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# Vascular Progenitor Cells Isolated From Human Embryonic Stem Cells Give Rise to Endothelial and Smooth Muscle–Like Cells and Form Vascular Networks In Vivo

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***Abstract***—We report that human embryonic stem cells contain a population of vascular progenitor cells that have the ability to differentiate into endothelial-like and smooth muscle (SM)-like cells. Vascular progenitor cells were isolated from EBs grown in suspension for 10 days and were characterized by expression of the endothelial/hematopoietic marker CD34 ($CD34^+$ cells). When these cells are subsequently cultured in EGM-2 (endothelial growth medium) supplemented with vascular endothelial growth factor-165 (50 ng/mL), they give rise to endothelial-like cells characterized by a cobblestone cell morphology, expression of endothelial markers (platelet endothelial cell-adhesion molecule-1, CD34, KDR/Flk-1, vascular endothelial cadherin, von Willebrand factor), incorporation of acetylated low-density lipoprotein, and formation of capillary-like structures when placed in Matrigel. In contrast, when $CD34^+$ cells are cultured in EGM-2 supplemented with platelet-derived growth factor-BB (50 ng/mL), they give rise to SM-like cells characterized by spindle-shape morphology, expression of SM cell markers ($\alpha$-SM actin, SM myosin heavy chain, calponin, caldesmon, SM $\alpha$-22), and the ability to contract and relax in response to common pharmacological agents such as carbachol and atropine but rarely form capillary-like structures when placed in Matrigel. Implantation studies in nude mice show that both cell types contribute to the formation of human microvasculature. Some microvessels contained mouse blood cells, which indicates functional integration with host vasculature. Therefore, the vascular progenitors isolated from human embryonic stem cells using methods established in the present study could provide a means to examine the mechanisms of endothelial and SM cell development, and they could also provide a potential source of cells for vascular tissue engineering. **(*Circ Res*. 2007;101:286-294.)**

**Key Words:** human embryonic stem cells ■ vascular progenitor cells ■ stem cell differentiation ■ endothelial cells ■ smooth muscle cells

The vascularization of tissue constructs remains a major challenge in regenerative medicine, as the diffusional supply of oxygen can support only 100- to 200-$\mu$m thick layers of viable tissue.[1,2] The formation of a mature and functional vascular network requires communication between endothelial cells (ECs) and smooth muscle cells (SMCs).[3–5] Isolating a population of human progenitor cells with potential for cell number expansion and differentiation into both ECs and SMCs with high efficiency could benefit the area of tissue engineering.[2,5,6]

Embryonic stem cells (ESCs) are a potential cell source for induction of tissue vascularization.[7] Prior studies have derived ECs and SMCs cells from a common progenitor ($Flk\text{-}1^+$ cells) from mouse[6] and monkey ESCs,[8] but not from human ESCs (hESCs). We previously reported that hESCs can spontaneously generate ECs with definitive properties.[9] These cells were isolated based on the expression of platelet EC-adhesion molecule-1 (PECAM1) from embryoid bodies (EBs) grown in suspension for 13 to 15 days. Using the same endothelial[10] or other (eg, CD34[11,12]) markers, others have isolated endothelial progenitor cells with the ability to differentiate into mature endothelium. In addition, it has been reported that hESCs can differentiate into mesodermal cells that can give rise to ECs and SMCs[13]; however, it is not clear that these cells were derived from the same progenitor.

Here we report that cells isolated from EBs at day 10 and expressing the hematopoietic/endothelial marker CD34 are vascular progenitor cells that can be selectively induced to

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From the Department of Chemical Engineering (L.S.F., H.F.S., R.L.), Massachusetts Institute of Technology, Cambridge, Mass; Center of Neurosciences and Cell Biology (L.S.F.), University of Coimbra, Portugal; Biocant-Biotechnology Innovation Center (L.S.F.), Cantanhede, Portugal; Harvard–Massachusetts Institute of Technology Division of Health Sciences and Technology (S.G., G.V.-N., R.L.), Cambridge, Mass; Whitehead Institute of Biomedical Research (N.W.), Cambridge, Mass; Division of Vascular Biology (M.A.R., S.M.D.), Children's Hospital, Boston, Mass; Division of Cardiovascular Medicine (M.A.R.), Brigham and Women's Hospital, Boston, Mass; and Department of Biomedical Engineering (G.V.-N.), Columbia University, New York.

Correspondence to Robert Langer, Department of Chemical Engineering, E25-342 MIT, 77 Massachusetts Ave, Cambridge, MA 02139. E-mail rlanger@mit.edu.



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differentiate into either endothelial-like (EL) (using endothelial growth medium [EGM-2] containing vascular endothelial growth factor-165 [$VEGF_{165}$]), or smooth muscle–like (SML) cells (using EGM-2 containing platelet-derived growth factor-BB [$PDGF_{BB}$]). When implanted in nude mice, these cells contributed to the formation of functional microvessels containing mouse blood cells. This study describes a potential source of cells for vascular tissue engineering and provides a model for the study of vascular differentiation.

## Materials and Methods

An expanded Materials and Methods section is provided in the online data supplement at http://circres.ahajournals.org

### Cell Culture

hESC lines H9 and H13 with normal karyotype (Figure I in the online data supplement) were grown (passages 25 to 45; WiCell, Madison, Wis) on an inactivated mouse embryonic feeder layer (Cell Essential, Boston, Mass) as previously described.[14] The studies were performed with H9 cell line unless otherwise stated. In some cases, $CD9^+GCTM2^+$ cells isolated by fluorescence-activated cell sorting (FACS) from hESCs were used to characterize the undifferentiated fraction of these cells.[15] EB formation and culture, as well as culture of relevant primary cells, can be found in the online data supplement.

### Isolation and Culture of $CD34^+$ Cells

Selection of $CD34^+$ cells at day 10 was performed by labeling the hESCs with the anti-CD34 antibody (QBEND/10, Miltenyi Biotec) conjugated with magnetic beads. The magnetically labeled cells were separated into $CD34^+$ and $CD34^-$ populations using a LS-MACS column (Miltenyi Biotec). CD34 enrichment was confirmed by flow cytometry analysis using a different anti-CD34 antibody (AC136; Miltenyi Biotec). Isolated $CD34^+$ cells were grown on 24-well plates ($3\times10^4$ cells/well) coated with 1% gelatin and containing EGM-2, or EGM-2 supplemented with $VEGF_{165}$ (50 ng/mL, R&D Systems) or $PDGF_{BB}$ (50 ng/mL, R&D Systems).

### Transplantation in Nude Mice

EL or SML cells alone (third passage, $0.5\times10^6$ cells in $\approx$20 $\mu$L of EGM-2), or EL cells mixed with SML cells (3:1; $0.5\times10^6$ cells in total, in 20 $\mu$L of EGM-2) were suspended in 0.350 mL of Matrigel (BD Biosciences) on ice. The cell suspension was injected subcutaneously (23-gauge needle) in each side of the dorsal region of a 4-week-old male balb/c nude mice (2 implants per mouse; 3 mice per experimental condition). Matrigel without cells was used as control. After 28 days, the implants were removed, fixed overnight in 10% (vol/vol) buffered formalin at 4°C, embedded in paraffin, and sectioned for histological examination.

### Histological Examination

Immunohistochemical staining of explants from animal studies was performed using the EnVision+/HRP kit (Dako) with prior heat treatment at 95°C for 20 minutes in ReVeal buffer (Biocare Medical) or trypsin (1 mg/mL) for epitope recovery. For immunofluorescent staining, anti-mouse IgG Cy3 conjugate was used as secondary antibody followed by DAPI (4′,6-diamidino-2-phenylindole) nuclear staining. The primary antibodies were anti-human PECAM1 (1:20), anti-human collagen type IV (1:500, Sigma), anti–$\alpha$-smooth muscle actin ($\alpha$-SMA) (1:50), anti-human nuclei (1:20, Chemicon), $\beta_2$-microglobulin (1:50, BD Pharmingen), and the corresponding isotype controls. Biotinylated *Ulex europaeus* agglutinin-1 (UEA-1, 1:100; Vector Laboratories) was also used for histological staining. The number of microvessels that were immunoreactive for human collagen type IV was counted in 7 random fields from at least four implants (2 sections for each implant) at $\times$20 magnifications (corresponding to an area of $3.4\times10^5$ $\mu m^2$).

### FACS Analysis

Undifferentiated hESCs, HUVECs, or $CD34^+$ cells grown in different growth media were dissociated with nonenzymatic cell dissociation solution (Sigma) for 10 minutes. EBs were dissociated with 0.4 U/mL collagenase B (Roche Diagnostics) for 2 hours in a 37°C incubator, followed by treatment with cell dissociation solution for 10 minutes, followed by gentle pipetting. Single cells were aliquoted (1.25 to $2.5\times10^5$ cells were used per condition) and stained with either isotype controls or antigen-specific antibodies. A detailed list of the antibodies used and the staining procedure can be found in the online data supplement.

### Western Blot Analysis

Cells differentiated for 3 passages were harvested using trypsin and lysed as reported elsewhere.[16] Briefly, sample loading buffer and reducing agent (both from Bio-Rad) were added to the lysates. Samples were heated (5 minutes, 95°C) and loaded on 4% to 15% Tris-HCl Criterion gels (Bio-Rad), separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were probed for smooth muscle myosin heavy chain (SM-MHC) (8.5 $\mu$g/mL, DakoCytomation), $\alpha$-SMA (0.7 $\mu$g/mL, DakoCytomation), and PECAM1 (2 $\mu$g/mL; Santa Cruz Biotechnology). Blot blocking and development procedures can be found in the online data supplement.

### RT-PCR Analysis

Total RNA was extracted using TRIzol (Invitrogen) according to the instructions of the manufacturer. Total RNA was quantified by a UV spectrophotometer, and 1 $\mu$g was used for each reverse-transcription sample. RNA was reversed transcripted with M-MLV and oligo (dT) primers (Promega) according to the instructions of the manufacturer. PCRs were done with BIOTAQ DNA polymerase (Bioline) using 1 $\mu$L of reverse-transcription product per reaction. To ensure semi-quantitative results of the RT-PCR assays, the number of PCR cycles for each set of primers was verified to be in the linear range of the amplification. In addition, all RNA samples were adjusted to yield equal amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal standard. Primer sequences, reaction conditions, and optimal cycle numbers are published as supporting information (supplemental Table I). The amplified products were separated on 2% agarose gels with ethidium bromide.

### Statistical Analysis

An unpaired Student $t$ test or 1-way analysis of variance with Bonferroni post test were performed for statistical tests by using GraphPad Prism 4.0 (San Diego, Calif). Results were considered significant when $P\leq0.05$.

## Results

### Vascular Differentiation During EB Development: Effects of Serum Supplements

EBs were grown in medium containing knockout serum (KO-SR) or FBS and analyzed over a 2-week period for expression of well-characterized EC (PECAM1, CD34 and KDR/Flk-1),[6,7,9,17–19] SMC ($\alpha$-SMA and SM-MHC),[6,8,13,17–19] and undifferentiated ESC markers (SSEA4, Nanog, and alkaline phosphatase)[20] at the gene and protein levels. Initially, hESCs expressed low or undetectable levels of CD34 and PECAM1, significant levels of KDR/Flk-1, and moderate levels of $\alpha$-SMA and SM-MHC (Figure 1A and 1B). The expression of KDR/Flk-1 coexisted with the expression of undifferentiated stem cell markers Nanog (Figure 1A), SSEA4, and alkaline phosphatase, showing that cells are undifferentiated.

The removal of undifferentiated hESCs from mouse embryonic feeder layers and subsequent culture as EBs in differentiation medium containing KO-SR reduced the ex-

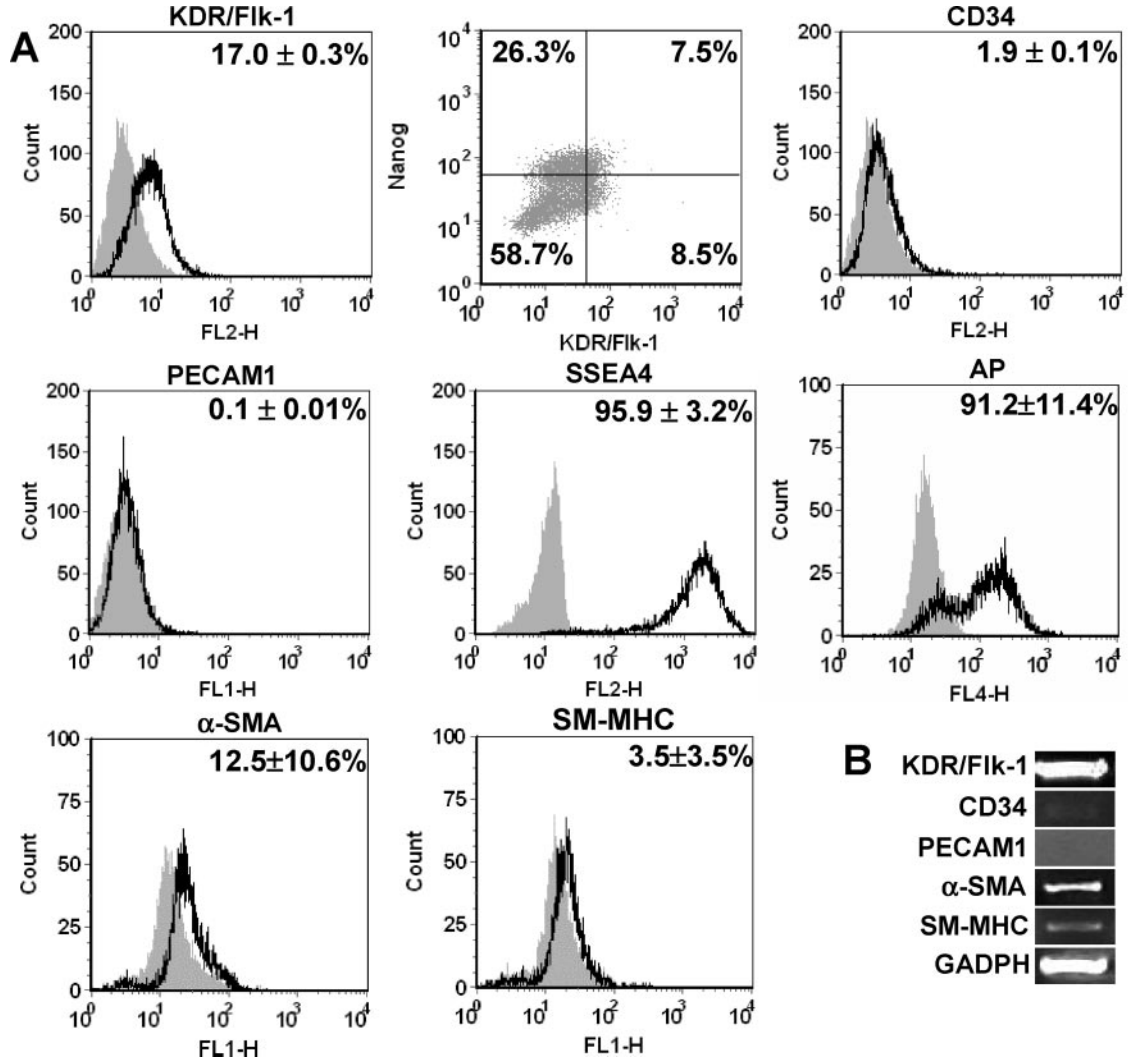


**Figure 1.** Expression of vascular and undifferentiated stem cell markers in hESCs. A, Flow cytometric analysis of undifferentiated and vascular markers on undifferentiated hESCs. The percentages of positive cells were calculated based on the isotype controls (gray plot) and are shown in the histogram plots. Values in histogram plots indicate averages±SD from 3 independent experiments. AP indicates alkaline phosphatase. B, Gene analysis for vascular markers on undifferentiated hESCs.

pression of alkaline phosphatase and SSEA4, indicating that cells were undergoing differentiation (Figure 2A.1). During this differentiation process, α-SMA and SM-MHC were highly expressed for 10 days (Figure 2A.1), expression of CD34 peaked around day 10, KDR/Flk-1 expression decreased by day 4 and remained low thereafter, and PECAM1 expression was low through the 12 days of differentiation (Figure 2A.2).

Next, we evaluated the effect of serum supplementation on EB differentiation. Use of FBS instead of KO-SR resulted in a slightly accelerated differentiation process, as indicated by the further decrease of alkaline phosphatase and SSEA4 levels and a significant ($P<0.05$) increase in the expression of CD34 (Figure 2A.1). EBs grown in medium containing FBS showed lower expression of α-SMA and SM-MHC than EBs grown in medium containing KO-SR. Taken together, medium supplementation with FBS enhanced the vascular differentiation of cells in EBs and contributed to high yields of $CD34^+$ cells.

## Formation of Vessel-Like Structures in EBs

Confocal analysis of EBs cultured for 10 days showed that $CD34^+$ cells formed extensive vascular networks (Figure 2B.1). The vessel-like structures resembled those we previously observed in $PECAM1^+$ cells[9]; however, these structures were more frequent for $CD34^+$ than for $PECAM1^+$ cells (Figure 2B.1 and 2B.2). FACS analysis confirmed that all $PECAM1^+$ cells coexpressed CD34 (supplemental Figure II).

## Isolation of $CD34^+$ Cells

A CD34 marker was used to isolate vascular progenitor cells by magnetic selection from EBs grown in differentiation medium with FBS for 10 days (Figure 3A). These conditions were selected because of high expression of CD34 during EB development (Figure 2A.1 and 2A.2). The cells isolated were 92.5±6.7% (n=3) pure for CD34 antigen (approximately a 9-fold enrichment of the initial cell population). At this stage, $CD34^+$ cells coexpressed high levels of PECAM1 (≈55%), α-SMA (≈45%), and SSEA4 (≈43%), moderate levels of

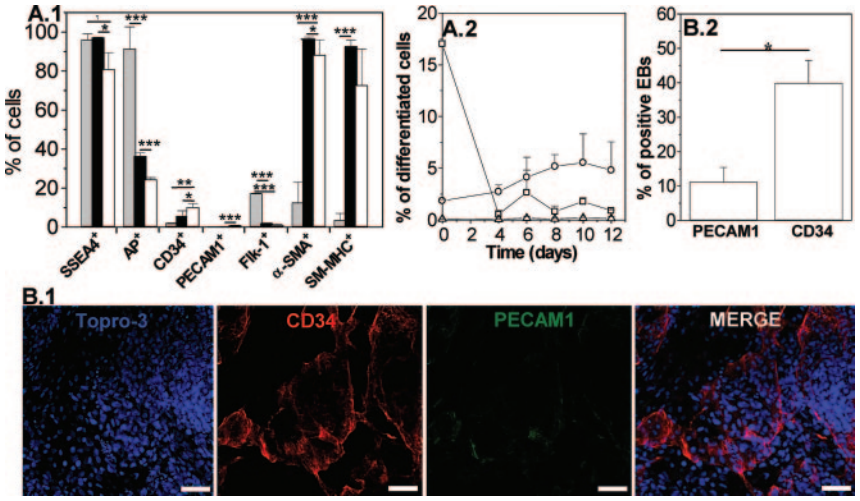


**Figure 2.** Expression of vascular and undifferentiated stem cell markers during hESCs differentiation through EBs. A, Summary of flow cytometric analysis for: the expression of undifferentiating markers and vascular markers in hESCs (gray columns) and EBs grown in differentiation medium containing KO-SR (black columns) or FBS (white columns) for 10 days (A.1); the time-course expression of KDR/Flk-1 (□), CD34 (○), and PECAM1 (△) in EBs grown in differentiation medium containing KO-SR (A.2). In all graphs, values indicate averages±SD from 3 independent experiments. *$P<0.05$, **$P<0.01$, and ***$P<0.001$. AP indicates alkaline phosphatase. B, Confocal microscopy of stained 10-day-old human EBs grown in differentiation medium containing FBS. $CD34^+$ and $PECAM1^+$ cells forming vascular networks along the EBs (B.1; magnification, ×25). Bar=50 μm. Quantification of EBs that stained for PECAM1 and CD34 (B.2). At least 100 EBs were scored (average±SD; n=3). *$P<0.05$.

KDR/Flk-1 (≈16%), and low levels of the hematopoietic marker CD45 (≈1%) (Figure 3B). The presence of these markers was also confirmed at gene level (Figure 3C).

## Induction of $CD34^+$ Cell Differentiation Into Endothelial and SMC Lineages

The isolated $CD34^+$ cells were cultured with EGM-2 alone or medium supplemented with $VEGF_{165}$ (50 ng/mL) or $PDGF_{BB}$ (50 ng/mL) (Figure 3A) because $VEGF_{165}$ and $PDGF_{BB}$ have been reported to facilitate the differentiation of stem cells into ECs and SMCs, respectively.[6,19] $CD34^+$ cells cultured in VEGF-supplemented EGM-2 for 1 passage (10 to 15 days after cell seeding) expressed high levels of EC markers (Figure 4A). Similar results were obtained with H13 cell line (supplemental Figure III). As compared with human umbilical vein ECs (HUVECs), $CD34^+$ cells had slightly lower expression of PECAM1 and KDR/Flk-1 (Figure 4A), and higher expression of CD34. At this stage, the cells lost nearly all expression of the marker SSEA4, indicating their differentiated state. $CD34^-$ cells grown in the same conditions as $CD34^+$ cells showed minimal expression of the endothelial markers (supplemental Figure IV), indicating that $CD34^+$ cells, but not the $CD34^-$ cells, can be effectively induced toward an endothelial lineage. $CD34^+$ cells cultured in EGM-2 or EGM-2 supplemented with $PDGF_{BB}$ for 1 passage showed a much lower expression of PECAM1 (26% and 18%, respectively) than the $CD34^+$ cells cultured in VEGF-supplemented medium (94%) (supplemental Figure IV). As EGM-2 contains <5 ng/mL $VEGF_{165}$ (as measured by

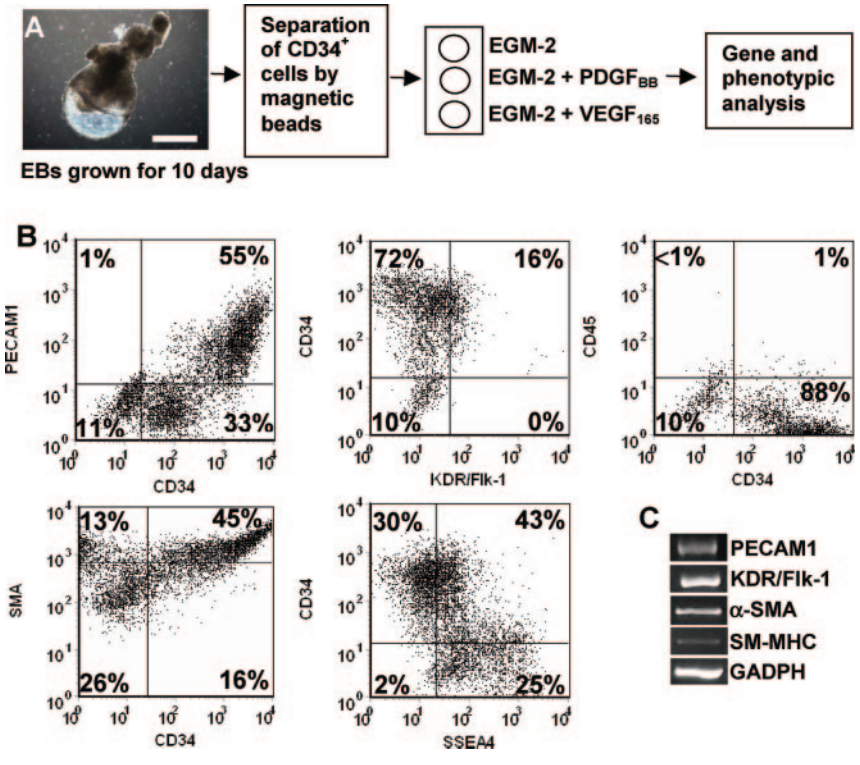


**Figure 3.** Isolation and characterization of $CD34^+$ cells. A, Scheme for the isolation and differentiation of $CD34^+$ cells. Bar corresponds to 500 μm. B, Phenotypic analysis of $CD34^+$ cells after MACS separation. Values within dot plots indicate percentage of cells in respective quadrants. C, Gene analysis of $CD34^+$ cells after MACS separation.

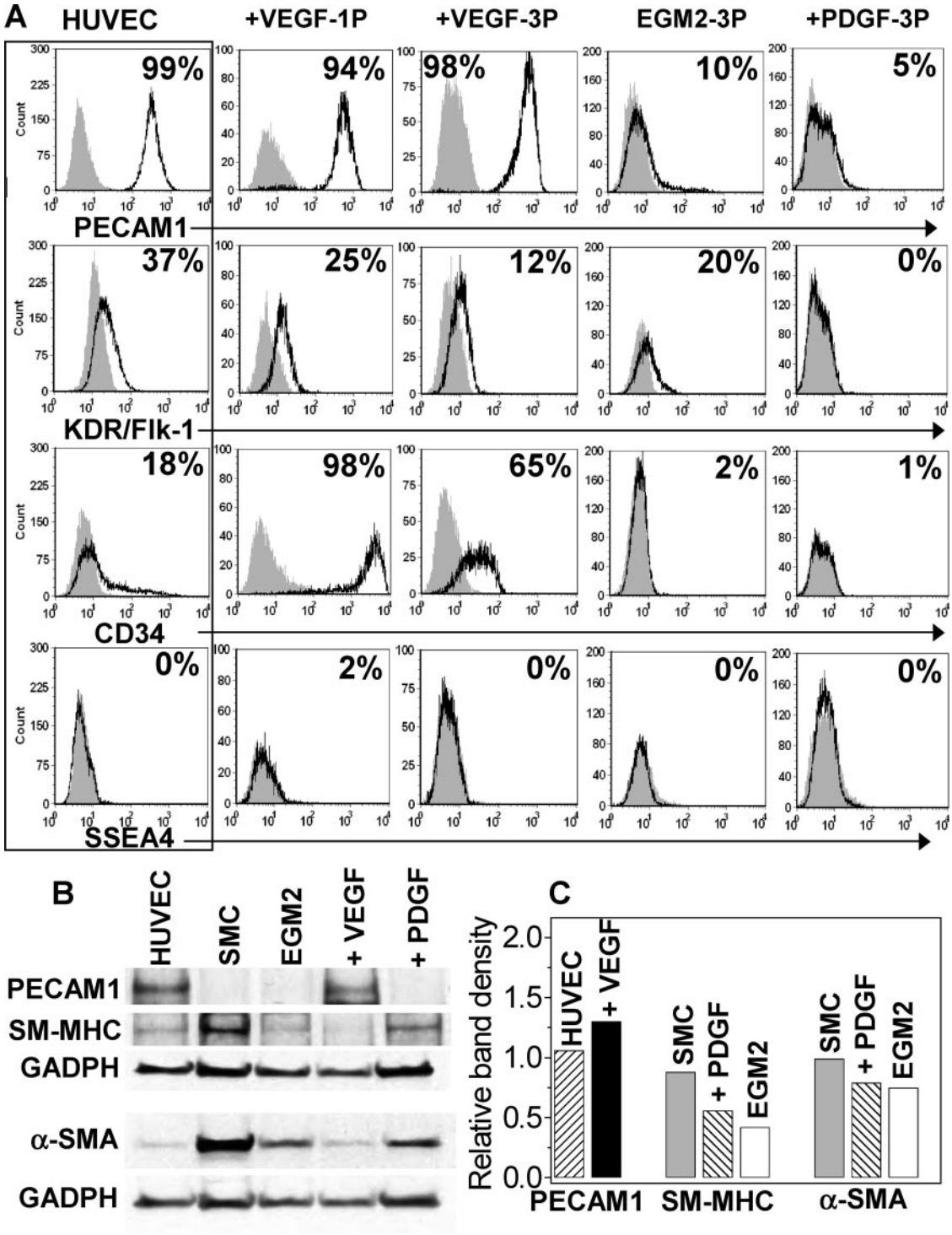


**Figure 4.** Endothelial and SMC differentiation of $CD34^+$ cells. A, FACS analysis of HUVECs and $CD34^+$ cells isolated from EBs grown in differentiation medium with FBS for 10 days and further differentiated in EGM-2, EGM-2 supplemented with 50 ng/mL $VEGF_{165}$, or EGM-2 supplemented with 50 ng/mL $PDGF_{BB}$ for 1 passage (1P; 10 to 15 days after cell seeding) or 3 passages (3P; ≈28 days after cell seeding). In all graphs, the percentages of positive cells were calculated based in the isotype controls (gray plot) and are shown in each histogram plot. B, Western blot analysis for $CD34^+$ cells differentiated in EGM-2, EGM-2 supplemented with 50 ng/mL $VEGF_{165}$, or EGM-2 supplemented with 50 ng/mL $PDGF_{BB}$ for 3 passages. HUVECs and SMCs are included for reference. GADPH was used as standard. C, Relative band density for vascular markers using GADPH as a control protein.

ELISA), VEGF concentration appears to have an important role in the endothelial differentiation of $CD34^+$ cells.

The proliferation rate of $CD34^+$ cells cultured in VEGF-supplemented medium is high, achieving 20 population doublings over a 2-month period. FACS analyses of $CD34^+$ cells cultured for 3 passages (Figure 4A) showed the expression of PECAM1 comparable to that in HUVECs (similar results were obtained by Western blot; Figure 4B and 4C),

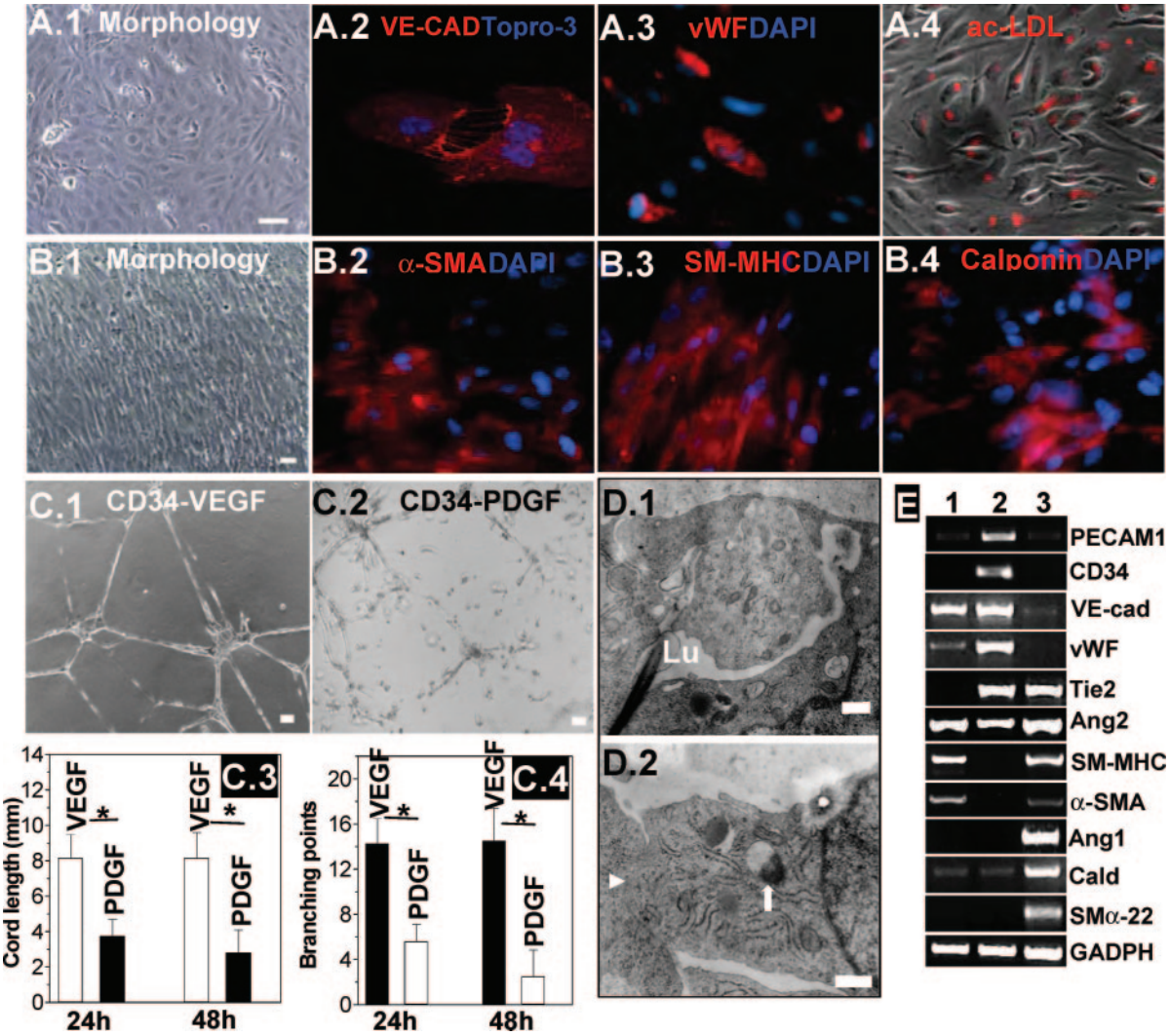


**Figure 5.** Characterization of hES-derived endothelial cells and SMCs grown in culture. A, EL cells have cobblestone morphology (A.1; light microscopy, bar corresponds to 50 $\mu$m), they show VE-cadherin at cell–cell junctions (A.2; magnification, ×40), von Willebrand factor (vWF) in the cell cytoplasm (A.3; magnification, ×40), and have the ability to uptake acetylated low-density lipoprotein (ac-LDL) (A.4; magnification, ×40), as shown by immunofluorescence staining. B, SML cells exhibit spindle-shaped morphology (B.1; light microscopy; bar corresponds to 50 $\mu$m) and highly express smooth muscle markers including $\alpha$-SMA (B.2; magnification, ×40), SM-MHC (B.3, ×40), and calponin (B.4; magnification, ×40), as shown by immunofluorescence staining. C, EL cells form cords when placed in Matrigel for 24 hours (C.1), whereas SML cells showed limited ability to form them during the same period of time (C.2). Scale bar=50 $\mu$m. The cord length (C.3) and branching points (C.4) on the cord-like structures formed by EL is statistically higher than the values found for SML cells during 24 or 48 hours. The counts were performed using an objective of ×10. Results are averages±SD, n=4. *$P<0.001$. D, Transmission electron microscopy images of cord sections formed by EL cells in Matrigel, showing lumen (Lu) formation (D.1). The cells present Weibel–Palade–like bodies (D.2, arrow) in the cytoplasm and form tight intercellular junctions (D.2; arrowhead). Scale bar=0.47 $\mu$m. D, RT-PCR analysis for endothelial and SMC markers in $CD34^+$ cells differentiated in EGM-2,[1] EGM-2 supplemented with 50 ng/mL $VEGF_{165}$,[2] and EGM-2 supplemented with 50 ng/mL $PDGF_{BB}$.[3] Ang indicates angiopoietin; Cald, caldesmon.

albeit different regarding the expression of CD34 and KDR/Flk-1 markers. Karyotyping analyses showed that genetic integrity was preserved during differentiation (supplemental Figure V). Differentiated $CD34^+$ cells stained positively for vascular endothelial (VE)-cadherin at cell–cell adherent junctions, produced von Willebrand factor, and were able to incorporate acetylated low-density lipoprotein (Figure 5A), typical markers found in ECs (supplemental Figure VI). Genetic analysis demonstrated that these cells express PECAM1, CD34, VE-cadherin, von Willebrand factor, and Tie2 receptor[19] but are negative for SMC markers including SM-MHC, SM$\alpha$-22, and angiopoietin-1[6,17,21] (Figure 5E). $CD34^+$ cells isolated from H13 cell line and differentiated in VEGF-supplemented medium presented lower levels of PECAM1 (39% versus 98%) and CD34 (14% versus 65%) compared with the H9 cell line (supplemental Figure III), suggesting slightly different differentiation profiles in the 2 cell lines.

Cells cultured in EGM-2 or $PDGF_{BB}$-supplemented medium for 3 passages expressed high levels of $\alpha$-SMA, SM-MHC, and calponin (Figures 4 and 5B), low levels of endothelial markers ($\leq$20%), and no detectable expression of the undifferentiating stem cell marker SSEA4. Western blot analysis showed that expressions of SM-MHC and $\alpha$-SMA were higher in cells differentiated in EGM-2 supplemented

with $PDGF_{BB}$ (Figure 4B and 4C). As confirmed by RT-PCR (Figure 5E), $PDGF_{BB}$-supplemented EGM-2 upregulated the expression of definitive SMC markers including caldesmon and SM$\alpha$-22,[17,21] and the expression of angiopoietin-1, a ligand produced by SMCs that activates the receptor Tie-2 found on ECs.[22] This indicates that the presence of $PDGF_{BB}$ contributed to cell maturation toward SMC phenotype. However, this process is not complete because cells express the endothelial markers angiopoietin-2 and Tie2. To examine whether these SML cells were functional, they were subjected to the effects of carbachol and atropine[23] (supplemental Figure VII). After exposure to carbachol ($10^{-5}$ mol/L) the cells contracted 30% after 30 minutes. In addition, the muscarinic antagonist atropine was shown to block the carbachol-mediated effects. Similar results were obtained in human vascular SMCs (hVSMCs). $CD34^+$ cells grown in the presence of PDGF had higher proliferation rates than $CD34^+$ cells grown in the presence of VEGF, with 42 population doublings over a 2-month period. Karyotyping analyses showed that genetic integrity was preserved during differentiation (supplemental Figure V).

The ability of $CD34^+$ cells differentiated in VEGF or PDGF-supplemented medium to form cord-like structures was also assessed by culturing these cells in the extracellular matrix basement membrane, Matrigel.[9,13,24] $CD34^+$ cells differentiated in VEGF-supplemented medium were able to spontaneously reorganize into cord-like structures when maintained in culture for 24 hours (Figure 5C and supplemental Figure VIII). In contrast, $CD34^+$ cells differentiated in EGM-2 containing $PDGF_{BB}$ have limited ability to form cord-like structures (Figure 5C). Transmission electron micrographs of cord sections formed by $CD34^+$ cells differentiated in $VEGF_{165}$-supplemented medium showed the presence of a lumen (Figure 5D.1), thus confirming the capacity of these cells to form vascular networks in vitro. In addition, these cells presented typical endothelial features (supplemental Figure VI) such as the presence of round or rod-shaped structures that resemble Weibel–Palade bodies and tight junctions between cells (Figure 5D.2). Based on the phenotype and genotype expression, the $CD34^+$ cells differentiated in $VEGF_{165}$ or $PDGF_{BB}$-supplemented medium were designated EL and SML cells, respectively.

### Transplantation of EL and SML Cells Into Nude Mice Resulted in Formation of Microvessels

EL or SML cells alone or EL mixed with SML cells (3:1 ratio) were suspended in Matrigel and injected subcutaneously in the dorsal region of nude mice. After 28 days, the mice were injected intravenously with fluorescein isothiocyanate–dextran solution. The Matrigel implants were then removed and imaged. Microvessels that support blood flow were observed in Matrigel implants containing EL or SML cells but rarely in Matrigel without cells (supplemental Figure IX). Matrigel implanted in the absence of cells showed no microvessels inside of the matrix, only at the periphery (Figure 6A). The constructs with EL cells showed the presence of microvessels within the Matrigel (Figure 6B.1), most of which (≈95%) were patent with empty lumens, whereas a small percentage (≈5%; Figure 6B.2) contained mouse red blood cells. These microvessels were reactive for UEA-1 (specific for human ECs[25]), anti-human PECAM1, anti-human nuclei, and anti-human collagen type IV (collagen IV is a component of the extracellular matrix actively produced by ECs[26]) (Figure 6.B and supplemental Figures X and XI), indicating that they were composed of human ECs. In general, the cells and microvessels inside Matrigel were not reactive for $\alpha$-SMA (Figure 6B.5). On the other hand, implants formed by a mixture of EL and SML showed the presence of microvessels that were immunoreactive to the same human markers described above (Figure 6C). A fraction of these microvessels (≈5% to 6%) contained mouse blood cells (Figure 6C.1). Cells inside Matrigel stained positively for PECAM1 (≈41%) or $\alpha$-SMA (≈20%); in this last case, they formed small tubules (Figure 6C.4) or surrounded human microvessels (Figure 6C.5; supplemental Figure XI). Thus, these cells have properties of SM cells. Constructs with only SML cells stained for $\alpha$-SMA (supplemental Figure XI) showing the differentiation of these cells into the SMC lineage.

## Discussion

We established a protocol for the isolation and differentiation of vascular progenitor cells from hESCs. We show that a $CD34^+$ population (of 93% purity) contains progenitors that can give rise to both EL and SML cells. This procedure includes 3 different steps: (1) the differentiation of hESCs through EBs for 10 days; (2) the isolation of $CD34^+$ cells by immunomagnetic beads; and (3) the culture of these cells in gelatin-coated dishes in the presence of EGM-2 enriched with $VEGF_{165}$ or $PDGF_{BB}$ for EC or SMC differentiation, respectively.

One of the specific limitations of our study is that single cell isolation and parallel divergence of its progeny was not performed. This issue should be addressed in future studies to show that the EB-derived $CD34^+$ cell that becomes a SML with PDGF exposure is the same cell that becomes an EL with VEGF exposure.

CD34 marker was selected to isolate vascular progenitor cells for several reasons. First, previous studies showed that $CD34^+$ cells from human blood cells could give rise to ECs and SMCs.[18,19,27,28] Second, human EBs express this marker at higher levels than other endothelial markers including KDR/flk-1 and PECAM1. Third, CD34 is upregulated during differentiation of human EBs, in contrast to KDR/Flk-1, and all the cells that stained positively for PECAM1 on day 10 coexpress CD34. Fourth, $CD34^+$ cells form vessel-like structures within EBs.

The composition of differentiation medium exerts a significant effect on the differentiation of EBs and yield of $CD34^+$ cells. EBs grown in differentiation medium containing KO-SR yield fewer $CD34^+$ cells than EBs grown in differentiation medium containing FBS. This suggests that factors present in FBS but not in KO-SR may play an important role in the vascular differentiation of hESCs. Furthermore, our data indicate that EBs grown in FBS media differentiate more rapidly than EBs grown in KO-SR media. This agrees with previous studies showing that KO-SR contribute for an increase growth rate of undifferentiated cells.[29]

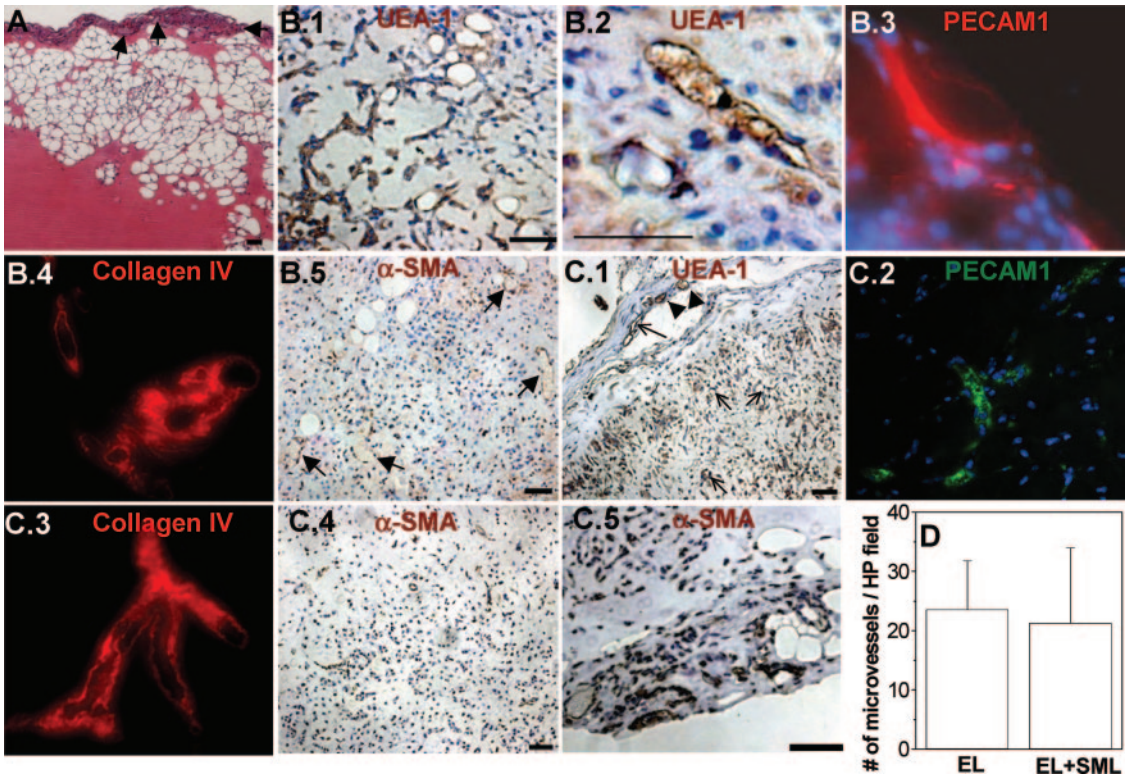


**Figure 6.** Transplantation of EL or SML cells in Nude mice. Matrigel alone (A), Matrigel containing EL cells (B), or a mixture of EL and SML cells (3:1) (C) was injected subcutaneously in the dorsal region of the nude mice (n=3, for each condition). After 28 days, the implants were removed, fixed, and processed for histological evaluation. A, Hematoxylin and eosin staining of Matrigel construct without cells showing mouse microvessels at the periphery of the implant (arrows) but not within Matrigel. B, The implants with EL cells show microvessels that are reactive for human UEA-1 (B.1 and B.2), anti-human PECAM1 (B.3; magnification, ×64), and anti-human collagen type IV (B.4; magnification, ×64); in some cases, they have mouse blood cells in their lumen (B.2). These microvessels are not reactive for anti-human α-SMA (B.5). B, The constructs with a mixture of EL and SML cells show microvessels that are reactive for human UEA-1 (C.1), anti-human PECAM1 (C.2; magnification, ×25), and anti-human collagen type IV (C.3; magnification ×64). The microvessels presented either an empty lumen (C.1, open arrowhead) or a lumen with mouse blood cells (C.1, closed arrowhead). $\alpha$-SMA$^+$ cells were observed inside of the Matrigel, and, in some cases, they formed small tubules (C.4). In the periphery of the Matrigel (C.5), $\alpha$-SMA$^+$ cells surrounded human ECs and formed microvessels carrying mouse blood. Scale bar=50 $\mu$m. D, Counts of human type IV collagen immunoreactive annular structures per 5 random high-power fields.

Recently, it has been reported that CD34$^+$CD31$^+$KDR$^+$[12] or CD34$^+$[11] cells isolated from hESCs and differentiated in the absence[12] or presence[11] of $VEGF_{165}$, respectively, can give rise to ECs. Our data show that CD34$^+$ cells cultured in the presence of $VEGF_{165}$ differentiated into EL cells, as confirmed by their morphology, biochemical markers, and functional studies. We further demonstrate that the levels of VEGF have an important role in the differentiation of CD34$^+$ cells into ECs. This effect has not been previously described. When CD34$^+$ cells are cultured in EGM-2 (low levels of VEGF), only ≈26% express PECAM1 marker after the first passage, and they start to lose this marker after several passages. This may indicate that other cell types take over the cell culture likely attributable to a high proliferation rate, or that the starting cells may differentiate into other cell types. It should be noted that only CD34$^+$ cells, not CD34$^-$, cells express significant levels of endothelial markers when exposed to VEGF-enriched medium, which shows that medium alone is not sufficient for the differentiation of hESCs into the vascular cell lineage. Our results also show that the differentiation of CD34$^+$ cells into the endothelial lineage is slightly different for H9 and H13 cell lines. It is unclear whether this is attributable to the presence of different populations of ECs, as shown in other ESCs,[24] or differences in the differentiation profile in both cell lines.

In this study, we showed the transplantation of EL cells into nude mice using Matrigel as scaffold contributed for the formation of human microvessels (Figure 6). In some cases, these microvessels contained mouse blood cells and supported blood flow, suggesting that these vessels anastomosed with the host vasculature. Our data agree with a study published during the reviewing process of this work, reporting that the transplantation of CD34$^+$ cells, isolated from hESCs, in mice contributed to the formation of blood vessels that integrated into the host circulatory system.[30]

We also demonstrated that CD34$^+$ cells can give rise to SML cells and that PDGF plays an important role in this differentiation process. It has been reported that $PDGF_{BB}$ promotes the differentiation of mouse ESCs and CD34$^+$ cells isolated from human blood into SMCs.[6,13,19] CD34$^+$ cells cultured in EGM-2 containing $PDGF_{BB}$ for 3 passages show minimal expression of EC markers but significant expression of SMC markers. The expression of SMC markers was also observed in cells grown in EGM-2 alone. However, the

expression of SM-MHC, a later marker in SMC differentiation that is not detected in other cell types,[31] was higher in PDGF conditions. In addition, upregulation of SM$\alpha$-22, caldesmon, and angiopoietin-1, known markers for maturing SMCs,[17,21] was achieved only in differentiating CD34$^+$ cells in PDGF-enriched medium. Furthermore, these cells seem functionally different from those differentiated in EGM-2 or VEGF-supplemented EGM-2 because they rarely form cord-like structures on Matrigel. Our data also suggest that the differentiation of SML is not complete because these cells express a low percentage of PECAM1 ($\approx$5%) and CD34 ($\approx$1%) markers and genotypically express Tie2 and angiopoietin-2 markers known to be displayed by ECs. SML cells have the ability to contract or relax in response to a variety of pharmacological agents like SMCs[18,23] and thus are functional. When SML cells were transplanted into nude mice, using Matrigel as scaffold, $\alpha$-SMA$^+$ cells were observed, forming either small tubules or surrounding microvessels.

Future studies should include further analysis of the molecular mechanism underlying vascular lineage differentiation and the influence of other growth factors in this process. It would be also important to test new scaffolds to improve the in vivo engraftment of these cells with the host vasculature of ischemic animal models.

## Acknowledgments

We thank Shulamit Levenberg and Jeffrey Karp for valuable suggestions.

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## Disclosures

None.

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## Supporting Information for:

# Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle-like cells and form vascular networks *in vivo*

**Lino S. Ferreira[*,†], Sharon Gerecht[‡], Hester F. Shieh[*], Watson, N.[§], Rupnick, M.A.[¶,‖], Dallabrida, S.M.[¶], Gordana Vunjak-Novakovic[‡,**], Robert Langer[*,‡,††]**

[*]*Department of Chemical Engineering, Massachusetts Institute of Technology, Massachusetts 02139,* [†]*Center of Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra and Biocant- Biotechnology Innovation Center, 3060-197 Cantanhede, Portugal* [‡]*Harvard-M.I.T. Division of Health Sciences and Technology Cambridge, Massachusetts 02139.* [§]*Whitehead Institute of Biomedical Research, Cambridge, MA 02142 USA.* [¶]*Division of Vascular Biology, Children's Hospital, Boston, MA 02115, USA.* [‖]Brigham and Women's Hospital, Division of Cardiovascular Medicine, Boston, MA. [**]*Columbia University, Department of Biomedical Engineering, New York NY 10027*

## Expanded Materials and Methods

**Cell culture.** To induce the formation of human EBs, the undifferentiated hESCs were treated with 2 mg/mL type IV collagenase (Invitrogen) for 2 h and then transferred (2:1) to low attachment plates (Ø=10cm, Ref: 3262, Corning) containing 10 mL of differentiation medium [80% Knockout- Dulbecco´s Modified Eagle Medium (Invitrogen), 20% Knockout-serum (KO-SR, Invitrogen) or fetal bovine serum (FBS, Hyclone), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol and 1% nonessential amino acid stock (all from Invitrogen)]. EBs were cultured for 12 days at 37 ºC, and 5% $CO_2$ in a humidified atmosphere, with media changes performed every 3-4 days. To serve as controls, human vascular smooth muscle cells (hVSMCs) and human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex and cultured in EGM-2 or SmGM-2 media (Cambrex). Medium was changed every other day.

**Transmission electron microscopy.** Cells seeded in Matrigel-coated 24-well plate were fixed for 1 h in 2.5% (w/v) glutaraldehyde, 3% (w/v) paraformaldehyde, and 5.0% (w/v) sucrose in 0.1 M sodium cacodylate buffer (pH 7.4) and then post-fixed in 1% (w/v) $OsO_4$ in veronal-acetate buffer for 1 h. The cells were stained en bloc overnight with 0.5% uranyl acetate in veronal-acetate buffer (pH 6.0), dehydrated, and embedded in Spurrs resin. Sections were cut on a Reichert Ultracut E at a thickness of 70 nm with a diamond knife. Sections were examined with a Philips EM410 electron microscope.

**Immunostaining.** For staining, EBs were transferred to gelatin-coated cover slips with differentiation medium containing 20% (v/v) fetal bovine serum (FBS), allowed to attach overnight, and then, fixed with 4% (w/v) paraformaldehyde for 30 minutes at room

temperature. For the evaluation of SMC or EC phenotypes in differentiated $CD34^+$ cells a similar fixation procedure was adopted. After blocking with 3% BSA solution, the cells were stained for 1 h with the following anti-human primary antibodies: PECAM1 (JC70A), CD34 (QBEnd 10), vWF (F8/86), α-SMA (1A4), SM-MHC (SMMS-1), calponin (CALP) (all from Dako) or VE-cad (F-8; Santa Cruz Biochemicals). In each immunofluorescence experiment, an isotype-matched IgG control was used. Binding of primary antibodies to specific cells was detected with anti-mouse IgG Cy3 conjugate (Sigma). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) or Topro-3 (Sigma). Immunostaining was examined with either a fluorescence microscope (Nikon) or Zeiss LSM 510 confocal microscope.

For uptake of Dill-labelled acetylated low-density lipoprotein (ac-LDL), differentiated $CD34^+$ cells were incubated with 10 mg/mL Dill-labelled ac-LDL (Biomedical Technologies) for 4 h at 37 ºC. After incubation, cells were washed three times with PBS, fixed with 4 % (w/v) paraformaldehyde for 30 min and visualized with a fluorescent microscope.

**Matrigel assay.** For Matrigel differentiation assay, a 24-well plate was coated with 0.4 mL of Matrigel per well and incubated for 30 minutes at 37 ºC. $CD34^+$ cells differentiated in EGM-2 medium, or EGM-2 medium supplemented with $VEGF_{165}$ or $PDGF_{BB,}$ for 3 passages, were seeded on top of the Matrigel at a concentration of $2.5 \times 10^4$ - $1 \times 10^5$ per 300 μL of culture medium. After 1 h of incubation at 37 ºC, 1 mL of medium was added. Cord formation was evaluated by contrast-phase microscopy 24 or 48 h after seeding the cells.

**Fluorescence-activated cell sorting (FACS) analysis.** Single cells were aliquoted (1.25-2.5 $\times 10^5$ cells were used per condition) and stained with either isotype controls or antigen-specific antibodies: SSEA-4-PE (MC813-70, R&D Systems), PECAM1-FITC (30884X, BD Pharmingen), CD34-PE/CD34-FITC (AC136, Miltenyi Biotec), KDR/Flk1-PE (89106,

R&D) and CD45-FITC (HI30, BD Pharmingen). Cells were analyzed without fixation on a FACScan (Becton Dickinson) using propidium iodide to exclude dead cells. For α–SMA, SM-MHC (all from Dako) and alkaline phosphatase-APC (R&D systems) markers, an intrastain kit (Dako) was used for the fixation and permeabilization of cell suspensions. In case of α–SMA and SM-MHC, the monoclonal antibodies were conjugated with a FITC-secondary antibody (Dako). Data analysis was carried out using CellQuest software.

**Western Blot Analysis.** Blots were blocked (30 min), incubated in primary antibody in block (1 h, Pierce), rinsed three times in 10 mM Tris-base /150 mM NaCl / 0.1% Tween20 (TBST), pH 7.6, incubated in appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG or anti-rabbit IgG, 1:1500, Cell Signaling) in block (1 h), and rinsed three times (TBST). Blots were developed using enhanced chemiluminescent kits (Amersham) and exposed to BioMax XAR film (Kodak). Blots were similarly reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2 μg/ml, Santa Cruz Biotechnology).

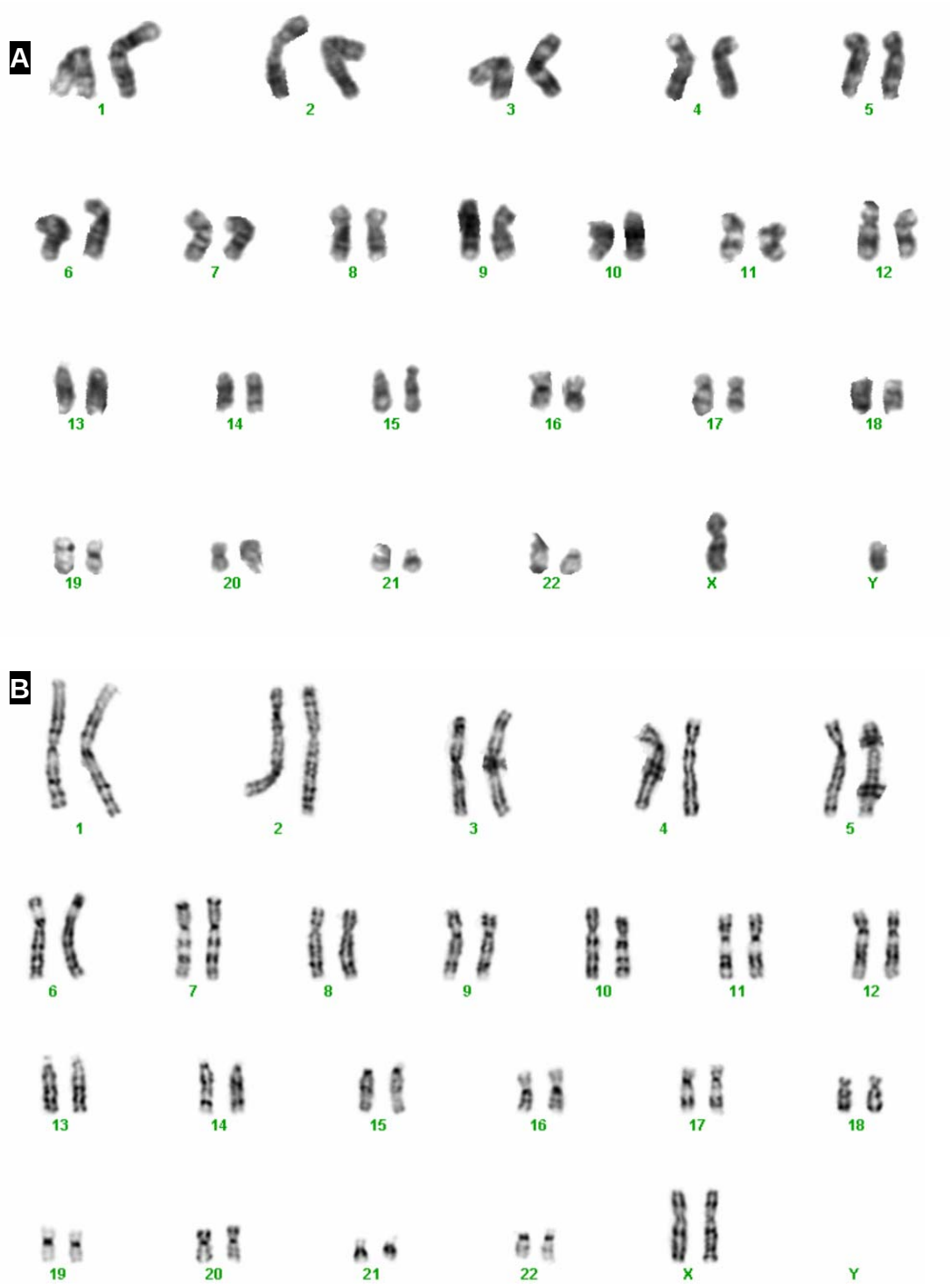


**Figure 1- Karyotyping analyses of H13 (A) and H9 (B) cell lines.** In H13 cells the karyotype obtained was 46, XY and is characteristic of a chromosomally normal male. In H9 cells the karyotype obtained was 46, XX and is characteristic of a chromosomally normal female. Cells were prepared and analysed as previously described (Cowan, C.A. *New England Journal of Medicine* 2004; 350:1353-1356). Approximately 20 metaphases spreads were counted and 5 metaphases analysed for each sample. Karyotyping analysis was performed by the Dana Faber /Harvard Cancer Research Center, Cytogenetics Laboratory, Cambridge, MA.

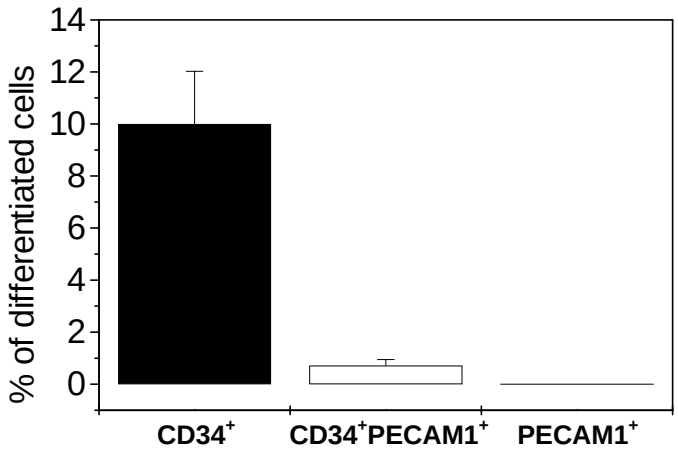


**Figure 2 – Expression of CD34 and PECAM1 in EBs grown in differentiation medium with FBS for 10 days, as assessed by FACS analysis.** Values indicate average ± SD, from 3 independent experiments.

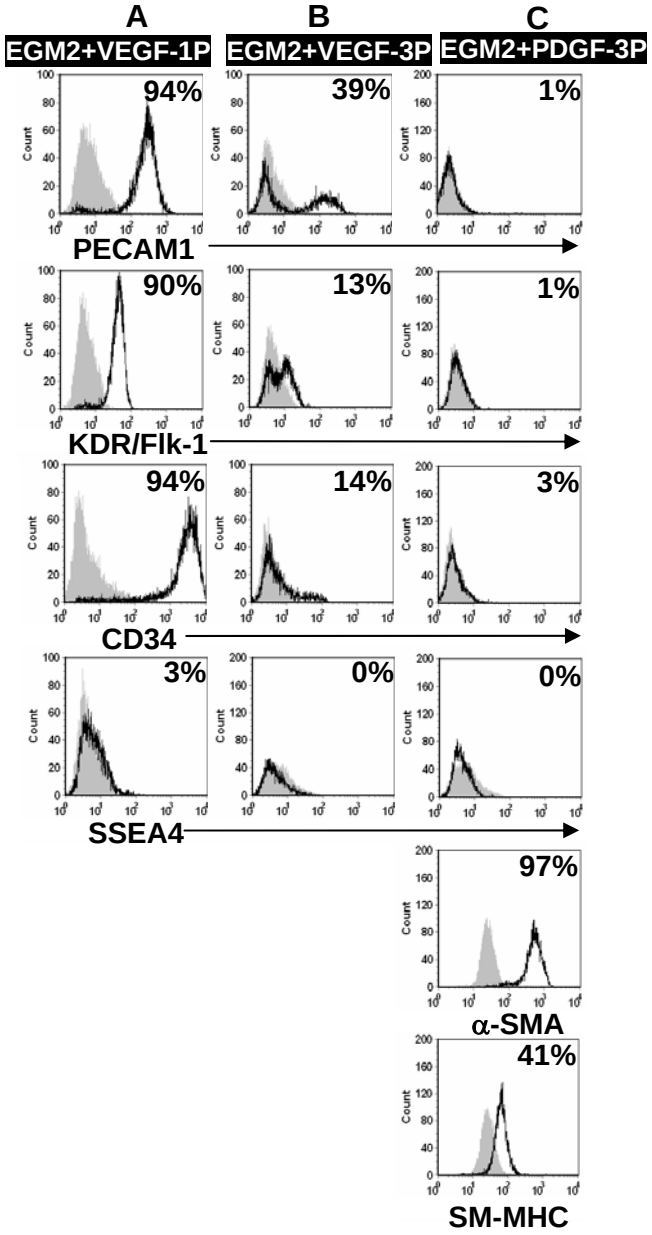


**Figure 3- FACS analysis of endothelial and smooth muscle cell markers in differentiated CD34$^+$ cells isolated from H13 cell line.** The cells were isolated from EBs grown in differentiation medium containing FBS for 10 days and then cultured in EGM-2 medium supplemented with 50 ngmL$^{-1}$ of $VEGF_{165}$ (A, B) or $PDGF_{BB}$ (C), for 1 (A) or 3 passages (B,C). Percent of positive cells were calculated based in the isotype controls (grey plots) and are shown in each histogram plot.

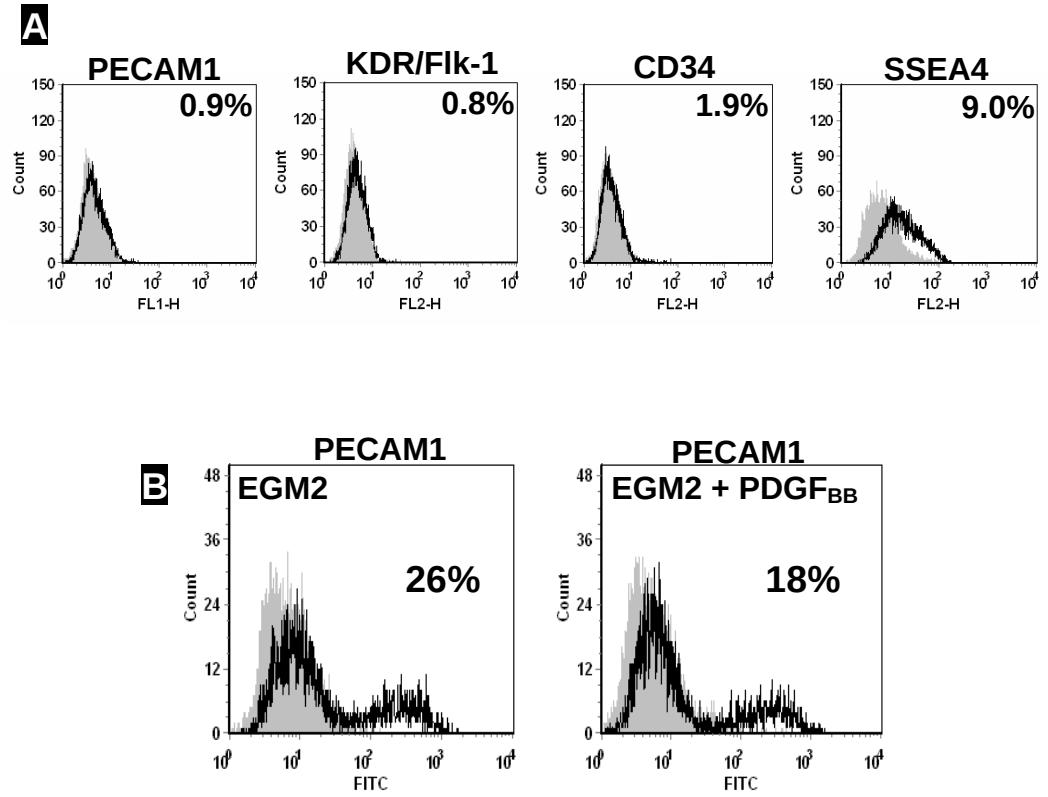


**Figure 4- Expression of endothelial markers in differentiated CD34$^-$ and CD34$^+$ cells.** A) FACS analysis of CD34$^-$ cells isolated from EBs grown in differentiation medium with FBS for 10 days and further differentiated in EGM-2 medium supplemented with 50 ngmL$^{-1}$ VEGF$_{165}$ (A), for 1 passage (10-15 days after cell seeding). In all graphs, the percents of positive cells were calculated based in the isotype controls (grey plot) and are shown in each histogram plot. B) FACS analysis of CD34$^+$ cells isolated from EBs grown in differentiation medium with FBS for 10 days and further differentiated in EGM-2 medium (A) or EGM-2 medium supplemented with 50 ngmL$^{-1}$ PDGF$_{BB}$ (B), for 1 passage (10-15 days after cell seeding).

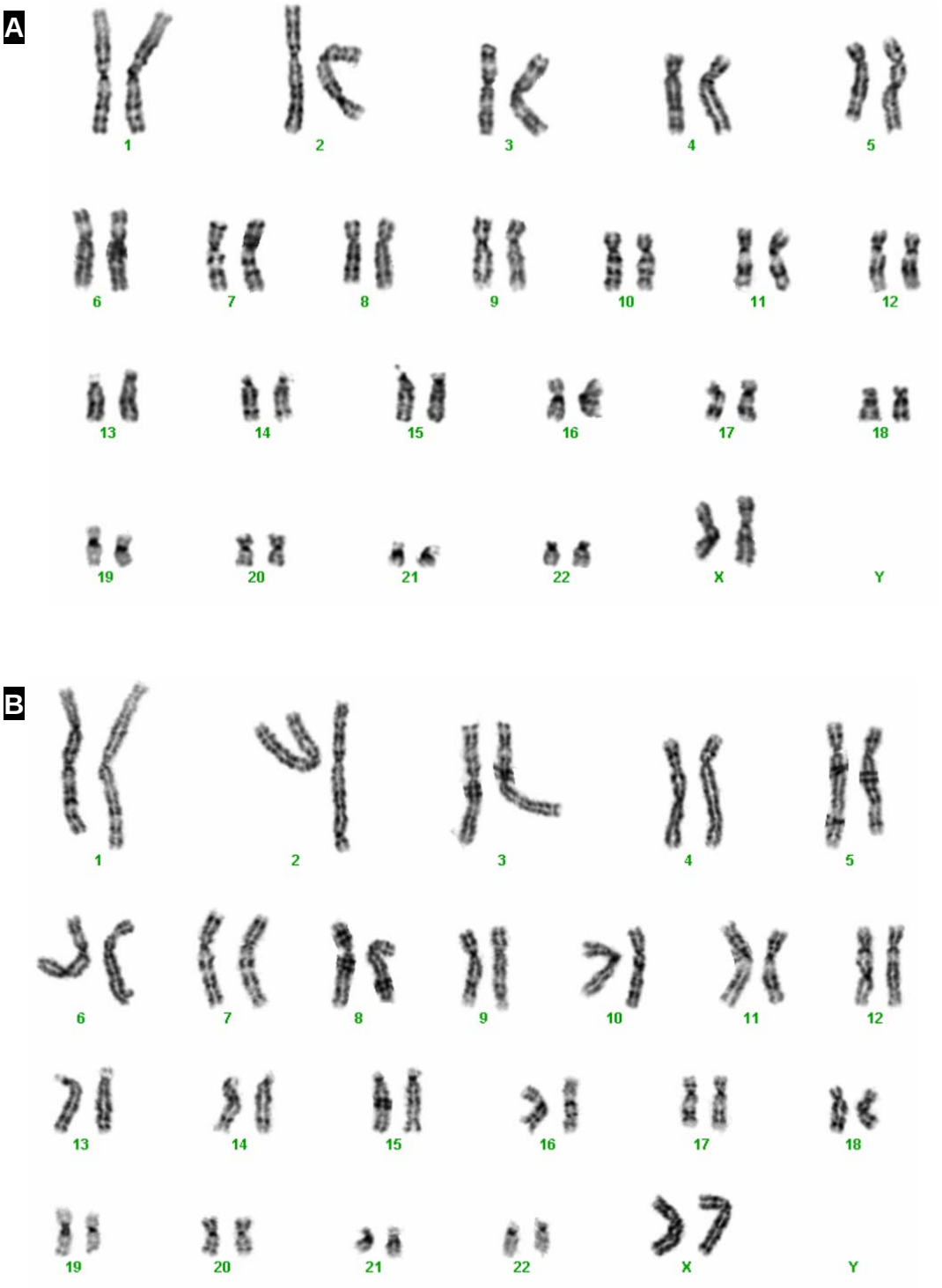


**Figure 5- Karyotyping analyses of CD34$^+$ cells differentiated in VEGF (A) or PDGF (B) supplemented media for three passages.** In both differentiated cells the karyotype obtained was 46, XX, and no clonal aberrations were observed in 20 cells examined.

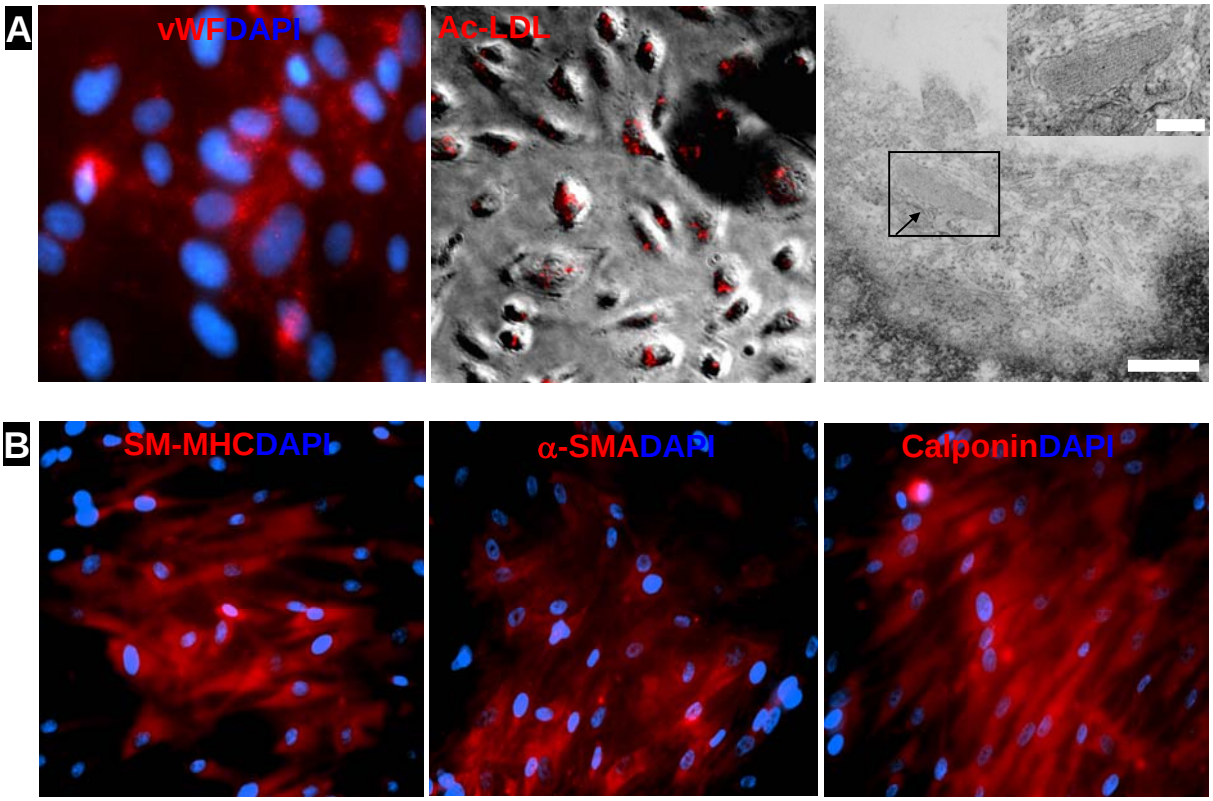


**Figure 6- Characterization of HUVECs and human vascular smooth muscle cells (hVSMCs).** A) HUVEC cells show vWF (× 125), have the ability to uptake ac-LDL (× 125) and present Weibel-Palade bodies (arrow) in the cytoplasm as shown by electron microscopy. Bar corresponds to 0.47 μm and in the inset 0.26 μm. B) hVSMCs express SM-MHC (× 40), α-SMA (× 40) and calponin (× 40).

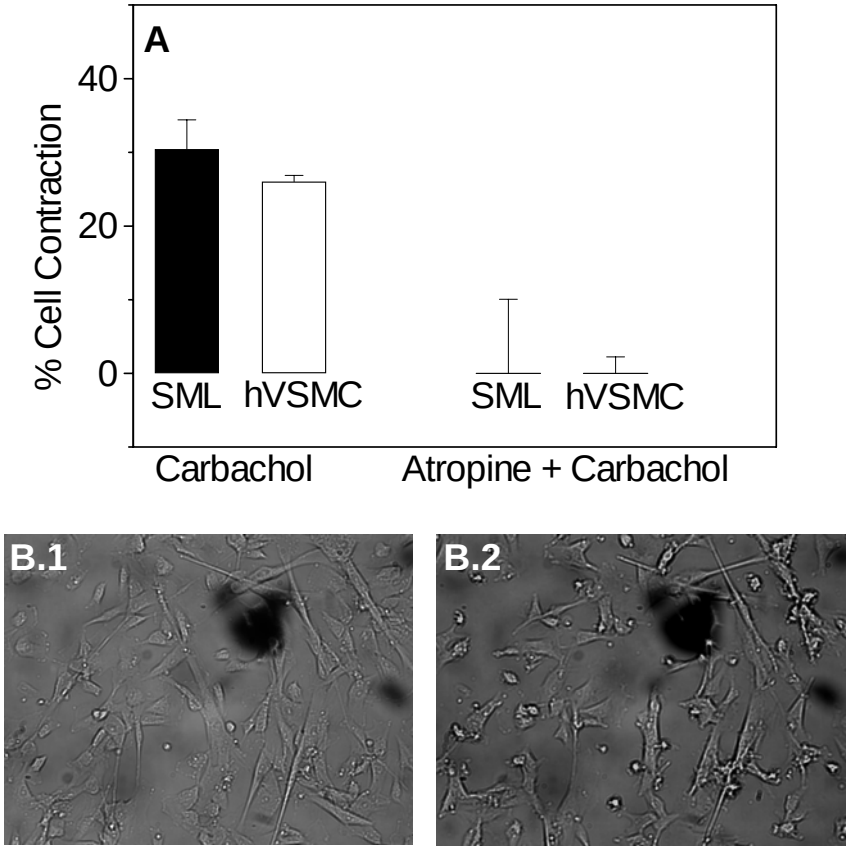


**Figure 7- SML cells have the ability to contract to carbachol as hVSMCs. A,B)** SML cells cultured for 3 passages were washed and contraction was induced by incubating these cells with $10^{-5}$ M Carbachol in DMEM medium for 30 min. Contraction was calculated by the difference of cell area at time zero and time 30 minutes. Bright-field images (×10 or ×20) were used for this purpose. In a separate experiment, the cells were induced to relax by incubation with $10^{-4}$ M atropine in DMEM for 1 h and then induce to contract with $10^{-5}$ M Carbachol. Contraction was calculated as before. hVSMCs (3rd passage) were used as controls. In B, morphological changes when SML were stimulated by carbachol (B.1 and B.2: before and after treatment, respectively).

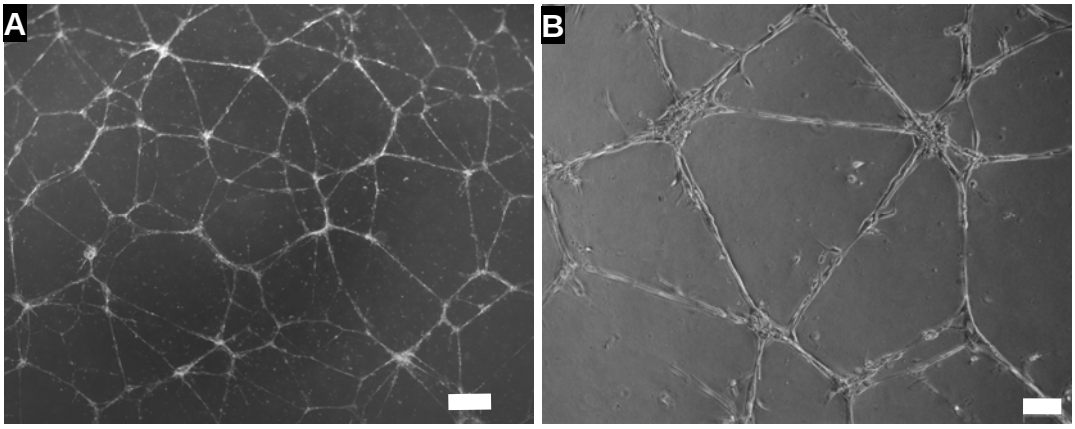


**Figure 8- Cord-like structures formed by differentiated $CD34^+$ cells (isolated from H13 cell line) on matrigel.** $CD34^+$ cells differentiated on EGM-2 medium supplemented with 50 $ngmL^{-1}$ $VEGF_{165}$ form continuous and complex cords after their seeding on matrigel for 24 h. Bar corresponds to 400 and 100 μm in A and B, respectively.

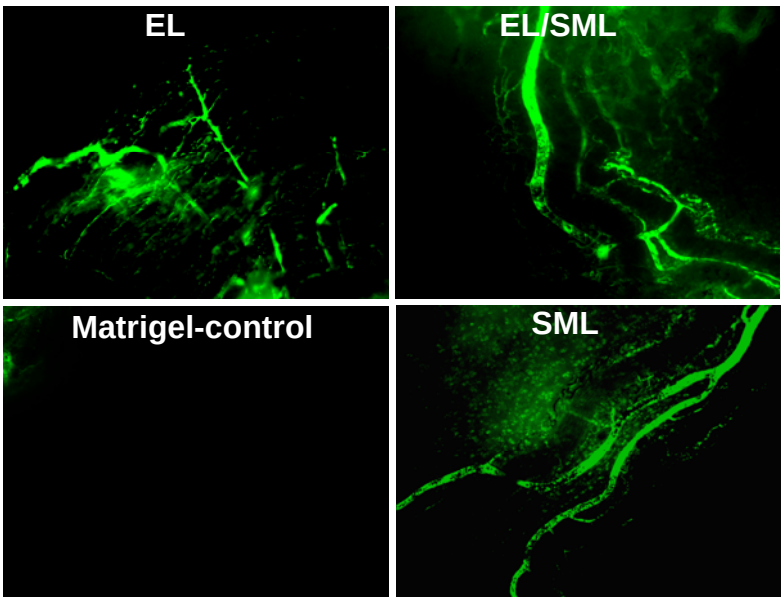


**Figure 9- Formation of vessels in matrigel implants that support blood flow.** EL and SML cells alone or EL cells mixed with SML cells were suspended in Matrigel and injected subcutaneously in the dorsal region of a balb/c nude mice. After 28 days, the mice were injected intravenously, through the tail vein, with 0.2 mL of PBS containing 50 mg/mL of FITC-dextran ($M_w$ 145 kDa). Animals were sacrificed 10 min following injection and the Matrigel implant removed and imaged. Microvessels that support blood flow were observed in Matrigel implants containing EL (×10), SML (×10) or a mixture of EL and SML (×10) cells, but rarely in Matrigel without cells.

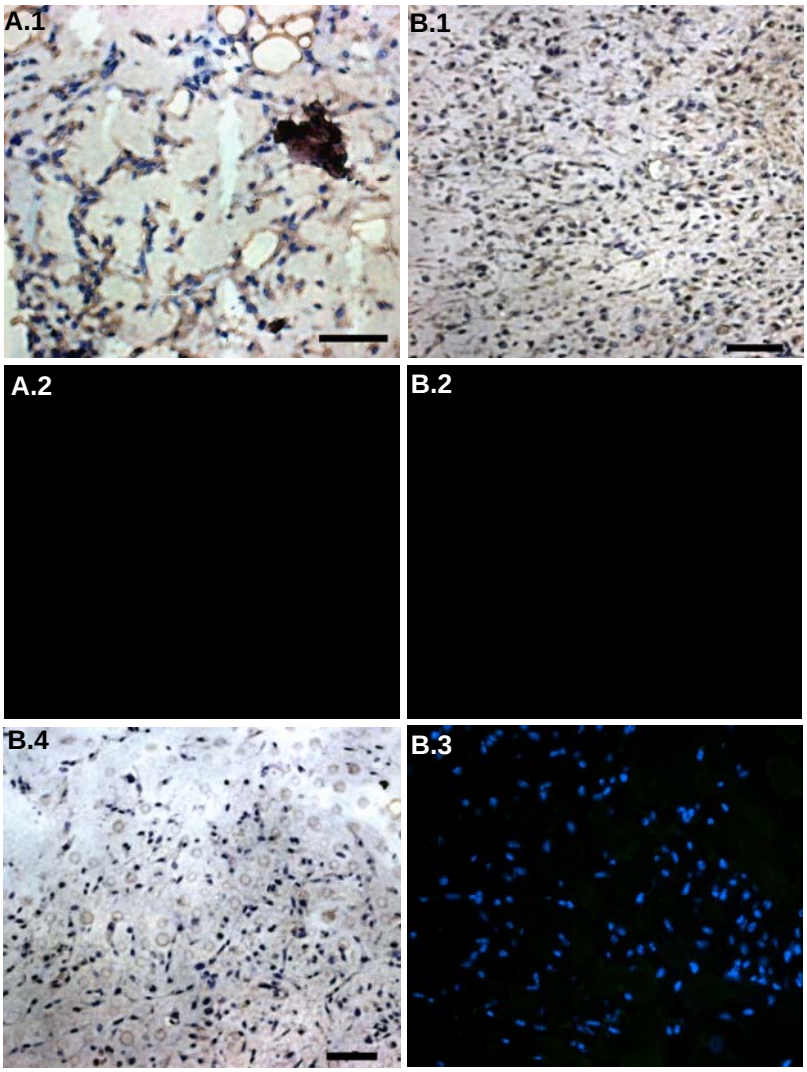


**Figure 10- Transplantation of EL and SML cells in balb/c nude mice**. Negative controls for samples of matrigel containing EL cells (A), or a mixture of EL and SML cells (3:1) (B). Negative controls for UEA-1 (A.1 and B.1), collagen type IV (A.2 and B.2, ×64), PECAM1 (B.3, ×25), and α-SMA (B.4, ×64). Bar represents 50 μm. In case of UEA-1, the negative control was prepared according to the manufacturer specifications, i.e., by inhibiting the UEA-1 with 100 mM L-(-)-fucose (Sigma) in 10 mM HEPES, pH 7.5 containing 0.15 M NaCl, for 30 min, at room temperature.

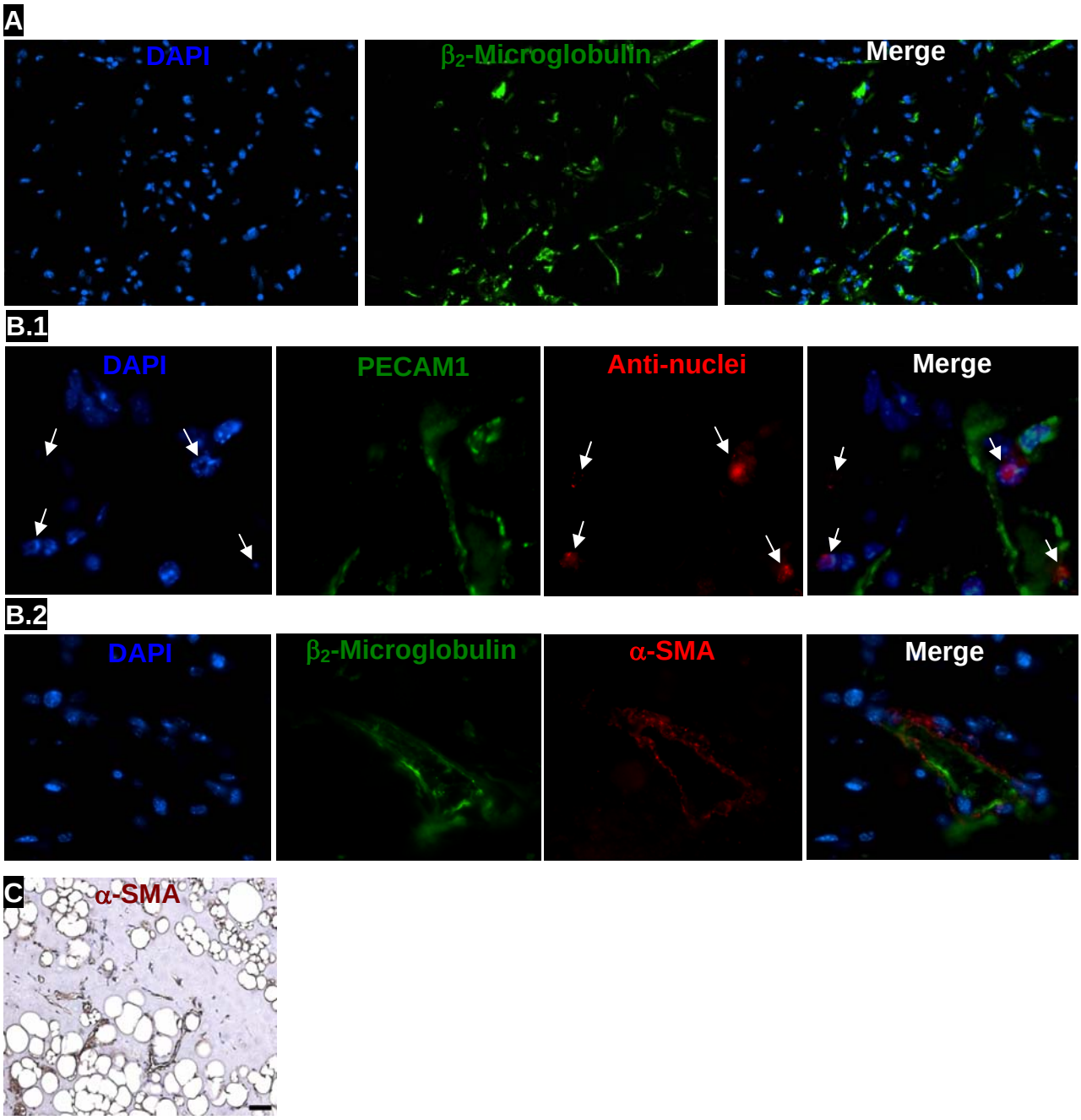


**Figure 11- Transplantation of EL and SML cells in balb/c nude mice.** A) The constructs with EL and SML cells contained regions that stained positively for human $\beta_2$-microglobulin, a specific human protein involved in the HLA class I antigen complex. B) Cells in these constructs stained positively for human PECAM1 and human anti-nuclei (B.1), and thus have properties of human endothelial cells while others stained positively for α-SMA and $\beta_2$-microglobulin (B.2) and thus have properties of human smooth muscle cells. C) The constructs with SML cells stained positively for α-SMA. Bar corresponds to 50 μm.

**Supplemental Table 1-** Primer sequences, reaction conditions[1] and optimal cycle numbers used for the RT-PCR analyses of vascular markers.

| Gene transcript | Primer sequences (5' to 3', $F_w/R_v$) | Product (bp) | *Cycles* | *Annealing temp. (°C)* | *[$MgCl_2$] (mM)* |
|---|---|---|---|---|---|
| **PECAM1**[2] | GCTGTTGGTGGAAGGAGTGC<br>GAAGTTGGCTGGAGGTGCTC | **620** | 28 | 55 | 1.5 |
| **CD34**[2] | TGAAGCCTAGCCTGTCACCT<br>CGCACAGCTGGAGGTCTTAT | **200** | 30 | 55 | 1.5 |
| **KDR/Flk-1** | CTGGCATGGTCTTCTGTGAAGCA<br>AATACCAGTGGATGTGATGGCGG | **790** | 35 | 60 | 1.5 |
| **Angiopoietin-1** | GGGGGAGGTTGGACTGTAAT<br>AGGGCACATTTGCACATACA | **362** | 35 | 60 | 1.5 |
| **Angiopoietin-2** | GGATCTGGGGAGAGAGGAAC<br>CTCTGCACCGAGTCATCGTA | **535** | 35 | 60 | 1.5 |
| **Tie2** | ATCCCATTTGCAAAGCTTCTGGCTGGC<br>TGTGAAGCGTCTCACAGGTCCAGGATG | **512** | 35 | 60 | 1.5 |
| **VE-cad** | ACGGGATGACCAAGTACAGC<br>ACACACTTTGGGCTGGTAGG | **596** | 35 | 60 | 1.5 |
| **Von Willebrand Factor (vWF)** | ATGTTGTGGGAGATGTTTGC<br>GCAGATAAGAGCTCAGCCTT | **656** | 40 | 55 | 1.0 |
| **SM-MHC** | GGACGACCTGGTTGTTGATT<br>GTAGCTGCTTGATGGCTTCC | **670** | 35 | 60 | 1.5 |
| **α-SMA** | CCAGCTATGTGAAGAAGAAGAGG<br>GTGATCTCCTTCTGCATTCGGT | **965** | 35 | 60 | 1.5 |
| **Caldesmon** | AACAACCTGAAAGCCAGGAGG<br>GCTGCTTGTTACGTTTCTGC | **530** | 35 | 60 | 1.5 |
| **SMα-22** | CGCGAAGTGCAGTCCAAAATCG<br>GGGCTGGTTCTTCTTCAATGGGG | **928** | 35 | 60 | 1.5 |
| **GAPDH** | AGCCACATCGCTCAGACACC<br>GTACTCAGCGCCAGCATCG | **302** | 27 | 60 | 1.5 |

[1]PCR conditions consisted of the following: 5 minutes at 94 ºC (hot start), 30 to 40 cycles (actual number noted in the table); 94 ºC for 30 seconds, annealing temperature (noted in the table) for 30 seconds; 72 ºC for 30 seconds. A final 7 minutes extension at 72 ºC was performed at the end.
[2]PCR conditions: 15 minutes at 95ºC, 1 minute at 94ºC, annealing temperature for 1 minute, 72ºC for 1 minute. A final 10 minutes extension at 72ºC was performed at the end.