Stem Cell Reports, Volume *2*

Supplemental Information

Defining Differences among Perivascular Cells Derived from Human Pluripotent Stem Cells

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Supplementary Figures and Figure Legends

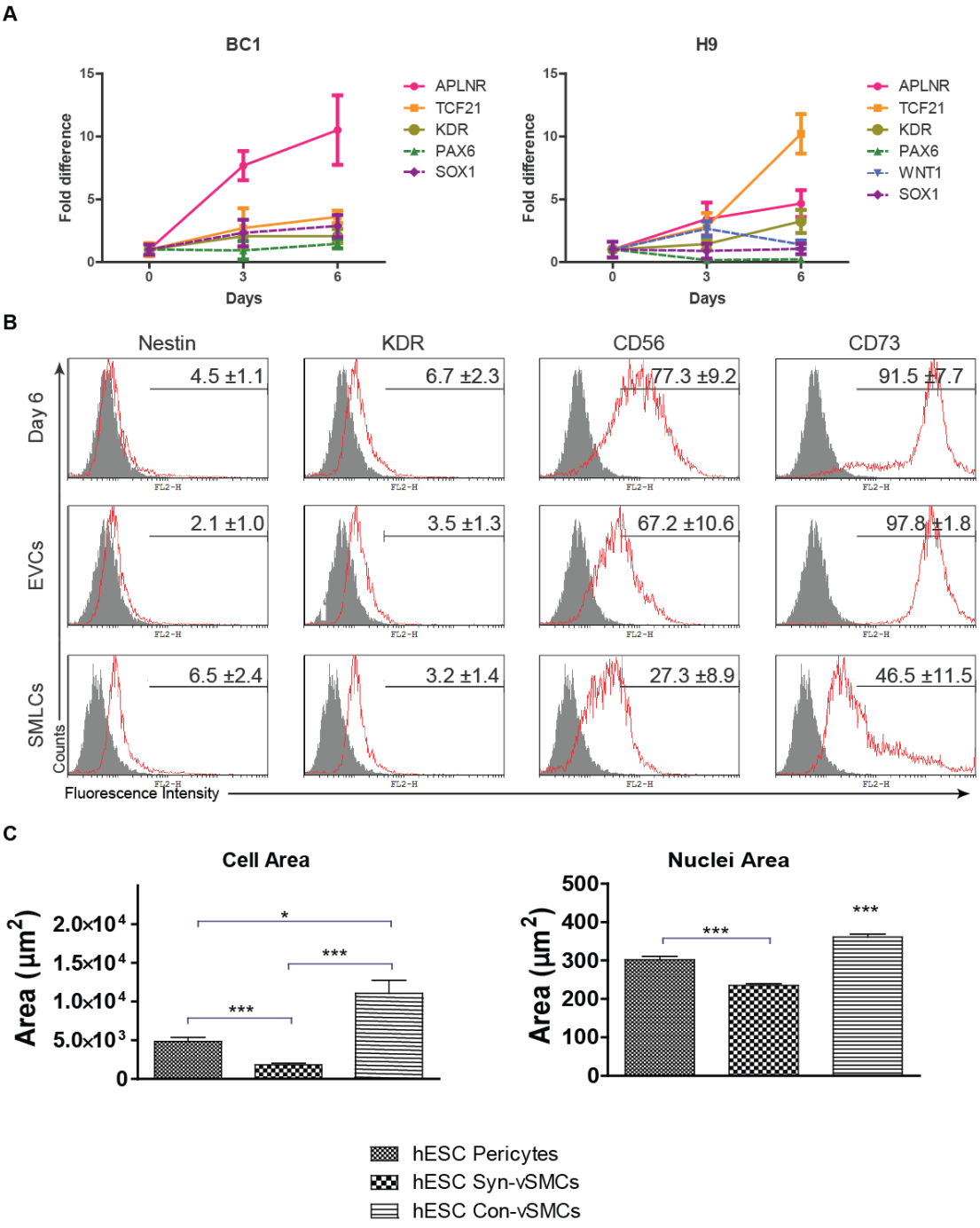

Figure S1. Marker assessment of perivascular derivatives related to Figure 1. (A) Quantitative real time RT-PCR analysis of mesoderm (in solid lines) and neural crest (in dashed lines) genes at days 0, 3 and 6 along differentiation for BC1 and H9 (n=3 biological replicates). **(B-C)** H9 differentiating 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.

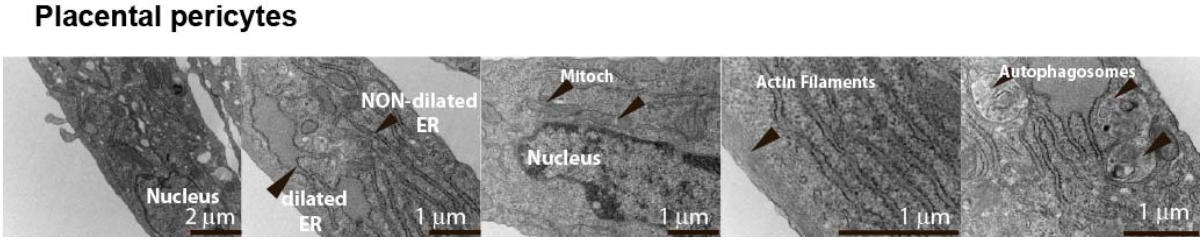

Figure S2. Control pericytes, related to Figure 2. Placenta pericytes were analyzed for sub-cellular organelle organization using TEM.

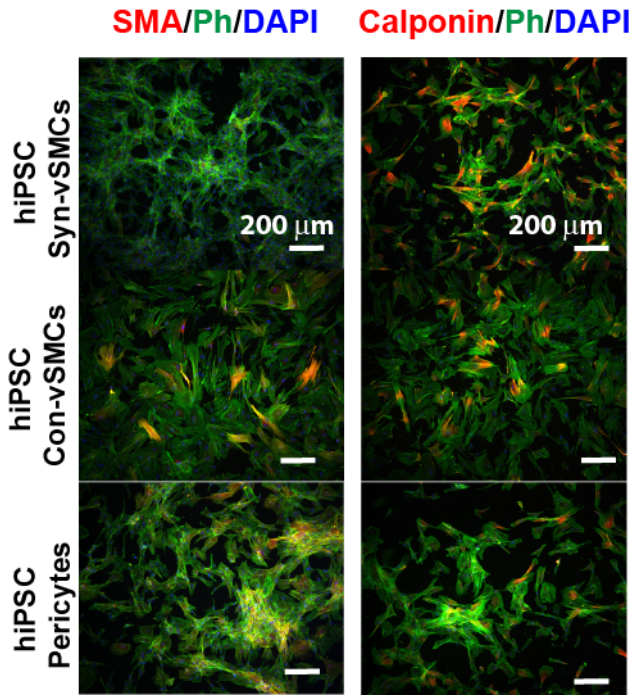

Figure S3. Differences in stress fiber and contractile marker expression, related to Figure 3. Perivascular derivatives were assessed by immunofluorescence for αSMA and calponin (in red; phalloidin in green; nuclei in blue). Figures shown are low magnification representative images of Fig. 3A.

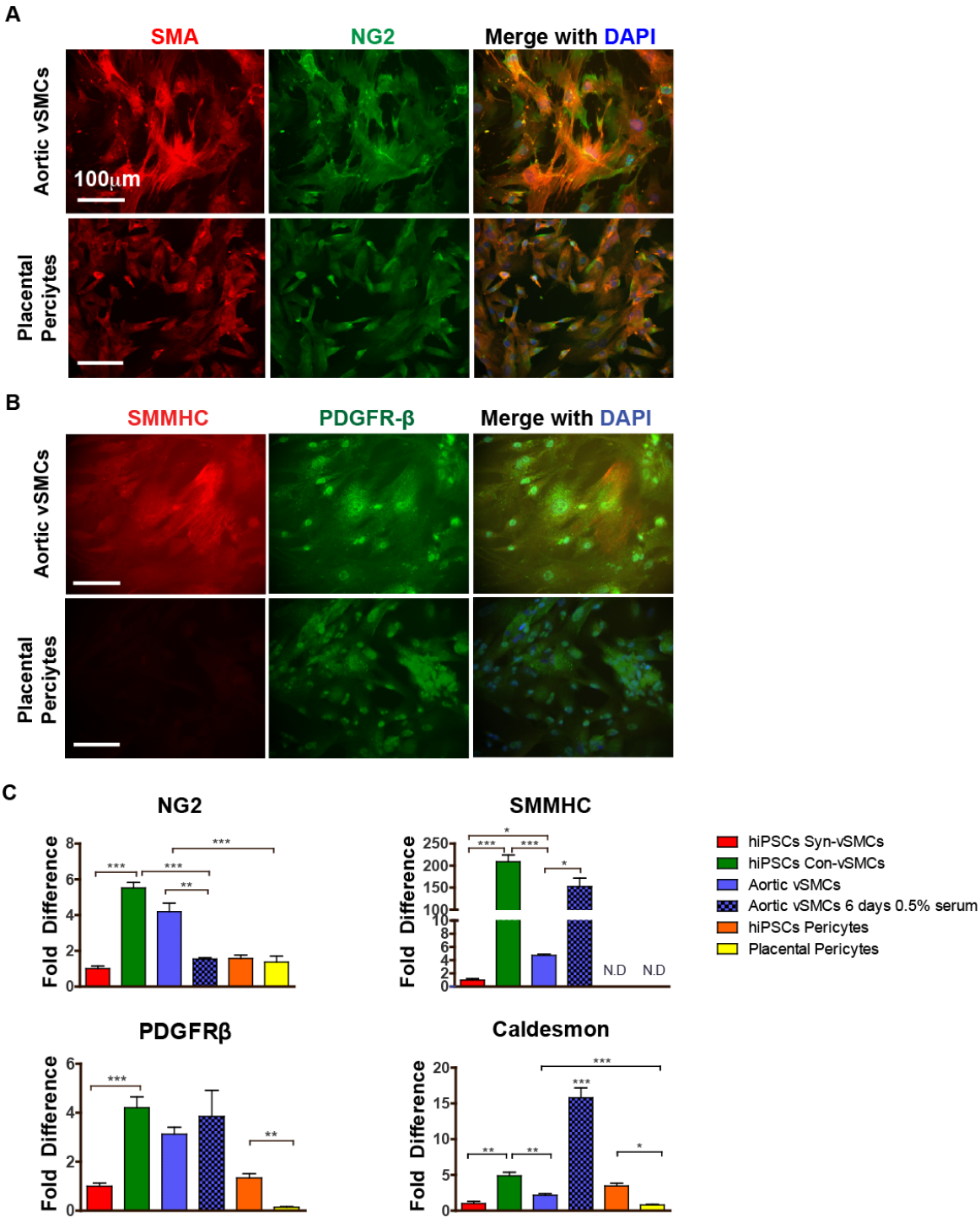

Figure S4. Perivascular marker expression in control cells related to Figure 3. Organization of **(A)** SMA (red) and NG2 (green; nuclei in blue) and **(B)** SMMHC (red) and PDGFRβ (green; nuclei in blue) in aortic vSMCs and placenta pericytes. **(C)** Expression of NG2, SMMHC, PDGFRβ and caldesmon in 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.

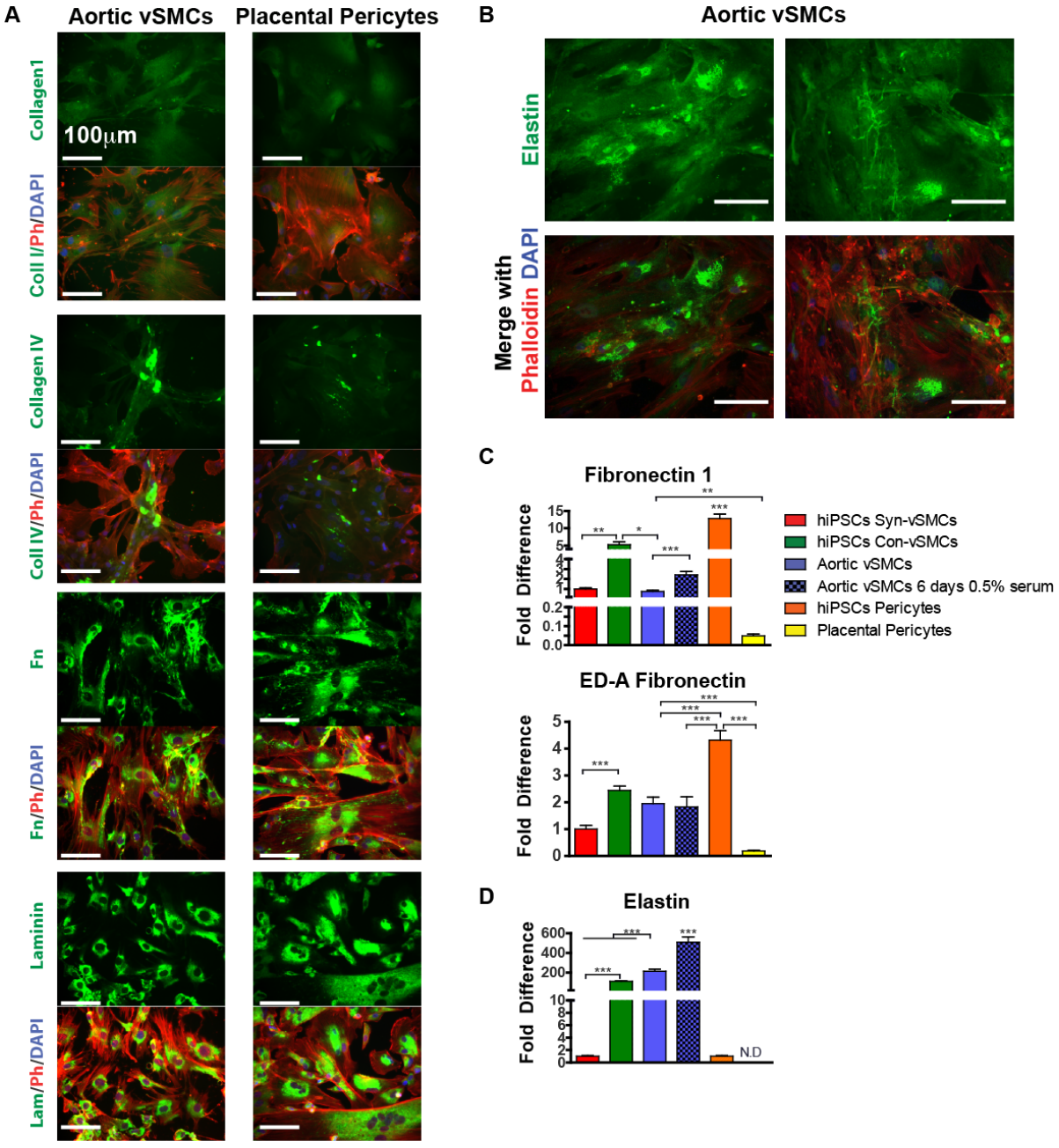

Figure S5. ECM in control cells related to Figure 4. (A) Deposition of the ECM proteins collagen I, collagen IV, fibronectin, and laminin (all in green; phalloidin in red; nuclei in blue.) in placenta pericytes and aortic vSMCs. **(B)** Elastin organization in aortic vSMCs (in green; phalloidin in red; nuclei in blue) showing interacellular (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 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.

Supplemental Experimental Procedures

Cell Culture

All cells were cultured in humidified incubators, with atmospheres at 37°C and 5% CO_2.

Human PSCs. hiPSC line BC1 (Cheng et al., 2012; Chou et al., 2011) 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 (Wanjare et al., 2013). 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 detatchment kit (Promocell).

vSMC differentiation protocol

vSMCs were derived as previously described (Wanjare et al., 2012). 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 (Kusuma et al., 2013; Orlidge and D'Amore, 1987). 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.

Flow cytometry

Flow cytometry was performed as previously described (Kusuma et al., 2012). 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.

Immunofluorescence

Cells were prepared for immunofluorescence as previously described (Kusuma et al., 2012; Wanjare et al., 2012). 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).

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.

Transmission electron microscopy (TEM)

Differentiated cells, placental pericytes, and aortic vSMCs were prepared for TEM analysis as described previously (Hanjaya-Putra et al., 2011). 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).

Stress fiber quantification

The number of stress fibers per cell was quantified using line intensity profiles of cells in ImageJ (Wei et al., 2011). 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.

Real-time quantitative RT-PCR

Two-step RT-PCR was performed on differentiated hPSCs at various time points as we previously described (Wanjare et al., 2012). 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, COL4A1, 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' (Forte et al., 2013; van der Straaten et al., 2004).

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.

Zymography

Zymography was performed to determine MMP activities as previously (Hanjaya-Putra et al., 2012). 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% commasie 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.

Mesenchymal differentiation (adipogenic and osteogenic)

We followed our previously published protocol for mesenchymal differentiations (Kusuma et al., 2013). For adipogenic differentiation (Pittenger et al., 1999), we cultured derived pericytes at 10,000cells/cm^2 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 H_2O, for 10 minutes and imaged using an inverted light microscope (Olympus).

For osteogenic differentiation (Grayson et al., 2010), we cultured derived pericytes at 5,000cells/cm^2 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 H_2O. Samples were incubated with Alizarin Red S (40mM in DI H_2O, pH ~4.2; Sigma) for 10-20 minutes.

Subcutaneous Matrigel implantation

hiPSC-derived perivascular cells were trypsinized, collected and stained with PKH26 (Sigma-Aldrich) membrane dye. We encapsulated a total of 0.5×10^6 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.

Wound healing assay

Migration of the derived hiPSC perivascular cells was assessed using a wound healing assay (Rodriguez et al., 2005). 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).

Invasion toward ECs

A downward invasion toward ECs assay was used to assess invasion of perivascular cells. Human umbilical vein endothelial cells (HUVECs) were seeded on 16 well detachable wells (Fisher). After 24 h, 150 ul of collagen gel was added on top of the HUVECs. Stock solution was used to prepare collagen gels at a density of 2.5 mg/ml. Gel formation was achieved by simultaneously decreasing the solution's pH and increasing the temperature to 37°C. To prepare 150 ul of collagen gels, we mixed 66.1 ul M199 1X with 6.44 ul M199 10x. To this, we added 57.8 ul collagen type I. After the addition of approximately 2 ul 1M NaOH, the solution was thoroughly mixed and added to the HUVEC monolayer. The gel was allowed to polymerize for 1h at 37°C in a CO_2 incubator. Upon polymerization, hiPSC perivascular cells were cultured on top of the gels to allow downward invasion. After 48h the gels were fixed using 3% gluteraldehyde for 30min, stained with 0.1% toluidine blue dye for 15 min, and washed with distilled water. Cross-sections of the gels were imaged using Accuscope. Quantification of invasion distance into the collagen gel was performed using ImageJ.

Functional contraction studies

Contraction studies in response to pharmacological drugs were done, as previously described (Vo et al., 2010; Wanjare et al., 2012). 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 (Vo et al., 2010) by eliminating the need for image compression and by increasing the consistency of cell selection within each set of images.

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.

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