermore, long-term uniaxial strain deterred collagen type III gene expression while long-term circumferential strain upregulated both collagen type III and elastin gene expression. Finally, long-term uniaxial strain downregulated ECM expression in more mature vSMC derivatives while upregulating elastin in less mature vSMC derivatives. Mechanical stimulation of hPSC-derived vSMCs in vessel constructs using uniaxial and circumferential strain may potentially modulate derived vSMC function and ultimately improve the construct mechanical properties needed for therapeutic purposes. With advancing technologies, opportunities to harness perivascular potential for regenerative medicine are envisioned to dramatically increase.

6.2 Suggestions for future work

Our studies have presented new questions that require further experimental work. In Chapter 3 there were discrepancies between our in vitro results compared to in vivo findings. Specifically, our finding that derived syn-vSMCs had a lower expression of fibronectin splice variant ED-A than con-vSMCs differed from results of in vivo studies. These results drive the need for continued study on the derivation of specialized cell types to rebuild tissue in a three dimensional environment. Additionally, studies in a
three dimensional environment would allow further investigation of morphological features such as nucleic size that may better match in vivo properties.

Additionally, further investigation of the biochemical cues and the molecular pathways activated is needed to understand the switch between the two phenotypes. Research has also suggested that microRNA is involved in the genetic regulation of growth factors and transcription factors involved in phenotypic switching [254]. For instance experiments tracking the miR-143 and miR-145 through PC maturation would elucidate microRNA role in determing the contractile or synthetic phenotype in vSMCs.

Finally, the role of biophysical cues in PC differentiation and maturation should be further studied. All of our differentiation studies were based on cell expansion on plastic tissue culture plates. However, the microenvironment of cells has been shown to affect cell function and phenotype. For instance, in the body, vSMCs are located in specific niches consisting of different ECM proteins that provide the cells with an appropriate mechanical environment to function properly. Altering substrate stiffness is a popular approach to emulate different mechanical microenvironments, in contrast to genetic modulation, that control the vSMC phenotype [255, 256]. Therefore, the development of biomaterials with properties that promote perivascular maturation and mimic in vivo microenvironments could promote proper perivascular function in tissue constructs. Additionally, micropatterning the surfaces of these biomaterials could control PC maturation by controlling cell shape. Experimentation with micropatterning by presenting micro- and nanometer scale topological features in distinct spatial patterns that serve as instructive cues may affect PC function. Ultimately, approaches balancing biochemical, biomechanical, and biophysical cues could yield optimal perivascular function in engineered blood vessels.
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- Optimized biochemically defined media to derive cells intended for vascular therapy
- Applied biomechanical force on cells using commercial instrumentation
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Honors/Awards

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2012 – 2014  NIH-NHLBI: Supplement for diversity in health related research to R01Graduate Research Supplement Awardee (stipend, tuition & fees/year for 2 years). Role: Graduate student
2012  Carl Storm Underrepresented Minority (CSURM) Fellowship to support participation in the 2012 Signal Transduction by Engineered Extracellular Matrices GRC

2011 – 2012  Integrative Graduate Education and Research Traineeship (IGERT) Fellowship in Chemical and Biomolecular Engineering. Johns Hopkins University. Full support and stipend

2007  Chemical Engineering Sponsor Undergraduate Research Award, a monetary award for exceptional performance by a senior student or students in a senior thesis research project

2007  Science and Engineering Research Scholar Award given to sophomores participating in research with a University of Delaware faculty member

2005  RISE (Resources to Insure Successful Engineers) Corporate Friends Award, a monetary award provided by industry contributions to the RISE program

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Publications


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