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Smooth-Muscle-Like Cells Derived from Human Embryonic Stem Cells Support and Augment Cord-Like Structures In Vitro

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Abstract Engineering vascularized tissue is crucial for its successful implantation, survival, and integration with the host tissue. Vascular smooth muscle cells (v-SMCs) provide physical support to the vasculature and aid in maintaining endothelial viability. In this study, we show an efficient derivation of v-SMCs from human embryonic stem cells (hESCs), and demonstrate their functionality and ability to support the vasculature in vitro. Human ESCs were differentiated in monolayers and supplemented with platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-beta 1 (TGF-β1). Human ESC-derived smooth-muscle-like cells (SMLCs) were found to highly express specific smooth muscle cell (SMC) markers—including α-smooth muscle actin, calponin, SM22, and smooth muscle myosin heavy chain—to produce and secrete fibronectin and collagen, and to contract in response to carbachol. In vitro tubulogenesis assays revealed that these hESC-derived SMLCs interacted with human endothelial progenitor cell (EPCs) to form longer and thicker cord-like structures in vitro. We have demonstrated a simple protocol for the efficient derivation of highly purified SMLCs from hESCs. These in vitro functional SMLCs interacted with EPCs to support and augment capillary-like structures (CLSs), demonstrating the potential of hESCs as a cell source for therapeutic vascular tissue engineering.

Keywords Smooth muscle cells · Blood vessels · Differentiation · Human embryonic stem cells · Tissue engineering

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Introduction

The vascularization of tissue constructs remains a major challenge in regenerative medicine. Without its own blood supply, an engineered construct relies mainly on diffusional oxygen supply, which can only support a thin layer of viable tissue. Therefore, vascularization of a tissue construct is crucial for its successful implantation, survival, and integration with the host tissue [1]. The formation of mature and functional vascular networks requires interaction between endothelial cells (ECs) and vascular smooth muscle cells (v-SMCs). During early vascular development, ECs line the vessel wall and organize into an immature vasculature [2]. To further stabilize these nascent vessels, ECs secrete platelet-derived-growth-factor (PDGF) to induce the differentiation of specialized mesenchymal stem cells (MSCs) into pericytes in capillaries or SMCs in larger vessels [3]. At this later stage, transforming growth factor-beta 1 (TGF-β1) regulates vessel maturation by inducing v-SMC differentiation and the generation of extracellular matrix (ECM) molecules, such as collagen, fibronectin, and laminin [4, 5]. Embedded within this ECM, v-SMCs

provide physical support to the vasculature and aid in the maintenance of endothelial viability. This process of vascular morphogenesis involving ECs interacting with both the ECM and v-SMCs has been widely studied in vitro using Matrigel assays [5, 6]. When grown on Matrigel, a basement membrane matrix enriched with laminin, ECs and v-SMCs interact to form capillary-like structures (CLSs) that resemble tube formation in vivo [7]. Thus, v-SMCs are key components in engineering vascularized tissue.

One major limitation of this therapeutic approach has been the lack of a reliable source of v-SMCs. Since v-SMCs isolated from patients are usually derived from diseased organs that have limited proliferative capacity and reduced collagen production, they have impaired mechanical strength and cannot support vascular function. Alternatively, bone marrow-derived MSCs have been used to engineer small-diameter vessel grafts [8] and blood vessels [9] which are stable and functional in vivo [9, 10]. Adipose tissue [11, 12] and neural crest tissue [13] also contain populations of multipotent cells that can be differentiated into functional v-SMCs. Another promising source of v-SMCs is human embryonic stem cells (hESCs), which are pluripotent, have high proliferative capacity, exhibit low immunogenicity, and have been shown to repair ischemic tissues and restore blood flow [14]. Studies demonstrating the derivation of v-SMCs from embryonic or induced pluripotent stem cells (human or mouse) have utilized various approaches to guide differentiation—such as coculture on OP9 feeder layer [14–16] or retinoic acid supplementation [17]—and to purify derivatives by sorting for specific vascular progenitors or mature markers [18, 19], selecting for stable expression of SMC promoter [20], or isolating the outgrowth of embryoid bodies (EBs) [21]. In our previous studies, we have demonstrated that the derivation of vascular lineages from hESCs can be achieved by administration of angiogenic growth factors, either by monolayer, two-dimensional (2D) differentiation protocol, or by isolation of vascular progenitor cells or $CD34^+$ cells from 10-day old EBs, followed by selective induction into either endothelial-like cells (using vascular endothelial growth factor; VEGF) or smooth-muscle-like cells (SMLCs; using PDGF-BB) [22, 23]. These SMLCs were shown to express v-SMC markers, such as alpha smooth muscle actin (α-SMA), calponin, and smooth muscle myosin heavy chain (SM-MHC) and to support vascular networks in vivo [22].

In this study, we differentiated hESCs into SMLCs using a simple step-wise monolayer differentiation protocol, resulting in highly purified cultures of SMLCs. We demonstrated that these SMLCs expressed v-SMCs markers, produced ECM components, and contracted in response to carbachol. Further in vitro tubulogenesis assays revealed that these hESC-derived SMLCs interacted with human endothelial progenitor cells (EPCs) to support and augment CLS formation. Thus, we demonstrated that SMLCs derived from hESCs are a potential cell source for therapeutic vascular tissue engineering.

Materials and Methods

Cell Culture

All cells were cultured in humidified incubators (37°C) in atmospheres maintained with 5% CO_2.

Human ESCs Human ESC line H9 was grown (passages 15 to 40; WiCell Research Institute, Madison, WI) on an inactivated mouse embryonic fibroblast feeder layer (Globalstem, Rockville, MD) in growth medium consisting of 80% ES-DMEM/F12 (Globalstem) supplemented with 20% knockout serum replacement (Invitrogen, Carlsbad, CA) and 4 ng/ml basic fibroblast growth factor (bFGF; Invitrogen), as previously described [23]. Human ESCs were passaged every 4 to 6 days with 1 mg/ml of type IV collagenase (Invitrogen). Media were changed daily.

Human v-SMCs Human aorta v-SMCs (ATCC, Manassas, VA) served as the control cell type and were grown in the specified ATCC complete SMC growth medium, consisting of Kaighn's Modification of Ham's F-12 Medium (F-12K Medium; ATCC), 10% fetal bovine serum (FBS; Hyclone), 0.05 mg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), 0.01 mg/ml insulin (Sigma), 0.01 mg/ml transferring (Sigma), 10 ng/ml sodium selenite (Sigma), 0.03 mg/ml Endothelial Cell Growth Supplement (Sigma), HEPES (Sigma) to a final concentration of 10 mM, and TES (Sigma) to a final concentration of 10 mM. Human v-SMCs were passaged every 3 to 4 days with 0.25% trypsin (Invitrogen). Media were changed every 2 to 3 days.

Human EPCs Human umbilical cord EPCs isolated from outgrowth clones, kindly provided by Dr. Yoder, Indiana University School of Medicine, were expanded and used for experiments between passages 3 and 10. For the current study, EPCs were isolated from seven healthy newborns (three females and four males; gestational age range, 38–40 weeks), pooled, expanded, and characterized according to a previously established protocol by Yoder and colleagues [24–28], and as we describe in detail in our recent publication [29]. Briefly, EPCs were expanded in flasks coated with type I collagen (Roche Diagnostics, Basel, Switzerland), in endothelial growth medium (EGM; PromoCell Heidelberg, Germany) supplemented with 1 ng/ml $VEGF_{165}$ (Pierce, Rockford, IL), and incubated in a

humidified incubator at 37°C in an atmosphere containing 5% CO_2. EPCs were passaged every 3 to 4 days with 0.05% trypsin (Invitrogen, Carlsbad, CA) and characterized for the positive expression of cell-surface antigens CD31, CD141, CD105, CD144, vWF and Flk-1, and the negative expression of hematopoietic-cell surface antigens CD45 and CD14. Single cell colony forming assays were used to characterize their robust proliferative potential, secondary and tertiary colony formation upon replating.

V-SMC Differentiation Protocol

Human ESCs were digested with TrypLE (Invitrogen). Cells were separated into an individual cell suspension using a 40-μm mesh strainer. The individual hESCs were plated onto collagen-type-IV-coated plates (R&D Systems, Minneapolis, MN) in a concentration of 5×10^4 cells/cm^2. These cells were cultured in a differentiation medium of alpha-MEM (Invitrogen) with 10% FBS and 0.1 mM β-mercaptoethanol (Invitrogen) for 6 days. Media were changed every day. On day six, differentiated cells were removed using trypLE, filtered through a 40 μm mesh strainer (BD Biosciences, San Jose, CA), and recultured onto collagen-type-IV-coated plates in cell concentrations of 1.25×10^4 cells/cm^2 in differentiation medium supplemented with PDGF-BB (10 ng/ml) and TGF-β1 (1 ng/ml) for 6 days (both from R&D Systems). Media were changed every second day.

Real-time Quantitative RT-PCR

Two-step RT-PCR was performed on hESCs, v-SMCs, and differentiated SMLCs after 6 days in the growth-factor-supplemented differentiation medium. Total RNA was extracted by using TRIzol (Gibco, Invitrogen), according to the manufacturer's instructions. Total RNA was quantified by an ultraviolet spectrophotometer, and the samples were validated for having no DNA contamination. RNA (1 μg per sample) was subjected to reverse transcriptase using M-MLV (Promega Co., Madison, WI) and oligo(dT) primers (Promega), using the manufacturer's instructions. We used TaqMan Universal PCR Master Mix and Gene Expression Assay (Applied Biosystems, Foster City, CA) for *COL, FN1, KDR, PDGFRB, NEUROPILIN, SMA, ANG-1, FLT-1, VE-CAD, β-ACTIN*, and *HPRT1*, according to the manufacturer's instructions. The TaqMan PCR step was performed with an Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems), following the manufacturer's instructions. The relative expression of *COL1A1* or *FN1* was normalized to the amount of *HPRT1* or β-*ACTIN* in the same cDNA by using the standard curve method described by the manufacturer. For each primer set, the comparative computerized tomography method (Applied Biosystems) was used to calculate amplification differences between the different samples. The values for experiments were averaged and graphed with standard deviations.

Immunofluorescence

After 6 days in growth-factor-supplemented differentiation medium, hESC-derived SMLCs were fixed using 3.7% formaldehyde fixative for 15 min and washed with phosphate buffered saline (PBS). After, cells were permeabilized with a solution of 0.1% Triton-X (Sigma) for 10 min, washed with PBS, and incubated for 1 h with anti-human SMA (1:200; Dako, Glostrup, Denmark), anti-human calponin (1:200; Dako), anti-human SM22 (1:200, Abcam, Cambridge, MA), and anti-human SM-MHC (1:100; Dako). For ECM staining, cells were incubated with anti-human fibronectin (1:200; Sigma) or anti-human collagen (1:200; Abcam) for 1 h. Cells were rinsed twice with PBS and incubated with anti-mouse IgG Cy3 conjugate (1:50; Sigma) or anti-rabbit IgG Alexa Fluor 488 conjugate (1:1000; Molecular Probes, Eugene, OR) for 1 h, rinsed with PBS, and incubated with DAPI (1:1000; Roche Diagnostics) for 10 min. 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).

Fluorescence-activated Cell Sorting (FACS)/Flow Cytometry

After 6 and 12 days of differentiation, hESC-derived SMLCs cells were treated with 0.25% trypsin for 5 min, counted, and separated into approximately 2×10^5 cells per vial. They were then incubated in either FITC- or PE-conjugated antigen specific antibodies for VEGFR-2/KDR, PDGFR-B, α-SMA (R&D systems) for 1 h on ice. For detection of intracellular markers, cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.1% Triton-X for ten min prior to incubation with antibodies. For other SMC markers, mouse anti-human SMA (1:200), mouse anti-human calponin (1:200), mouse anti-human SM-MHC (1:10; Dako), and rabbit anti-human SM22 (1:2000; Abcam) were used. Cells were rinsed with 0.1% bovine serum albumin (BSA), and then incubated in the anti-mouse IgG FITC conjugate (1:50; Molecular Probes) or anti-rabbit IgG Alexa Fluor 488 conjugate (1:1000; Molecular Probes) for 1 h. Afterwards, cells were strained and suspended in 0.1% BSA. All analysis was done using isotype controls corresponding to each specific antibody. User guide instructions were followed to complete the FACS analysis.

Tube Formation Assay on Matrigel

Matrigel (BD Bioscience) was cast into each well of a μ-Slide Angiogenesis (iBidi, Munich, Germany) and allowed to polymerize inside the incubator for 1 h. For each well, 100,000 cells/cm^2 of EPCs and hESC-derived SMLCs were seeded with respective ratios of 100:0, 60:40, 40:60, 20:80, and 0:100 in EGM (PromoCell) supplemented with 1 ng/ml $VEGF_{165}$ (Pierce). Visualization and image acquisition were performed using an inverted light microscope (Olympus IX50) at time intervals of 12, 24, and 48 h.

Quantification of CLSs

After 12 h of culture on Matrigel, the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) was used to visualize CLSs, following the manufacturer's protocol. Briefly, calcein AM dye was diluted in phenol-red-free DMEM (Invitrogen) to obtain a final concentration of 2 μM. The constructs were incubated with the dye solution for 30 min. After replacing with fresh phenol-red-free DMEM, CLSs were visualized using a fluorescent microscope with a 10× objective lens (Axiovert; Carl Zeiss Inc., Thornwood, NY). As previously described [29], we analyzed four image fields per construct from three distinct experiments ($n=3$) performed in triplicate, using Metamorph software 6.1 (Universal Imaging Co., Downingtown, PA) to quantify and compare CLSs formed on each substrate.

Spatial Organization of EPCs and SMLCs in CLSs

To analyze the position of EPCs and hESC-derived SMLCs within the forming CLSs, both cell types were labeled: EPCs with PKH2 (green) and SMLCs with PKH-26 (red) (Sigma), according to the manufacturer's protocol. Briefly, EPCs and differentiated SMLCs suspensions in diluent C were mixed with PKH2 and PKH-26, respectively, for 5 min. The staining was stopped by adding Heat Inactivated-FBS (Globalstem), and the cells were washed three times with EGM medium (PromoCell) supplemented with 1 ng/ml $VEGF_{165}$ (Pierce) and 2% HI-FBS (Globalstem). The cells were seeded for tube formation assays on Matrigel (BD Bioscience) in EGM media and cultured for 12 h. A sequence of z-stack images was obtained using confocal microscopy (LSM 510 Meta; Carl Zeiss) to determine the spatial arrangement of the cells in the CLSs.

Functional Contraction Studies

Contraction studies in response to pharmacological drugs were done, as previously described [12, 22]. Briefly, hESC derived-SMLCs cultured for three passages were washed, and contraction was induced by incubating with 10^{-5} M carbachol (Calbiochem, Darmstadt, Germany) in DMEM medium (Invitrogen) for 30 min. In a separate experiment, the cells were induced to relax by incubating with muscarinic antagonist 10^{-4} M atropine (Sigma) in DMEM for 1 h and then induced to contract with 10^{-5} M carbachol. The cells were visualized using cytoplasm-viable fluorescence dye, as described in the "*Quantification of CLSs*" section, above. A series of time-lapse images were taken using a microscope with a 10× 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 min).

Statistical Analysis

We performed statistical analyses of CLS quantification, fibronectin and collagen production, and contractility data using GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, CA). Unpaired Student's t-tests were performed, and significance levels were set at $*p<0.05$, $**p<0.01$, and $***p<0.001$, respectively. No significant difference ($p>0.05$) was indicated with #. All graphical data were reported.

Results

Derivation of SMLCs from hESCs

Our previous studies demonstrated that a 2D differentiation strategy and supplementation of PDGF-BB induced differentiation towards v-SMCs [21, 23]. Recent publications have suggested that autocrine signaling within the niche maintains the pluripotency of hESCs [30–32]. We sought to improve our protocol and efficiently derive SMLCs from hESCs (Fig. 1). At the first stage, to reduce the pluripotency associated with autocrine signaling, we utilized TrypLE to ensure a single-cell suspension and decreased cell seeding concentrations to 2.5×10^4 cells/cm^2. In addition to supplementation of culture media with 10 ng/ml PDGF-BB, we added 1 ng/ml TGF-β1, as it has been suggested to support SMC lineage differentiation from adult stem cells [8, 33].

Characterization of hESC-derived SMLCs

After 12 days of differentiation, hESC-derived SMLCs were analyzed for specific v-SMC markers and compared to human aorta v-SMCs. The chosen markers are proven indicators of v-SMC lineage, including α-SMA, an actin isoform typical of SMCs and present in high amounts in v-SMCs [8]; calponin, a calcium-binding protein that normally functions to inhibit ATPase activity in v-SMCs [34]; SM22 alpha, an actin cross-linking/gelling protein that

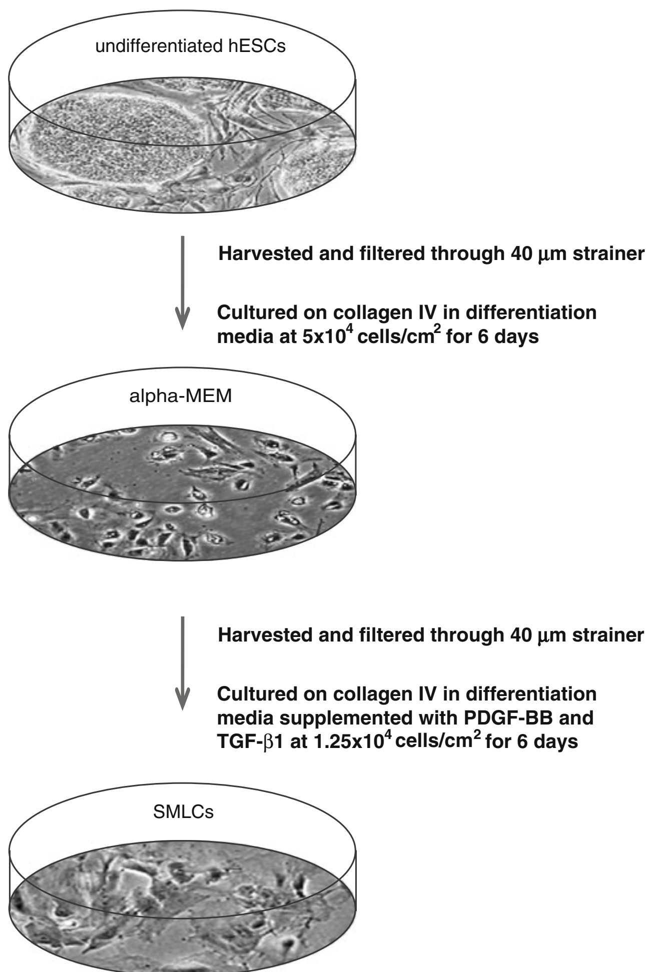

Fig. 1 Protocol for the derivation of SMLC from hESCs

belongs to the calponin family [35]; and SM-MHC, a contractile protein specific for the SMC lineage [36, 37]. Human ESC-derived SMLCs, like human aorta v-SMCs, were found to express SMA, calponin, SM22, and SM-MHC within the cell cytoplasm (Fig. 2a and Supplementary Figure 1). Flow cytometry analysis (using indirect labeling) further showed high expression levels of most markers in hESC-derived SMLCs, which were comparable to their expression levels in human aorta v-SMCs—including SMA (99 vs. 98%, respectively), calponin (99 vs. 98%, respectively), and SM22 (98 vs. 90%, respectively)—while SM-MHC was expressed in hESC-derived SMLCs to a lesser extent than its expression in human aorta v-SMCs (52 vs. 70%, respectively) (Fig. 2b). To better understand the kinetics of gene regulation, we further analyzed differentiating cells at day 6 and day 12 for markers known to be involved in mesodermal/vascular differentiation. It was previously demonstrated that KDR is expressed in undifferentiated hESCs and continues to be expressed during differentiation associated with embryoid body formation [14, 38–40]. We found that using our differentiation protocol, KDR is downregulated, as demonstrated by both FACS and qRT-PCR analyses (Supplementary Figure 2A). Other markers related to SMC specification are upregulated throughout the differentiation including: PDGFR-B, Neuropilin, and SMA, as well as Angiopoietin 1 (Ang-1) production (Supplementary Figure 2B-E). We also show that VE-Cad and FLT-1, which are known to be involved in endothelial cell commitment, are downregulated along the SMC lineage commitment (Supplementary Figure 2F-G).

ECM production by hESC-derived SMLCs

To explore the potential of hESC-derived SMLCs to support engineered vasculatures, we examined the production of the ECM molecules fibronectin and collagen. Real-time PCR analysis revealed that, compared to undifferentiated hESCs, hESC-derived SMLCs produced 565-fold more collagen and 52-fold more fibronectin (Fig. 3a). We found no significant difference in collagen production between hESC-derived SMLCs and human aorta v-SMCs, while slightly lower, but significant, expression levels of fibronectin were observed in hESC-derived SMLCs compared to human aorta v-SMCs (Fig. 3a). It should be noted that culturing human aorta v-SMCs in differentiation media of hESC-derived SMLCs resulted in decreased expression of fibronectin, suggesting an inhibitory effect of differentiation media compared to v-SMC media (Supplementary Fig. 3A). Immunofluorescence analysis revealed that hESC-derived SMLCs lay down their own ECM, including fibronectin and collagen (Fig. 3b). However, unlike human aorta v-SMCs, where fibrous fibronectin was observed mainly within the cells' cytoplasm, fibronectin produced by hESC-derived SMLCs was found both within the cells and outside on the Petri dish (Fig. 3c), indicating ECM secretion by hESC-derived SMLCs. No significant differences in the ECM secretion pattern were observed when human aorta v-SMCs were cultured in differentiation media (Supplementary Fig. 3B). Overall, this data may provide insight into the early developmental stage of hESC-derived SMLCs and their potential to support developing vasculatures.

Contraction of hESC-derived SMLCs

The primary function of v-SMCs is to contract and relax within the blood vessel wall to maintain its integrity. To examine whether hESC-derived SMLCs can contract, the cells were subjected to carbachol, which induces contraction in v-SMCs, and atropine, which blocks contractility [12]. Human ESC-derived SMLCs were found to contract in response to carbachol (10^{-5} M), as demonstrated by a series of time-lapse images (Supplementary Movie 1). Morphological changes of hESC-derived SMLCs were clearly observed following treatment with carbachol, with

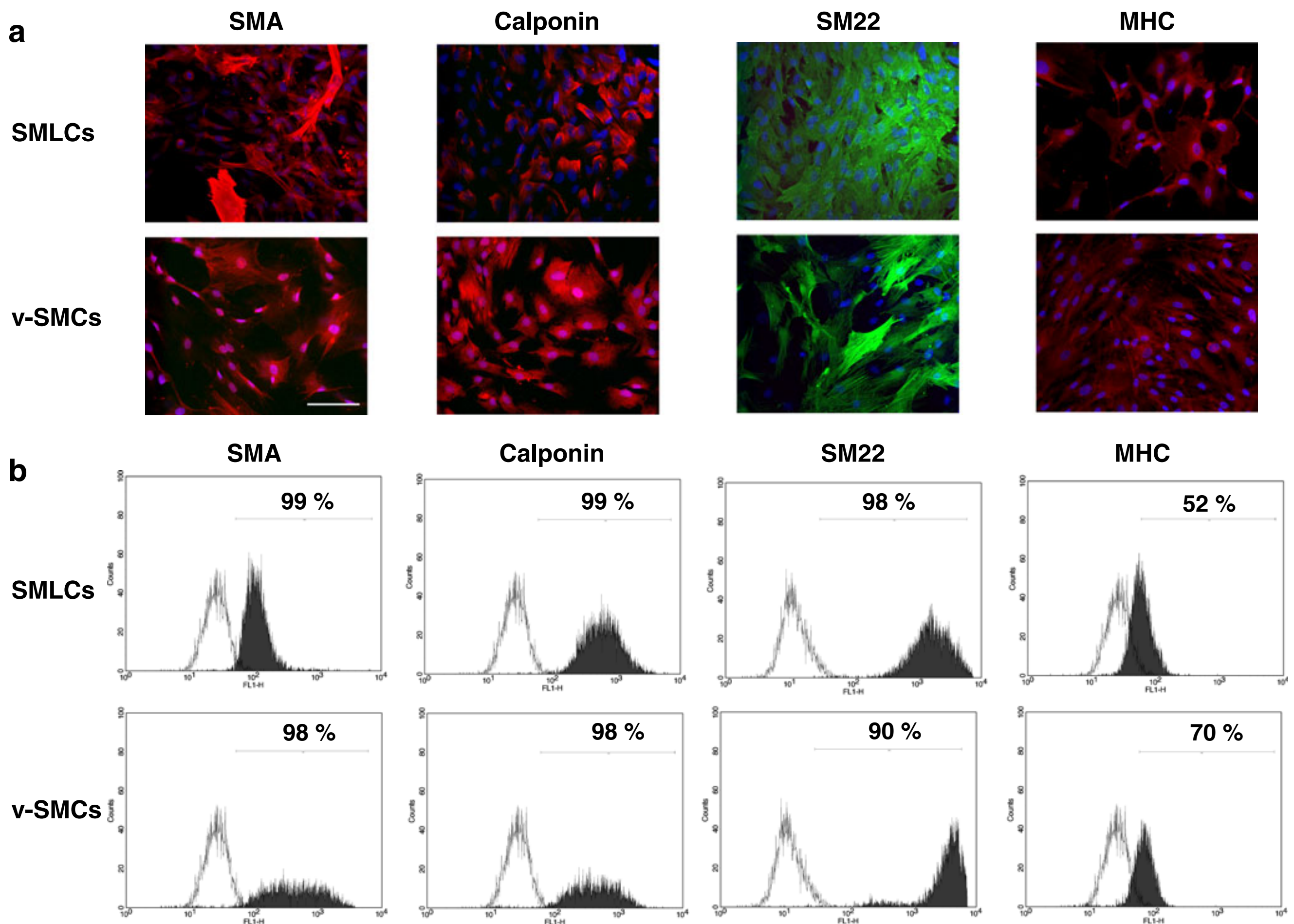

Fig. 2 Characterization of hESC-derived SMLCs. Human ESC-derived SMLCs were analyzed for the expression of specific SMCs markers, including SMA, calponin, SM22 and SM-MHC, using **a**. immunofluorescence staining (SMA, calponin, and SM-MHC in red; SM22 in *green*; and nuclei in *blue*); and **b**. flow cytometry. Scale bar is 100 µm

cytoplasm-viable fluorescence dye showing shrinkage of contracting cells after treatment (Fig. 4a). Contraction was quantified by the difference in cell area between time zero and time 30 min. Contractions of hESC-derived SMLCs were not significantly different than those of human v-SMCs (Fig. 4b). Furthermore, the muscarinic antagonist atropine (10^{-4} M) was shown to significantly block the carbachol-mediated contractility (Fig. 4b).

Human ESC-derived SMLCs Augmented CLS Phenotype

Cord-blood-derived hEPCs have been shown to form functional and stable blood vessels [41]. We previously used EPCs to study in vitro capillary tube formation induced by substrate nanotopography and viscoelasticity [29, 42]. Therefore, to study the ability of hESC-derived SMLCs to support an engineered vascular network, we examined in vitro formation of CLSs from cocultures of human EPCs and SMLCs. We seeded human EPCs and hESC-derived SMLCs at ratios of 100:0, 60:40, 40:60, 20:80, and 0:100 (EPCs:SMLCs) on Matrigel. After 12 h, CLS formation was observed in all conditions (Fig. 5a), while SMLCs supported longer and thicker tubes, with less complex networks (Fig. 5b). Moreover, SMLCs were found to stabilize and prolong CLS formation on Matrigel, which otherwise collapsed after 48 h (Supplementary Fig. 4). It should be noted that CLSs formed by both EPCs and SMLCs were found to break down by clumping at around 60 to 72 h after seeding (data not shown).

Human ESC-derived SMLCs Coherently Positioned with EPCs During CLS Formation

To examine whether SMLCs participate in CLS formation, a series of confocal z-stack analyses was performed on CLSs formed by EPCs and SMLCs (20:80) which showed the longest and thickest tubes (Fig. 5b). We found that all CLSs contained both SMLCs and EPCs (Fig. 6a), where, in

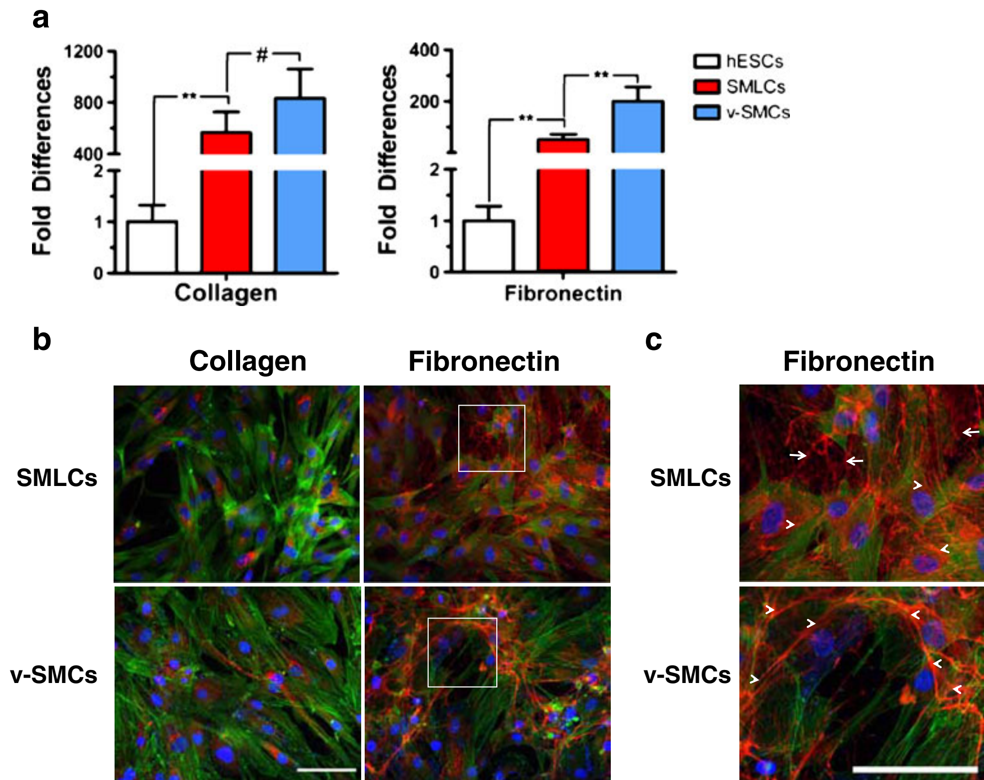

Fig. 3 ECM production and secretion. **a**. Expression levels of fibronectin and collagen were examined in hESC-derived SMLCs, and v-SMCs compared to undifferentiated hESCs, using real-time RT-PCR; **b**. immunofluorescence staining further demonstrated the expression pattern of fibronectin and collagen (in *red*; phalloidin in *green*; and nuclei in *blue*) in hESC-derived SMLCs and v-SMCs. **c**. Higher magnification images (of *squares* in B) demonstrate the intracellular expression (*arrowheads*) and the secretion (*arrows*) of fibronectin from hESC-derived SMLC, while fibronectin is expressed intracellular in the v-SMC (arrowheads). Significance levels were set at: # $p>0.05$ and **$p<0.01$. Scale bar is 100 μm

most cases, SMLCs were found to wrap the inner lining EPCs, providing a supportive layer for the developing network (Fig. 6b).

Discussion

One of the major issues in therapeutic vascularization is finding a reliable source from which cells can be isolated with high efficiency, purity, and minimal manipulation. In addition to the emerging sources of SMCs from MSCs [8, 9, 41], adipose tissues [11, 12], and neural crests [13], hESCs offer a unique source of cells for understanding signaling during vascular development and for therapeutics to treat the vasculature. Our previous study demonstrated that vascular progenitors derived from hESCs could be induced to differentiate into both ECs and SMLCs by exposure to VEGF or PDGF-BB, respectively [22]. This differentiation protocol required sorting of the vascular progenitors from developing EBs, followed by their seeding as a monolayer to induce lineage commitment [22].

In the current study, we built on our earlier protocol to differentiate hESCs into vascular lineages using a monolayer differentiation protocol [23]. We utilized this method to better control the differentiation processes and tuning of the supplemented growth factors. We have shown here that when hESCs are seeded as single cells in a certain cell density on collagen-IV-coated plates, efficient guidance of

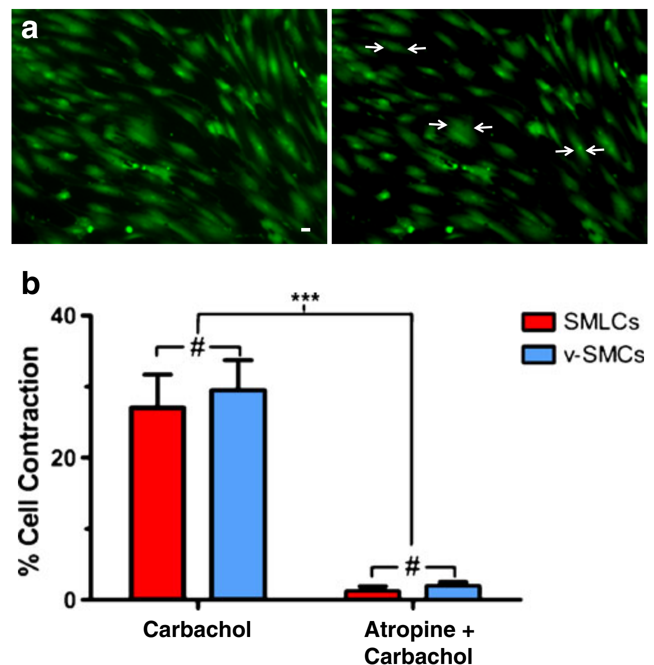

Fig. 4 Contractility of hESC-derived SMLCs. **a**. Human ESC-derived SMLCs (dyed with cytoplasm-viable fluorescent green dye; some indicated by *arrows*) were found to shrink in response to treatment with carbachol; imaged before (*left*) and after (*right*) treatment. **b.** Quantitative contractility of hESC-derived SMLCs and human aorta v-SMCs. Significance levels were set at: # $p>0.05$ and **$p<0.01$. Scale bar is 20 μm

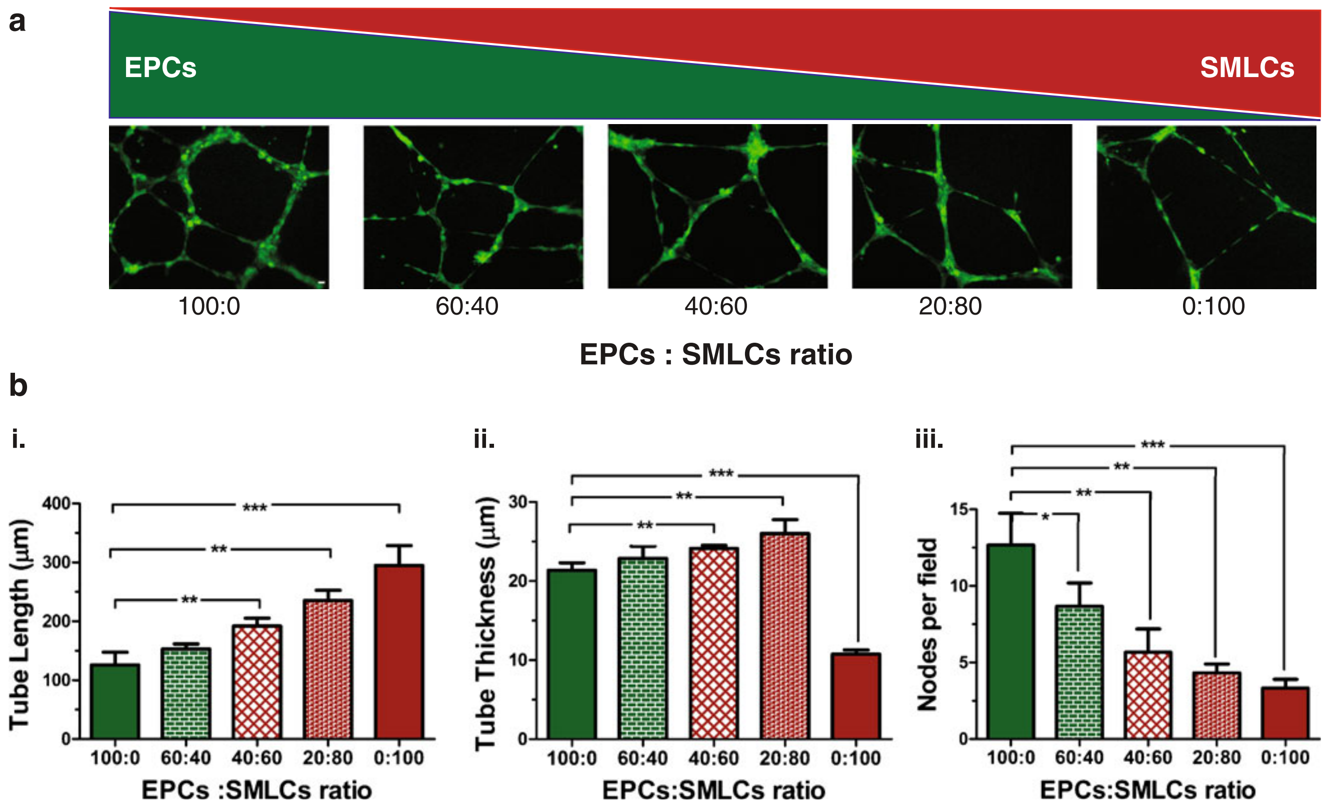

Fig. 5 Human ESC-derived SMLCs support in vitro vascular network formation and stabilization. **a**. Fluorescent microscopy images of viable CLS (*green*) formed on Matrigel following seeding with ratios of 100:0, 60:40, 40:60, 20:80, and 0:100 (EPCs:SMLCs). **b**. Metamorph analysis of CLSs revealed a significant increase of mean tube length (*i*) and mean tube thickness (*ii*) and a decrease in complexity (*iii*) as the ratio of EPC to SMLCs decreased. Significance levels were set at: $*p<0.05$, $**p<0.01$, and $***p<0.001$. Scale bar is 20 μm

their differentiation is achieved probably due to reduced autocrine signaling. These adherent cells grew for 6 days in differentiation media without growth factor supplementation to promote lateral mesoderm cell differentiation [23]. After 6 days, the cells were harvested, sorted through a 40-μm strainer, and recultured on collagen-IV-coated plates in differentiation media containing 10 ng/ml PDGF-BB and 1 ng/ml TGF-β1 for an additional 6 days to induce lineage commitment to v-SMCs. PDGF-BB has been reported to induce SMC differentiation of vascular progenitors derived from hESCs [22, 23]. TGF-β1 has been shown to induce differentiation of multipotent embryonic fibroblasts [5], neural crest cells [43], and hMSCs [8] into SMCs. Along the differentiation period, expression of PDGFR-B, Neuropilin, α-SMA, and Ang1 are upregulated, while VE-Cad and FLT1, which are known to be involved in the endothelial lineage, are downregulated. By the end of 12 days, FACS and immunofluorescence analysis revealed highly purified, differentiated SMLCs, more than 90% of which expressed levels of α-SMA, calponin, and SMC-SM22 that were comparable to levels in human aorta v-SMCs. However, only 52% of these SMLCs expressed SM-MHC, a mature marker of SMCs, compared to 70% expression by human aorta v-SMCs. Our data also indicate that KDR+ cells give rise to these SMLCs, as its expression is downregulated throughout the differentiation period.

A recent study reported that inhibition of TGF-β1 during hESC differentiation sustains stability and expansion of endothelial cells [44]. Hence, the addition of TGF-β1, which has been widely demonstrated to derive SMC lineage commitment [5, 8, 43, 45], enabled the robust SMC commitment achieved here, using a relatively simple differentiation procedure.

Real-time RT-PCR showed that these hESC-derived SMLCs highly expressed collagen and fibronectin, which are crucial for SMCs to support vascular development. SMLCs produced collagen in levels comparable to human aorta v-SMC while producing fibronectin to a lesser extent than human aorta v-SMCs. This could be attributed to the differentiation media, which also yielded reduced expression of fibronectin in human aorta v-SMCs cultured in this differentiation media. Interestingly, immunofluorescence analysis revealed that, in hESC-derived SMLCs, fibrous fibronectin was located both within the cells and outside the cells on the Petri dish, indicating that hESC-derived SMLCs secrete fibronectin to the extracellular space. This

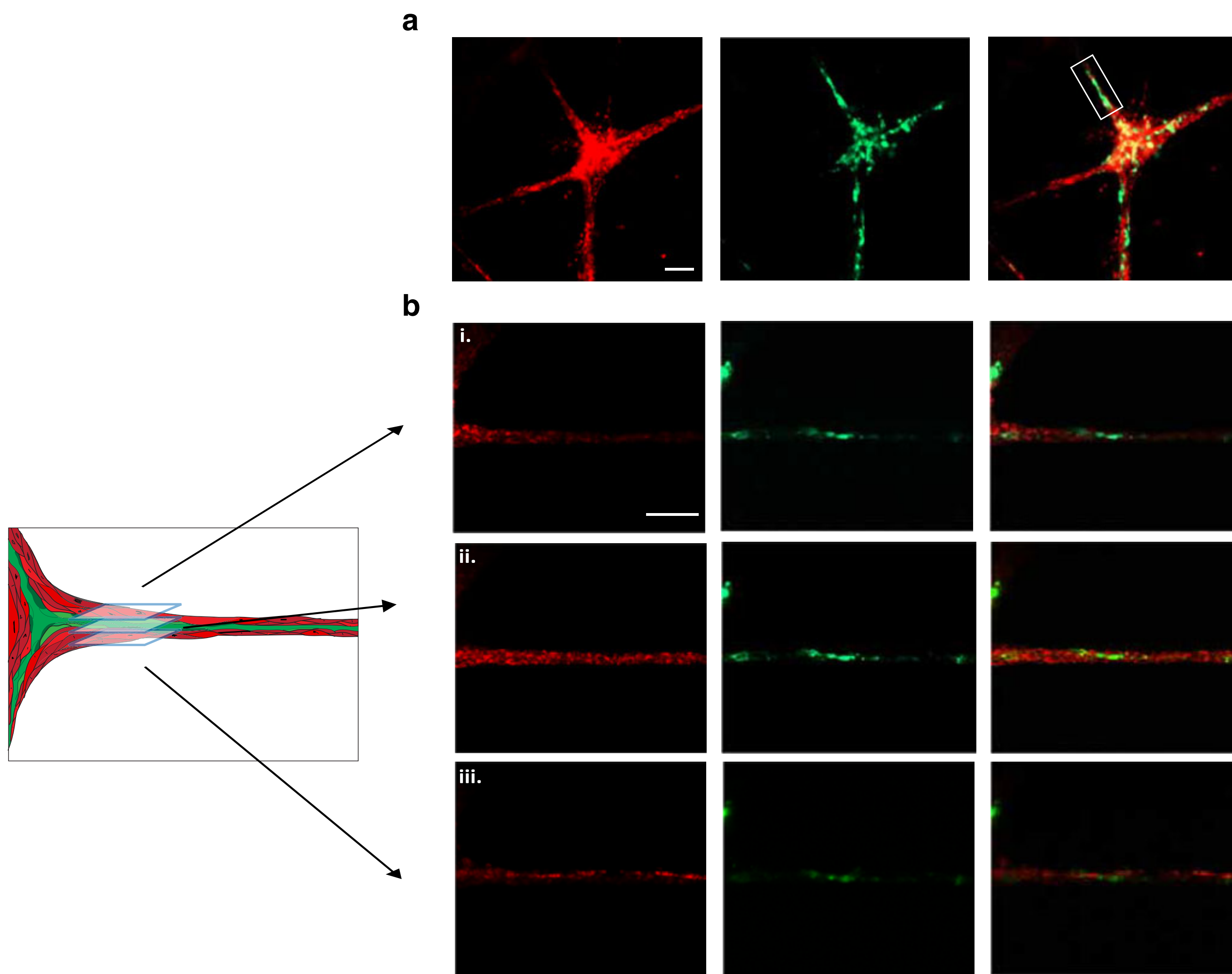

Fig. 6 Organization of SMLCs and EPC in CLS. EPCs and hESC-derived SMLCs were dyed in *green* and *red* (respectively) and seeded at ratio of 20:80 (EPCs:SMLCs) on Matrigel. **a**. Representative image showing that CLSs formed from both EPCs and SMLCs. **b**. Higher magnification Z-stack confocal images (of the *squares* in A) from *top* (*i*), *middle* (*ii*), and *bottom* (*iii*) show the outer localization of SMLCs and the inner lining EPCs. Scale bar is 100 µm

favorable early result suggests that hESC-derived SMLCs are promising for therapeutic vascularization, as SMCs tend to show a decrease in ECM production as they age [46]. Furthermore, hESC-derived SMLCs were found to contract in response to carbachol, while atropine blocks this contraction, a response similar to that observed in human aorta v-SMCs.

Mature vascular SMCs are highly specialized cells which can perform both synthetic function, to support blood vessels, and contractile function, to regulate blood pressure [47]. During early vascular morphogenesis, SMCs and mural cells are recruited to stabilize the nascent capillary through cytokine interactions and ECM production [4, 48]. In later stages, in response to their local environment (i.e., shear stress), these SMCs acquire a contractile phenotype [49]. Hence, engineering functional vascular networks requires both phenotypes, synthetic at the early stage of vascular morphogenesis and contractile at the later stage of vascular stabilization. Here, we report the derivation of SMLCs from hESCs with emerging synthetic SMC phenotype and contractility responsiveness. Together, these results indicate that hESC-derived SMLCs, although in an early stage of development, are functional and may be able to support in vitro engineered vasculature.

To study the ability of hESC-derived SMLCs to support engineered vascular networks, we examined in vitro formation of CLSs from cocultures of human EPCs and SMLCs. As the ratio of EPC to SMLCs decreased, we found that SMLCs supported longer and thicker tubes, with less complex networks. Moreover, SMLCs were able to stabilize and prolong CLS formation on Matrigel, which would otherwise collapse after 48 h of culture. These results support previous observations suggesting that pericytes might stabilize CLSs by altering the EC pheno-

type to reflect a more differentiated state [50, 51]. SMLCs may stabilize CLSs in vitro by both cytokine interactions and physical arrangement, by wrapping the inner lining of EPCs, providing a supportive layer for the developing network [4, 5, 7, 48, 52, 53]. These in vitro results also agree with previous in vivo studies where co-cultured, differentiated MSCs were shown to stabilize vessel formation of EPCs [9, 10].

Collectively, these results indicate that the improved derivation protocol could lead to highly purified cultures of hESC-derived SMLCs. Such SMLCs have early stage v-SMC characteristics, and hESC-derived SMLCs have the potential to support engineered vascular networks in vitro and therefore should be considered as a potential cell source for therapeutic vascularization.

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Conflict of interest None

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