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Supplemental Information

Tissue-Engineered Vascular Rings from Human iPSC-Derived Smooth Muscle Cells

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SUPPLEMENTAL FIGURES:

Figure S1. Related to Figure 1.

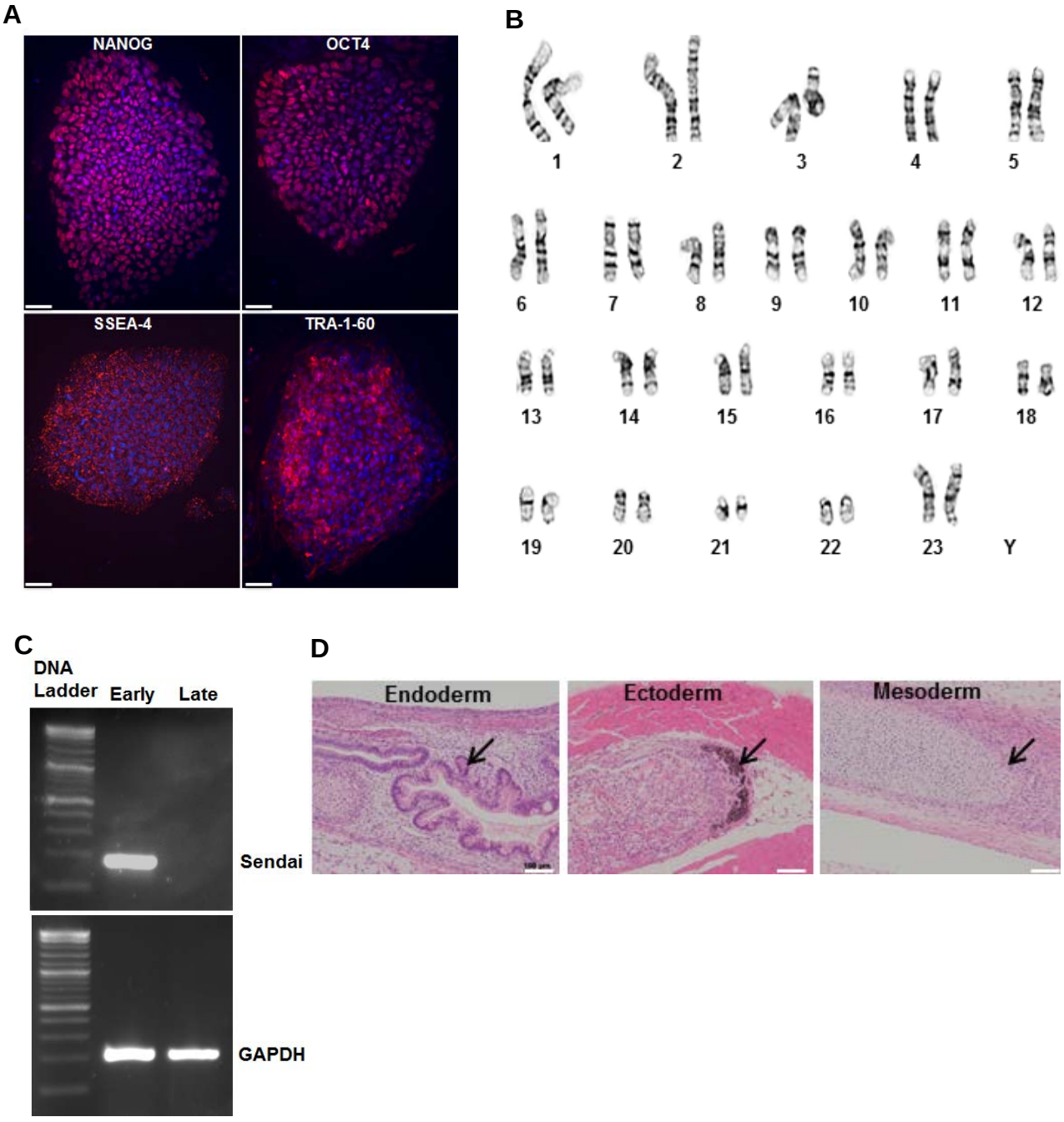

Figure S1. H**uman induced pluripotent stem cells (Y6) were generated using a Sendai virus vector and reprogramming factors OCT3/4, KLF4, c-MYC and SOX2.** (**A**) Human iPSC clones were selected and characterized for major pluripotency markers OCT4, SSEA4, NANOG, and Tra-1-60. Blue stains for nuclei, red stains for the pluripotency markers. Scale bar represents 100 µm. (**B**) G band staining for karyotype analysis of hiPSCs. (**C**) Detection of Sendai virus in the hiPSCs by RT-PCR analysis. GAPDH gene was used as the internal loading control.

Early passage hiPSCs (passage 2, “Early”; still containing virus) served as a positive control, while late passage hiPSCs (typically after passage 15, “Late”) lost the Sendai virus vector transgenes. (**D**) Teratoma formation after injection of undifferentiated hiPSCs in immunodeficient Rag2-/-GammaC-/- mice. Note the formation of gastrointestinal epithelium (endoderm, left), pigmented epithelium (ectoderm, center), and hyaline cartilage (mesoderm, right), as identified by the arrows. Scale bar represents 100 μm.

Figure S2. Related to Figure 1 and 2.

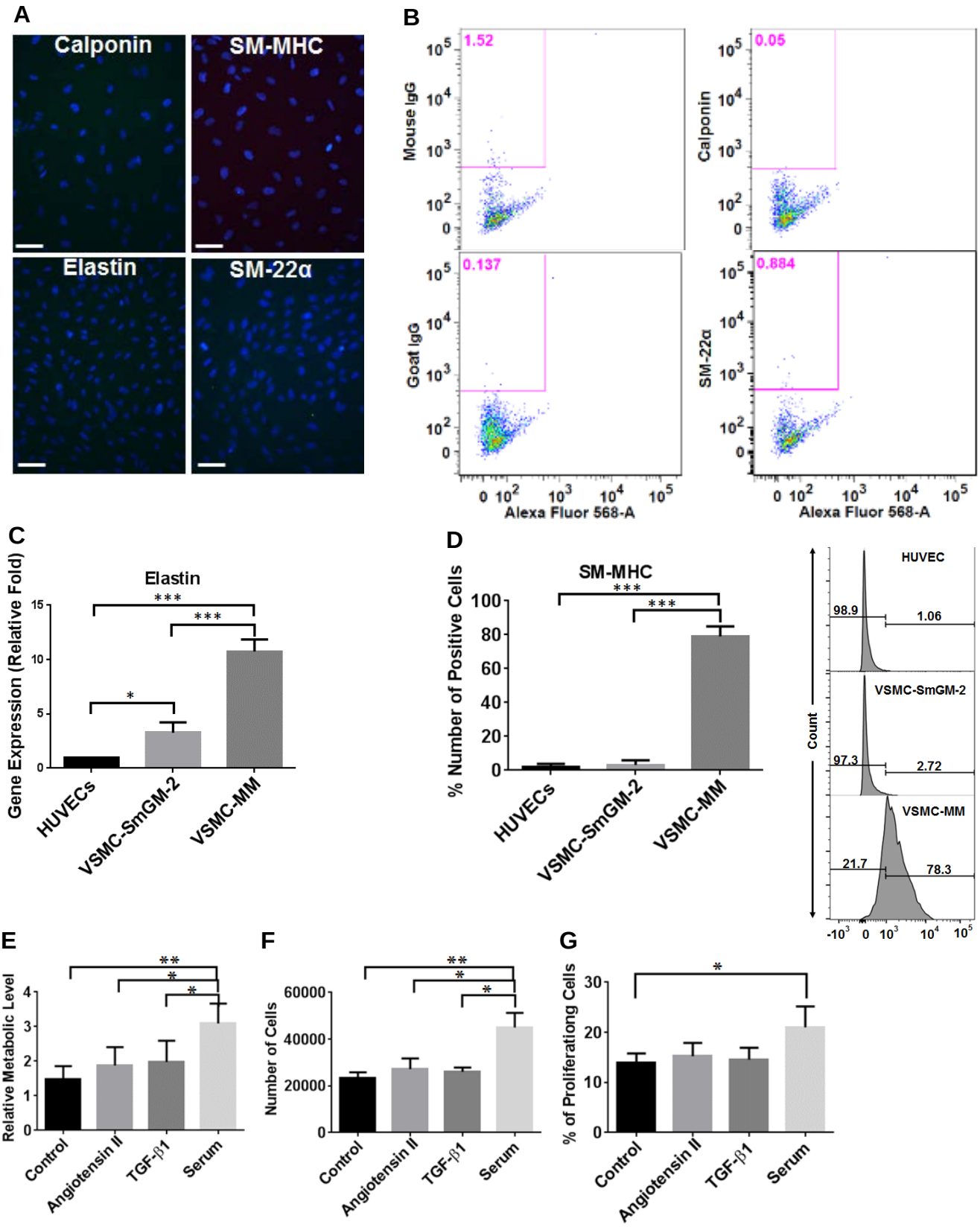

Figure S2: Analysis of hiPSC-VSMCs. (**A**) Immunofluorescence images of HUVECs stained for calponin, SM-22, elastin and SM-MHC. Blue stains for nuclei. Scale bar represents 50 µm. (**B**) FACS data showing HUVECs as a negative control for major smooth muscle cell specific markers SM-22 and calponin. Goat and mouse IgGs were used as experimental negative controls. Alexa Fluor 488 was used to gate positively stained cells. (**C**) qRT-PCR analysis of relative transcript amounts of elastin genes in hiPSC-VSMCs cultured in SmGM-2 and maturation (MM) medium (mean ± SD; n=3 independent experiments, *$p < 0.05$, ***$p<0.001$). HUVEC was used as a negative control. Values in the y-axis represent fold changes relative to human 18S expression. (**D**) Graph showing percentage of VSMCs positive for SM-MHC in three independent FACS experiments in SmGM-2 and maturation (MM) medium. HUVECs and rabbit IgG were used as negative and experimental control respectively (mean ± SD; n=3 independent experiments, ***$p<0.001$). Representative FACS plots showing SM-MHC quantification for HUVEC, VSMC-SmGM-2 and VSMC–MM. **(E-G)** Proliferative responses of hiPSC-VSMCs to different factors. Y6-VSMC proliferation was measured in response to control (chemically defined medium only), angiotensin II (10µM), TGF-β1 (5ng/ml), fetal bovine serum (10%) using (**E**) MTT assay (mean ± SD; n=4 independent experiments, *$p < 0.05$, **$p<0.01$), (**F**) cell counting (mean ± SD; n=3 independent experiments, *$p < 0.05$, **$p<0.01$) and (**G**) cell cycle analysis (mean ± SD; n=3 independent experiment, *$p < 0.05$) after 3 days in culture. Note that % of proliferating cells with cell cycle analysis (**G**) was quantified as the % of cells in S and G2-M phase. The data were represented as mean ± SD. Unpaired Student t-test was performed to determine statistical significance between control and treatment groups.

Figure S3. Related to Figure 1

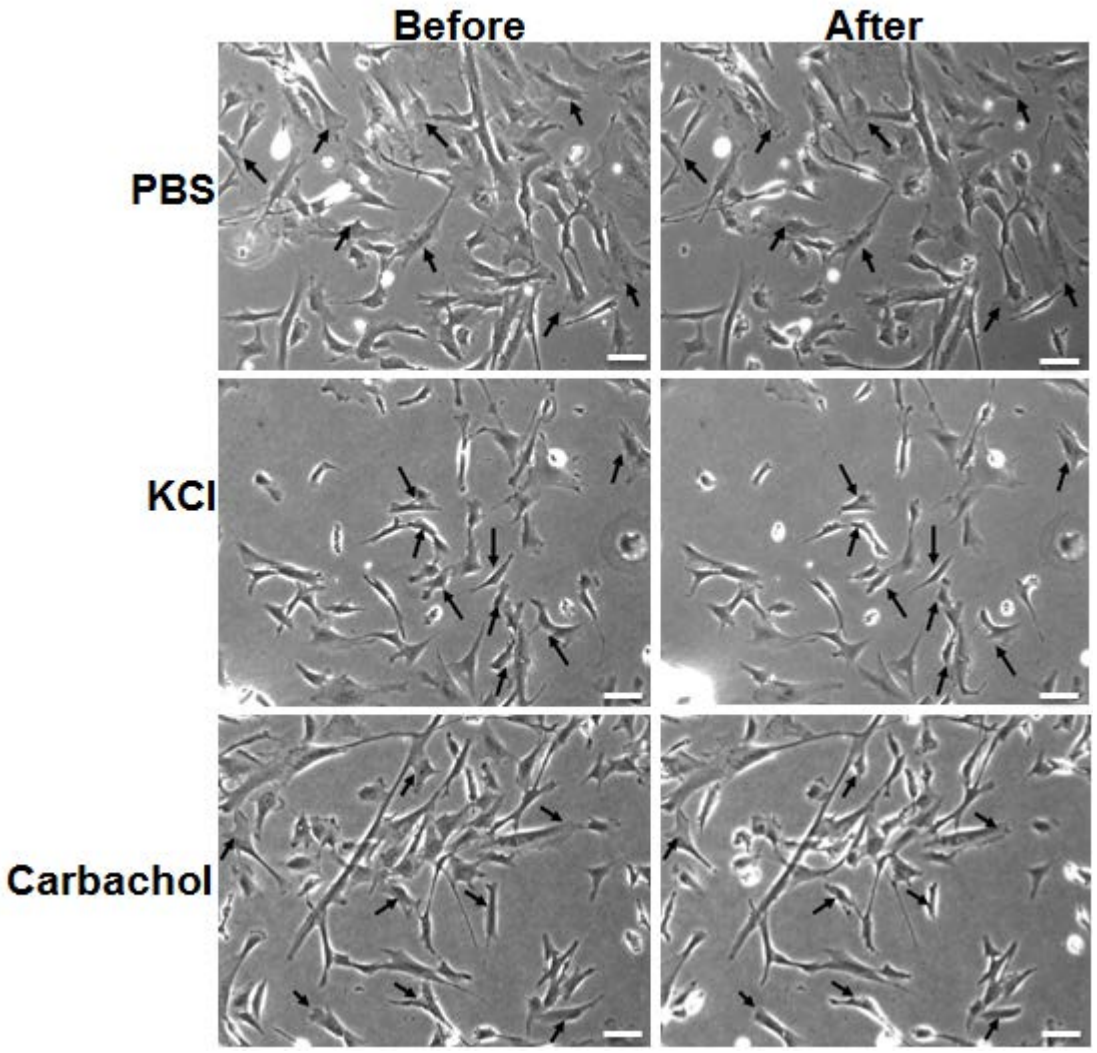

Figure S3: 2D Contractility response of VSMCs. Bright field images of Y6-VSMCs taken before and 15 minutes after adding agonists: KCl (50mM) and Carbachol (1mM). PBS was used as a negative control. Black arrows mark the cells quantified for decrease in the cell area. Scale bar represents 100 µm.

Figure S4. Related to Figure 3 and 4.

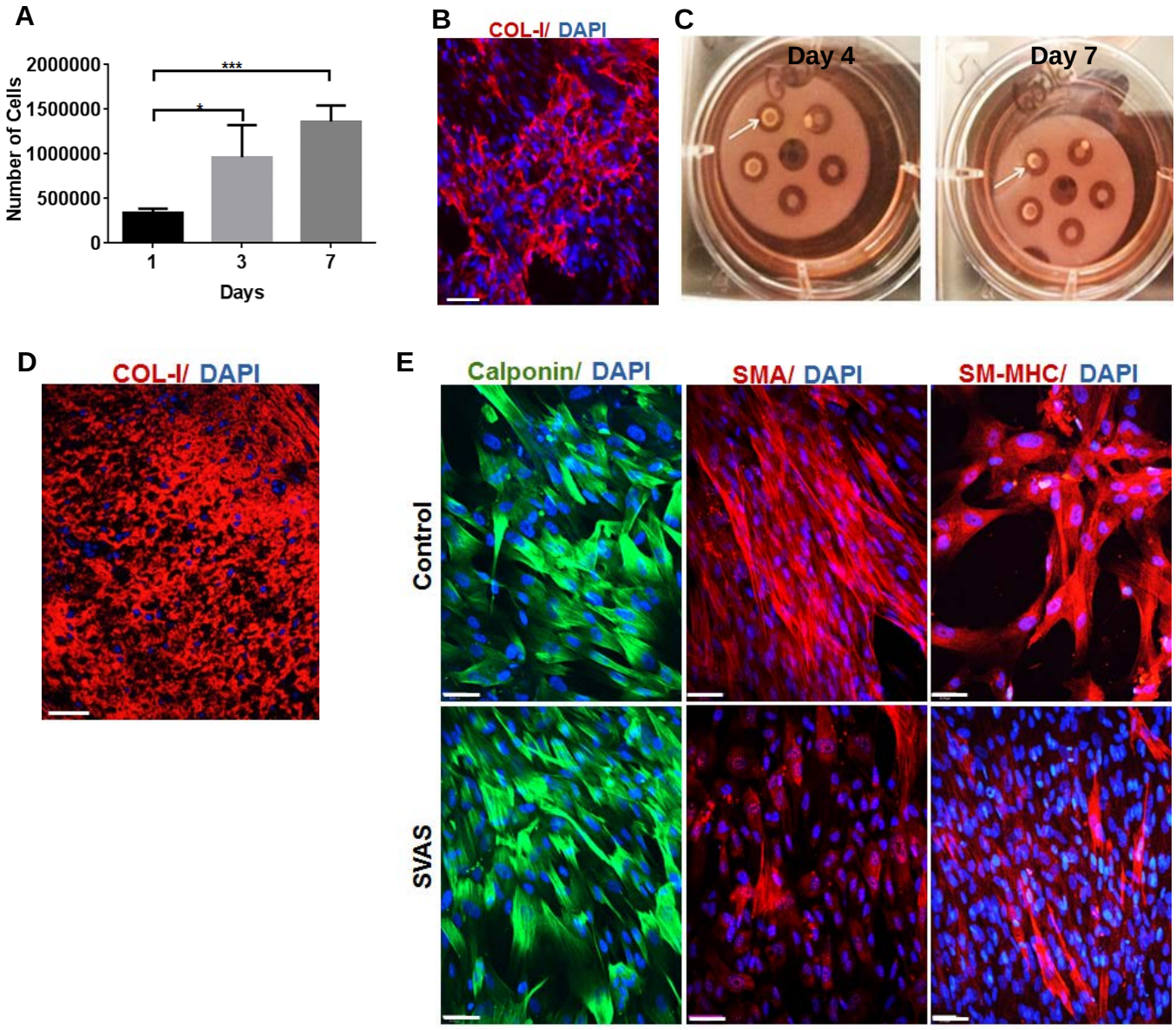

Figure S4. Tissue Ring and SVAS-VSMC characterization. (**A**) Proliferation of hiPSC-VSMCs in ring culture medium for day 1, 3 and 7 (mean ± SD; n=3 independent experiments,*p < 0.05, ***p < 0.001). (**B**) Immunofluorescence analysis of Y6-VSMCs cells producing type-I collagen in ring culture medium in a tissue culture plate within 7 days of culture. Collagen-1 (COL-1) stains red, and blue stains for nuclei). Scale bar represents 100 µm**. (C)** Photographs of Y6-VSMCs rings seeded in agarose wells cultured in SmGM-2 medium in a 6-well plate for 7 days. White arrows point to a typical tissue ring, which formed on day 4 (top panel) but was broken on day 7 (bottom panel). **(D)** Immunofluorescence analysis of Y6-VSMCs cells producing type-I collagen in the tissue ring after 14 days in culture. Collagen-1 (COL-1) stains red, and blue stains for nuclei). Scale bar represents 50 µm**. (E)** Analysis of control and SVAS iPSC-derived VSMCs in ring culture medium. Immunofluorescence images of cells stained with calponin and SMA and SM-MHC. Green stains for calponin and red for both SMA and SM-MHC. Blue stains

for nuclei. Scale bar represents 50 μm.

Table S1: Antibodies used in immunostaining

Antigen	catalog#	Isotype	Company
ES cell characterisation kit	SCR001	Mouse	EMD Millipore
α-SMA	Ab5694	Rabbit	Abcam
Calponin	C2687	Mouse	Sigma
SM-22α	Ab10135	Goat	Abcam
SM-MHC	Ab53219	Rabbit	Abcam
Collagen-I	Ab34710	Rabbit	Abcam
Elastin	Ab9519	Mouse	Abcam
Ki67	Ab15580	Rabbit	Abcam
Mouse IgG1 isotype control	MA1-10405	Mouse	Thermo Scientific
Goat IgG isotype control	02-6202	Goat	Thermo Scientific
Rabbit IgG isotype control	sc-2027	Rabbit	Santa Cruz Biotechnology
Alexa 488 goat anti-mouse IgG	A11029	N/A	Thermo Scientific
Alexa 555 goat anti-rabbit IgG	A21428	N/A	Thermo Scientific
Alexa 555 Donkey anti-goat IgG	A21432	N/A	Thermo Scientific

Table S2: List of primers used in qPCR assay.

human elastin F	AAGATGGTGCAGACACTTC
human elastin R	AGAGCGAATCCAGCTTTGG
human 18S F	GGAAGGGCACCACCAGGAGT
human 18S R	TGCAGCCCCGGACATCTAAG

EXPERIMENTAL PROCEDURES

Human iPSC Generation and Characterization

Fibroblasts were derived from discarded female neonatal skin tissue under Yale Institutional Review Board approval. The cells were plated at a density of 1×10^5 cells per well in a 6-well plate one day before transduction at approximately 60-70% confluence. CytoTune® Sendai tubes (3×10^6 CIU each; ThermoFisher, USA) were thoroughly mixed with 1 mL of pre-warmed fibroblast medium at 37°C. Then, culture medium was aspirated and replaced with 1 ml of the medium containing virus to each well. Twenty-four hours after transduction, the medium was replaced with fresh fibroblast medium. Seven days after transduction, fibroblast cells were harvested using 0.05% trypsin/EDTA and plated on a mitotically arrested mouse embryonic fibroblast (MEF) feeder layer. Twenty-four hours later, the MEF medium was replaced with iPSC medium (20% KSR in DMEM/F12 medium supplemented with 10ng/ml bFGF, 1% non-essential amino acid (v/v), and 2 mM L-Glutamine; all from ThermoFisher, USA). The medium was changed every day thereafter. Three weeks later, the iPSC colonies were manually picked and transferred onto Matrigel-coated plates and cultured in the mTeSR™1 medium (Stem Cell Technologies, USA) to expand the colonies (a clone named Y6 was used in this study). Total RNA was extracted from selected iPSC colonies from both early and late passage to determine the presence of Sendai RNA. RT-PCR analyses were performed to detect the expression of Sendai RNA using primers specific for its genomic DNA (Forward primer: GGATCACTAGGTGATATCGAGC; Reverse primer: ACCAGACAAGAGTTTAAGAGATATGTATC). GAPDH was used as the internal loading control (Forward primer: GAAGGTGAAGGTCGGAGTCA; Reverse primer: TTGAGGTCAATGAAGGGGTC). Passage 2 iPSC cultures were used as a positive control.

Pluripotency markers were analysed by immunofluorescence. Briefly, colonies of undifferentiated human iPSCs were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and blocked with 5% fetal bovine serum. Specimens were incubated overnight at 4°C with primary antibodies for Tra-1-60, OCT4, NANOG and SSEA4 (EMD Millipore, USA), then incubated with secondary antibodies (1:1000 in PBS) for one hour at room temperature. DAPI (Life Technologies) was used to stain cell nuclei. The samples were examined using a fluorescent microscope. G-band staining for karyotype analysis was performed. Small clumps of human iPSC were seeded on glass slides pre-coated with Matrigel and fed with mTeSR™1 medium for three days. Then, medium was switched to DMEM basal medium supplemented with 10% of FBS (ThermoFisher, USA) for another 3 days. The slide was transferred to the Yale Cytogenetic Laboratory for G-band staining.

Teratoma formation was performed to assess iPSC pluripotency. Briefly, $1x10^6$ human iPSC cells were collected by collagenase treatment, and resuspended in 100 µl of DMEM/F12, collagen, and Matrigel mix (2:1:1 ratio). Cells were intramuscularly injected into immunodeficient Rag2-/-GammaC-/- mice. After 8 weeks, teratomas were harvested, fixed, and subjected to paraffin-embedding and hematoxylin and eosin (H/E) staining. All animal studies were approved by the Yale IACUC protocol (2013-11347) and performed in accordance with the NIH guidelines for care and use of laboratory animals.

Human iPSC-VSMC Derivation

Human iPSC-VSMCs were derived using an embryoid body (EB) method (Ge et al., 2012; Xie et al., 2007). Briefly, hiPSC lines including Y6 derived from healthy skin fibroblasts in this study; Control 1 derived from healthy VSMCs as describe previously (Ge et al., 2012) ; and Control 2 derived from healthy peripheral blood mononuclear cells as described previoiusly (Chen et al., 2013), were cultured under feeder-free conditions (mTeSR™1 medium) until they reached 80% confluency in a 6-well tissue culture plate (~4 days). hiPSC clumps of uniform sizes were harvested by cutting the colonies using a sharp glass tip and treating them with dispase (1mg/ml) for 15 min at 37 °C. To prepare EBs, hiPSC clumps were seeded in a 6-well low attachment plate with mTeSR™1 medium. The next day, the mTeSR™1 medium was replaced with a mixture of mTeSR™1 and EB differentiation medium (DMEM high glucose + 10% FBS + 1% non-essential amino acid (v/v) + 2mM L-glutamine and 0.012 mM 2-mercaptoethanol) in a ratio of 1 to 3. On day 3, the medium was replaced with EB differentiation medium. The EBs were cultured for the next 3 days in EB differentiation medium. By the end of day 5, the EBs were collected and seeded on a gelatin-coated 6-well plate for 5 days using EB differentiation medium. Medium was replaced with fresh EB medium every day. After the 5-day culture, the cells were harvested using 0.25% trypsin, seeded on Matrigel coated T75 flasks, and cultured until the VSMCs reached 80% confluence (~7 days). SmGM-2 medium (Lonza, USA) was used during the culture and was replaced with fresh medium every other day. Cells were subsequently cultured in either SmGM-2 (Lonza, USA), or a ring culture medium composed of Dulbecco's Modified Eagle Medium (DMEM; high glucose) with 20% FBS (Gibco® ThermoFisher, USA), PDGF-BB (10 ng/ml) (R&D Systems,

USA), TGF-β1 (1 ng/ml) (R&D Systems, USA), penicillin G (100U/ml) and supplemented with proline (50 µg/ml), glycine (50 µg/ml), alanine (20 µg/ml), $CuSO_4$ (3ng/ml) and ascorbic acid (50 µg/ml).

Immunofluorescence and Flow Cytometry

Human iPSC-VSMCs were characterized for smooth muscle cell protein expression by staining for smooth muscle α-actin (SMA), SM-22α, calponin, myosin heavy chain (SM-MHC) and elastin. Briefly, cells were fixed using 4% paraformaldehyde for 10 min, washed 3 times using PBS, and incubated with 10% goat serum or 5% bovine serum albumin (BSA) and 0.25% of Triton X-100 at 37 ºC for 1 hour. The cells were washed once with PBS and then incubated with 1:500 of polyclonal goat anti-SM-22α (Abcam, USA), 1:200 of monoclonal mouse anti-calponin (Sigma, USA), 1:100 of polyclonal rabbit anti-SM-MHC (Abcam, USA) and 1:100 of monoclonal mouse anti-elastin (Abcam, USA) in separate wells for overnight at 4 ºC. Alternatively, cells were stained with 1:200 polyclonal rabbit anti-collagen I primary antibody (Abcam, USA). After the primary antibody incubation, cells were washed with PBS/Tween-20 (PBST) three times for 5 minutes each wash. Finally, the cells were incubated with secondary antibodies (1:1000 in PBS): goat anti-mouse Alexa Fluor® 488 (ThermoFisher, USA), goat anti-rabbit Alexa Fluor® 568 (ThermoFisher, USA), or donkey anti-goat Alexa Fluor® 488 (ThermoFisher, USA) and DAPI (ThermoFisher, USA) for one hour. The cells were washed three times for 5 minutes each before fluorescence imaging.

For flow cytometry, harvested cells were fixed using 2% paraformaldehyde for 30 min at room temperature and washed with PBS three times followed by blocking with 5% BSA and 0.1% Triton-X-100. The primary antibodies SM-22α (Abcam, USA),calponin (Sigma, USA) and SM-MHC (Abcam, USA) were used in a final concentration of 1 µg/ml. Mouse,goat IgG and rabbit isotype controls (ThermoFisher, USA; Santa Cruz Biotechnology, USA) and non-expressing cell control (HUVECs) were used for both immunofluorescence and flow cytometry. Cell fluorescence was measured by a flow cytometer (BD, LSR II), and cell cycle analysis was done using FlowJo software. Refer to Table S1 for the catalog numbers for the antibodies used.

qRT-PCR Analysis

The HUVEC and human iPSC-derived VSMCs were subjected to RNA extraction and RT-PCR assay to evaluate the gene expression levels of elastin. RNA extraction and purification was completed by using the NucleoSpin RNA XS Total RNA Isolation Kit (Macherey-Nagle), following the manufacturer's instructions. Total RNA was then subjected to reverse transcription using an iScript cDNA synthesis Kit (Bio-rad). The primer sequences for the genes used in qPCR are listed in Table S2. qRT-PCR was performed using Bio-Rad IQ SYBR green supermix. The expression of elastin was normalized to that of human 18S.

VSMC Proliferation Assays

To determine the lineage of hiPSC-VSMCs, we measured cell proliferation in response to TGF-β1, angiotensin II and 10% FBS using MTT assay, cell counting and cell cycle analysis. For MTT assay, hiPSC-VSMCs were seeded 6,000 cells/well into 96-well plates using basal chemically defined medium (CDM) as previously described (Cheung et al., 2014; Cheung et al., 2012). Briefly, 500 ml of basal CDM contains 250 ml of IMDM, 250 ml of DMEM/F12, 5 ml of lipid concentrate, 1mg/ml of polyvinyl alcohol, 250 µl of transferrin, 350 µl of insulin and 20 µl of monothioglycerol. After incubation overnight, TGF-β1 (5 ng/ml, R&D System), angiotensin II (10 µM, Sigma) or 10% FBS (Gibco® ThermoFisher, USA) was added. Cell proliferation was measured as a function of the metabolic activity using MTT (Sigma) on different time points: Day 0 and 3. At every time point, MTT (0.5 mg/ml) was incubated with the cells at 37 °C for 2 h and then solubilized using DMSO (AmericanBIO, USA) at 37 °C for 15 min. Absorbance was measured at 540 nm using the Synergy 2 multi-mode plate reader (BioTek). To perform cell cycle analysis, hiPSC-VSMCs were harvested, and the cell suspension was fixed by ice cold 70% ethanol for 30 min at 4 °C followed by incubation with 100 µg/ml of RNase. Finally, cells were stained with 50 µg/ml of propidium iodide in the dark at 25 °C for 30 min. Cell fluorescence was measured by a flow cytometer (BD, LSR II), and cell cycle analysis was done using FlowJo software.

Tissue Ring Fabrication

Custom agarose molds for tissue ring formation were made as previously described, with minor modifications (Dikina et al., 2015; Gwyther et al., 2011a; Gwyther et al., 2011b). Briefly, a machined custom polycarbonate mold was created with 5 annular wells, each with posts 2 mm in diameter, designed to fit in one well of a 6-well plate. A polydimethylsiloxane (PDMS) mold was cured on the polycarbonate template, and was used to make wells from a 2% agarose solution in un-

supplemented DMEM (high glucose). The agarose molds were equilibrated in SmGM-2 medium overnight before rings were seeded. The hiPSC-VSMCs were harvested and seeded at 600,000 cells/well in SmGM-2. The rings were allowed to aggregate for 24 hours and were then cultured in ring culture medium composed of Dulbecco's Modified Eagle Medium (DMEM; high glucose) with 20% FBS (Gibco® ThermoFisher, USA), PDGF-BB (10 ng/ml) (R&D Systems, USA), TGF-β1 (1 ng/ml) (R&D Systems, USA), penicillin G (100U/ml) and supplemented with proline (50 µg/ml), glycine (50 µg/ml), alanine (20 µg/ml), $CuSO_4$ (3ng/ml) and ascorbic acid (50 µg/ml). The rings were harvested on either 14 or 17 days and analyzed by mechanical testing and histology.

2D and 3D Contractility Study

Two-dimensional (2D) SMC contraction studies were carried out using hiPSC-VSMCs cultured on tissue culture polystyrene. KCl (50mM) and carbachol (1mM) were used as agonists. Briefly, VSMCs were treated with agonists for 15 min, and images of pre- and post-treatments were acquired and decrease in the cell area was measured by ImageJ software.

For 3D contractility study, VSMC tissue ring was carefully removed from the culture and immersed in a temperature-controlled perfusion bath. The ring was picked up by two motorized micromanipulators with hook-like extensions, leaving the ring suspended between an anchoring attachment on one end and a force transducer (KG7, SI Heidelberg) on the other (see Figure 4C). Throughout measurements, the ring was incubated in Tyrode's solution (NaCl 140mM, KCl 5.4mM, MgCl2 1mM, HEPES 25mM, Glucose 10mM and 1.8mM $CaCl_2$, pH 7.3) freshly aerated with carbogen: 20% of oxygen,5% of carbon dioxide and 75% nitrogen. Force measurements were taken at the slack length before the manipulators were moved apart by a distance of 1.14 mm to take a second reference force measurement before agonist treatment. Either carbachol or KCl solutions were used in a final concentration of 1mM or 50mM respectively. The readings were taken for 15 min. Customized Matlab software was used to calculate the resulting changes in the force. For each ring, a baseline measurement was taken in PBS. Furthermore, cross sectional areas of the VSMC rings were acquired to calculate the changes in tension (Pa). The cross-section of the tissue rings were assessed using an optical coherence tomography (Ganymede-II-HR, Thorlabs). An index of refraction of 1.38 was used for each of the tissue rings. Four evenly distanced images were taken and their cross-sectional area were determined using Image J and averaged to give the overall cross sectional area of the tissue ring.

Tissue Ring Mechanical Testing

Before mechanical testing, the rings were removed from the posts and ring thickness measurements were taken as previously described (Gwyther et al., 2011a; Gwyther et al., 2011b). Briefly, four measurements were obtained at four different locations around the ring circumference using edge detection software (Framework, DVT), and were averaged to find the mean ring thickness. Mechanical testing was performed using a uniaxial tensile testing machine (Electropuls E1000, Instron) as previously described (Gwyther et al., 2011b). Briefly, rings were mounted on custom grips, loaded with a 5mN tare load, pre-cycled 8 times from 5mN to 50kPa, and pulled to failure at a rate of 10mm/min. Data were analyzed using a custom MATLAB program (The MathWorks Inc.) to calculate ultimate tensile strength (UTS) and maximum tangent modulus (MTM).

Histology and Immunohistochemistry

Tissue ring samples were paraffin-embedded and blocks were cut into sections of 5 µm thickness. Slides were stained with H/E and Masson's trichrome using standard protocols. VSMCs were identified by immunofluorescence staining of the VSMC specific markers calponin, SMA, SM-MHC, SM-22α and elastin using standard protocols (Refer to previous section). Briefly, tissue sections from the paraffin embedded samples were deparaffinized and processed through a gradient of alcohol from 100% to 50% ethanol and finally in water. Antigen retrieval was carried out at 100 ^{o}C using sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0). The primary and secondary antibodies were used as mentioned above in the immunofluorescence section. Slides were stained for type-I collagen using a similar protocol as above. The primary antibody used was polyclonal rabbit anti-type I collagen (1:200 in PBS containing 10% goat serum) and the secondary antibody was goat anti-rabbit Alexa Fluor® 568. Cell proliferation was measured between control and SVAS rings by staining slides with ki67 primary antibody (Abcam, USA; 1:200 in PBS containing 10% goat serum) and the goat anti-rabbit Alexa Fluor® 568 secondary antibody (1:1000 in PBS).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6. All the data were presented as mean ± SD. One-way ANOVA was used to compare the cell proliferation, elastin gene expression and SM-MHC FACS analysis, and unpaired Student t-test was used for comparisons.

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