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Supplemental Information

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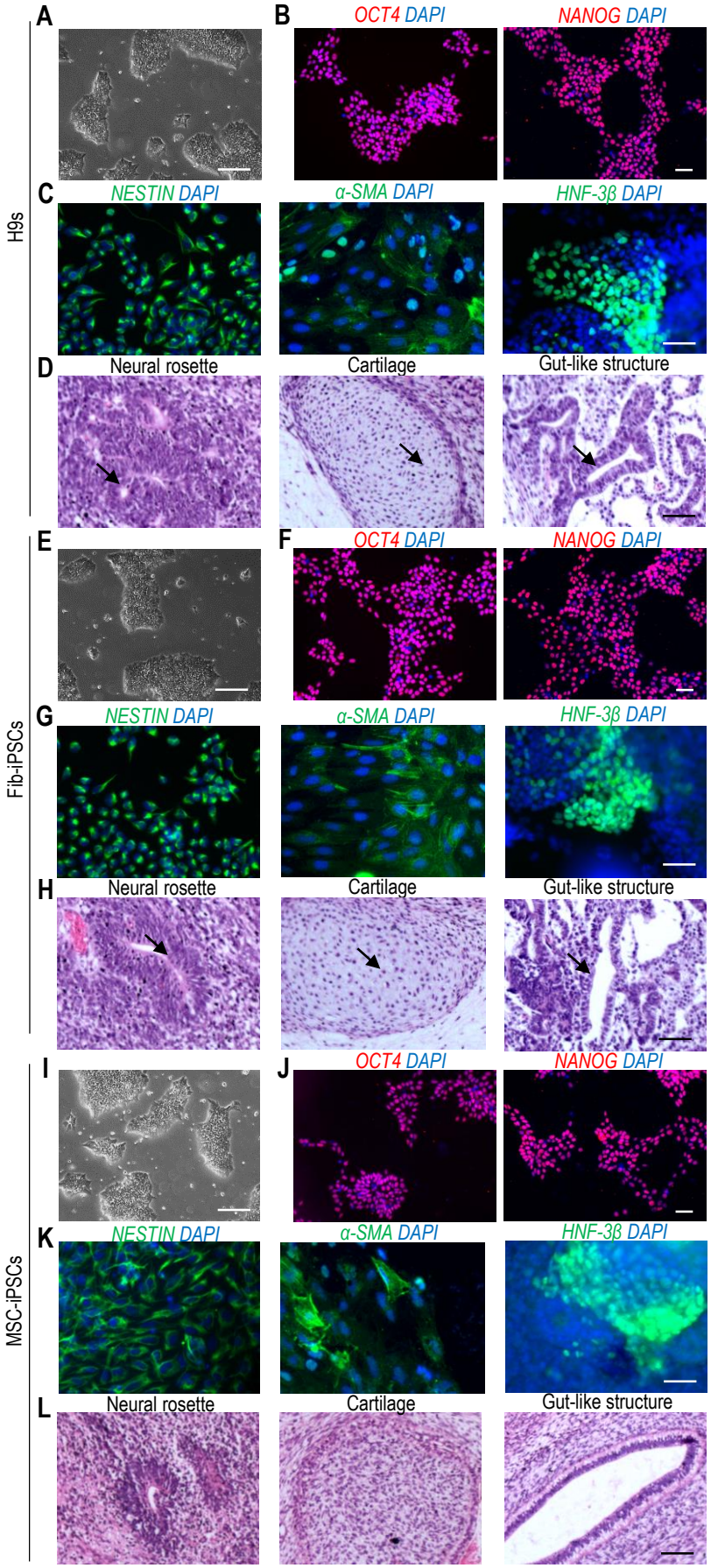


Figure S1. Starting hPSCs (H9s, Fib-iPSCs and MSC-iPSCs).
(**A, E, I**) Phase images. Scale bar, 200 μm.
(**B, F, J**) Majority of the cells expressed the pluripotency markers, *OCT4* and *NANOG*. Scale bar, 50 μm.
(**C, G, K**) They could be differentiated into all three germ layer cells such as *NESTIN*+ ectodermal, *α-SMA*+ mesodermal and *HNF-3β*+ endodermal cells in EB assay. Scale bar, 50 μm.
(**D, H, L**) They formed teratomas containing all three germ layer tissues (arrows) in SCID mice. Scale bar, 50 μm.

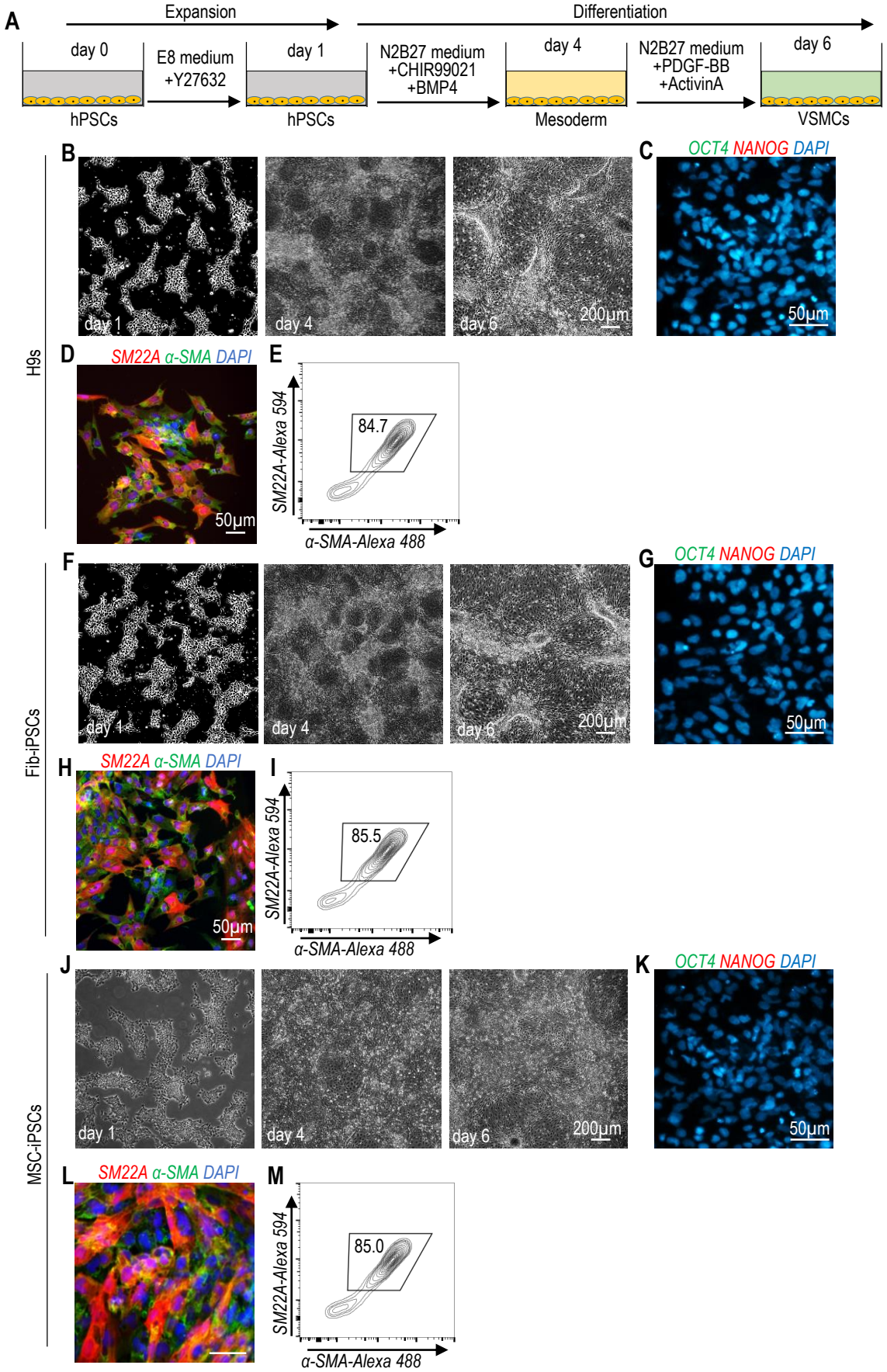


Figure S2. Differentiate hPSCs (H9s, Fib-iPSCs and MSC-iPSCs) into VSMCs in 2D cultures.
(**A**) Illustration of the VSMC differentiation protocol in 2D cultures.
(**B, F, J**) Phase images of day 1, 4 and 6 cells during VSMC differentiation. Scale bar, 200 μm.
(**C, G, K**) Immunostaining of day 6 cells for hPSCs markers, *OCT4* and *NANOG*. Scale bar, 50 μm.
(**D, H, L**) Immunostaining of day 6 cells for VSMC markers, *SM22A* and *α-SMA*. Scale bar, 50 μm.
(**E, I, M**) Flow cytometry analysis of day 6 hPSC-VSMCs for *SM22A* and *α-SMA*.

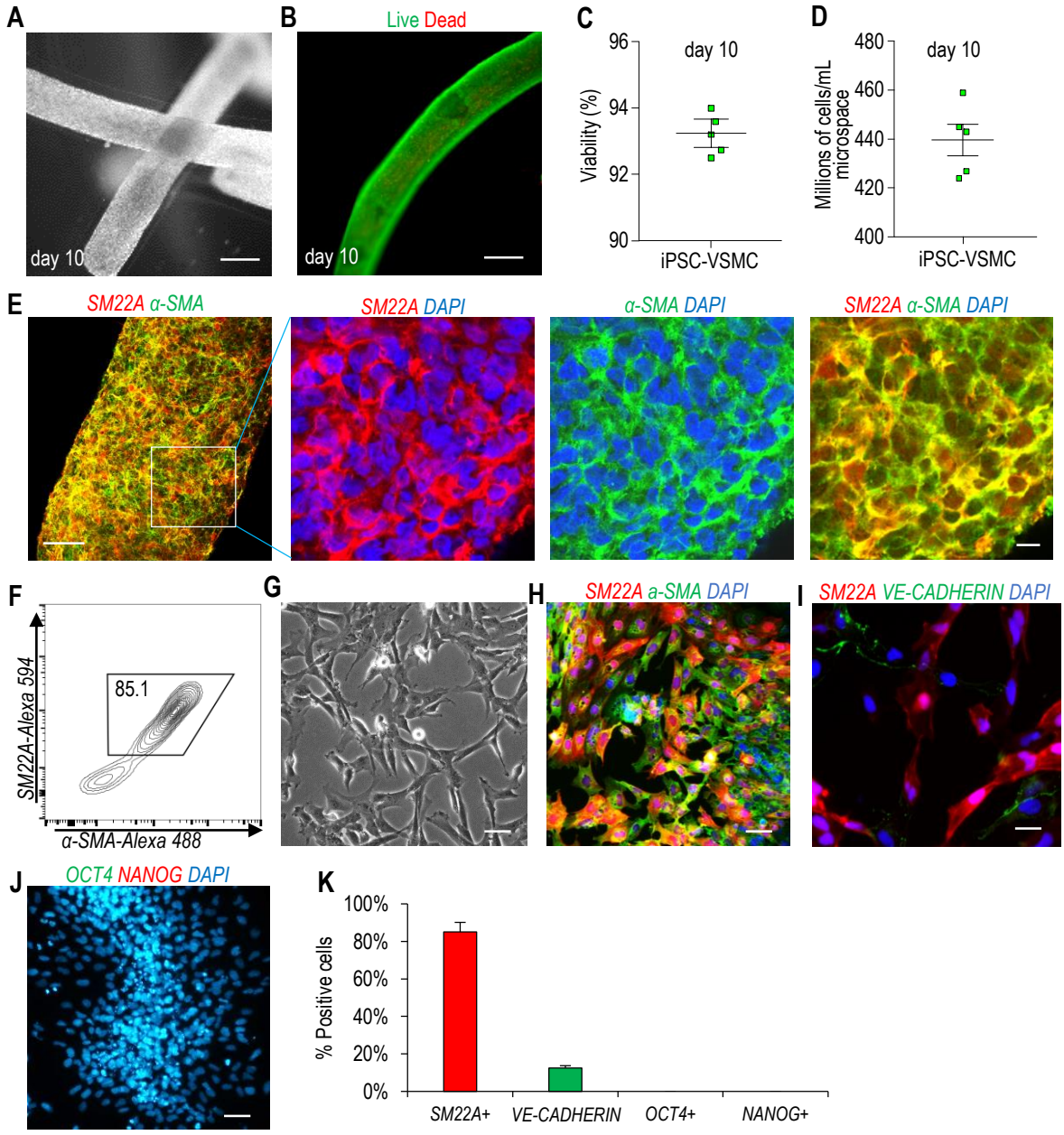


Figure S3. Differentiating Fib-iPSCs into VSMCs in alginate hydrogel tubes.
(**A**) Phase images of day 10 cells. Scale bar, 200 µm.
(**B, C**) Live/dead staining and cell viability analysis of day 10 cells. Data are represented as mean ± SD (n=5). Scale bar, 200 µm.
(**D**) Volumetric yield on day 10. Data are represented as mean ± SD (n=5).
(**E, F**) Immunostaining and flow cytometry analysis of VSMC markers *SM22A* and *α-SMA* on day 10 cells. Scale bar, 50 µm and 10 µm.
(**G-K**) The day 10 cell masses were dissociated into single cells and plated on 2D surface overnight. Phase image (**G**), immunostaining showed majority of the cells were *SM22A+/α-SMA+* (**H**), and ~10% *VE-CADHERIN+* endothelial cells (**I, K**), but no *OCT4+/NANOG+* undifferentiated Fib-iPSCs (**J, K**). Data are represented as mean ± SD (n=3). Scale bar, 50 µm.

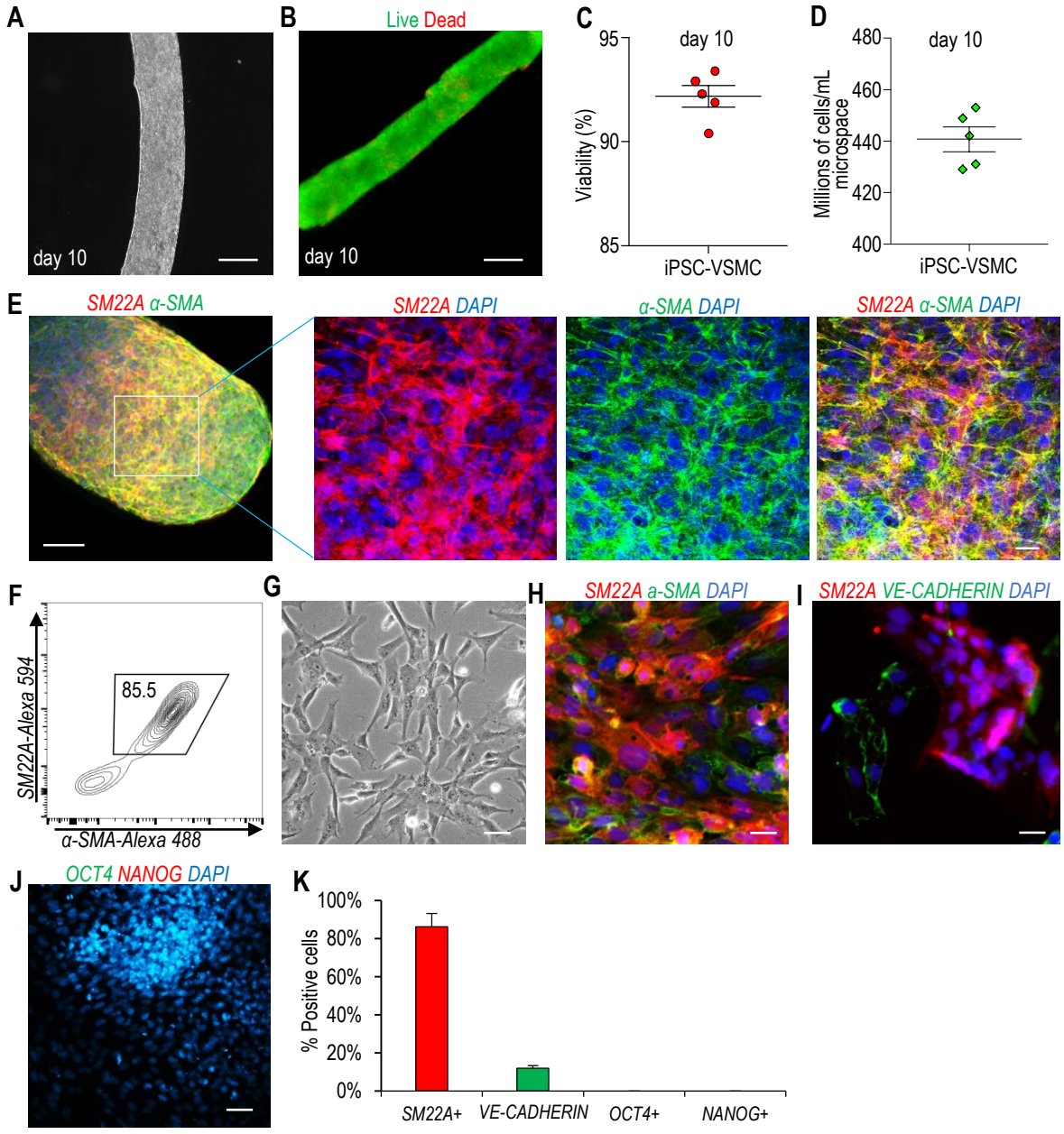


Figure S4. Differentiating MSC-iPSCs into VSMCs in alginate hydrogel tubes.
(**A**) Phase images of day 10 cells. Scale bar, 200 μm.
(**B, C**) Live/dead staining and cell viability analysis of day 10 cells. Data are represented as mean ± SD (n=5). Scale bar, 200 μm.
(**D**) Volumetric yield on day 10. Data are represented as mean ± SD (n=5).
(**E, F**) Immunostaining and flow cytometry analysis of VSMC markers *SM22A* and *α-SMA* on day 10 cells. Scale bar, 50 μm and 10 μm.
(**G-K**) The day 10 cell masses were dissociated into single cells and plated on 2D surface overnight. Phase image (**G**), immunostaining showed majority of the cells were *SM22A+/α-SMA+* (**H**), and ~10% *VE-CADHERIN+* endothelial cells (**I, K**), but no *OCT4+/NANOG+* undifferentiated MSC-iPSCs (**J, K**). Data are represented as mean ± SD (n=3). Scale bar, 50 μm and 20 μm.

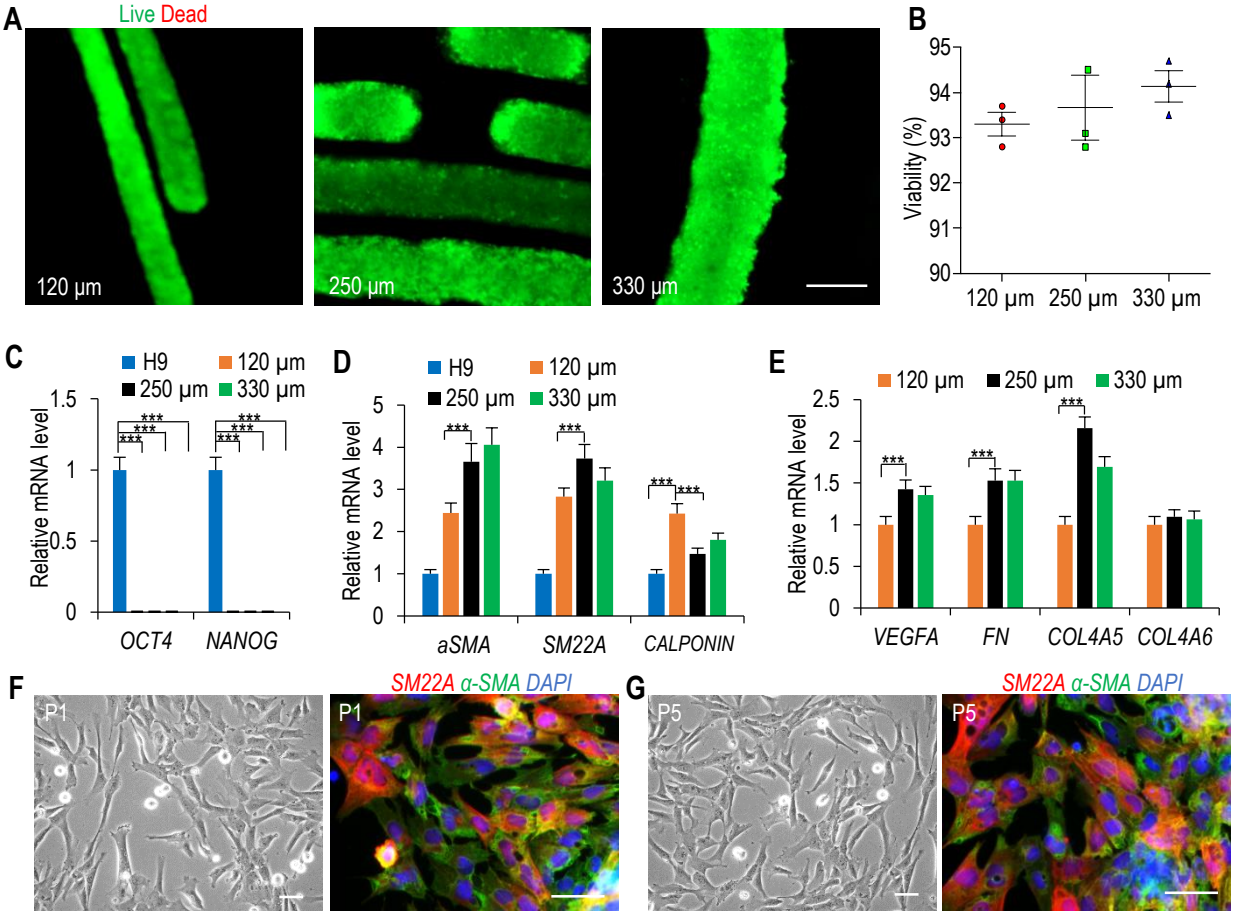


Figure S5. Effect of hydrogel tube diameter on VSMC differentiation.
(**A, B**) Live/dead staining and cell viability analysis of day 10 hPSC-VSMCs. Data are represented as mean ± SD (n=3). Scale bar, 200 μm.
(**C-E**) qRT-PCR analysis of gene expression of H9s and day 10 hPSC-VSMCs in 120 μm, 250 μm and 330 μm hydrogel tubes, including pluripotency markers *OCT4* and *NANOG*, VSMC markers *α-SMA, SM22A* and *CALPONIN*, growth factor *VEGFA*, and extracellular matrices *FN, COL4A5* and *COL4A6*. Data are represented as mean ± SD (n=3). ****p*<0.001.
(**F, G**) Phase and immunostaining images of VSMCs at passage 1 (P1) and 5 (P5) after bioreactor-VSMCs were plated on 2D plates. Scale bar, 50 μm.

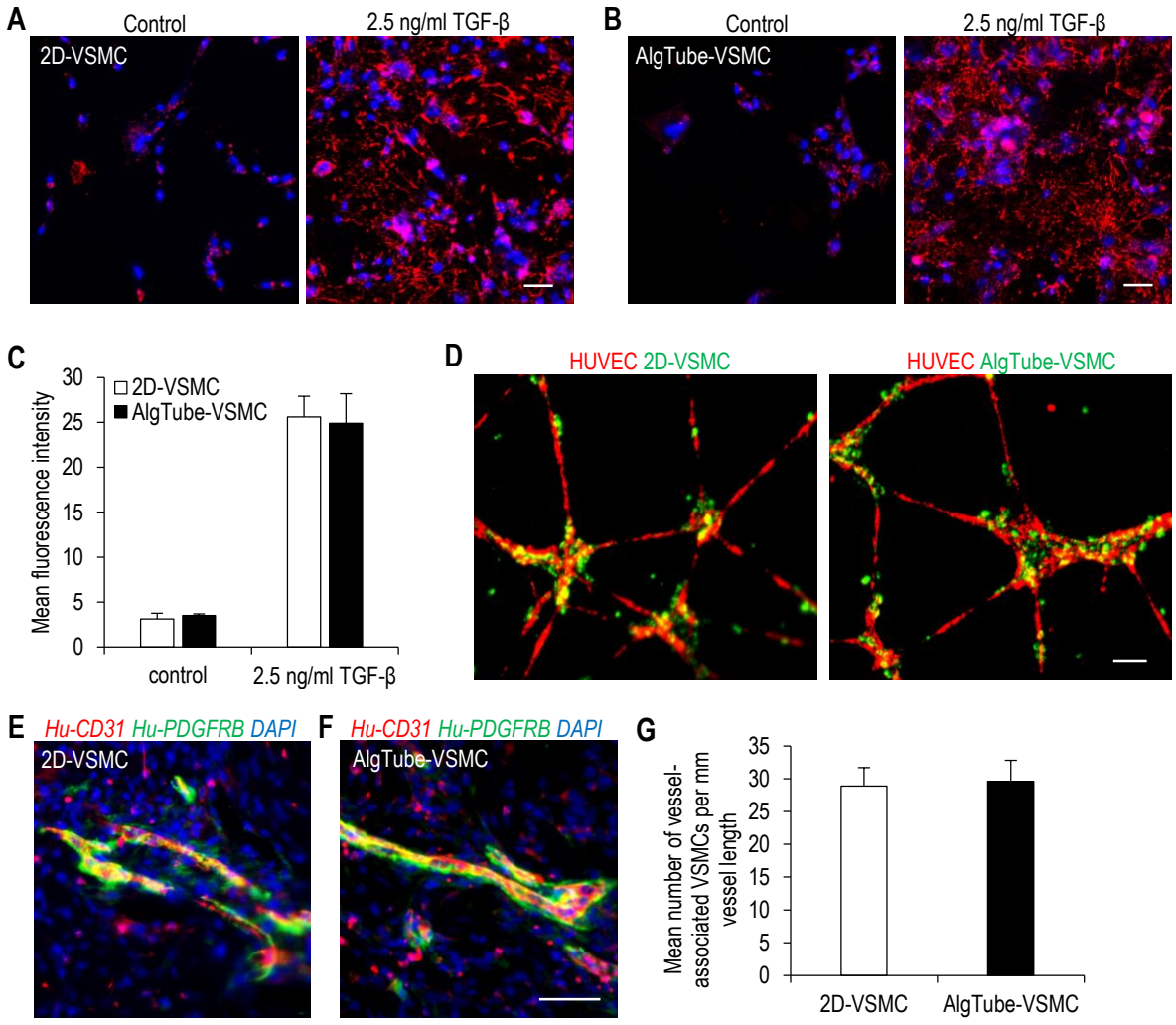
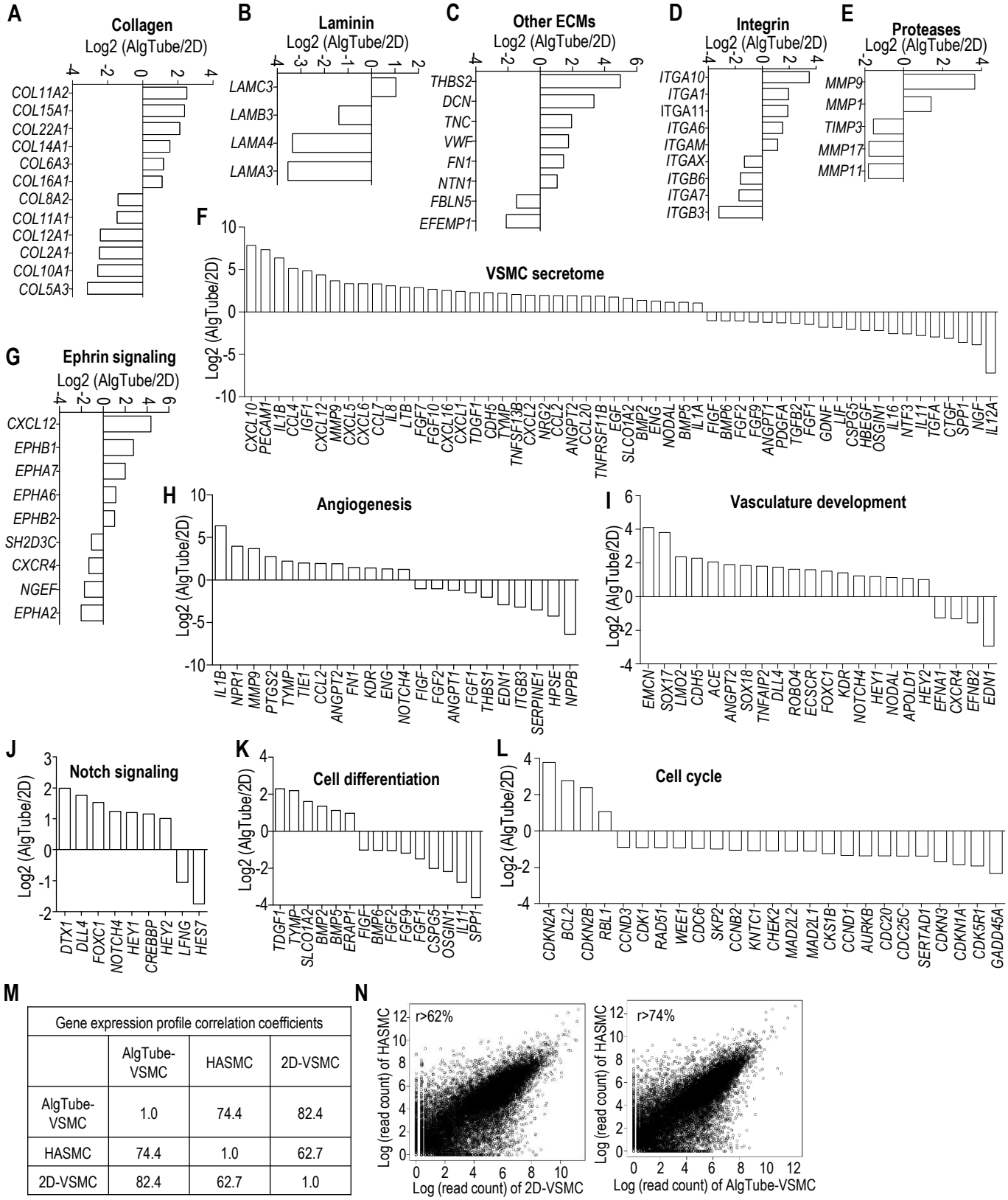


Figure S6. Properties of VSMCs derived from Fib-iPSCs in 2D (2D-VSMCs) and alginate hydrogel tubes (AlgTube-VSMCs).
(**A-C**) Fibronectin production after 24 hours of 2.5 ng/mL TGF-β treatment. Data are represented as mean ± SD (n=3). Scale bar, 50 μm.
(**D**) Co-culture of VSMCs and HUVECs. Scale bar, 50 μm.
(**E, F**) When VSMCs and HUVECs were co-transplanted subcutaneously, 2D-VSMCs (**E**) and AlgTube-VSMCs (**F**) formed nice vascular structures. Scale bar, 50 μm.
(**G**) 2D-VSMCs and AlgTube-VSMCs showed similar number of VSMCs attached to vessel.



Gene expression profile correlation coefficients			
	AlgTube-VSMC	HASMC	2D-VSMC
AlgTube-VSMC	1.0	74.4	82.4
HASMC	74.4	1.0	62.7
2D-VSMC	82.4	62.7	1.0

Figure S7. Differential gene expression analysis of H9s, AlgTube-VSMCs and 2D-VSMCs derived from H9s. (**A-L**) Log2 (expression level in AlgTube-VSMCs/expression level in 2D-VSMCs) analysis of genes related to extracellular matrices (**A-E**), VSMC secretome (**F**), Ephrin signaling (**G**), angiogenesis (**H**), vasculature development (**I**), Notch signaling (**J**), cell differentiation (**K**) and cell cycle (**L**). (**M, N**) The global gene expression correlation coefficients (**M**) and scatter plot in log scale (**N**) of gene expression between AlgTube-VSMCs or 2D-VSMCs and HASMCs.

Table S1. Antibodies used in this study (Related to Figure 1, 2, 3, 6 and Figure S1-6).

Antibody	Supplier	Catalog. No	Species	Dilution
VE-Cadherin	Santa Cruz	sc-9989	Mouse	1:100 (IF&FC)
α-SMA	Santa Cruz	sc-130616	Mouse	1:100 (IF&FC)
SM22A	Abcam	ab14106	Rabbit	1:100 (IF&FC)
SSEA4	R&D System	962648	Mouse	1:200 (IF&FC)
ALP	R&D System	962647	Mouse	1:200 (IF&FC)
HNF-3β	Santa Cruz	sc-101060	Mouse	1:200 (IF)
OCT4	R&D System	962649	Goat	1:200 (IF&FC)
NANOG	R&D System	963488	Goat	1:200 (IF&FC)
NESTIN	BioLegend	809801	Mouse	1:500 (IF&FC)
Fibronectin	Abcam	ab2413	Rabbit	1:500 (IF)
Human-PDGFRB	ThermoFisher	PA5-14718	Rabbit	1:100 (IF)
Human-CD31	BD Biosciences	555444	Mouse	1:200
Secondary antibody	Jackson ImmunoResearch	715-545-151	Donkey	1:500
Secondary antibody	Jackson ImmunoResearch	711-585-152	Donkey	1:500
Secondary antibody	Jackson ImmunoResearch	711-165-152	Donkey	1:500
Secondary antibody	Jackson ImmunoResearch	705-605-147	Donkey	1:500

Table S2. Real time qPCR primers used in this study (Related to Figure 3, 6 and Figure S3).

Primer Name	**Forward 5'-3'**	**Reverse 5'-3'**
SM22A	TGAAGGCGGCTGAGGACTAT	TCTGTTGCTGCCCATCTGAA
α-SMA	CTGGGACGACATGGAAAA	ACATGGCTGGGACATTGA
Calponin	AGCATGGCGAAGACGAAAGGAA	CCCATCTGCAGGCTGACATTGA
SMTN	CGGCCTGCGCGTGTCTAATCC	CTGTGACCTCCAGCAGCTTCCGAA
Elastin	CAGCTAAATACGGTGCTGCTG	AATCCGAAGCCAGGTCTTG
MYH11	AGATGGTTCTGAGGAGGAAACG	AAAACTGTAGAAAGTTGCTTATTCACT
MMP9	GCTTTTCTTCTTCTCTGGGCGCC	CGGTCCTGGCAGAAATAGGCTTT
VEGFA	TCACAGGTACAGGGATGAGGACAC	TCCTGGGCAACTCAGAAGCA
VEGFB	GCTTAGAGCTCAACCCAGACACC	CAAGTCACCCTGCTGAGTCTGAA
VEGFC	CAGCACGAGCTACCTCAGCAAG	TTTAGACATGCATCGGCAGGAA
Fibronectin	GCACCACAGCCATCTCACAT	TCCAACGGCCTACAGAATTT
Collagen4A5	AAAAGAGCCCACGGTCAAG	GGGGTAGAGAGCCAGTAAGAA
Collagen4A6	ACCCTGCTGAGATCTGCTGT	GGCCCATCAAATCTTTCTGA
PECAM1	GCAGCATCGTGGTCAACATAA	GCAGGACAGGTTCAGTCTTTCA
ANGPT2	ACCCCACTGTTGCTAAAGAAGA	CCATCCTCACGTCGCTGAATA
NOTCH4	CCTGGCTCCTTCAACTGCC	GCAAGTAGGTCCAGACAGGT
DLL4	GTCTCCACGCCGGTATTGG	CAGGTGAAATTGAAGGGCAGT
OCT4	CCCCAGGGCCCCATTTTGGTACC	ACCTCAGTTTGAATGCATGGGAGAGC
NANOG	TACCTCAGCCTCCAGCAGAT	CCTTCTGCGTCACACCATT
GAPDH	TCGACAGTCAGCCGCATCTTCTTT	ACCAAATCCGTTGACTCCGACCTT

Supplemental Experimental Procedures

Routine Cell Culture

H9 hESCs (#WA09, WiCell) were purchased from WiCell Research Institute. Fib-iPSCs and MSC-iPSCs reprogrammed from fibroblasts and mesenchymal stem cells, respectively, were obtained from Human Embryonic Stem cell core, Harvard Medical School and have well characterized and described in the literature(Park et al., 2008). hPSCs (H9s and iPSCs) were maintained in 6-welll plate coated with Matrigel in Essential 8^{TM} medium. Cells were passaged every 4 days with 0.5 mM EDTA. Medium was changed daily. Cells were routinely checked for the expression of pluripotency markers, OCT4 and NANOG, their capability to form teratomas in immunodeficient mice, their karyotypes, and bacterial or mycoplasma contamination. HUVECs (#00191027, Lonza) were obtained from Lonza.

Processing Alginate Hydrogel Tubes

A custom-made micro-extruder was used to process the alginate tubes according to our previous publication(Li et al., 2018a). Briefly, a hyaluronic acid (HA) solution containing single cells and an alginate solution was pumped into the central and side channel of the home-made micro-extruder, respectively, and extruded into a $CaCl_2$ buffer (100 mM) to make alginate hydrogel tubes. Subsequently, $CaCl_2$ buffer was replaced by cell culture medium. We made the hydrogel tubes with diameter of 120, 250 and 330 μm and found 250 μm was optimal for cell culture. We decided to use 250 μm hydrogel tubes with 40 μm shell thickness for all the cell culture studies. To make these tubes, the flow rates for the cell suspension and alginate solution were 30 μL/min and 30 μL/min, respectively.

Culturing hPSCs in Alginate Hydrogel Tubes

For a typical cell culture, 20 μL cell solution in alginate hydrogel tubes were suspended in 3 mL E8 medium with 10 μM ROCK inhibitor in a 6-well plate and cultured in an incubator with 5% CO_2, 21% O_2 at 37 °C. Medium was changed daily. To passage cells, medium was removed, and alginate hydrogels were dissolved with 0.5 mM EDTA for 5 minutes. Cell masses were collected by centrifuging at 100 g for 5 minutes, and treated with Accutase at 37 ºC for 10 minutes and dissociated into single cells if needed.

Making hPSC-VSMCs in 2D Culturing or Alginate Hydrogel Tubes

For VSMCs differentiation in 2D, hPSCs were dissociated with Accutase and plated on Matrigel-coated plate at a density of 40,000 cells/cm^2 in E8 medium with 10 μM ROCK inhibitor. After 24 hours, the medium was replaced with differentiation medium, consisting of N2B27 medium (1:1 mixture of DMEM/F12 (#SH30004.04, HyClone) with Glutamax-I (#35050061, Life Technologies) and Neurobasal medium (#21103049, Life Technologies) supplemented with N2 (#17502048, Life Technologies) and B27 minus vitamin A (#12587010, Life Technologies) with 8 μM CHIR99021 (#C6556, LC laboratories) and 25 ng/mL BMP4 (#314BP010, R&D Systems). After 3 days, the differentiation medium was replaced by VSMC induction medium consisting of N2B27 medium supplemented with 10 ng/mL PDGF-BB (#100-14B, PeproTech) and 2 ng/mL ActivinA (#338-AC, R&D Systems). The medium was exchanged every day. VSMCs were harvested for analysis on day 6.

For VSMCs differentiation in AlgTubes, single hPSCs were encapsulated in AlgTube ($1.0x10^6$ cells/mL) and cultured in E8 medium for 5 days. E8 medium was removed and replaced with VSMC differentiation medium. After 3 days, the differentiation medium was replaced by VSMC induction medium. The induction medium was changed after 1 day. On day 10, cell masses were collected for following analysis.

Producing hPSC-VSMCs in Alginate Hydrogel Tubes in the Prototype Bioreactor

1 mL of hPSCs solution in AlgTube were suspended in a bioreactor. hPSCs were cultured in E8 medium with 5% CO_2, 21% O_2 at 37 °C for 5 days. E8 medium was removed and replaced with VSMC differentiation medium for 3 days, followed by VSMC induction medium for 2 days. For bioreactor, medium was stored in a bellow bottle that was periodically pressed to flow the medium into, or released to withdraw the medium from bioreactor. On day 10, hydrogel tubes were dissolved by adding 0.5 mM EDTA buffer. Cell masses were pelleted by centrifugation. Cell masses were dissociated into single cells through incubating in Accutase at 37 °C for 10 minutes. Magnetic beads coated with anti-CD144 antibodies were added to remove CD144+ hPSC-ECs with a magnetic cell separator. The bioprocess was repeated 2 times.

Immunocytochemistry and Flow Cytometry

For 2D immunostaining, the 2D cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 20 minutes, permeabilized with 0.25% Triton X-100 for 30 minutes, and blocked with 5% donkey serum for 1 hour before incubating with primary antibodies (**Table S1**) at 4 °C overnight. After extensive washing, secondary antibodies (**Table S1**) and 10 µM 4', 6-Diamidino-2-Phenylindole (DAPI) in 2% BSA were added and incubated at room temperature for 4 hours. Cells were washed with PBS for 3 times before imaging with A1 confocal microscope. For cell mass immunostaining, after fixation for 30 minutes, cell masses were incubated with PBS + 0.25% Triton X-100 + 5% (vol/vol) goat serum + primary antibodies at 4 °C for 48 hours. After extensive washing, secondary antibodies in 2% BSA were added and incubated at 4 °C for 24 hours. Cells were washed with PBS three times before imaging with a confocal microscope.

For flow cytometry analysis, the harvested cells were dissociated into single cells with Accutase, then fixed with 4% PFA at room temperature for 20 minutes. Single cells were stained with primary antibodies (**Table S1**) at 4 °C overnight. After washing 3 times with 1% BSA in PBS, secondary antibodies were added and incubated at room temperature for 2 hours. Cells were washed with 1% BSA in PBS and analyzed using a Cytek DxP10 flow cytometer. Single-color and isotype controls were used for compensation and negative gating.

RNA Extraction, cDNA Synthesis and Quantitative PCR

Total RNAs for qPCR and RNA sequencing were extracted from undifferentiated hPSCs, 2D-VSMCs and AlgTube-VSMCs on day 5 of the differentiation using Trizol (#15596018, Invitrogen), according to the manufacturer's instructions. Reverse transcription was done with the Maxima First Strand cDNA Synthesis Kit (#K1642, Life Technologies). Quantitative real-time PCR was carried out in an Eppendorf MasterCycler RealPlex4 (ThermoFisher Scientific) using the Power SYBR Green PCR Master Mix (#4367659,

ThermoFisher), according to the manufacturer's instructions. The data were normalized to the endogenous GAPDH. Primer sequences are listed in **Table S2**.

Embryoid Body (EB) Differentiation

hPSCs were suspended in DMEM + 20% FBS + 10 μM β-mercaptoethanol in low adhesion plate for 6 days. The cell masses were then transferred into plates coated with 0.1% gelatin and cultured in the same medium for another 6 days, followed by fixation and staining as described above.

Teratoma Formation *in vivo*

The animal experiments were carried out following the protocols approved by the University of Nebraska–Lincoln Animal Care and Use Committee. $1.0x10^6$ hPSCs were suspended in 25 μL PBS + 25 μL Matrigel and injected subcutaneously at the back of the neck of the NOD-SCID mice (female, age 7 weeks, Charles River Laboratory). Three mice (two teratomas per mouse) were used for teratoma assay for each hPSC line. Teratomas were harvested when their size reached 2 cm. Teratomas were fixed with 4% PFA for 48 hours, dehydrated with 70%, 95% and 100% ethanol sequentially, and de-fated with xylene for 2 hours before being embedded in paraffin. 10 μm thick sections were cut and stained with hematoxylin and eosin. The structures from all 3 germ layers were identified by a trained specialist.

Co-Culture Assay of HUVECs and hPSC-VSMCs

200 μL of Matrigel was added into each well of a 12-well plate and incubated for 30 minutes at 37 °C to allow the formation of a thin layer of hydrogel. For the functional tube formation and association assays *in vitro*, the HUVECs and the hPSC-VSMCs were prestained with DiI (red) and DiO (green), respectively, according to the manufacturer's instructions. Cells were grown in EGM-2 medium supplemented with 50 ng/mL VEGF-A. For the experiment, $2.0x10^4$ HUVECs/cm^2 and $2.0x10^4$ hPSC-VSMCs/cm^2 were co-cultured for 24 hours in the incubator. Cells were fixed with 2% PFA for 10 minutes and analyzed using Zeiss fluorescence microscope.

Fibronectin Production

After 5 days of differentiation, 2D-VSMCs and AlgTube-VSMC were seeded in N2B27 containing 10 ng/mL PDGF-BB at 40,000 cells/cm^2 on gelatin-coated wells. After 24 hours, the medium was changed to N2B27 with 10 ng/mL PDGF-BB supplemented with DMSO or 2.5 ng/mL TGF-β. After 24 hours, cells were washed with PBS, fixed with 4% PFA for 10 min at room temperature, and immunofluorescence staining of deposited Fibronectin was performed (**Table S1**).

Contraction Study

2D-VSMCs and AlgTube-VSMCs were seeded in N2B27 containing 2 ng/mL ActivinA and 2 μg/mL at 40,000 cells/cm^2 on Collagen-coated wells according to previous studies(Matsumoto et al., 2007; Patsch et al., 2015). After 48 hours, they were stained with 2.5 μM Fluo-4 AM (#50018, Biotium) at 37 °C for 1 hour. Contraction was induced by treating the cells with 100 μM carbachol (#2810, Tocris). Contraction images of VSMCs were acquired by a Zeiss fluorescence microscope. The fluorescence intensity of

intracellular calcium flux, cell surface area (mm^2) and percent change of cell surface area was assessed by ImageJ software.

Matrigel Plug Assay

Animal procedures were performed in accordance with an IACUC-approved protocol reviewed by the University of Nebraska–Lincoln Animal Care and Use Committee. 6-8 week old female SCID mice (Charles River Laboratory) were used. HUVECs and hPSC-VSMCs were added to the Matrigel mixture to a final concentration of 10 million cells/mL. The Matrigel mixture (300 µL) was then immediately engrafted subcutaneously into the dorsal flank of the mouse. Two implants were engrafted per animal. Implants were recovered after 14 days, then the implants were excised. They were fixed in 4% PFA. Hematoxylin & eosin staining, and immunostaining was performed to analyze the tube formation potential *in vivo*. Six mice were used for the Matrigel plug assay. Mean number of vessel-associated VSMCs per mm vessel length was assessed by ImageJ software.

RNA Sequencing and Data Analysis

Total RNA of day 6 VSMCs from 2D culture and day 10 VSMCs from AlgTubes (note: both VSMCs were differentiated for 5 days) were prepared with RNeasy mini kit (cat # 74104 QIAGEN) according to the manufacturer's instruction. Prior to RNA sequencing, magnetic beads coated with anti-CD144 antibodies were added to remove CD144+ hPSC-ECs with a magnetic cell separator. VSMCs reached 95% purity after purification. Libraries were prepared with TruSeq Stranded mRNA Library Prep Kit and sequenced with Illumina NextSeq 500. 20 million 75 bp paired-end reads were generated for each sample. The thresholds for differential expression were set at fold-change > 2 and adjusted P-values < 0.001 for the null hypothesis. Methods for the data processing, heatmap generating, PCA analysis, differential gene expression analysis have been described in our previous publication(Li et al., 2018a).

Statistical Analysis

The data are presented as the mean ± standard deviation (SD) from three independent experiments. We used an unpaired t-test to compare two groups and one-way ANOVA to compare more than two groups. A sample size of 3 was selected so that at a significance level of 0.05 there was at least 95% chance of detecting two SD's difference in outcome between the groups. All data were processed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA).

Data Availability

The final processed data and raw fastq files were submitted to Gene Expression Omnibus (GEO) with the accession number GSE99776 and GSE109683. All other data supporting the findings of this study are available within the paper and its Supplemental Information.

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